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The Distribution and Morphological Characteristics of Serotonergic Cells in the Brain of Monotremes

Paul R. Manger[a,c] Heidi M. Fahringer[a] John D. Pettigrew[b]
Jerome M. Siegel[a]

[a]Department of Psychiatry, University of California, Los Angeles, Neurobiology Research 151A3, Sepulveda VAMC, North Hills, Calif., USA, [b]Vision, Touch and Hearing Research Centre, University of Queensland, St Lucia, Australia, [c]Department of Neuroscience, Division of Neuroanatomy and Brain Development, Karolinska Institutet, Stockholm, Sweden

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Mammals · Monotremes · Platypus · Echidna · Serotonin · Evolution · Sleep

Abstract
The distribution and cellular morphology of serotonergic neurons in the brain of two species of monotremes are described. Three clusters of serotonergic neurons were found: a hypothalamic cluster, a cluster in the rostral brainstem and a cluster in the caudal brainstem. Those in the hypothalamus consisted of two groups, the periventricular hypothalamic organ and the infundibular recess, that were intimately associated with the ependymal wall of the third ventricle. Within the rostral brainstem cluster, three distinct divisions were found: the dorsal raphe nucleus (with four subdivisions), the median raphe nucleus and the cells of the supralemniscal region. The dorsal raphe was within and adjacent to the periaqueductal gray matter, the median raphe was associated with the midline ventral to the dorsal raphe, and the cells of the supralemniscal region were in the tegmentum lateral to the median raphe and ventral to the dorsal raphe. The caudal cluster consisted of three divisions: the raphe obscurus nucleus, the raphe pallidus nucleus and the raphe magnus nucleus. The raphe obscurus nucleus was associated with the dorsal midline at the caudal-most part of the medulla oblongata. The raphe pallidus nucleus was found at the ventral midline of the medulla around the inferior olive. Raphe magnus was associated with the midline of the medulla and was found rostral to both the raphe obscurus and raphe pallidus. The results of our study are compared in an evolutionary context with those reported for other mammals and reptiles.

Introduction

The serotonergic system is thought to be involved in many different and varying behavioral and physiological actions. However, despite this diversity the serotonergic system is not unconditionally necessary for any of these functions [Jacobs and Azmitia, 1992]. Thus, the role of the serotonergic system has been difficult to ascertain, but it is believed to have a 'tonic modulatory influence' within the central nervous system [Jacobs and Azmitia, 1992]. One example of this is the activity of serotonergic neurons

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Paul Manger
School of Anatomical Sciences, Faculty of Health Sciences
Wits Medical School, 7 York Road, Parktown, 2193
Johannesburg (South Africa)
Tel. ■■■■; Fax ■■■■; E-Mail Paul.Manger@neuro.ki.se

near the end of but not during a bout of REM sleep presumably alerting the brain to be ready to respond to, and act upon, sensory information [Fornal and Jacobs, 1988].

The serotonergic system has been found to project to almost the entire central nervous system [Jacobs and Azmitia, 1992], however, the major serotonergic nuclei are located within the brainstem mostly near the midline. The serotonergic nuclei can be grouped into a rostral and caudal cluster, each with distinct nuclear subdivisions. A third cluster of serotonergic cells has been reported in the hypothalamus of various vertebrate species [for reviews see Smeets and Steinbusch, 1988; Meek, 1999]. However, this cell group has not been reported for several species, which include the marmoset [Hornung and Fritschy, 1988], pygmy marmoset [Jacobowitz and MacLean, 1978], squirrel monkey [Hubbard and Di Carlo, 1974], cat [Poitras and Parent, 1978; Leger et al., 2001], rabbit [Bjarkam et al., 1997], wallaby [Ferguson et al., 1999], pigeon [Fuxe and Ljunggren, 1965], chicken [Dubé and Parent, 1981], painted turtle [Parent, 1973a], frog [Parent, 1973b], and sunfish [Parent et al., 1978]. Despite this disparate reporting, Smeets and Steinbusch [1988], conclude that this group of neurons is found only in non-mammalian vertebrates. A study of intraventricular injections of serotonin demonstrated that cells in the hypothalamus of the rat did accumulate this catecholamine [Fuxe and Ungerstedt, 1968], but these cells do not appear to produce serotonin, and have not been identified with serotonin immunohistochemistry.

The majority of anatomical studies of the serotonergic system have been undertaken in typical laboratory animals: rat [e.g., Lidov and Molliver, 1982]; rabbit [e.g., Bjarkam et al., 1997]; cat [e.g., Jacobs et al., 1984]; monkeys [e.g., Hornung and Fritschy, 1988]; and in humans [e.g., Törk, 1990]. Few comparative studies of the serotonergic system of other vertebrates are available [garfish – Parent and Northcutt, 1982; salamander – Dubé and Parent, 1982; chicken – Ikeda and Gotoh, 1971; ranid frog – Parent, 1973b; reptile review – Smeets, 1988; varanid lizard – Wolters et al., 1985; wallaby – Ferguson et al., 1999].

The comparative anatomical studies along with the anatomical studies of typical laboratory animals have allowed the proposal of two evolutionary trends. The first trend is seen as increasing myelination of the serotonergic axons with a concomitant decrease in the collateralization of the axons as one compares less complex central nervous systems with more complex central nervous systems [Bowker and Abbott, 1990]. The second trend is the 'lateralization' of the cells within the serotonergic nuclei. This lateralization trend is derived from comparisons between the rodent and primate serotonergic nuclei, but other studies lend support to this view also [Bjarkam et al., 1997]. This trend is shown as an increase in the number of serotonergic cells located away from the midline in more complex brains. If this trend is a real phenomenon, one would expect the serotonergic nuclei of the monotremes, as the earliest branch of mammals [Musser and Archer, 1998; Kirsch and Mayer, 1998], to be concentrated on the midline of the brainstem, as would those of non-mammalian vertebrates.

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% saline, followed by 4% paraformaldehyde in 0.1 *M* phosphate buffer.

Serial 50-μm sections of hemi-sectioned 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 the ChAT and TH immunohistochemistry are presented elsewhere [Manger et al., 2002a, b]. For serotonin immunohistochemistry, the sections were rinsed 5 times in 0.1 *M* PO_4 buffer prior to a 1 h incubation in a blocking buffer solution of 0.1% Triton X-100 (TX), 0.1% bovine serum albumin and 2% normal goat serum (NGS) in 0.1 *M* phosphate-buffered saline (PBS). The primary and secondary antibodies were diluted in the blocking buffer solution. The sections were then incubated for 48 h at 4°C in a 1/100,000 dilution of rabbit antiserum to 5HT (Incstar, Stillwater, Minnesota). This was followed by 2 h in 1/500 biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA) and then a 1/100 solution of avidin biotin complex (Vector) for 2 h. Sections were rinsed in PBS following each incubation. For visualization, the sections were treated for approximately 6 min with a 0.05% solution of 3,3′diaminobenzidine and 0.01% hydrogen peroxide. The sections were then rinsed in phosphate buffer, mounted on gel-coated slides, cleared in xylene and coverslipped in 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. Ordinary light microscopic examination and photomicroscopy allowed us to make descriptions of cellular morphology. High power photomicrographs were taken of approximately 100 cells in each region and the somatal area was determined using the program Image Tools. Only cells in which a clear nucleus 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

Cells reactive to serotonin immunohistochemistry were located within the hypothalamus and brainstem of both species of monotremes. The distribution of these cells was similar in both species, thus the following description is applicable to the platypus and the echidna. Terminology employed in this description is derived from a review by Jacobs and Azmitia [1992], and from recent papers by Bjarkam et al. [1997] and Ferguson et al. [1999]. Serotonergic cells could be divided into a hypothalamic cluster, a rostral brainstem cluster and a caudal brainstem cluster. The hypothalamic cluster was intimately associated with the ependymal wall of the third ventricle, and consisted of the periventricular hypothalamic organ and the infundibular recess. The rostral brainstem cluster consisted of three nuclei, the dorsal raphe nucleus (composed of four divisions), the median raphe nucleus, and the cells of the supralemniscal region. The rostral cell cluster was located between the decussation of the brachium conjunctivum, and continued posterior to the level of the trigeminal motor nucleus. The caudal cluster also consisted of three nuclei, the raphe obscurus nucleus, the raphe pallidus nucleus, and the raphe magnus nucleus. The anterior limit of the caudal cluster was found at the posterior end of the trigeminal motor nucleus, the posterior limit being the rostral border of the cervical spinal cord.

Hypothalamic Cell Cluster

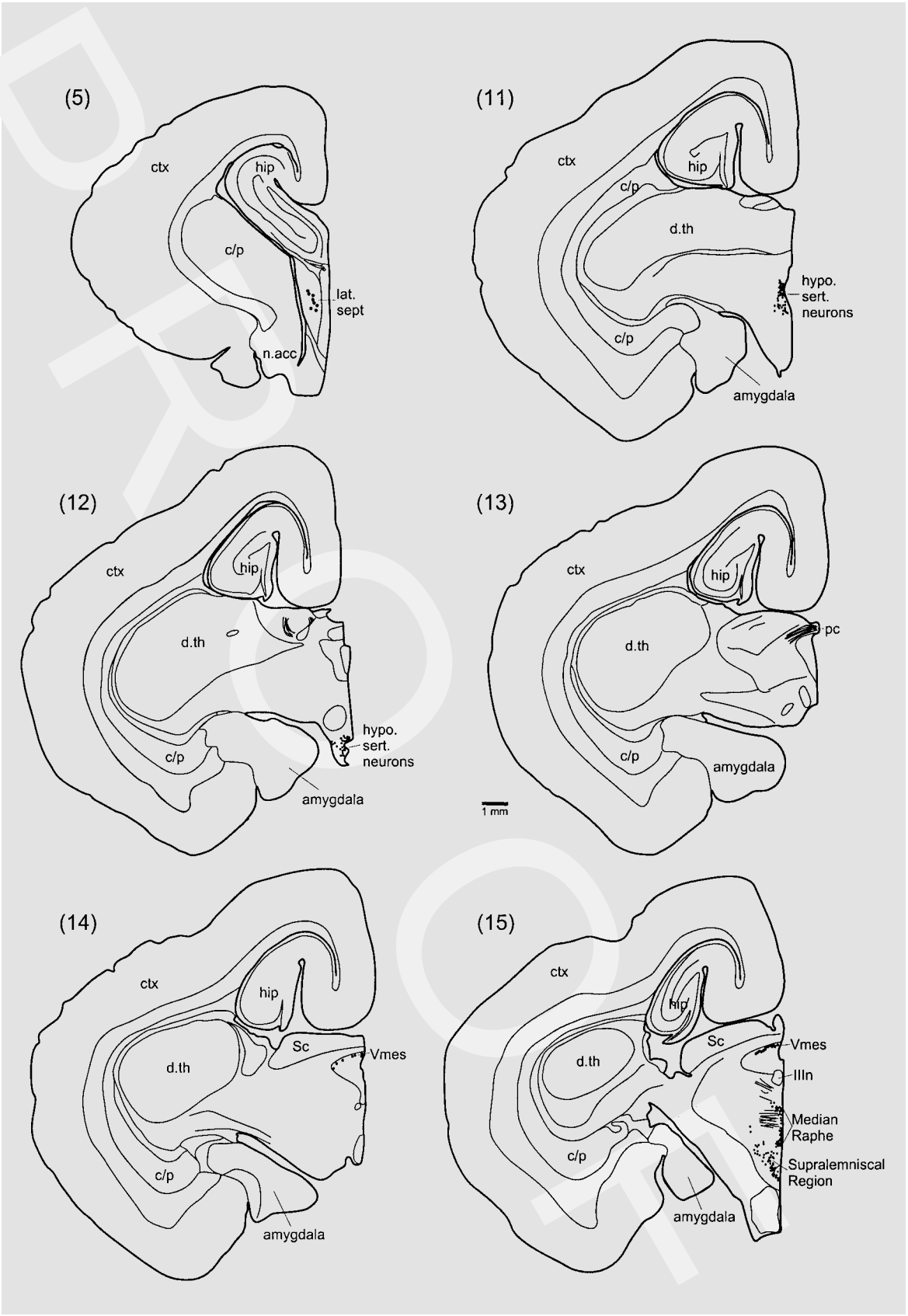
The serotonergic neurons within the hypothalamus are closely associated with the ependymal wall of the third ventricle and span an antero-posterior distance of around 1.5 mm (fig. 1: 11–12; 2: 1–3). Within the periventricular hypothalamic organ, there are two types of serotonergic neurons, one closely associated with the ependymal wall, and a second found at a small distance from the ependymal wall (fig. 3A). The first type, associated with the ependymal wall, has a small circular cell body, from the ependymal side of which a club-like process extends to the ependymal wall. From the opposite end of the cell body the axon extends laterally away from the midline. The second type forms an arc surrounding the cell cluster of the first type, and is located at a short distance from the ependymal wall. Cells of the second type have small oval shaped cell bodies and have two primary dendrites. The first dendrite projects to the cells associated with the ependymal wall, and the second dendrite projects laterally away from the midline along with the axon. The axons of both these cell types form a fasciculus that appears to traverse the zona incerta and comingle within the serotonergic plexus that arises from the brainstem and passes through the substantia nigra. Ventral to the periventricular hypothalamic organ is a second group of serotonin immunoreactive cells that surround the infundibular recess. These cells are similar in morphology to those of the first type in the periventricular hypothalamic organ in that they possess a club-like extension which is associated with the ependymal wall. The axon projects from the other side of the small circular cell body, away from the ependymal wall.

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
hip	hippocampus
hypo. sert. neurons	hypothalamic serotonergic neurons
i. olive	inferior olive
ic	inferior colliculus
lat. sept.	lateral septal nucleus
IIIn	oculomotor nucleus
med. sept.	medial septal nucleus
n. acc.	nucleus accumbens
NDR if	dorsal raphe nucleus, interfascicular division
NDR lat	dorsal raphe nucleus, lateral division
NDR m	dorsal raphe nucleus, median division
NDR vm	dorsal raphe nucleus, ventromedial division
NMR	median raphe nucleus
NRM	raphe magnus nucleus
NRO	raphe obscurus nucleus
NRP	raphe pallidus nucleus
NSL	supralemniscal region
olf. tub.	olfactory tubercle
pc	posterior commissure
Sc	superior colliculus
Vmes	fifth mesencephalic nucleus
Vmot	trigeminal motor nucleus
Vsens	trigeminal sensory nucleus
VIIn	facial nucleus
Xn	dorsal motor vagus nucleus
XIIn	hypoglossal nucleus

For figure 1 see page ■■■■

Fig. 1. Serial drawings of coronal sectionsthrough the platypus brain showing the distribution of serotonergic cells (small black dots). In diagram 5 the dots represent the pericellular basket formations of serotonergic terminals in the lateral septal nucleus. Sections are approximately 1000 μm apart. The number on each diagram corresponds to the numbers on the diagrams in the accompanying papers [Manger et al., 2002a, b]. See list for abbreviations.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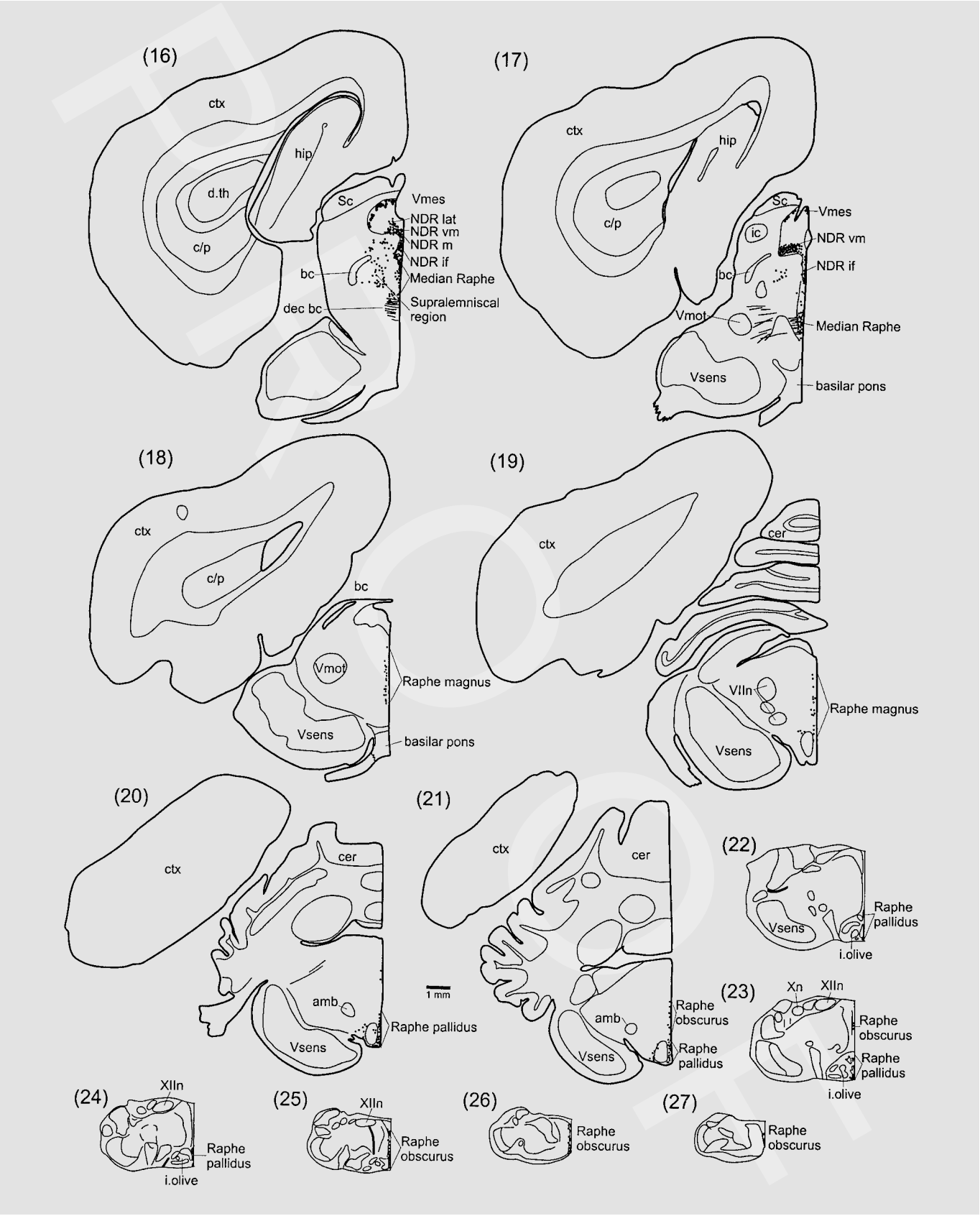
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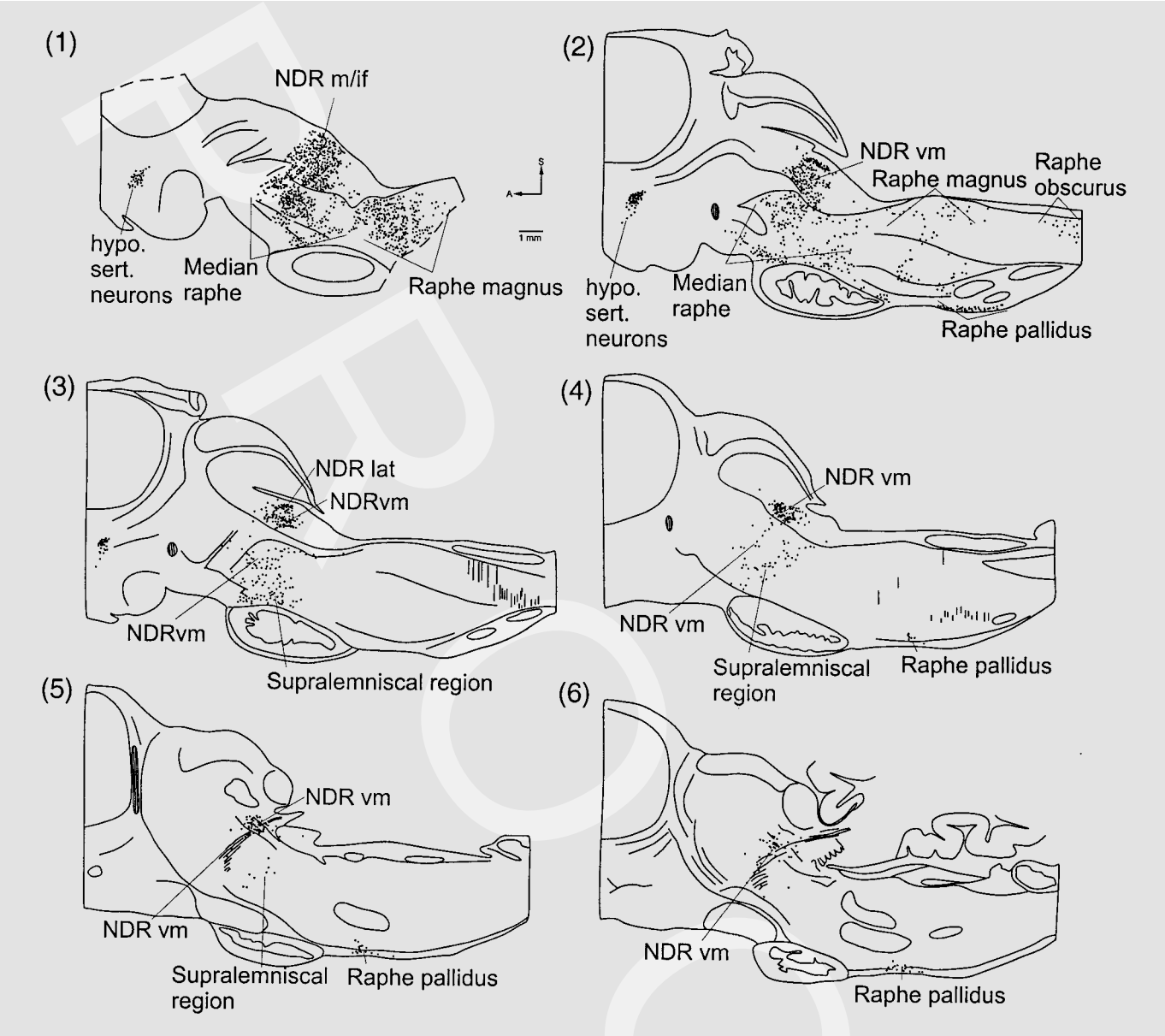


Fig. 2. Serial drawings of sagittal sections through the echidna brainstem and thalamus, showing the distribution of serotonergic cells (small black dots). Only the region posterior to the level of the thalamus is shown in these figures. Sections are approximately 250 μm apart. See list for abbreviations.

Rostral Cell Cluster

Dorsal Raphe Nucleus. The dorsal raphe nucleus (NDR) is located within the periaqueductal gray matter, below the fourth ventricle at the midline of the anterior portion of the brainstem (fig. 1: 16–17; 2: 1–6). It extends from the anterior-most border of the trigeminal motor nucleus to the level of the oculomotor nucleus and is comprised of four divisions, lateral, median, ventromedial and interfascicular. The interfascicular division is found at the midline, between the medial longitudinal fasciculi (fig. 1: 16–17; 2: 1). The cells of this division form a dorso-ventral column of a few cells thickness, the base of this column is found slightly inferior to the inferior boundary of the medial longitudinal fasciculus. The top of the column extends slightly higher than the superior limit of the medial longitudinal fasciculus. The cell bodies of this

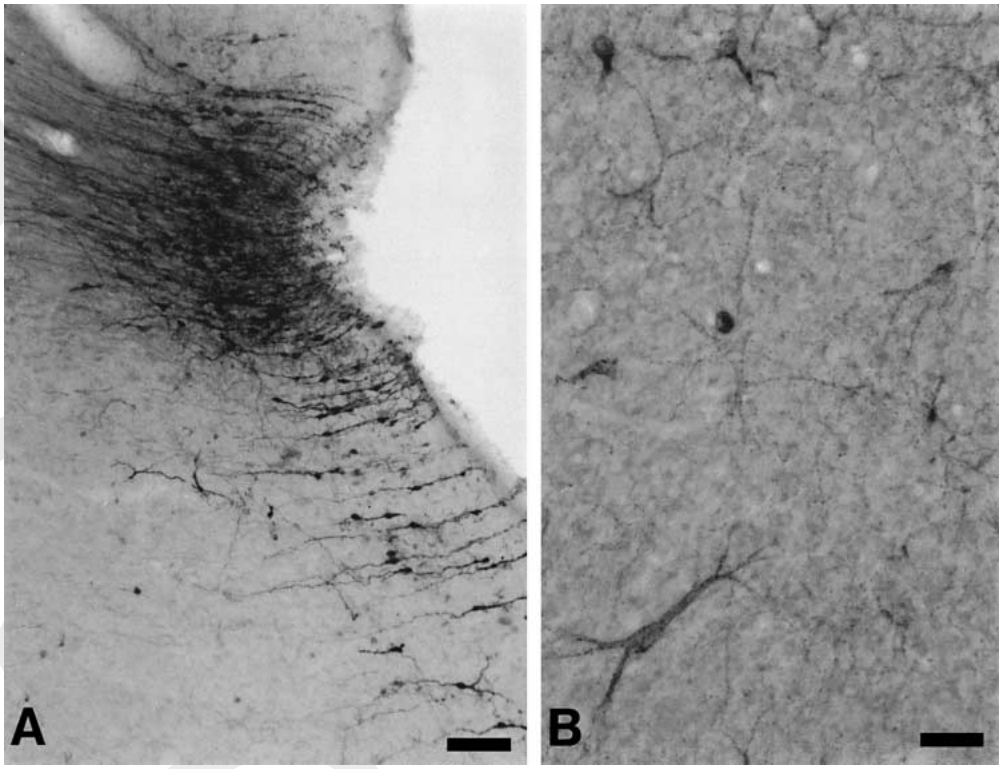


Fig. 3. A Photomicrograph of serotonergic cells in the periventricular hypothalamic organ of the echidna. Note the two cell types, one with a club-like extension associated with the ependymal wall, and the other further from the wall, unassociated with it. Scale bar = 100 μm. B Photomicrograph of the pericellular basket serotonergic terminal configuration in the lateral septal nucleus of the platypus. Note the manner in which the serotonergic terminals form pericellular baskets, as well as providing general innervation to the surrounding region. Scale bar = 50 μm.

division are close to spherical in shape, the sphere being distorted by the bipolar nature of these cells (fig. 4C). The bipolar cells of the interfascicular division appear to have their primary dendrites oriented in an approximate dorso-ventral plane. The ventromedial division of the NDR is located at the lateral and ventral aspect of the periaqueductal gray (fig. 1: 16–17; 2: 2–6). This is the largest of the four divisions and has a high density of serotonergic cells. The cells of this division are monopolar, but are roughly similar in shape to those of the interfascicular division (fig. 4A). The dendrites of these cells project in the same ventro-lateral direction, towards the location of the locus coeruleus and the pendunculopontine nuclei. Numerous cells of this division, with similar somatal morphology, are located outside the periaqueductal gray and are found comingled with the cells of the locus coeruleus and pedunculopontine nuclei. These cells are more scattered than those found within the periaqueductal gray, and are separated from the cells of the supralemniscal region by the brachium conjunctivum. The median division is located at the midline, immediately beneath the fourth ventricle (fig. 1: 16; 2: 1). The cells of this division are small and bipolar. The cell bodies are flattened in a horizontal plane, the same plane in which the dendrites appear to project, on either side of the cell body (fig. 4B). The lateral division of the NDR contains the smallest number of serotonergic cells. It is located dorsal to the ventromedial division of the NDR, and lateral to the median division, and is spatially segregated from these divisions (fig. 1: 16; 2: 3). The majority of the cells are multipolar and have extravagant dendritic branching in comparison to the other cells of the NDR (fig. 4B). The NDR is dominated by the cells of the ventromedial and interfascicular divisions, and both these are readily visible in Nissl-stained sections.

Median Raphe Nucleus. The median raphe nucleus (NMR) is found along the midline of the brainstem extending from the trigeminal motor nucleus to the decussation of the brachium conjunctivum, forming a dorsoventral column (fig. 1: 15–17; 2: 1–2). It is bordered superiorly by the medial longitudinal fasciculus and inferiorly

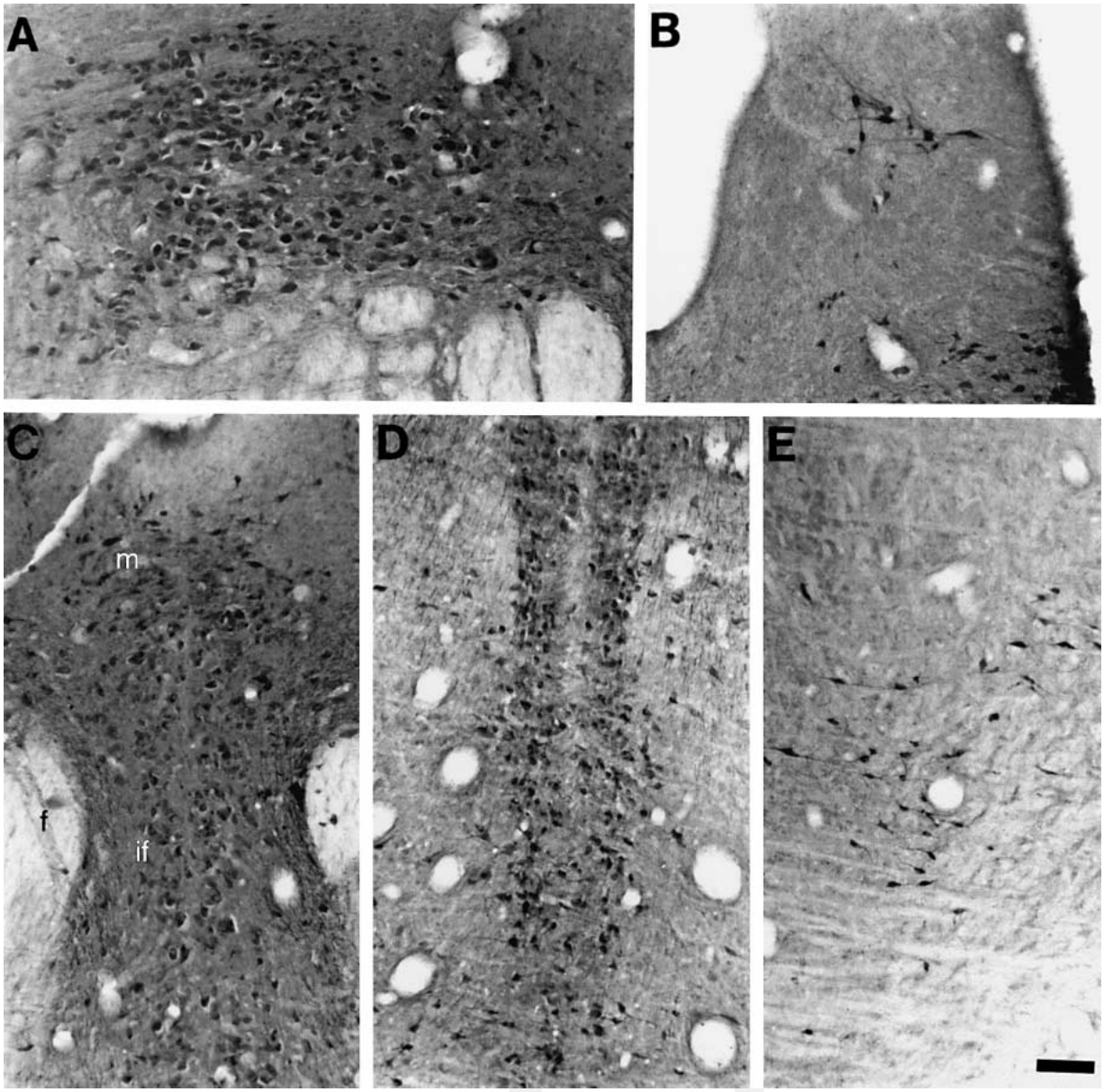


Fig. 4. Photomicrographs of serotonergic cells in the rostral cell cluster of the platypus brain. A Serotonergic cells in the ventromedial division of the dorsal raphe nucleus. This division shows the highest density and number of serotonergic cells in the monotreme brain. B Serotonergic cells in the lateral division of the dorsal raphe nucleus. Note the more dorsal position of this division, and the more apparent dendritic arborization due to the lower neuronal density. C Photomicrograph of the median (m) and interfascicular (if) divisions of the dorsal raphe nucleus. The medial longitudinal fasciculus (f) is apparent on either side of the interfascicular division. The cells of the median division are located dorsal to the interfascicular division, however, the ventral-most portion of this division is not clearly separated from the interfascicular division. D Serotonergic cells of the median raphe nucleus show the typical bilateral columns. Also note the cells that are lateral to the main columns. E A group of serotonergic cells in the supralemniscal region located just dorsal to the decussation of the brachium conjunctivum. This group of cells is very lateralized in both species of monotremes, and extends dorsally to reach the lateralized portions of the ventromedial division of the dorsal raphe nucleus. Scale bar = 100 μm (for all photomicrographs).

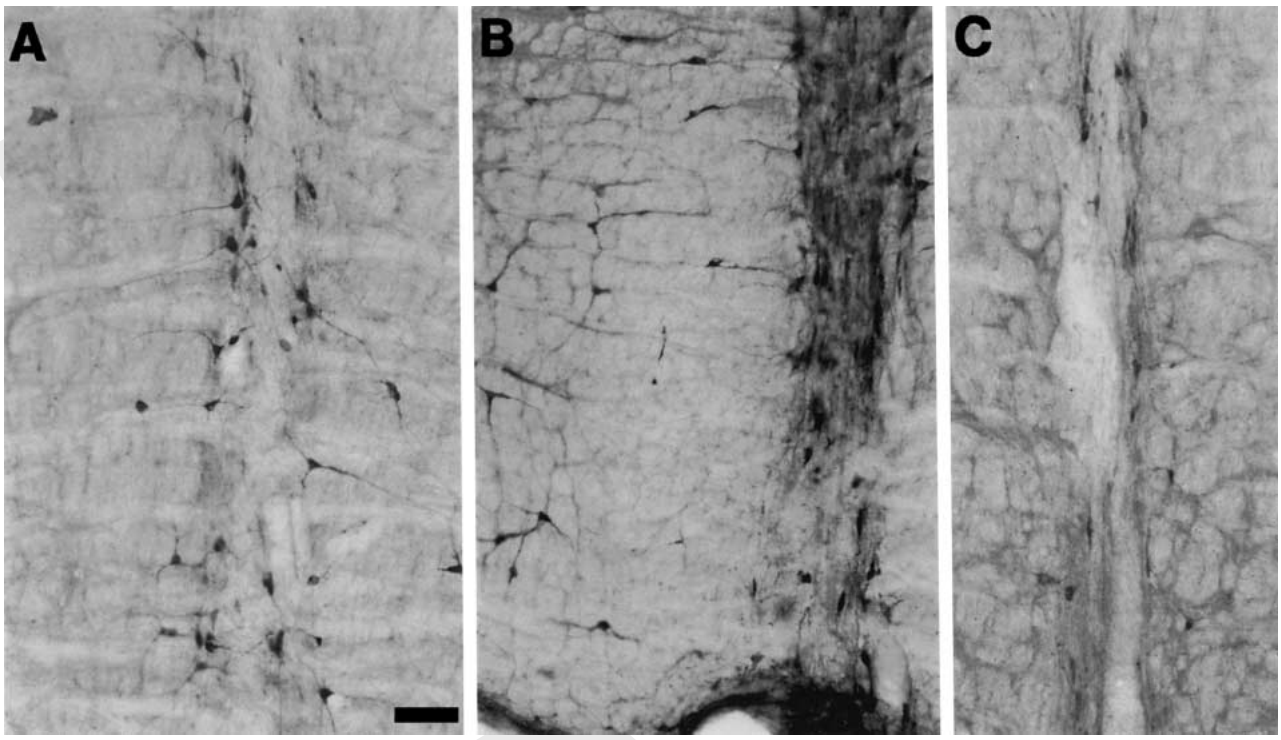


Fig. 5. Photomicrographs of the caudal serotonergic nuclei from the brain of the platypus. A Serotonergic cells of the raphe magnus nucleus show bilateral columns on either side of the midline. Similar to the median raphe nucleus, there are a secondary group of more lateralized cells associated with this nucleus. B The cells of the raphe pallidus nucleus were found in the midline between the two inferior olives. The typical bilateral column appearance of the midline serotonergic nuclei is not so clear in this particular nucleus. Lateralized cells of this nucleus are also seen surrounding the inferior olive. C The cells of the raphe obscurus nucleus are not numerous, but do form the distinct bilateral columns of midline serotonergic nuclei. As with the other midline nuclei some serotonergic cells are found lateral to the midline columns. Scale bar = 100 μm (for all photomicrographs).

by the medial lemniscus. The cells of this nucleus are smaller than those of the other serotonergic nuclei. These cells have a spherical cell body and are monopolar (fig. 4D). The dendrites are oriented either dorsally or ventrally, parallel to the midline. The density of cells throughout this nucleus appeared to be consistent.

Supralemniscal Region. The supralemniscal region (NSL) is found lateral to the midline of the brainstem. It is bordered inferiorly and laterally by the medial lemniscus and superiorly and anteriorly by the brachium conjunctivum (fig. 1: 15–16; 2: 3–5). The brachium conjunctivum distinguishes the cells of the ventromedial division of the NDR from those of the NSL. The NSL extends from just anterior to the trigeminal motor nucleus to just posterior to the decussation of the brachium conjunctivum. The cell group as a whole forms a crescent shape, paralleling the arc of the medial lemniscus. The cell bodies are flattened in a plane corresponding with the arc of the medial lemniscus (fig. 4E). The cells are mostly bipolar, and the dendrites project in the same plane as the cell body. The density of cells in the NSL is lower than the other nuclei in the rostral cluster, and the density of cells appears consistent throughout the nucleus.

Caudal Cell Cluster

Raphe Obscurus Nucleus. The raphe obscurus nucleus (NRO) is located between the rostral part of the cervical spinal cord and the mid-level of nucleus ambiguus. All cells within the NRO were located along the midline, a paramedian distribution, forming a vertical row of cells on either side of the midline (fig. 1: 21–27; 2: 2). At the posterior level of this nucleus the cells are located dorsal to the central canal of the spinal cord. Further anterior the cells are found dorsal to the inferior olive, forming a vertical column of cells dorsal to the raphe pallidus nucleus, but still located close to the midline. There are two distinct cell types found in this nucleus (fig. 5C). First, the cells forming the distinct vertical column are oriented in a

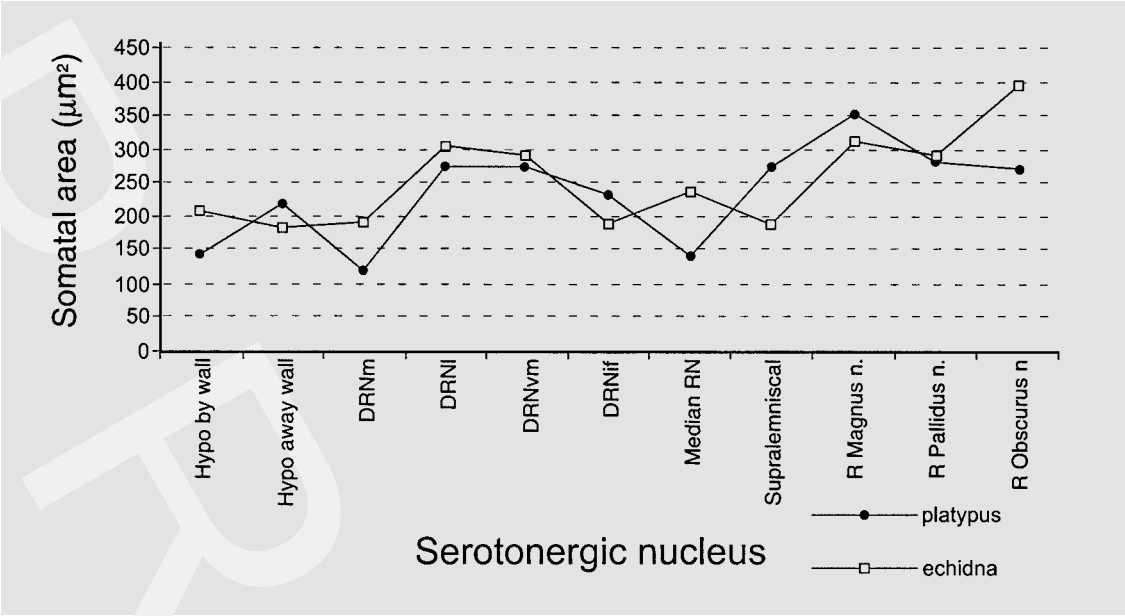


Fig. 6. Graphical representation of the data on somatal areas of serotonergic neurons given in table 1. Despite the almost threefold difference in brain size between platypus and echidna, the somatal areas of the serotonergic nuclei show a great deal of overlap.

Table 1. Somatal areas of serotonergic positive somata in µm² (and standard deviation) in the various subdivisions of the serotonergic system in the platypus and echidna

Serotonergic nuclear group	Platypus cell size (µm²)	Echidna cell size (µm²)
Periventricular organ		
By wall	145.08 (31.89)	208.10 (38.61)
Away from wall	220.14 (38.38)	183.00 (43.65)
Dorsal raphe nucleus		
Medial division	120.39 (30.57)	191.87 (21.63)
Lateral division	275.26 (83.09)	302.57 (49.90)
Ventral medial wing	275.39 (42.13)	292.30 (35.72)
Interfascicular division	234.89 (43.15)	191.32 (34.73)
Median raphe nucleus	144.53 (16.78)	236.97 (45.72)
Supralemniscal region	276.42 (47.74)	191.93 (23.38)
Raphe magnus nucleus	355.72 (62.01)	314.35 (58.31)
Raphe pallidus nucleus	287.20 (58.72)	293.38 (34.74)
Raphe obscurus nucleus	276.11 (54.46)	399.91 (58.86)

dorso-ventral plane, the cell bodies being elongated in this plane. These vertical column cells are bipolar and the axons and dendrites of these cells are oriented in the same dorso-ventral plane as the cell bodies. Lateral to the cells of the vertical column are a group of cells with smaller, triangular cell bodies. These cells have between three and four primary dendrites which are oriented in both a dorso-ventral plane and extend laterally toward the reticular formation as are the axons of these cells. These multipolar cells are less numerous than those forming the central column and are only found in the region of the NRO dorsal to the inferior olive.

Raphe Pallidus Nucleus. The raphe pallidus nucleus (NRP) is found medial and ventral to the inferior olive and pyramidal tract, from the posterior level of the hypoglossal nucleus to the anterior limit of the facial nucleus (fig. 1: 20–24; 2: 2). There are two cells types located within this nucleus (fig. 5B). The first type is located close to the midline and is generally bipolar, with the primary dendrite either coursing dorsal or ventral, parallel to the midline (see below). The second cell type is found ventral and lateral to the inferior olive and pyramidal tract along the inferior edge of the brainstem. These cells are bi- or tripolar, and the dendrites project either towards or away from the midline, parallel with the ventral edge of the brainstem (see below). Some of the cells of this second type are located as far lateral as the base of the fifth arcuate nucleus. Many of the axonal projections of these neurons are found to terminate on the cells of the nucleus ambiguus, the facial nucleus and the trigeminal motor nucleus. These cranial nerve nucleus projecting axons

course inferiorly along the midline, pass through the ventral-lateral cell group and begin to course dorsally towards the cranial nerve nuclei at the lateral-most part of the base of the fifth arcuate nucleus.

Raphe Magnus Nucleus. The raphe magnus nucleus (NRM) is found at the level of the facial nucleus. The NRM is located dorsal to the pyramidal tract and forms a loose column of cells close to the midline of the brainstem (fig. 1: 18–19; 2: 1–2). The two cells types found in the NRM are very similar to those seen for the NRO (fig. 5A). There is a column of bipolar cells located very close to the midline, the cell bodies are flattened in a dorso-ventral plane and the axonal projections are oriented in the same plane. Slightly lateral to the cell column are tripolar cells, the bodies of which are not flattened in any given plane. The projections of these cells are sent dorsal, ventral, and lateral into the reticular formation.

Somatal Areas of Serotonergic Neurons

For both species of monotremes the somatal areas of cells in the various nuclear subdivisions were determined (table 1). The somatal areas exhibit a consistency across the nuclei and between species. The data is given in table 1 and is presented in graphical form in figure 6.

Serotonergic Terminal Fields

Serotonergic terminals were located throughout the majority of the monotreme CNS, however, some regions were found to be densely innervated by serotonergic terminals. These regions included the lateral septal nucleus, the striatum, the basal forebrain, amygdala, hypothalamus, the cranial nerve nuclei and the periaqueductal gray matter. To a lesser extent serotonergic terminals were located in the cortex, hippocampus, dorsal thalamus, cerebellar cortex, superior colliculus, and the entire tegmentum of the brainstem. This pattern is similar to that seen in other mammals [e.g., Leger et al., 2001], so a full description is not given here, except for the following description of the lateral septal nucleus serotonergic terminals.

Lateral Septal Nucleus. The lateral septal nucleus was found to be densely innervated by serotonergic neuronal terminals. Terminals were found throughout this nucleus, but were mostly absent from the adjacent medial septal nucleus (which contains numerous cholinergic cells). There appeared to be two distributions of the serotonergic terminals within the lateral septal nucleus. The first involved a non-specific distribution of terminals that did not show a preference for a specific cell type or region within the nucleus. These terminals were located throughout the entire extent of the nucleus. A second, much more specific group of serotonergic neuronal terminals forming pericellular baskets, was also found (fig. 1: 5). These baskets were found to be associated with a specific cell type whose morphological features were clearly visible (fig. 3B). The cells on which these dense terminals were located had a large ovoid cell body, with a long axis. There was one large apical dendrite and two smaller distal dendrites.

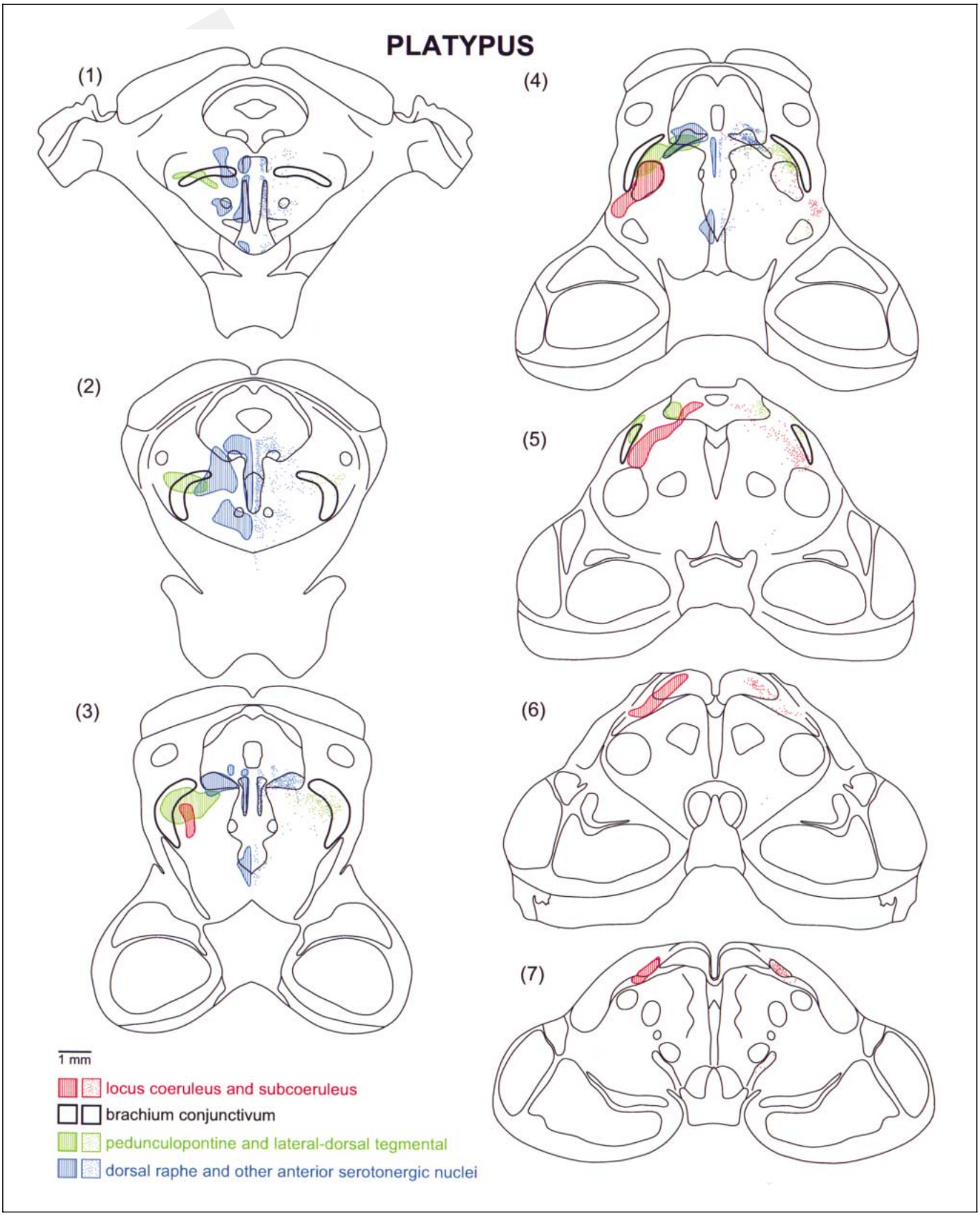
Distribution of the Dorsal Raphe Nuclei Relative to the Locus coeruleus, subcoeruleus, Lateral Dorsal Tegmental and Pedunculopontine Nuclei

The results of the present study, combined with those of the two preceding papers [Manger et al., 2002a, b], allows us to make a direct comparison of the distribution of the monoaminergic and cholinergic cells located in the pontine region of the monotreme brain. The topological location of these cell groups relative to one another are similar in both platypus and echidna, thus the description given below is applicable to both monotreme species, unless otherwise stated (fig. 7–9).

For figure 7 + 8 see pp ■■■

Fig. 7. Compilation diagram showing the distribution of serotonergic, cholinergic, and noradrenergic cells in the pontomesencephalon of the platypus. Apart from minor overlaps, there appears to be a distinct complementarity in the distribution of these different cell groups [see Manger et al., 2002a, b for details of cholinergic and catecholaminergic cells groups in the monotremes].

Fig. 8. Compilation diagram showing the distribution of serotonergic, cholinergic, and noradrenergic cells in the pontomesencephalon of the echidna. Although the complementarity in cellular distribution is evident in this species, and the overall similarity in distribution of the cellular groups, it differs from the platypus in having much greater areas of cellular location overlap. One clear difference is the overlapping distribution of locus coeruleus neurons and those of the lateral-dorsal tegmental nucleus. There is also much greater overlap in the distribution of subcoeruleus, pedunculopontine and the lateralized part of ventromedial division of the dorsal raphe nucleus.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(1)
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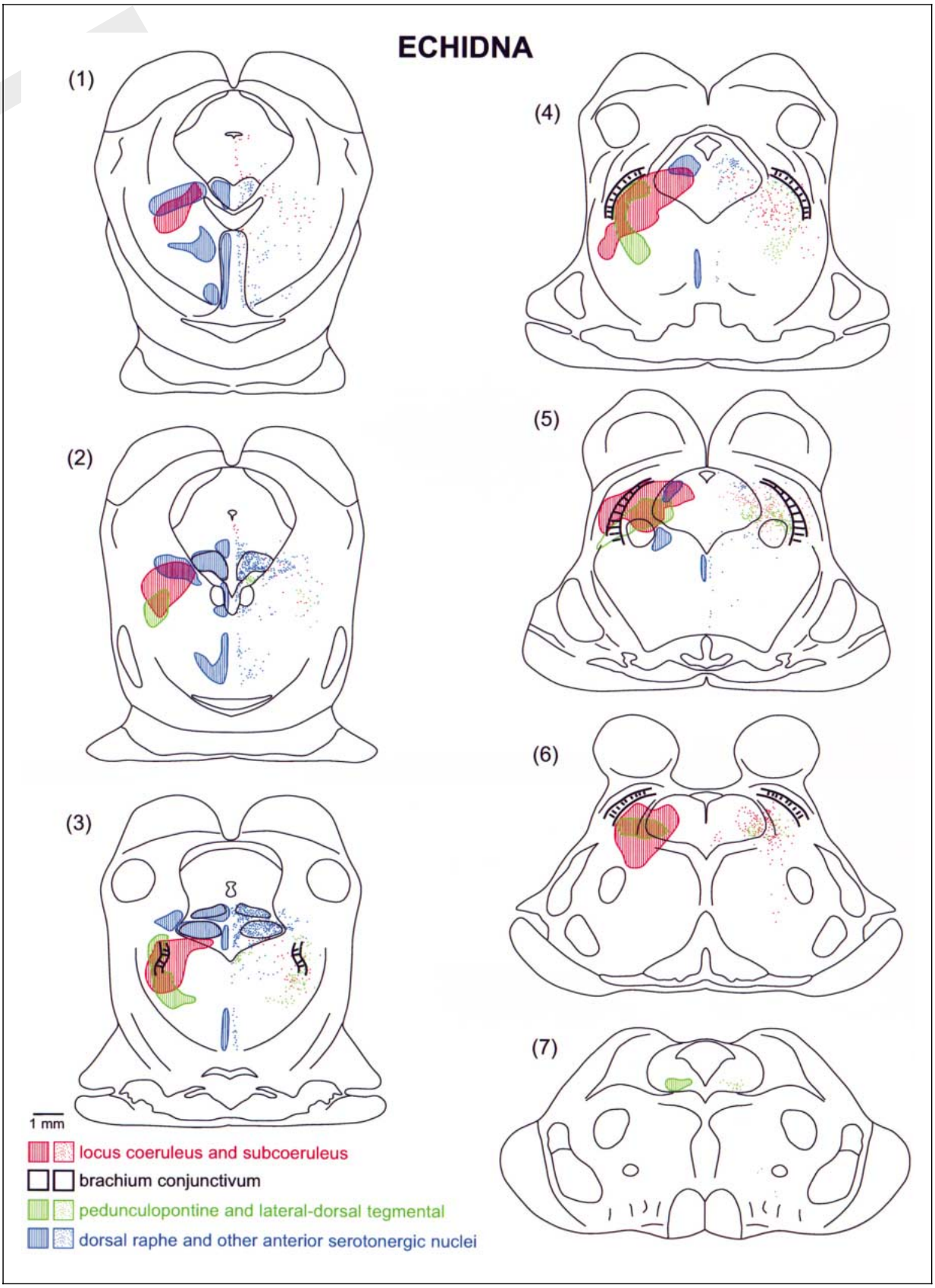
1 mm
locus coeruleus and subcoeruleus
brachium conjunctivum
pedunculopontine and lateral-dorsal tegmental
dorsal raphe and other anterior serotonergic nuclei

7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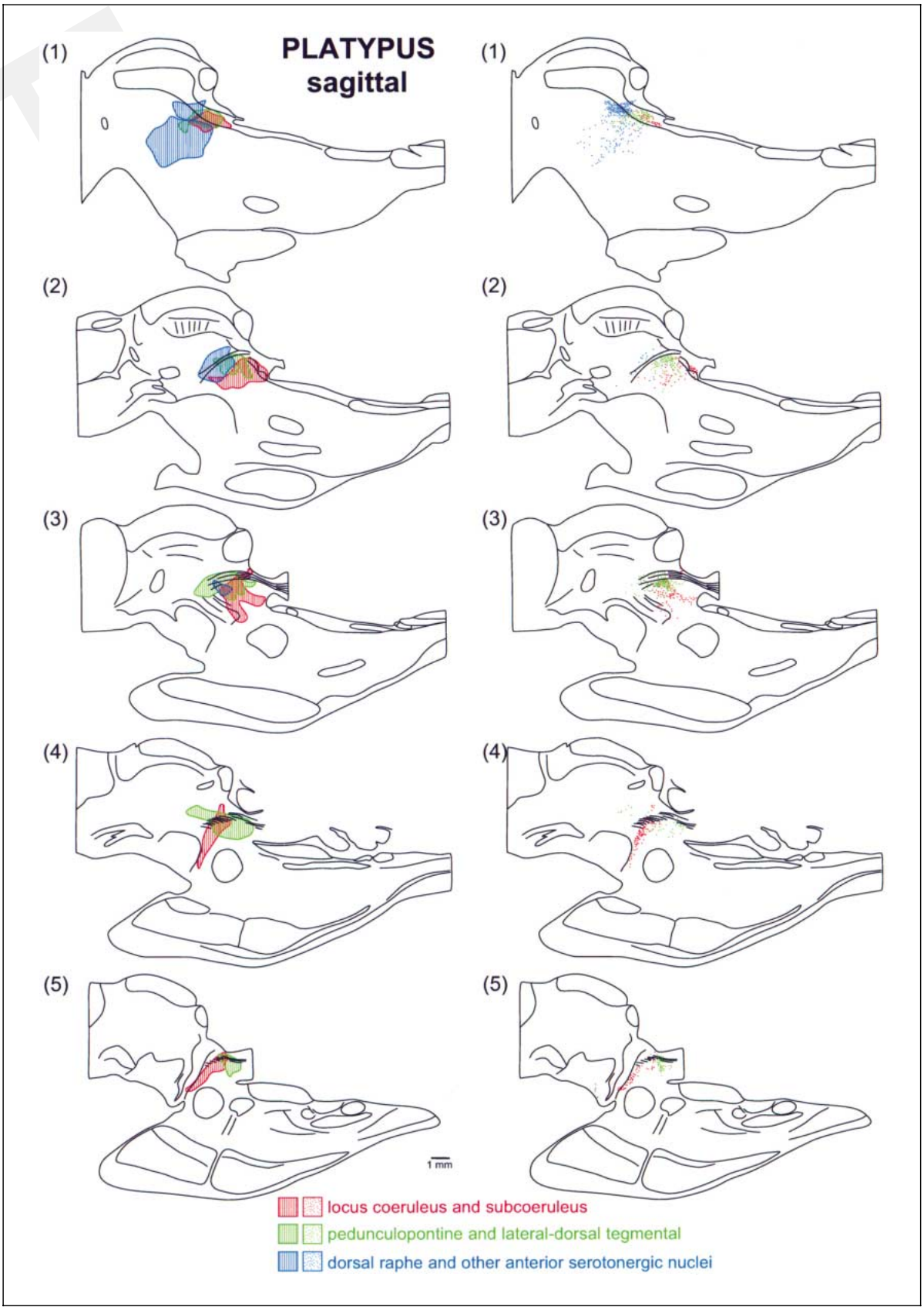
brachium conjunctivum
pedunculopontine and lateral-dorsal tegmental
dorsal raphe and other anterior serotonergic nuclei

8


PLATYPUS
sagittal
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locus coeruleus and subcoeruleus
pedunculopontine and lateral-dorsal tegmental
dorsal raphe and other anterior serotonergic nuclei

At the posterior level of the inferior colliculus, within the ventral lateral periaqueductal gray matter, there is limited overlap in the distribution of the ventromedial division of the dorsal raphe with the locus coeruleus and/or lateral dorsal tegmental nuclei. The antero-posterior extent of this overlap is small and limited to less than 500 μm. A slightly larger overlap is found between the cells of the ventromedial division of the dorsal raphe located outside the periaqueductal gray matter and the cells of the subcoeruleus and pedunculopontine nuclei. This is found at a more rostral location than the previously described overlap and extends for an anteroposterior extent of approximately 1 mm. These two regions of overlap are the only ones found between the serotonergic cells of the dorsal raphe and those of the catecholaminergic and cholinergic nuclei in this region of the brain.

Significant overlap in the distribution of cells of the subcoeruleus and pedunculopontine nuclei were observed. In the echidna these cells are found in the same location throughout the anteroposterior extent of both these nuclei, a distance of some 2.5 mm. The overlap in platypus is somewhat more restricted (around 1.5 mm) and reflects the fact that the pedunculopontine nucleus is located in a slightly more rostral position than the subcoeruleus in this animal. In the echidna the lateral dorsal tegmental nucleus and locus coeruleus were also seen to have a fully overlapping distribution. In the platypus these two nuclei were never seen to occupy the same region of the periaqueductal gray matter, however, they were located adjacent to one another over the majority of their distribution.

Discussion

The results from the present study demonstrate that there are three groups of serotonergic neurons in the brain of monotremes: the hypothalamic cluster, the rostral nuclear cluster and the caudal nuclear cluster. The rostral and caudal clusters appear to be consistent across mammals. The hypothalamic cluster has not been previously reported in mammals, however it is found in most other species of vertebrates studied (see references listed in introduction). However, despite this overall topological and morphological congruency, there are details that warrant further discussion.

One of the major reasons for investigating the distribution of the serotonergic cell clusters in monotremes is the evolutionary position of this order of mammals. Recent reviews of the evolutionary relationships of the monotremes indicate that they form an early branch of mammals with a very conservative evolutionary history [Musser and Archer, 1998]. However, it is has also been suggested that they have a special relationship with the marsupials [Kirsch and Mayer, 1998]. The high degree of structural and physiological conservatism over monotreme evolutionary history might lead us to believe that the soft tissue structures of the monotremes have also been greatly conserved over the course of time. Thus, by examining the brains of monotremes, we could be gaining a unique insight into the manner in which the mammalian brain was organized very early in its evolutionary history. Furthermore, the similarities in the brain structure of monotreme, marsupial and placental mammals provide clues regarding the probable brain structure of a common mammalian ancestor. However, we must also be mindful that we might be analyzing a very highly specialized brain that has had a long period of gradual evolution (over 100 million years).

The existence of a hypothalamic group of neurons with an affinity for serotonin was first described by Fuxe and Ungerstedt [1968] in the rat. They demonstrated that intraventricular injections of serotonin resulted in accumulation of serotonin in a group of neurons in contact with the ventricle. Reports omitting the description of hypothalamic serotonergic cells in a range of vertebrates conflict with reports indicating the presence of these cells [see the discussion of Smeets and Steinbusch, 1988]. Despite these conflicts, and the report of Fuxe and Ungerstedt [1968], Smeets and Steinbusch [1988] conclude that this group is found in all vertebrate species except mammals. The results of the present study show that both species of monotreme contain the hypothalamic serotonergic cell group, and is the first to demonstrate immunohistochemical identification of these neurons in a mammal. As with other species in which this cell group has been described, the cells are intimately associated with the ependymal wall of the third ventricle [Meek, 1999]. The morphology of the cells within this group also appears to be similar across species in which they have been described. Thus, the occurrence of these serotonergic neurons in mammalian species might need further investiga-

Fig. 9. Compilation diagram showing the distribution of serotonergic, cholinergic, and noradrenergic cells in the pontomesencephalon of the platypus in the sagittal plane. The distinction of the cellular groups is again clear (see fig. 7), however, the partial overlaps are more clearly demonstrated.

tion, despite several recent reports failing to identify this group [e.g., Leger et al., 2001]. It is possible that the ability of these neurons to produce serotonin is lost in non-monotreme (therian) mammals, however, therians could have a group of neurons in the hypothalamus with the ability to accumulate serotonin as demonstrated by Fuxe and Ungerstedt [1968].

Within the rostral cell group of serotonergic nuclei, we failed to identify a distinct caudal linear nucleus in either species of monotremes. Previous authors have defined the caudal linear nucleus by means of cellular morphology and location in relation to the brachium conjunctivum [Jacobs and Azmitia, 1992]. In the monotremes there appear to be very few serotonergic cells anterior to the brachium conjunctivum, and those that are present show a similar morphology to those found in the median raphe nucleus. In placental mammals, the caudal linear nucleus has projections to the basal ganglia motor system [Jacobs and Azmitia, 1992]. The effect of the lack of a caudal linear nucleus on basal ganglia activity in monotremes and subsequent motor activity is unknown. As Hines [1929] noted, the cranial nerve motor system of monotremes is exceedingly large, somewhat similar to that found in reptiles, and the present study shows the cranial nerve nuclei to be heavily innervated by serotonergic terminals. It is also worth noting here that there does not appear to be a reptilian homologue of the caudal linear nucleus [Wolters et al., 1985]. Thus, perhaps the lack of a caudal linear nucleus in the monotremes is not altogether too surprising.

As mentioned in the introduction to this paper, there are two proposed evolutionary trends exhibited in the serotonergic system, these being increased myelination of the serotonergic axons and increased lateral displacement of the serotonergic soma. As we did not investigate the serotonergic axons with electron microscopy we are unable to comment on this proposed evolutionary trend [Bowker and Abbott, 1990]. In their paper on the serotonergic system of the rabbit, Bjarkam et al., [1997] discuss the evolutionary phenomenon of lateralization of serotonergic cells in the brainstem. It appears that in more complexly organized brains, such as those of primates, there are increasing numbers of serotonergic cells located away from the midline (as compared to the rodent brain). This lateralization phenomenon is seen to occur in both the rostral and caudal cell clusters of primates and rabbits. However, in the rat the rostral cell cluster is not as clearly lateralized as the caudal cell cluster. The present study describes a substantially lateralized ventromedial division of the NDR in both platypus and echidna, but the cells of the caudal clusters of monotremes do not appear to stray far from the midline. Thus, it appears that monotremes exhibit a pattern opposite to that seen in rodents. Interestingly, lizards [Wolters et al., 1985] also exhibit a substantial degree of lateralization of serotonergic cell groups in the brainstem. For example, the cells of the nucleus reticularis superior pars lateralis, of the rostral cell cluster (probably homologous to the supralemniscal region of mammals), and the cells of the nucleus reticularis inferior, of the posterior group (probably homologous to the rostral and caudal ventrolateral medullary groups), show substantial deviation from the midline. This leads us to the question the validity of lateralization as an evolutionary trend. Do a few comparative studies allow the identification of such a trend? Perhaps at present it is more prudent to regard lateralization as a species specific phenomenon.

One clear conclusion we can draw from the present study is that all of the serotonergic nuclei of the monotremes, excepting those of the hypothalamic cluster, have direct homologues in other mammals. Within the rostral cell cluster of monotremes, we found all four subdivisions of the dorsal raphe, the median raphe nucleus and supralemniscal region. Within the caudal cell cluster we found the raphe obscurus nucleus, raphe pallidus nucleus and raphe magnus nucleus. There were three major differences found between monotremes and other mammals: the presence of a hypothalamic cluster; the lack of a caudal linear nucleus in the rostral cell cluster; and the lack of lateralization in the caudal cell cluster (the serotonergic cells of the caudal and rostral ventrolateral medulla). Thus it is possible to conclude that, apart from the hypothalamic cluster, the serotonergic nuclei we found in the monotremes represent the serotonergic nuclei common to all mammals, excluding the possibility of specific evolutionary loss of certain nuclei, or a functional alteration as appears to have occurred in the hypothalamic neurons of non-monotreme mammals.

It is somewhat difficult to compare data on mammalian serotonergic nuclei with those of reptiles as no previously published study has proposed a series of homologues for these two vertebrate groups and the nomenclature is quite dissimilar. However, there are two similarities that are readily identified. First, the caudal group of serotonergic nuclei, raphe magnus, obscurus and pallidus appear to have corresponding nuclei in the brainstem of turtles [unpublished observations] and lizards. In lizards they have been collectively termed the nucleus raphes inferior [Wolters et al., 1985]. Second, in reptiles there is the nucleus raphes superior that corresponds to the rostral

cell cluster of sertonergic nuclei. It is far more difficult to propose homologies for this region which might stem from the vast difference in telencephalic structure between reptiles and mammals. A series of coupled connectional and immunohistochemical studies appear to be required to elucidate mammalian homologies for the nucleus raphes superior of reptiles. This type of study might also help in determining reptilian homologues of the telencephalon with those of the mammalian telencephalon, a still contentious issue [Striedter, 1997].

Within this paper, and the two preceding papers [Manger et al., 2002a, b], the distribution of serotonergic, cholinergic, and catecholaminergic cells in the monotreme brain have been described. In the results section of the present paper we have also described the distribution of those nuclei in the pontomesencephalon relative to one another. The described overlap in the location of cholinergic and catecholaminergic cells in the monotremes has been shown previously for other mammals, for example the cat [Jones and Beaudet, 1987], and is thus not a surprising finding. However, given the extreme differences in the pattern of monotreme sleep [Siegel et al., 1996, 1998, 1999], one might have expected to find some species specific differences in the monotremes. The only significant difference found in the present series of studies is a hiatus in the distribution of cholinergic neurons. This hiatus is located between the cholinergic cells of the basal forebrain and those of the pontomesencephalon and is due to the lack of hypothalamic cholinergic neurons [see Manger et al., 2002a]. This hiatus might be related to the phenomenological differences between monotreme sleep and that in placental mammals.

One of the most interesting features of the distribution of these nuclei in monotremes and other mammalian species is the fact that they are all located very close to one another in the rostral-most portion of the rhombencephalon. All of these nuclear groups play an important role in the regulation of the sleep-wake cycle [Siegel, 1994], presumably by exerting tonic modulatory effects on the central nervous system. It is striking that these nuclei, that appear to have a unifying task of controlling the global excitability of the brain, are all located within close proximity of one another. This region of the rhombencephalon is known to develop from the same rhombomere – R1 [Puelles, 1995]. The correspondence of task and developmental congruency of these nuclei might be of some assistance in the understanding of the mechanisms of sleep and awareness in the brain, but further speculation is beyond the scope of the present study.

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