Nuclear organisation of some immunohistochemically identifiable neural systems in five Insectivorous species – *Crocidura cyanea, Crocidura olivieri, Sylvisorex ollula, Paraechinus aethiopicus* and *Atelerix frontalis*.

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Highlights

• Several neural systems in the brains of insectivores are described.

• All species show a similar global pattern of organization.

• Certain variations indicate a phylogenetic relationships between shrews and microchiropterans.

• This study support the diphyletic hypothesis of bat evolution.

Abstract

The organization of the cholinergic, catecholaminergic, and serotonergic neurons in the brains of five species of insectivores and the orexinergic (hypocretinergic) system in four insectivore species is presented. We aimed to investigate the nuclear complement of these neural systems in comparison to those of other mammalian species. Brains of insectivores were coronally sectioned and immunohistochemically stained with antibodies against cholineacetyltransferase, tyrosine hydroxylase, serotonin and orexin-A. The majority of nuclei were similar among the species investigated and to mammals in general, but certain differences in the nuclear complement highlighted potential phylogenetic interrelationships. In the cholinergic system, the three shrew species lacked parabigeminal and Edinger-Westphal nuclei. In addition, the appearance of the laterodorsal tegmental nucleus in all insectivores revealed a mediodorsal arch. All three of these features are the same as those present in microchiropterans. The catecholaminergic system of the three shrew species lacked the A4 and A15d nuclei, as well as having an incipient A9v nucleus, again features found in microchiropteran brains. The serotonergic and orexinergic systems of the insectivores are similar to those seen across most Eutherian mammals. The analysis of similarities and differences across mammalian species indicates a potential phylogenetic relationship between the Soricidae (shrews) and the microchiropterans.

Keywords: choline acetyltransferase; tyrosine hydroxylase; serotonin; hypocretin; orexin; evolution; mammal; Insectivora; Chiroptera; neural systems.

Introduction:

The Eutherian mammal order, Insectivora, comprises over 400 species making it the third most speciose mammalian order. It is subdivided into four families: Soricidae (shrews), Solenodontidae (solenodons), Talipidae (moles), and Erinaceidae (hedgehogs and gymnures) (Symonds, 2005). Symonds (2005) has indicated that the actual true derived characteristics of the order Insectivora (i.e. synapomorphies) are notoriously difficult to define, with few groupings having been as problematic in their classification and systematics as the Insectivora. The key reason behind the uncertainties of the phylogeny of this order stems from the large numbers of primitive Eutherian character states as well as a lack of unifying derived character states - it is only the individual families that exhibit derived character states. Many of the earlier phylogenies, based on morphology, assumed monophyly in the Insectivora, whereas molecular-based analyses conclude that this mammalian order is paraphyletic (Symonds, 2005). The paraphyletic insectivore grouping is now generally thought to belong to the Laurasiatheria super order, which is proposed to include, in addition to the insectivores, the Artiodactyls, Perissodactyls, Carnivores and the Chiropterans (Arnason et al., 2002; Asher et al., 2009; Lee and Camens, 2009; Meredith et al., 2011).

For Insectivorous species, the nuclear organization of the cholingeric system has been examined for the lab shrew (*Suncus murinus*) (Karasawa et al., 2003) and partially for the European hedgehog (*Erinaceus europaeus*) (Dinopolous et al., 1988). Additionally, the catecholaminergic (Michaloudi and Papadopoulos, 1996) and serotonergic (Michaloudi and Papadopoulos, 1995) nuclei of the European hedghog brain have been described; however, no reports of the orexinergic system in any Insectivore are available. Thus, there is limited data regarding the nuclear organization of these neural systems in the diverse and possibly paraphyletic grouping that encompasses the Insectivores. The present study addresses, in part, this paucity in the available data regarding these systems in the Insectivores by examining the brains of three shrews and two hedgehogs that have not been previously examined.

The insectivores, especially the shrews (family Soricidae) are also of interest in regards to questions surrounding Chiropteran phylogenetic relationships. While many phylogeneticists aver that the two suborders of the Chiroptera, the megachiropterans and microchiropterans, belong to the same monophyletic mammalian order (e.g.,Teeling et al., 2002, 2005; Murphy et al., 2001), others, notably beginning with Linneas (1758) and more recent analyses of the reproductive organs (Smith and Madkour, 1980), the retinotectal

pathways (Pettigrew, 1986; Pettigrew et al., 2008), and a suite of other neural and non-neural features (Pettigrew et al., 1989), have developed the concept of a dual and independent phylogenetic origin for the megachiroptera and microchiroptera. In the dual phylogenetic origin scenario, the megachiropterans have been proposed to be a sister group to the primates, and closely associated with the dermopterans (Pettigrew, 1986; Pettigrew et al., 1989). Unfortunately, the potential phylogenetic relationships of the microchiroptera remain unaccounted for in the dual origin scenario – if the megachiropterans are related to dermopterans and primates, to what group/s are the microchiroptera related? In this sense, the insectivores become an interesting target for study, as earlier analyses of the cholinergic, catecholaminergic, serotonergic and orexinergic systems (Maseko and Manger, 2007; Maseko et al., 2007; Kruger et al., 2010a, b; Dell et al., 2010, 2013) have indicated a strong similarity between the nuclear organization of these systems in the microchiropteran brain with that reported in the brains of the lab shrew and the European hedgehog. Thus, using immunohistochemical techniques, we have examined the brains of five species of insectivores to determine the organization and complement of the nuclei of the cholinergic, catecholaminergic, serotonergic and orexinergic systems. The results of this study are discussed in terms of the possible phylogenetic affinities between microchiropterans and the paraphyletic groupings of the insectivores.

Materials and methods:

Brains from *Crocidura cyanea* (the reddish-grey musk shrew, brain masses 0.42 and 0.46 g), *Crocidura olivieri* (the African giant shrew, brain masses 0.81 and 0.78 g), *Sylvisorex ollula* (the greater forest shrew brain masses, 0.45 and 0.43 g), *Paraechinus aethiopicus* (the desert hedgehog, brain masses 4.3 and 4.5 g) and *Atelerix frontalis* (the southern African hedgehog, brain masses 1.8 and 2.0 g), were collected for the present study. Permits were obtained from the relevant wildlife authorities in South African, the Democratic Republic of Congo and Saudi Arabia for the capture and euthanasia of the animals from their natural habitat, as well as from the Copenhagen zoo for *A. frontalis*. All animals were handled according to the guidelines of the University of the Witwatersrand Animal Ethics Committee. Each animal was weighed, anaesthetized and subsequently euthanized with mass appropriate doses of sodium pentobarbital (200 mg sodium pentobarbital/kg, i.p.). Upon cessation of respiration the animals were perfused intracardially with 0.9% saline followed by

4% paraformaldehyde in 0.1M phosphate buffer (PB), approximately 1 l/kg of each solution, both solutions having a temperature of approximately 4°C. The brains were then carefully removed from the skulls and post-fixed overnight in 4% paraformaldehyde in 0.1 M PB followed by equilibration in 30% sucrose in 0.1M PB. Each brain was then frozen in crushed dry ice and sectioned into 50 µm thick serial coronal sections on a freezing microtome. A one in five series of sections was made for Nissl substance, cholineacetyltransferase (ChAT), tyrosine hydroxylase (TH), serotonin (5-HT) and orexin (OxA) in all species except *C. cyanea*, which did not undergo orexin-A immunostaining. Sections used for the Nissl series were mounted on 0.5% gelatine-coated glass slides, cleared in a solution of 1:1 chloroform and absolute alcohol, then stained with 1% cresyl violet to reveal cell bodies.

For the immunohistochemical staining each section was treated with endogenous peroxidase inhibitor (49.2% methanol: 49.2% 0.1M PB: 1.6% of 30% H_2O_2) for 30 min and subsequently subjected to three 10 min 0.1M PB rinses. Sections were then preincubated for 2 h, at room temperature, in blocking buffer (containing 3% normal goat serum for the TH, 5-HT and OxA sections or 3% normal rabbit serum for the ChAT sections, plus 2% bovine serum albumin and 0.25% Triton-X in 0.1M PB). This was followed by three 10 min rinses in 0.1M PB. The sections were then placed in the primary antibody solution that contained the appropriate diluted primary antibody in blocking buffer for 48 h at 4°C under gentle agitation. Anti-choline acetyltransferase (AB144P, Millipore, raised in goat) at a dilution of 1:3000 was used to reveal cholinergic neurons. Anti-tyrosine hydroxylase (AB151, Millipore, raised in rabbit), at a dilution of 1:7500 (in all species except *A. frontalis* where the AB152 tyrosine hydroxylase antibody, Millipore, raised in rabbit, at a dilution of 1:3000 was used) was used to reveal the putative catecholaminergic neurons. Serotonergic neurons were revealed using anti-serotonin (AB938, Millipore, raised in rabbit) at a dilution of 1:5000. Orexinergic neurons were revealed using anti-Orexin A (AB3704, Millipore, raised in rabbit) at a dilution of 1:3000. This incubation was followed by three 10 min rinses in 0.1M PB and the sections were then incubated in a secondary antibody solution (1:1000 dilution of biotinylated anti-rabbit IgG, BA-1000, Vector Labs, for TH, 5-HT and OxA sections, or a 1:1000 dilution of biotinylated anti-goat IgG, BA-5000, Vector Labs, for ChAT sections, in a blocking buffer containing 3% NGS/NRS and 2% BSA in 0.1M PB) for 2 h at room temperature. This was followed by three 10 min rinses in 0.1M PB, after which sections were incubated for 1 h in avidin-biotin solution (at a dilution of 1:125, Vector Labs), followed by three 10 min rinses in 0.1M PB. Sections were then placed in a solution of 0.05%

diaminobenzidine (DAB) in 0.1M PB for 5 min, followed by the addition of 3 µl of 3% hydrogen peroxide to each 1 ml of solution in which each section was immersed. Chromatic precipitation was visually monitored and verified under a low power stereomicroscope. Staining was continued until such time as the background stain was at a level that would assist architectural reconstruction without obscuring the immunopositive neurons. Development was arrested by placing sections in 0.1M PB, followed by two more rinses in this solution. Sections were then mounted on 0.5% gelatine coated glass slides, dried overnight, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Depex. To test for non-specific staining of the immunohistochemical protocol, in selected sections the primary antibody and the omission of the secondary antibody were omitted, which resulted in no staining of the tissue.

Sections were examined under a low power stereomicroscope and using a camera lucida, the architectonic borders of the sections were traced following the Nissl stained sections. Sections containing the immunopositive neurons were matched to the drawings and the neurons were marked. All drawings were then scanned and redrawn using the Canvas 8 drawing program (Figs. 1, 2). The nomenclature used for the cholinergic nuclei was adopted from Woolf (1991), Limacher et al. (2008), Bhagwandin et al. (2008), Gravett et al. (2009), Pieters et al. (2010), and Calvey et al. (2013), the catecholaminergic nuclei from Hökfelt et al. (1984), Smeets and Gonzalez (2000), Limacher et al. (2008), Bhagwandin et al. (2008), Gravett et al. (2009), Pieters et al. (2010) and Calvey et al. (2013), the serotonergic nuclei from Törk (1990), Limacher et al. (2008), Bhagwandin et al. (2008), Gravett et al. (2009), Pieters et al. (2010) and Calvey et al. (2013), and the orexinergic nuclei from Kruger et al. (2010b), Bhagwandin et al. (2011), Gravett et al. (2011) and Calvey et al. (2013).

Abbreviations

III – oculomotor nucleus

IV – trochlear nucleus

Vmot – motor division of trigeminal nerve nucleus

Vsens – sensory division of trigeminal nerve nucleus

VI – abducens nucleus

VIId – dorsal division of facial nerve nucleus

VIIv – ventral division of facial nerve nucleus

X – dorsal motor vagus nucleus

XII – hypoglossal nucleus

3V – third ventricle

4V – fourth ventricle

7n – facial nerve

A1 – caudal ventrolateral medullary tegmental nucleus

A2 – caudal dorsomedial medullary nucleus

A4 – dorsolateral division of locus coeruleus

A5 – fifth arcuate nucleus

A6d – diffuse portion of locus coeruleus

A7d – nucleus subcoeruleus, diffuse portion

A7sc – nucleus subcoeruleus, compact portion

A8 – retrorubral nucleus

A9l – substantia nigra, lateral

A9m – substantia nigra, medial

A9pc – substantia nigra, pars compacta

A9v – substantia nigra, ventral, pars reticulata

A10 – ventral tegmental area

A10c – ventral tegmental area, central

A10d – ventral tegmental area, dorsal

A10dc – ventral tegmental area, dorsal caudal

A11 – caudal diencephalic group

A12 – tuberal cell group

A13 – zona incerta cell group

A14 – rostral periventricular nucleus

A15d – anterior hypothalamic group, dorsal division

A15v – anterior hypothalamic group, ventral division

A16 – catecholaminergic neurons of the olfactory bulb

ac – anterior commissure

Amyg – amygdala

AON – anterior olfactory nucleus

AP – area postrema

B9 – supralemniscal serotonergic nucleus

C1 – rostral ventrolateral medullary tegmental group

C2 – rostral dorsomedial medullary nucleus

ca – cerebral aqueduct

Cb - cerebellum

cc – corpus callosum

Cl – claustrum

CLi – caudal linear nucleus

CO – cochlear nuclei

C/P – caudate/putamen

CVL – caudal ventrolateral serotonergic group

DCN – deep cerebellar nuclei

dfu – dorsal funiculus

Diag.B – diagonal band of Broca

DRc – dorsal raphe, caudal division

DRd – dorsal raphe, dorsal division

DRif – dorsal raphe, interfascicular division

DRl – dorsal raphe, lateral division

DRp – dorsal raphe, peripheral division

DRv – dorsal raphe, ventral division

DT – dorsal thalamus

EW – Edinger-Westphal nucleus

f – fornix

fr – fasciculus retroflexus

GicRt – gigantocellular reticular nucleus

GC – central gray matter

GP – globus pallidus

Hbl – lateral habenular nucleus

Hbm – medial habenular nucleus

HIP – hippocampus

Hyp - hypothalamus

Hyp.d – dorsal hypothalamic cholinergic nucleus

Hyp.l – lateral hypothalamic cholinergic nucleus

Hyp.v – ventral hypothalamic cholinergic nucleus

IC – inferior colliculus

ic – internal capsule

io – inferior olivary nuclei

IP – interpeduncular nucleus

Is.Call/TOL – island of Calleja/olfactory tubercle

LDT – laterodorsal tegmental nucleus

lfu – lateral funiculus

LGv – ventral lateral geniculate nucleus

lot – lateral olfactory tract

LV – lateral ventricle

MB – mammillary bodies

Mc – main cluster of orexinergic neurons

mcp – middle cerebellar peduncle

MnR – median raphe nucleus

N.Acc – nucleus accumbens

N.Amb – nucleus ambiguus

N.Bas – nucleus basalis

NEO – cerebral neocortex

OB – olfactory bulb

OC – optic chiasm

OT – optic tract

OTc – optic tract cluster of orexinergic neurons

pVII – preganglionic motor neurons of the superior salivatory nucleus or facial nerve

pIX – preganglionic motor neurons of the inferior salivatory nucleus

PBg – parabigeminal nucleus

PC – cerebral peduncle

PcRt – parvocellular reticular nucleus

PIR – piriform cortex

PPT – pedunculopontine tegmental nucleus

Pta – pretectal area

py – pyramidal tract

pyx – decussation of the pyramidal tract

R – thalamic reticular nucleus

Rmc – red nucleus, magnocellular division

RMg – raphe magnus nucleus

ROb – raphe obscurus nucleus

RPa – raphe pallidus nucleus

RVL – rostral ventrolateral serotonergic group

S – septal nuclear complex

SC – superior colliculus

scp – superior cerebellar peduncle

Sep.m – medial septal nucleus

Sp5 – spinal trigeminal tract

TOL – olfactory tubercle

vfu – ventral funiculus

vh – ventral horn

VPO – ventral pontine nucleus

zi – zona incerta

Zic – zona incerta cluster of orexinergic neurons

Results:

The current study describes and defines the nuclear organization of the cholinergic, catecholaminergic and serotonergic neural systems in five species of Insectivorous mammals (the reddish-grey musk shrew – *Crocidura cyanea*, the African giant shrew – *Crocidura olivieri*, the greater forest shrew – *Sylvisorex ollula*, the desert hedgehog – *Paraechinus aethiopicus* and the southern African hedgehog – *Atelerix frontalis*) and the orexinergic system in the brain of the African giant shrew, the greater forest shrew (Fig. 1), the desert hedgehog and the southern African hedgehog (Fig. 2), all species for which these systems have not been previously described. For the most part, the systems investigated exhibited a nuclear organization that may be thought of as typically mammalian; however, we observed many points of departure from this typical organization that has a strong bearing on the phylogenetic relationships of these species (results summarized in a comparative context in Table 1). The following description applies to all species studied, with species differences noted and highlighted where they occurred.

3.1. Cholinergic nuclei

The cholinergic system is generally subdivided into six main regions containing a cluster of distinct nuclei: cortical interneurons; striatal; basal forebrain; diencephalic; pontomesencephalic; and cranial motor nerve nuclei (Woolf, 1991). Choline acetyltransferase immunoreactive neurons (ChAT+) were identified in all these subdivisions, except the cortical interneurons, which were absent in all five species of insectivore studied. The nuclei forming the cholinergic system in these species were similar to that observed in previously studied mammals (Woolf, 1991; Manger et al., 2002a; Maseko et al., 2007; Dell et al., 2010; Kruger et al, 2010a), but there were a few minor differences. The neurons forming the parabigeminal nucleus (PBg) were either not ChAT immunoreactive or absent in all three shrew species, but were present in the two hedgehog species. The neurons forming the Edinger-Westphal nucleus (EW) were not ChAT immunoreactive, or absent, in *Crocidura*

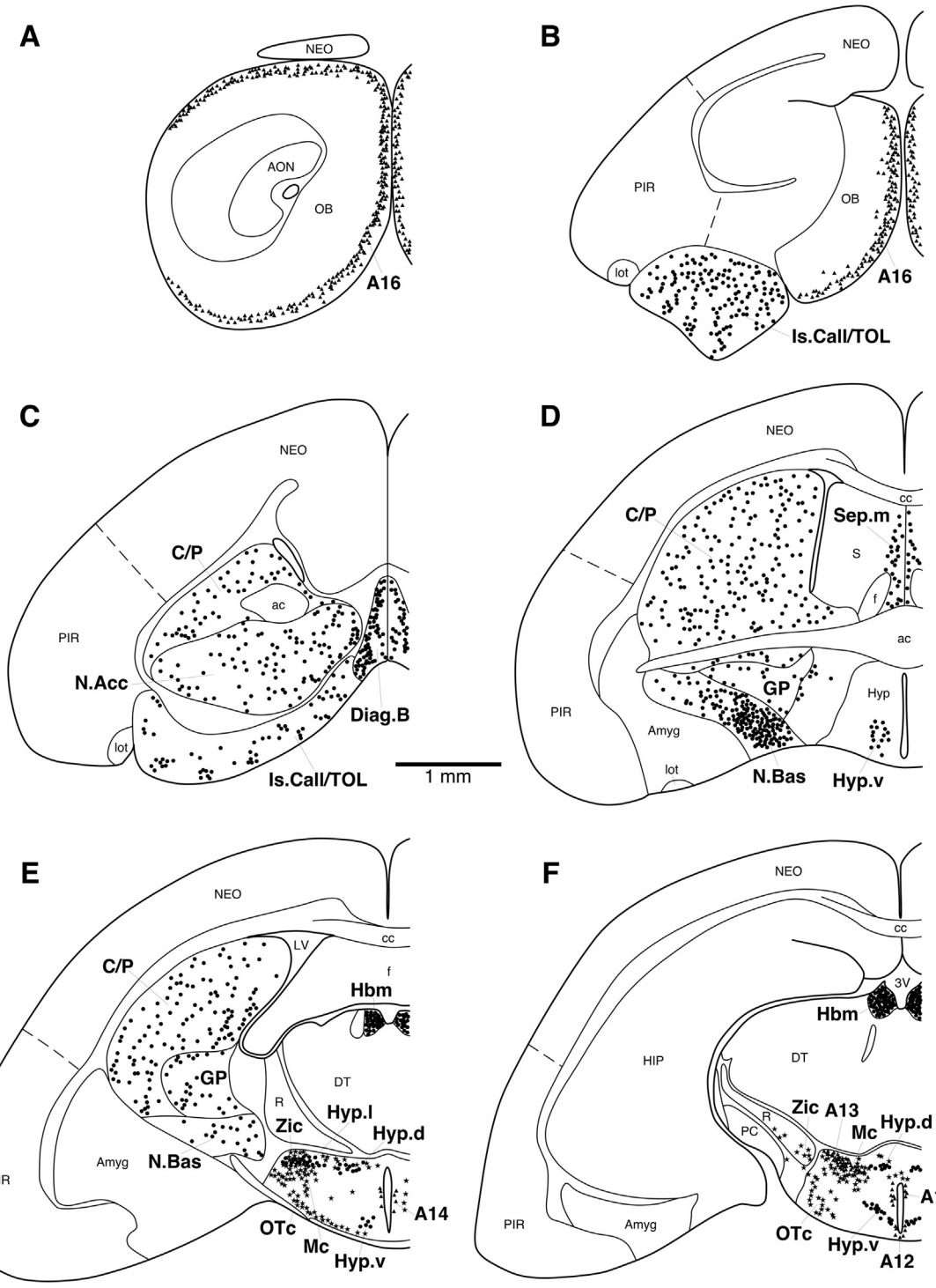

Fig. 1. Serial drawings of coronal sections through one half of the greater forest shrew (Sylvisorex ollula) brain from the olfactory bulb through to the spinomedullary junction. A is the most rostral section, P the most caudal. The outlines of the architectonic regions were drawn using Nissl stains and immunoreactive cells marked on the drawings. Solid black circles depict cholinergic neurons, solid triangles depict catecholaminergic neurons (those immunoreactive for tyrosine hydroxylase), open squares depict serotonergic neurons and closed stars represent orexinergic neurons. Each circle, triangle, square or star represents an individual neuron. The figures are approximately 500 mm apart. See list for abbreviations.

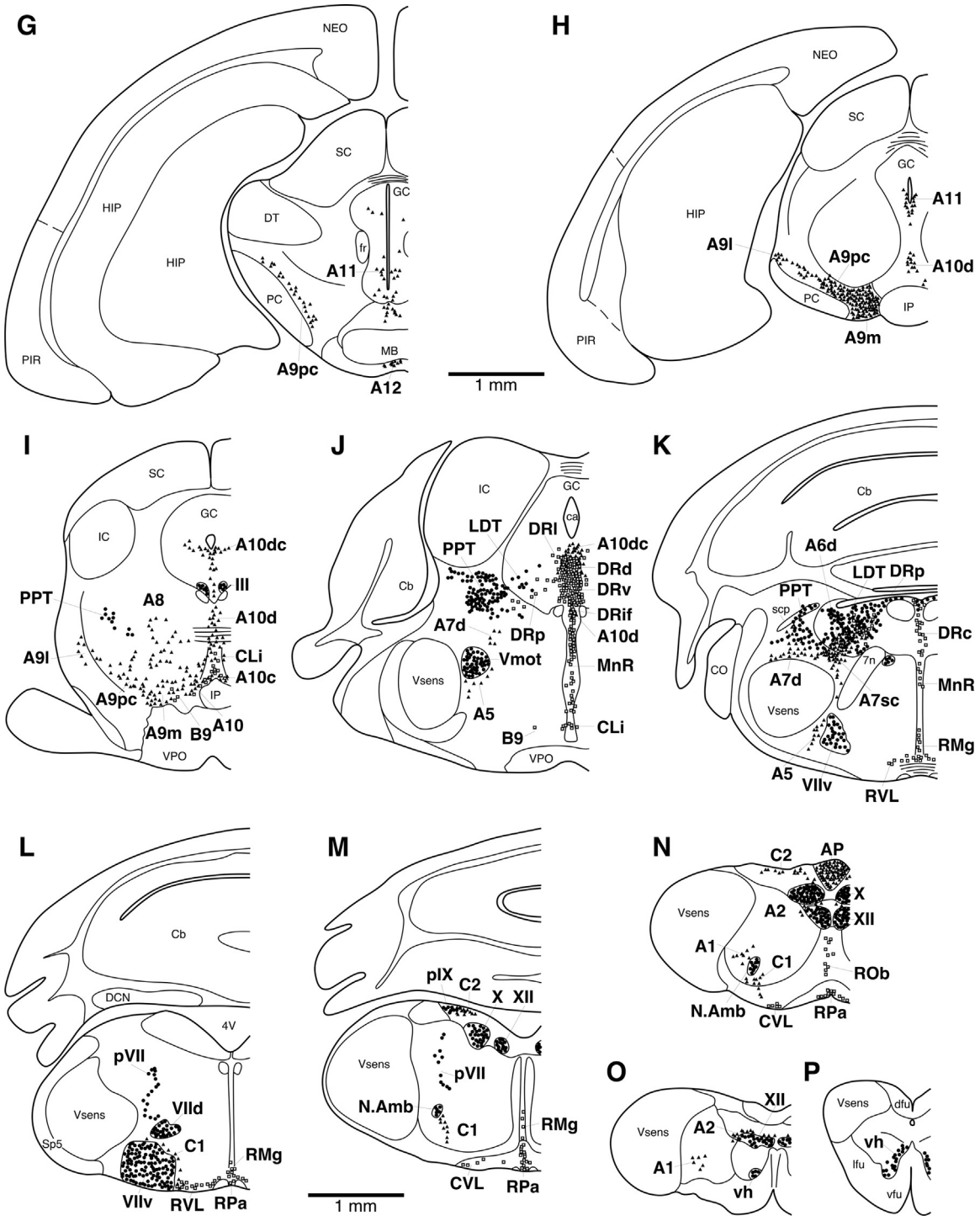

Fig. 1. (Continued)

Table 1: Summary of the nuclei delineated in the current study of insectivores in comparison to similar studies previously undertaken in Afroinsectiphilia (Calvey et al., 2013), Erinaceidae (Michaloudi and Papadopoulos,1995, 1996 and current study), Soricidae (current study), Microchirotpera (Maseko and Manger, 2007,b; Kruger et al., 2010a,b) and Megachiroptera (Maseko et al., 2007; Dell et al., 2010, 2013). Cells with a dark grey background indicate order distinguishing features. Cells with a light grey background indicate features that align Microchiroptera with shrews to the exception of megachiropterans and hedgehogs. See list for abbreviations.

	Afroinsectiphilia				**Erinaceidae**			**Soricidae**			**Microchiroptera**						**Megachiroptera**		
Species	*Amblysomus hottentotus*	*Potamogale velox*	*Petrodromus tetradactylus*	*Elephantulus myurus*	*Erinaceus europeaus*	*Paraechinus aethiopicus*	*Atelerix frontalis*	*Sylvisorex ollula*	*Crocidura olivieri*	*Crocidura cyanea*	*Miniopterus schreibersii*	*Chaerephon pumilis*	*Hipposideros commersoni*	*Cardioderma cor*	*Coleura afra*	*Triaenops persicus*	*Rousettus aegyptiacus*	*Eidolon helvum*	*Epomophorus wahlbergii*
Common name	Hottentot golden mole	Giant otter shrew	Four-toed sengi	Eastern rock elephant shrew	European hedgehog	Desert hedgehog	Southern African hedgehog	Greater forest shrew	African giant shrew	Reddish-grey musk shrew	Schreiber's long fingered bat	Little free-tailed bat	Commerson's leaf-nosed bat	Heart-nosed bat	African sheath-tailed bat	Persian trident bat	Egyptian Rousette	Straw coloured fruit bat	Wahlberg's epaletted fruit bat
Cholinergic																			
Cortical interneurons	+	-	-	-	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Olfactory bulb interneurons	+	-	-	-	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hippocampal interneurons	+	-	-	-	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amygdala interneurons	+	-	-	-	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Superior colliculus interneurons	-	-	+	+	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inferior colliculus interneurons	-	-	+	+	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cochlear nucleus interneurons	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Islands of Calleja	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Olfactory tubercle	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nucleus accumbens	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Caudate/Putamen	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Globus pallidus	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Medial septal nucleus	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Diagonal band of Broca	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nucleus basalis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dorsal hypothalamic	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ventral hypothalamic	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lateral hypothalamic	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Medial habenular	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Parabigeminal nucleus	+	+	+	+	?	+	+	-	-	-	-	-	-	+	+	-	+	+	+
Pedunculopontine nucleus	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Laterodorsal tegmental nucleus	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Edinger-Westphal nucleus	+/-	+	+	+	?	+	+	-	-	-	-	+	+	+	+	+	+	+	+
Oculomotor nucleus	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trochlear nucleus	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trigeminal motor nucleus	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Abducens nucleus	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Facial nucleus dorsal	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Facial nucleus ventral	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nucleus ambiguous	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Vagus motor nucleus	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hypoglossal nucleus	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ventral horn	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Superior salivatory nucleus	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inferior	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+

salivatory nucleus																			
Medullary tegmental field	-	-	-	-	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catecholaminergic																			
A1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C1	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C2	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Area postrema	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A4	+	+	+	+	?	+	+	-	-	-	-	-	-	-	-	-	+	+	+
A5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A6d diffuse	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A6c compact	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
A7sc compact	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A7d diffuse	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A9pc	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A9m	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A9v	+	+	+	+/-	+/-	+	+	+/-	+/-	+/-	-	+/-	+/-	+/-	+/-	+/-	+	+	+
A9l	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A10c	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A10dc	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A10d	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A15d	+	+	-	-	-	+	+	-	-	+	-	-	-	-	-	-	+	+	+
A15v	+	+	+	+	+	+	+	-	+	+	-	+	+	-	-	+	+	+	+
A16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Serotonergic																			
CLi	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MnR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DRl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DRv	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DRd	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DRif	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DRp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DRc	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RMg	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RPa	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RVL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CVL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ROb	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Orexinergic																			
Main cluster	?	+	+	+	?	+	+	+	+	?	?	+	+	+	+	+	+	+	+
Zona incerta cluster	?	+	+	+	?	+	+	+	+	?	?	+	+	+	+	+	+	+	+
Optic tract cluster	?	+	+	+	?	+	+	+	+	?	?	-	-	-	-	-	+	+	+

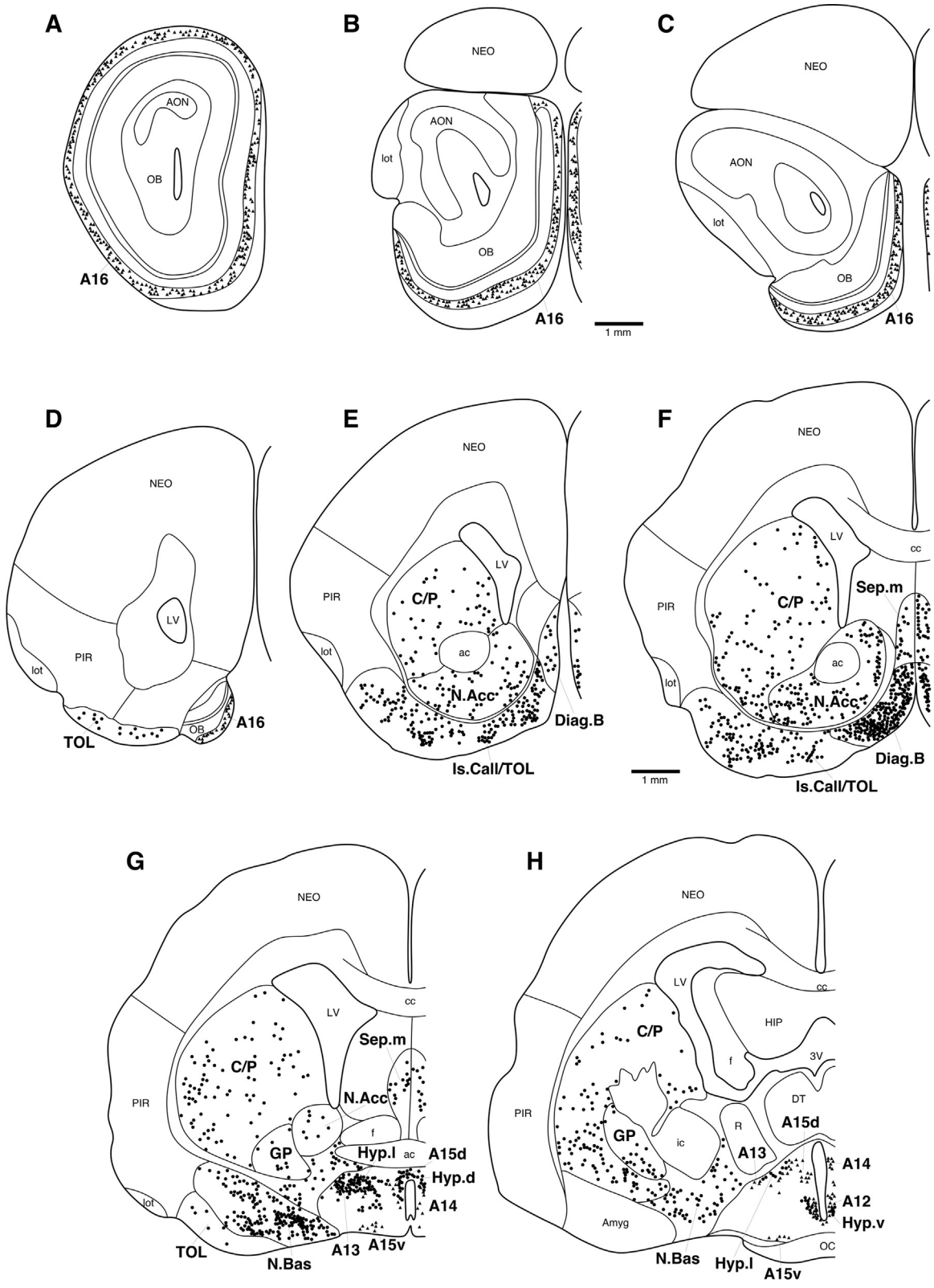

Fig. 2. Serial drawings of coronal sections through one half of the southern African hedgehog (Atelerix frontalis) brain from the olfactory bulb through to the spinomedullary junction. A is the most rostral section, U the most caudal. The outlines of the architectonic regions were drawn using Nissl stains and immunoreactive cells marked on the drawings. Solid black circles depict cholinergic neurons, solid triangles depict catecholaminergic neurons (those immunoreactive for tyrosine hydroxylase), open squares depict serotonergic neurons and closed stars represent orexinergic neurons. Each circle, triangle, square or star represents an individual neuron. The figures are approximately 1250 mm apart. See list for abbreviations.

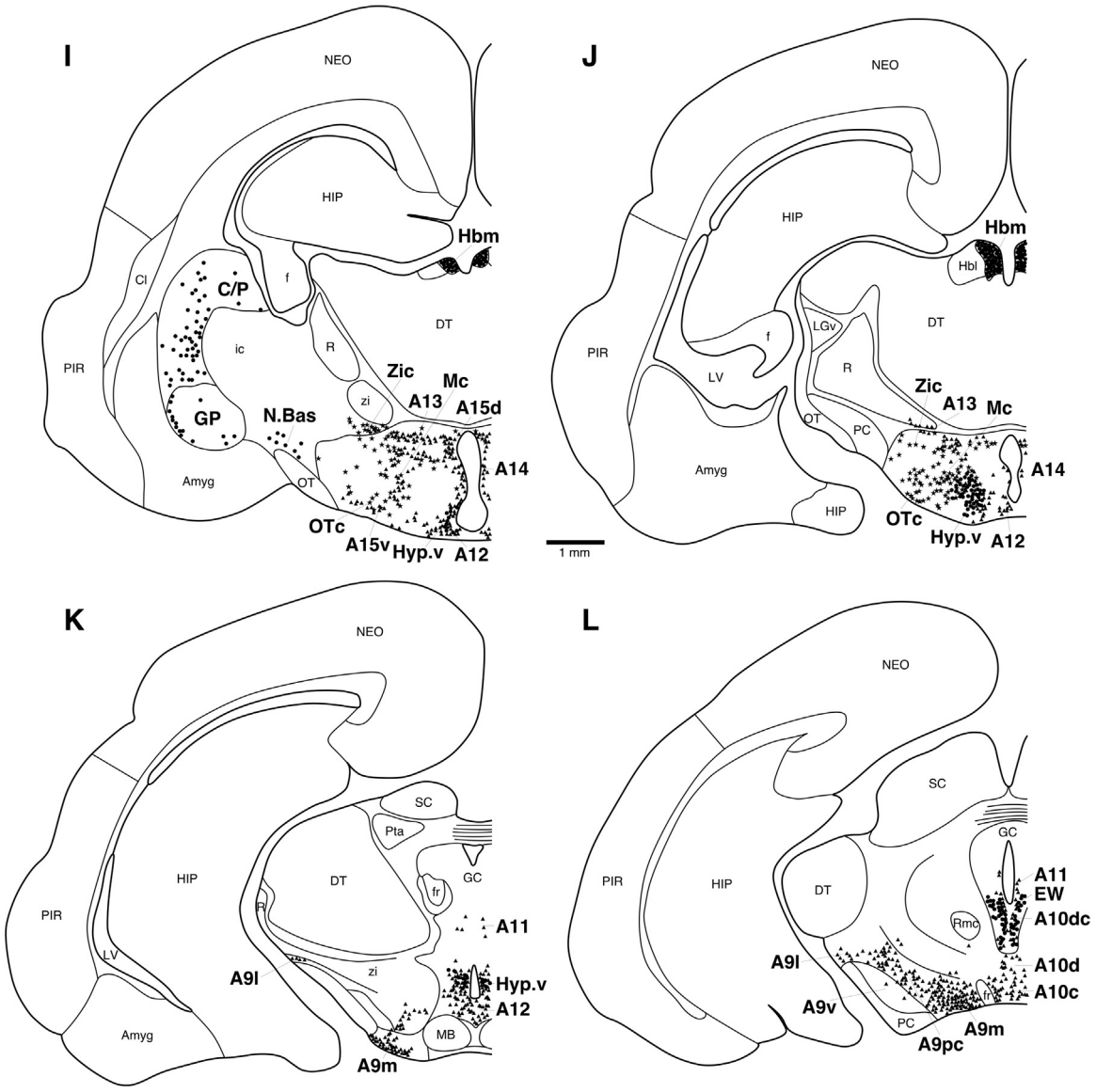

Fig. 2. (Continued)

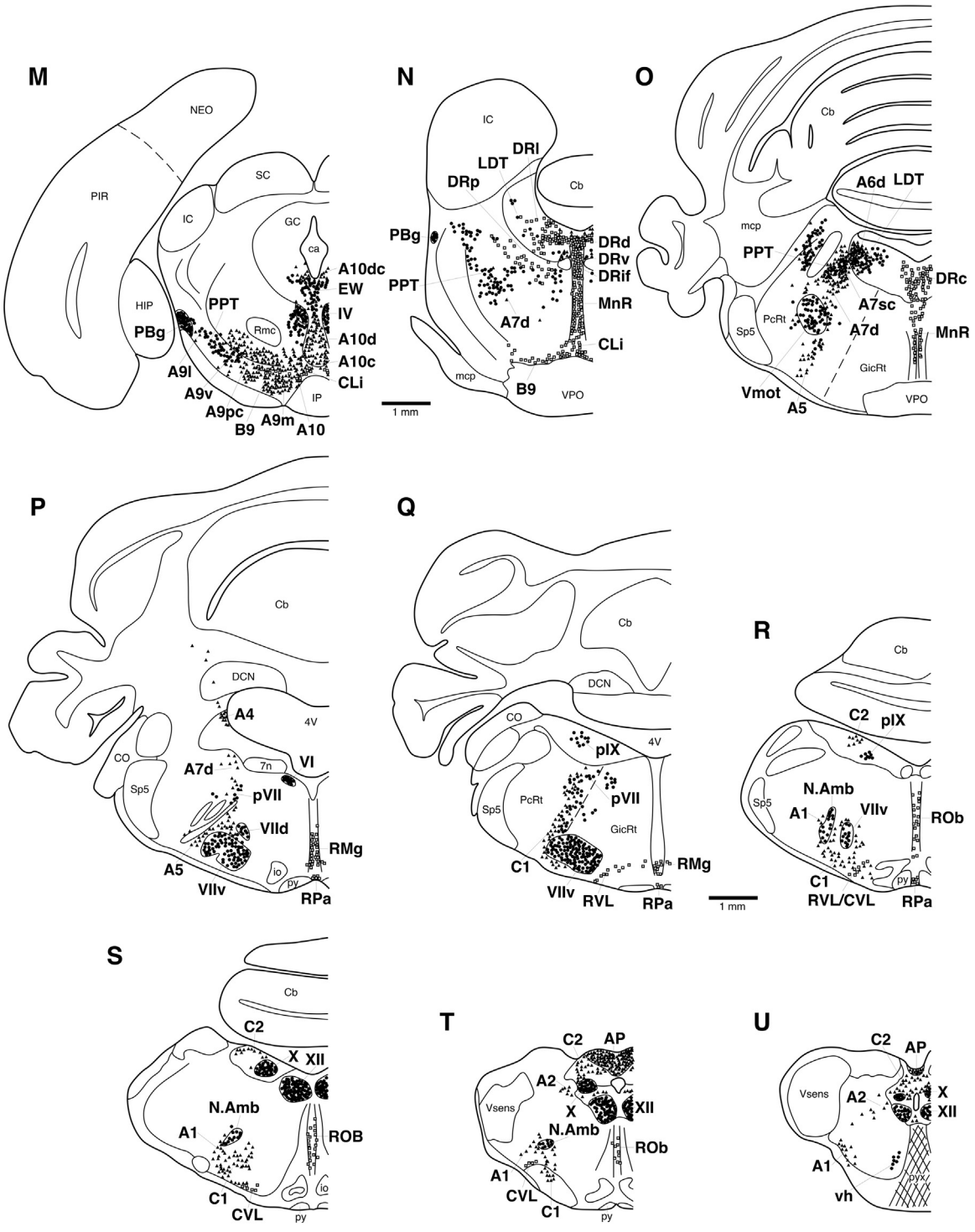

Fig. 2. (Continued)

olivieri and *Sylvisorex ollula* yet were present in the other shrew and both hedgehog species. The cholinergic neurons of the medullary tegmental field were absent in all five species.

3.1.1. Striatal cholinergic interneurons

ChAT+ neurons were found in the caudate/putamen complex, the globus pallidus, the nucleus accumbens, the Islands of Calleja and the olfactory tubercle in all five species (Figs. 1B – 1E, 2D – 2I). These nuclei occupied positions within the brain that could be considered typical of mammals. A low to moderate density of bipolar neurons were observed in the striatum with no clear internal capsule forming a distinction between the caudate and putamen nuclei. A low density of similar ChAT+ neurons were observed within the globus

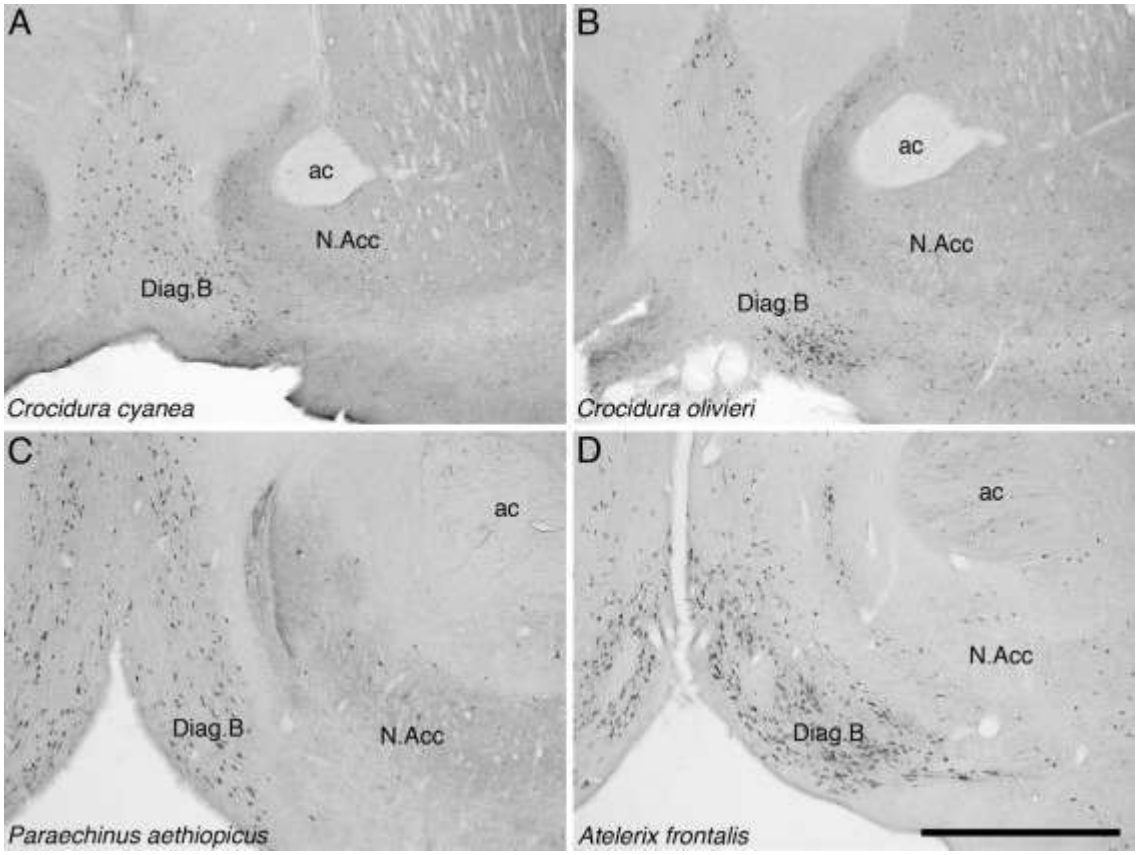

Figure 3: Photomicrographs showing neuronal groups immunoreactive for choline acetyltransferase in the diagonal band of Broca (**Diag.B**) and nucleus accumbens (**N.Acc**) in four of the species studied: (**A**) the reddish-grey musk shrew (*Crocidura cyanea*); (**B**) the African giant shrew (*Crocidura olivieri*); (**C**) the desert hedgehog (*Paraechinus aethiopicus*); and (**D**) the southern African hedgehog (*Atelerix frontalis*). Note the similarity in appearance of this region of the brain in all species. In the larger brain of the hedgehog, the numbers of neurons in the diagonal band appear to be greater. Scale bar in **D** = 1000 µm and applies to all. **ac** – anterior commissure. In all images, medial is to the left and dorsal to the top.

pallidus of all species, and while the majority of these neurons were found around the lateral edges of this nucleus, in the shrews they were spread throughout the nucleus more so than in the hedgehogs. A slightly higher density of bipolar ChAT+ neurons were observed in the nucleus accumbens, but in comparison to the hedgehogs, this nucleus was wider in the medio-lateral plane, but shorter in the rostrocaudal plane (Fig. 3). In all five species the ChAT+ neurons of the nucleus accumbens were sparser anteriorly, yet increased in density in the more caudal aspects of the nucleus. In all species, the ChAT+ neurons in the olfactory tubercle had regions of higher density that formed the islands of Calleja within this region of the brain. The hedgehogs generally had a higher density of ChAT+ neurons in this region of the brain than the shrews.

3.1.2. Cholinergic neurons of the basal forebrain

Cholinergic neurons were found in the diagonal band of Broca, the medial septal nucleus and the nucleus basalis in the basal forebrain of all five insectivore species (Figs. 1C – 1E, 2E – 2I). The dendrites of the bipolar neurons of the diagonal band of Broca ran parallel to the ventromedial border of the cerebral hemisphere in all species, although the number and density of neurons appeared to be more substantial in the hedgehogs than the shrews (Fig. 3). The medial septal nucleus, within the septal nuclear complex, had a far lower concentration of ChAT+ neurons than the diagonal band of Broca, but they did exhibit a similar morphology. The nucleus basalis in all species was large and exhibited many intensely ChAT+ bipolar neurons.

3.1.3 Diencephalic cholinergic nuclei

In all five species, ChAT+ neurons were found in the medial habenular nucleus, as well as the dorsal, lateral and ventral hypothalamic clusters (Figs. 1E – 1F, 2G – 2K). The medial habenular nucleus was located in the dorso-medial aspect of the diencephalon and the ChAT+ neurons within this nucleus were small with ovoid shaped soma and very densely packed. The three clusters of ChAT+ neurons within the hypothalamus all showed moderate to weak ChAT immunoreactivity, but were clearly observed in all species. The dorsal cluster was found in the dorso-medial aspect of the hypothalamus between the third ventricle and fornix, the lateral cluster was found in the dorsolateral aspect of the hypothalamus, lateral to

the fornix and extending to the lateral edge of the hypothalamus, while the ventral cluster was located in the ventral medial portion of the hypothalamus adjacent to the lateral ventricle.

3.1.4. Pontomesencephalic cholinergic nuclei

ChAT+ immunoreactive neurons delineated the pedunculopontine (PPT) and laterodorsal (LDT) nuclei in all five species investigated, but the neurons forming the parabigeminal nucleus (PBg) were ChAT immunoreactive only in the two hedgehog species and were either absent or not ChAT immunoreactive in all three shrew species (Figs. 1I – 1K,

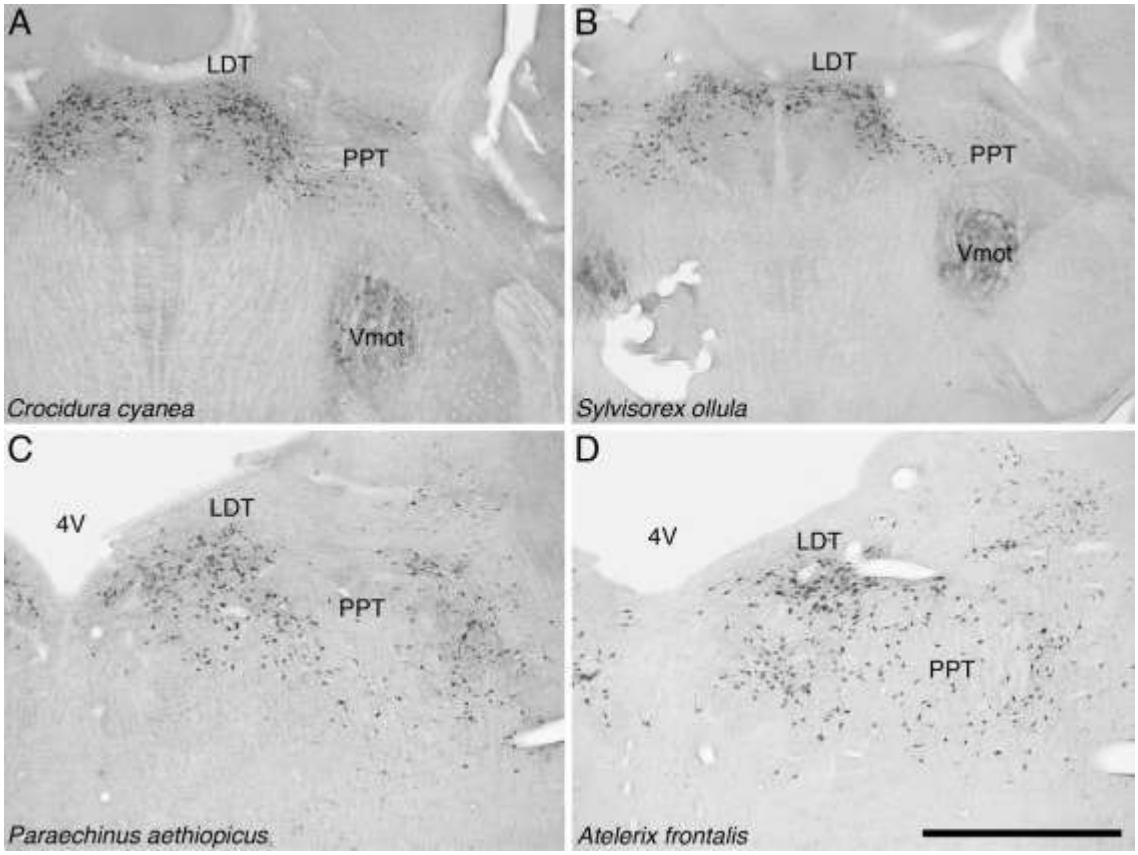

Figure 4: Photomicrographs showing neuronal groups immunoreactive for choline acetyltransferase in the pedunculopontine (**PPT**) and laterodorsal tegmental (**LDT**) nuclei in the pontine region in four of the species studied: (**A**) the reddish-grey musk shrew (*Crocidura cyanea*); (**B**) the greater forest shrew (*Sylvisorex ollula*); (**C**) the desert hedgehog (*Paraechinus aethiopicus*); and (**D**) the southern African hedgehog (*Atelerix frontalis*). Note the similarity in appearance of these nuclei in all species. Of specific interest is the medial extension of the LDT to the midline, a feature of this nucleus only previously observed in microchiropteran bats (Kruger et al., 2010a). Scale bar in **D** = 1000 µm and applies to all. **4V** – fourth ventricle, **Vmot** – motor division of the trigeminal nerve nuclei.

2M – 2O). In the two hedgehog species the parabigeminal nucleus was a small, but distinct nucleus located at the lateral margin of the pontine tegmentum dorsolateral to the lateral margin of the cerebral peduncle and ventral to the inferior colliculus. In all five species, the PPT nucleus exhibited a moderate to high density of multipolar ChAT+ neurons that were located throughout the dorsal aspect of the midbrain tegmentum surrounding the superior cerebellar peduncle and dorsal and anterior to the motor division of trigeminal nerve nucleus (Fig. 4). In all species, the ChAT+ neurons forming the LDT nucleus were found mostly in the ventrolateral periventricular gray matter, but were observed to extend through to the midline in all species (Fig. 4), a feature not typically observed in other mammals except for the microchiropterans (Kruger et al., 2010a). The neurons of the LDT had a similar appearance to those of the PPT, and the two nuclei appear to be continuous across the tegmental/gray matter border, being distinguished by location and the presence of the fifth mesencephalic tract.

3.1.5. Cholinergic cranial nerve motor nuclei

The following cranial nerve motor nuclei and other associated nuclei with ChAT immunopositive neurons were found in positions typical of all mammals in all five species (Woolf, 1991; Manger et al., 2002a; Maseko et al., 2007; Dell et al., 2010; Kruger et al, 2010a): the oculomotor nucleus (III), trochlear nucleus (IV), motor division of trigeminal nerve nucleus (Vmot) (Fig. 4A, 4B), adbucens nucleus (VI), dorsal and ventral subdivisions of the facial nerve nucleus (VIId and VIIv), nucleus ambiguus, dorsal motor vagus nucleus (X), hypoglossal nucleus (XII), the preganglionic motor neurons of the superior salivatory nucleus or facial nerve (pVII), the preganglionic motor neurons of the inferior salivatory nucleus (pIX) and the ventral horn of the spinal cord (vh) (Figs. 1I – 1P, 2L – 2U). These motor nuclei contained predominantly bipolar and multipolar ChAT+ neurons with no predominant dendritic direction. The Edinger-Westphal nucleus (EW) was present in both hedgehogs and *Crocidura cyanea*, yet was absent, or the neurons were not ChAT immunoreactive, in both *Sylvisorex ollula* and *Crocidura olivieri.* The cholinergic medullary tegmental field (mtf) was absent in all five species.

3.2. Catecholaminergic nuclei

Tyrosine hydroxylase (TH+) immunoreactivity revealed the putative catecholaminergic neurons in the brains of the five species examined. The nuclei formed by these neurons were arranged in a number of identifiable nuclear complexes that extended from the olfactory bulb through to the spinomedullary junction. These complexes correspond to that seen in other mammals (e.g., Smeets and Gonzalez, 2000; Manger et al., 2002b) and could be divided into the olfactory bulb, diencephalic, midbrain, pontine and medullary nuclear clusters. In the current description the nuclei are referred to using the nomenclature of Dahlström and Fuxe (1964) and Hökfelt et al. (1984), as no putatively catecholaminergic nuclei outside the classically defined nuclei (e.g., Smeet and Gonzalez, 2000) were observed. The TH+ nuclei found in the five species studied were similar to that seen in many other mammals, but there were some exceptions. The dorsal division of the anterior hypothalamic group (A15d) was missing in *Crocidura olivieri* and *Sylvisorex ollula*. The ventral division of the anterior hypothalamic group (A15v) was missing in *Sylvisorex ollula*, but was present in the other four species. The dorsal division of the locus coeruleus (A4) was absent in all three shrew species, but was present in both hedgehog species.

3.2.1. Olfactory bulb (A16)

The neurons forming the A16 nucleus were observed as dense clusters of TH+ cells in the glomerular layer of the olfactory bulb in all five species (Figs. 1A – 1B, 2A – 2D). These neurons likely represent the periglomerular dopaminergic neurons, were small in size, with a triangular shaped soma, and were found in equal density surrounding the ventral and lateral borders of the glomeruli throughout the olfactory bulb.

3.2.2. Diencephalic nuclei (A15-A11)

In the hypothalamus of many of the species studied, as in most mammals, six distinct nuclei formed of TH+ cells are normally found, these include: the dorsal division of the anterior hypothalamic group (A15d – except in *Crocidura olivieri* and *Sylvisorex ollula*, where this nucleus was absent), the ventral division of the anterior hypothalamic group (A15v – except in *Sylvisorex ollula*, where this nucleus was absent), the rostral periventricular cell

group (A14), the zona incerta (A13), the tuberal cell group (A12), and the caudal diencephalic group (A11) (Figs. 1E – 1H, 2G – 2L). In the species where it was present, the A15d nucleus was found in the dorsal anterior region of the hypothalamus, between the third ventricle and the fornix and contained a moderate density of TH+ neurons. In the species where it was present, the A15v nucleus, made up of a moderate density of TH+ neurons, was located in the ventrolateral portion of the anterior half of the hypothalamus. The ovoid shaped, bipolar TH+ neurons forming the A14 nucleus were found in a moderate to low density on either side of the third ventricle within the rostral two thirds of the hypothalamus. Within the dorsolateral aspect of the hypothalamus, lateral to the fornix and intermingling with the neurons forming the zona incerta of the ventral thalamus, was a moderate density of TH+ neurons forming to the A13 nucleus. A moderate to high density of TH+ neurons, forming the A12 nucleus, were located lateral and inferior to the third ventricle within the most ventral portion of the caudal third of the hypothalamus. Within the hypothalamic grey matter adjacent to the posterior pole of the third ventricle, a low number of TH+ neurons formed the A11 nucleus.

3.3.3. Midbrain nuclei (A10-A8)

Tyrosine hydroxylase immunoreactive neurons in the midbrain were found within the ventral tegmental area (the A10 complex, including the A10, A10c, A10d, A10dc nuclei), the substantia nigra (the A9 complex, including the A9pc, A9l, A9v, A9m nuclei) and the retrorubral nucleus (A8) within the midbrain tegmentum in all five species studied (Figs. 1G – 1J, 2K – 2M, 5). In all these nuclei, the TH+ neurons were either bipolar or multipolar showing no specific dendritic orientation, and exhibited a range of somal shapes (Fig. 5). A moderate to high density of TH+ neurons, found dorsal and dorsolateral to the interpeduncular nucleus, between this nucleus and the root of the oculomotor nerve, was assigned to the A10 nucleus. Immediately dorsal to the interpeduncular nucleus, in a location just anterior to the decussation of the superior cerebellar peduncle, a cluster of TH+ neurons formed the A10c nucleus. Dorsal to A10c, between it and the oculomotor nucleus, was a moderately dense bilateral parasagittal cluster of TH+ neurons that formed the A10d nucleus. The moderate to low density of TH+ neurons assigned to the A10dc nuclear complex were found within the periaqueductal grey matter surrounding the ventral aspect of the cerebral aqueduct.

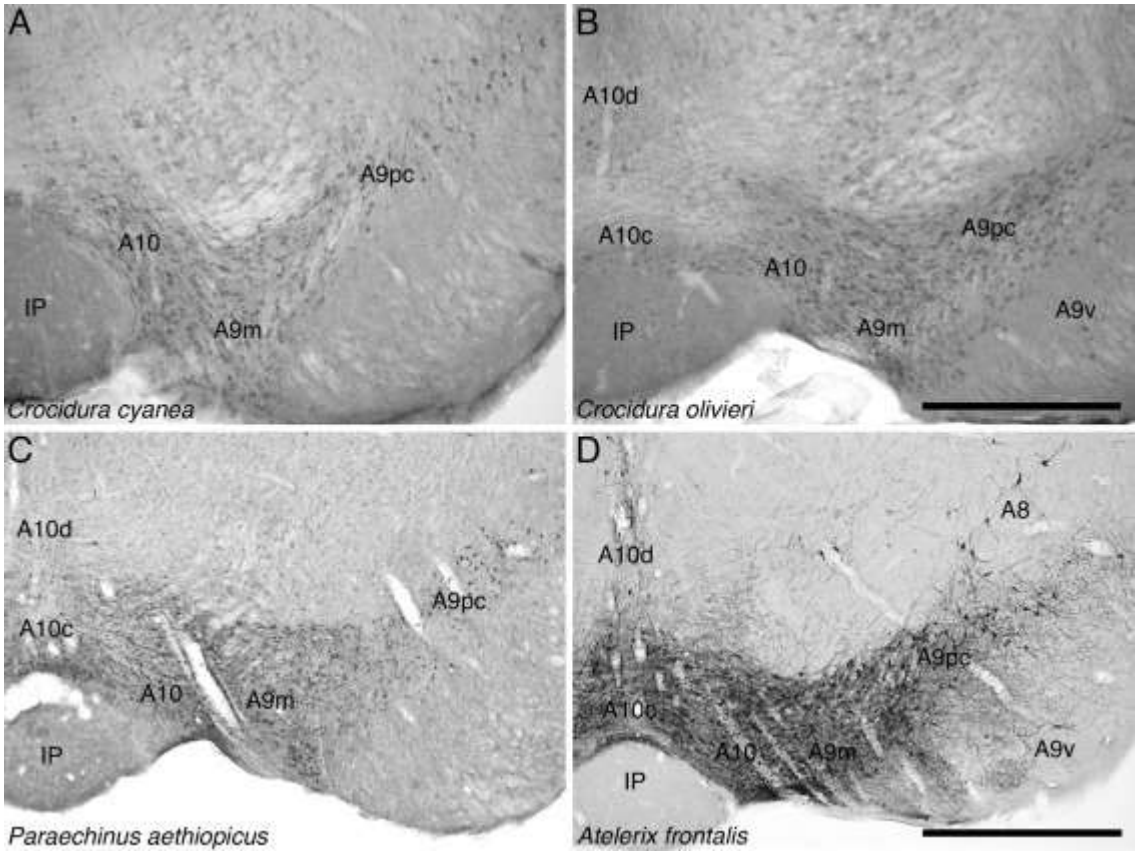

Figure 5: Photomicrographs showing the neuronal groups immunoreactive for tyrosine hydroxylase in the ventral tegemental and substantia nigra nuclear complex in four of the species studied: (**A**) the reddish-grey musk shrew (*Crocidura cyanea*); (**B**) the African giant shrew (*Crocidura olivieri*); (**C**) the desert hedgehog (*Paraechinus aethiopicus*); and (**D**) the southern African hedgehog (*Atelerix frontalis*). Note the similarity in the organization of these nuclear clusters across the species, and specifically note the lack of cells in the region of the substantia nigra ventral division (**A9v**). Scale bar in **B** = 500 µm and applies to **A** and **B**. Scale bar in **D** = 1000 µm and applies to **C** and **D**. **IP** – interpeduncular nucleus; for other abbreviations see list. In all images, medial is to the left and dorsal to the top.

The substantia nigra nuclear complex was observed in the ventral and lateral portions of the midbrain tegmentum, just dorsal to the cerebral peduncles (Fig. 5). The A9pc (pars compacta) was formed by a moderately dense band of TH+ neurons that ran from medial to lateral immediately ventral to the medial lemniscus. Throughout the grey matter (pars reticulata of the substantia nigra) ventral to A9pc, a moderate number of scattered TH+ neurons were assigned to the A9v (ventral) nucleus in both hedgehog species, but the number of these neurons in the three shrew species studied were very low. At the lateral edge of A9pc, a loose aggregation of TH+ neurons formed the A9l (lateral) nucleus. Medial to A9pc and lateral to the root of the oculomotor nerve (IIIn), a dense cluster of TH+ neurons formed the A9m (medial) nucleus. Scattered throughout the midbrain tegmentum, in a position

caudal to the magnocellular division of the red nucleus and dorsal to the A9 complex, a sparsely packed cluster of TH+ neurons formed the A8 nucleus (Fig. 5D).

3.2.4. Rostral rhombencephalon – the locus coeruleus complex (A7-A4)

Within the pontine region of all five species studied a large number of TH+ neurons forming the locus coeruleus complex were readily observed. These could be subdivided into the following five distinct nuclei: the subcoeruleus compact portion (A7sc), the subcoeruleus diffuse portion (A7d), the locus coeruleus diffuse portion (A6c), the fifth arcuate nucleus (A5) and the dorsolateral division of the locus coeruleus (A4 – although this nucleus was absent in all three shrew species) (Figs. 1J – 1K, 2N – 2P, 6). Within the dorsal portion of the pontine tegmentum adjacent to the ventrolateral border of the periventricular grey matter, a tightly packed cluster of TH+ neurons represented the A7 compact portion of the locus subcoeruleus (Fig. 6A, 6B). This division is the same as what was previously described as the subcoeruleus (Dahlström and Fuxe, 1964; Olson and Fuxe, 1972). Ventral and lateral to the A7sc, a diffusely organised aggregation of TH+ neurons formed the A7d nuclear complex (Fig. 6C, 6D). These neurons are located both medially and laterally around the trigeminal motor nucleus (Vmot) and the superior cerebellar peduncle. Within the lateral portion of the periventricular grey matter a loosely packed, moderate density of a moderate number of TH+ neurons were assigned to the A6d nucleus (Fig. 6). No compact division of the locus coeruleus (the A6c division as observed in Murid rodents, e.g., Kruger et al., 2012) was observed in any of the species examined. In the ventrolateral pontine tegmentum lateral to the superior olivary nucleus and ventrolateral to Vmot and A7d, a small cluster of TH+ neurons formed the A5 nucleus. These neurons formed a rough mesh-like dendritic network around the ascending fascicles located within the ventrolateral pontine tegmentum. Immediately adjacent to the wall of the fourth ventricle, in the dorsolateral portion of the periaqueductal grey matter, a dense, but small cluster of TH+ neurons represented the A4 nucleus in the two hedgehog species studied. In *A. frontalis*, a small number of TH+ cells appeared to extend dorsally from the A4 nucleus, investing into the white matter of the cerebellum.

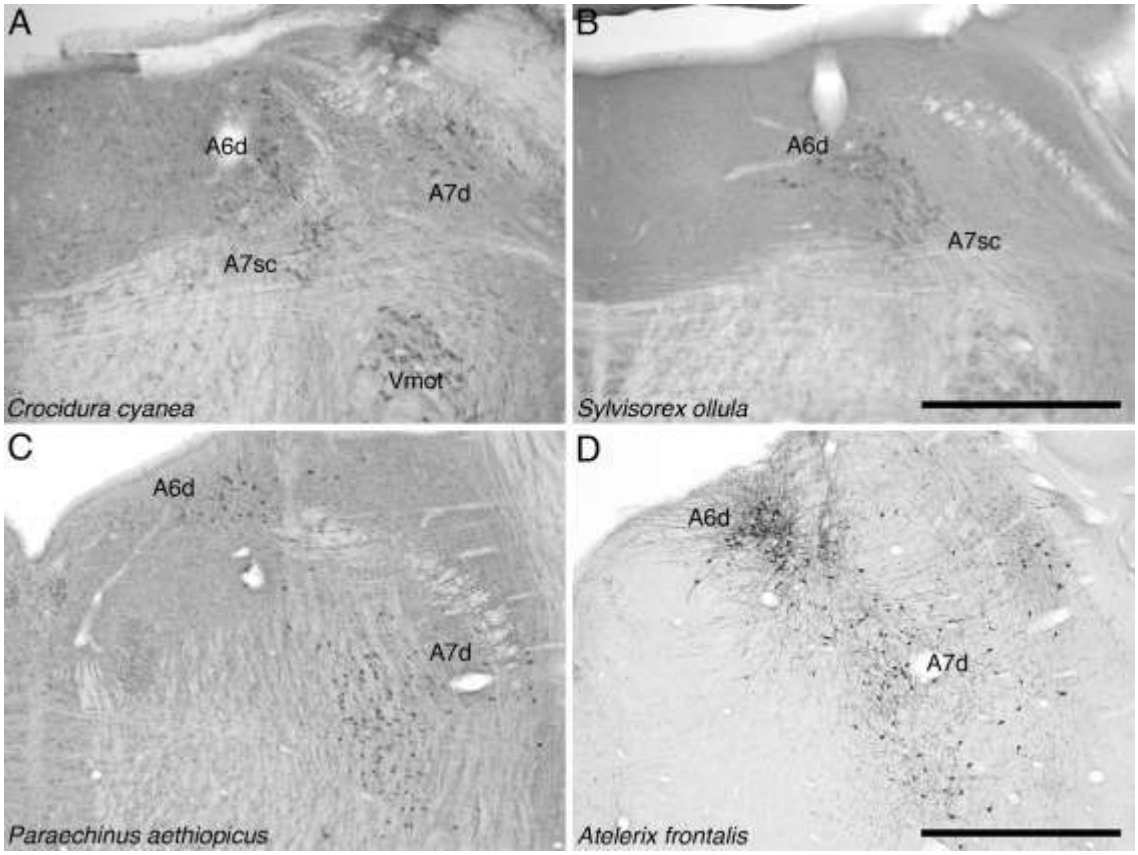

Figure 6: Photomicrographs showing the neuronal groups immunoreactive for tyrosine hydroxylase in the locus coeruleus nuclear complex in four of the species studied: (**A**) the reddish-grey musk shrew (*Crocidura cyanea*); (**B**) the greater forest shrew (*Sylvisorex ollula*); (**C**) the desert hedgehog (*Paraechinus aethiopicus*); and (**D**) the southern African hedgehog (*Atelerix frontalis*). Note the similarity in the organization of the nuclei within this complex across the species, and specifically note the diffusely packed cells of the locus coeruleus (**A6d**). Scale bar in **B** = 500 µm and applies to **A** and **B**. Scale bar in **D** = 1000 µm and applies to **C** and **D**. **Vmot** – motor division of the trigeminal nerve nuclei; for other abbreviations see list. In all images, medial is to the left and dorsal to the top

3.2.5. Medullary nuclei (C1, C2, A1, A2, area postema)

In the medulla oblongata of all five species, five catecholaminergic nuclei were observed: the rostral ventrolateral tegmental group (C1), the rostral dorsomedial group (C2), the caudal ventrolateral tegmental group (A1), the caudal dorsomedial group (A2), and the area postrema (AP) (Figs. 1L – 1O, 2Q – 2U). A low density of TH+ neurons found in the ventrolateral medulla from the level of the facial nerve nucleus to the mid-level of nucleus ambiguus were classified as the C1 nucleus. Continuing in the ventrolateral medulla, a column of TH+ neurons located laterally to the posterior most part of the C1 nucleus and

extending to the spinomedullary junction was designated as the A1 nucleus. The A1 column was distinguished from the C1 column by occupying a position lateral to the nucleus ambiguus, whereas the C1 nucleus was located medial to nucleus ambiguus. In the dorsal part of the medulla, in the region of the anterior part of the dorsal and medial border of the nucleus tractus solitarius, a distinct, but not particularly dense cluster of TH+ neurons was designated as the C2 nucleus. Within this nucleus there was a clear region close to the floor of the fourth ventricle termed the dorsal strip and a continuation of this cluster into the region of the tractus solitarius termed the rostral subdivision of the C2 nucleus. Between the caudal portions of the dorsal motor vagus and hypoglossal cranial nerve nuclei, a small number of TH+ neurons represented the A2 nucleus. Some of these A2 neurons were located a small distance into the dorsal caudal medullary tegmentum. Straddling the midline, dorsal to the central canal and the dorsal motor vagus nucleus, and between the most caudal aspect of the bilateral C2 nucleus, was a single large, densely packed, cluster of intensely stained TH+ neurons, the area postrema. The rodent specific rostral dorsal midline medullary nucleus (C3) was absent in all five species.

3.3. Serotonergic nuclei

The serotonergic nuclei (5HT+) identified in the brains of all three species of this study were found to be the same as other eutherian mammals studied to date (Steinbusch, 1981; Maseko et al., 2007; Dell et al., 2010; Kruger et al., 2012). These nuclei were all located within the brainstem and can be divided into a rostral and caudal cluster. Both of these clusters contained distinct nuclei found throughout the brainstem from the level of the decussation of the superior cerebellar peduncle through to the spinomedullary junction. All five species examined exhibited the same complement of serotonergic nuclei in both the rostral and caudal clusters.

3.3.1. Rostral serotonergic cluster

Within the rostral cluster we found evidence for the caudal linear nucleus (CLi), the supralemniscal serotonergic nucleus (B9), the median raphe nucleus (MnR) and the dorsal raphe complex formed of six distinct nuclei (see below) (Figs. 1I – 1K, 2M – 2O, 7). A moderate density of 5HT+ bipolar ovoid neurons on the ventral midline dorsal to the

interpeduncular nucleus represented the CLi nucleus. The dendrites of these neurons were oriented parallel to each other in a mediolateral direction. The B9 nucleus appears to be a lateral extension of the CLi nucleus into the ventrolateral midbrain tegmentum and the 5HT+ neurons have similar morphology. The few neurons that formed this nucleus were found lateral to the interpeduncular nucleus rostrally and dorsal to the ventral pontine nucleus caudally. The median raphe nucleus (MnR) was characterised by two distinct, densely packed 5HT+ neuronal columns on either side of the midline and was found from the caudal most part of the decussation of the superior cerebellar peduncle through to the trigeminal motor nucleus (Fig. 7). These neurons had round soma and the dendrites showed no specific orientation.

Within the dorsal raphe nuclear complex we identified six distinct nuclei in all five species: the dorsal raphe interfascicular nucleus (DRif), the dorsal raphe ventral nucleus (DRv), the dorsal raphe dorsal nucleus (DRd), the dorsal raphe lateral nucleus (DRl), the dorsal raphe peripheral nucleus (DRp) and the dorsal raphe caudal nucleus (DRc) (Fig. 7). These six nuclei were found, for the most part, within the periaqueductal and periventricular grey matter from the level of the oculomotor nucleus to the trigeminal motor nucleus. Two pararaphe columns of 5HT+ cells located between the bilaterally paired medial longitudinal fasciculi represent the DRif nucleus in all five species. The DRv was found immediately dorsal to the DRif and just caudal to the oculomotor nuclei. The DRv exhibited a high density of 5HT+ neurons which were ovoid in shape. Immediately dorsal to DRv and ventral to the inferior border of the cerebral aqueduct a high-density cluster of 5HT+ neurons was designated as the DRd nucleus. The morphology of these neurons was similar to the morphology of the DRv neurons. A moderate density of 5HT+ neurons representing the DRp, were located in the ventrolateral portion of the periaqueductal grey matter lateral to the DRd and DRv. Some neurons of the DRp were found in the adjacent midbrain tegmentum and were the only serotonergic immunopositive neurons of the dorsal raphe complex found outside the periaqueductal grey matter. The DRp nucleus was extensive in *Crocidura cyanea*, but was smaller in all other species studied. The 5HT+ neurons of the DRl were located dorsolateral to the DRd and adjacent to the ventrolateral edges of the cerebral aqueduct in a low to moderate density. The neurons of this nucleus were readily distinguishable from the remainder of the dorsal raphe nuclei since they were substantially larger and multipolar. As we followed the DRl caudally, where the cerebral aqueduct opened into the fourth ventricle and the DRd, DRv and DRif disappeared, the neurons of the DRl formed an arc across the

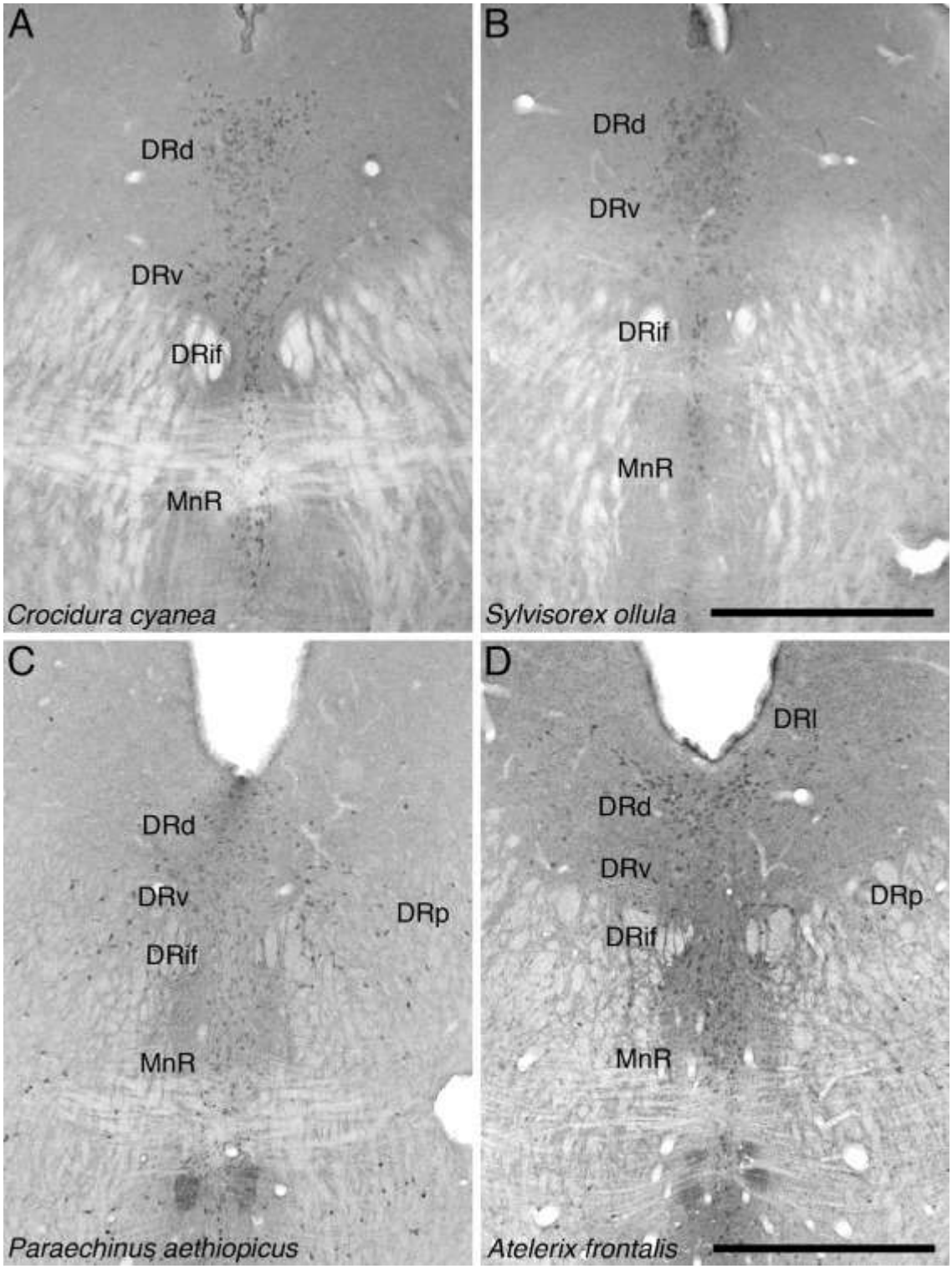

Figure 7: Photomicrographs showing the neuronal groups immunoreactive for serotonin in the dorsal raphe nuclear complex in four of the species studied: (**A**) the reddish-grey musk shrew (*Crocidura cyanea*); (**B**) the greater forest shrew (*Sylvisorex ollula*); (**C**) the desert hedgehog (*Paraechinus aethiopicus*); and (**D**) the southern African hedgehog (*Atelerix frontalis*). Note the similarity in the organization of the nuclei within this complex across the species. Scale bar in **B** = 500 µm and applies to **A** and **B**. Scale bar in **D** = 1000 µm and applies to **C** and **D**. See list for abbreviations.

midline of the dorsal portion of the periventricular grey matter. This caudal arc of the DRl was classified as the DRc nucleus. We classified this as an independent nucleus due to the lack of 5HT+ neurons in this region in the brain of monotremes (Manger et al., 2002c).

3.3.2. Caudal cluster

Within the caudal serotonergic cluster we found evidence for the raphe magnus nucleus (RMg), rostral and caudal ventrolateral serotonergic groups (RVL and CVL), the raphe pallidus nucleus (RPa) and the raphe obscurus nucleus (ROb) nuclei in all five species (Figs. 1K – 1N, 2P – 2T). The RMg was observed to be two columns of loosely aggregated moderate to large 5HT+ neurons of moderate density located on either side of the midline of the rostral medulla from the level of the caudal pole of the trigeminal motor nucleus to the caudal pole of the facial nerve nucleus. These neurons were ovoid in shape and bipolar, with dendrites oriented parallel to the midline. Within the left and right ventrolateral medullary tegmentum a distinct anteroposterior column of 5HT+ neurons of moderate density extending from the level of the facial nucleus to the spinomedullary junction were observed. These have previously been termed the rostral and caudal ventrolateral serotonergic columns (e.g., Maseko et al., 2007; Moon et al., 2007; Dwarika et al., 2008; Calvey et al., 2013). The RVL began as a lateroventral continuation of 5HT+ neurons from the lower portion of the RMg extending over the pyramidal tracts and trapezoid body, and consolidating as a distinct column lateral to the inferior olivary nuclear complex. At the approximate level of nucleus ambiguus the RVL becomes the CVL. The CVL continues in the caudal ventrolateral medullary tegmentum until the spinomedullary junction, marked by the decussation of the pyramidal tract, is reached. The 5HT+ neurons forming the RPa nucleus were found in the ventral midline of the rostral medulla oblongata. These neurons were for the most part located between the two pyramidal tracts, were ovoid in shape and bipolar with dorsoventrally oriented dendrites. Two loosely arranged bilateral columns of 5HT+ neurons located dorsal to the RPa on either side of the midline from the level of nucleus ambiguus to the spinomedullary junction were classified as the ROb. The dendrites of these neurons were oriented parallel to the midline.

3.4. Orexinergic (hypocretinergic) nuclei

Orexin-A immunohistochemistry was used to identify orexinergic neurons (OxA+) within the hypothalamus of all species studied except *Crocidura cyanea*. The vast majority of OxA+ neurons identified in the brains of the four species (two shrews and two hedgehogs) were localised within the hypothalamus. Within the hypothalamic region where the OxA+ neurons were located we could divide them into three distinct clusters: a main cluster (Mc), a zona incerta cluster (Zic) and an optic tract cluster (Otc) (Figs. 1E – 1F, 2I – 2J, 8). The orexinergic cells of the two hedgehog species were greater in number and density when compared to the two shrew species, making the three orexinergic clusters more distinct in the shrews when compared to the hedgehogs (Fig. 8). The main cluster (Mc) was identified as a large group of densely packed OxA+ neurons located in the perifornical region, with additional OxA+ neurons extending medially from this location into the medial hypothalamus and a larger number extending into the lateral hypothalamic areas. The difference between the shrews and the hedgehogs was the extent to which the cells extended medially towards the third ventricle, with the shrews having significantly less medially placed cells. From the main cluster, a group of OxA+ neurons, the zona incerta cluster (Zic) were observed to extend laterally into the dorsolateral region of the hypothalamus, with some neurons being found lateral to the hypothalamus and within or around the zona incerta. The Zic exhibited a moderate density of OxA+ neurons that were co-localized with the A13 catecholaminergic neurons (see above) in all four species studied. The optic tract cluster (Otc) was found ventral to the main cluster, in the ventral lateral hypothalamus where it borders with the optic tract. This cluster exhibited a low to moderate density of OxA+ neurons. In the four species studied, the orexinergic neurons were typically bipolar in nature and exhibited no clear dendritic orientation, except for those in the Zic where the dendrites were observed to run parallel to the superior border of the hypothalamus or the inferior border of the zona incerta.

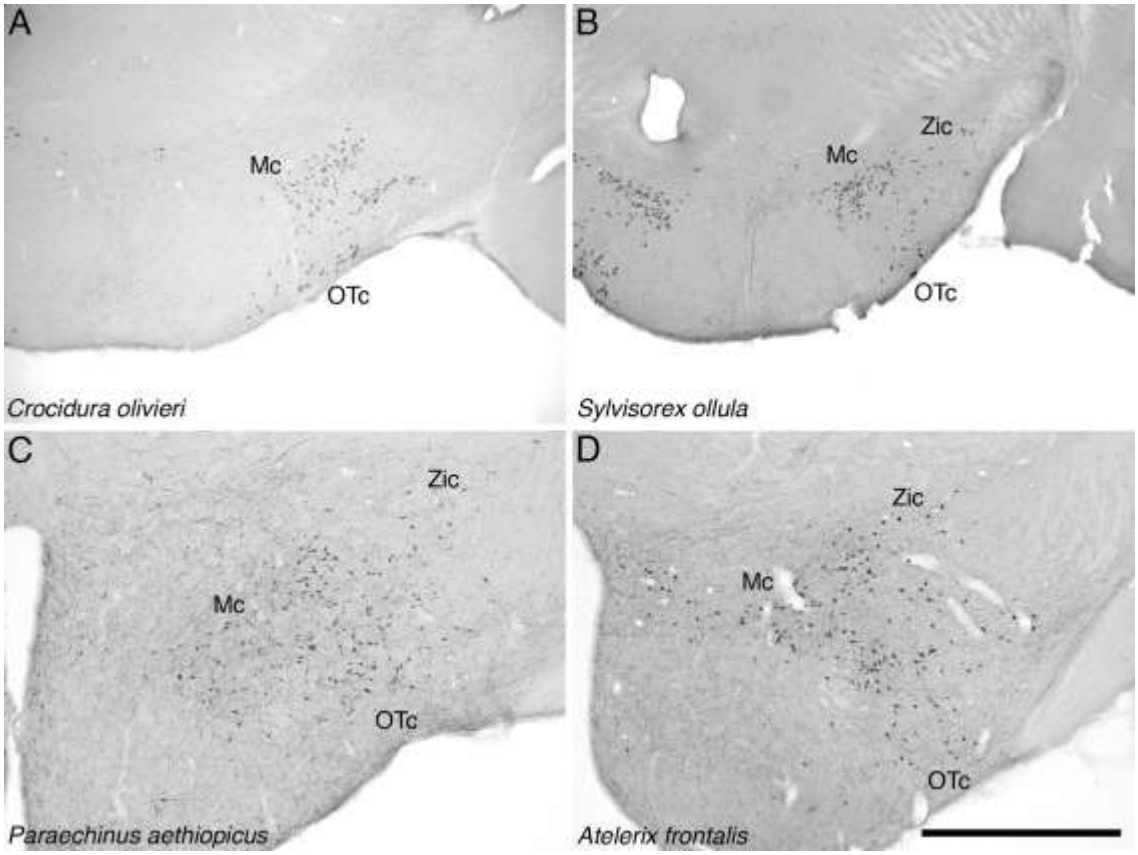

Figure 8: Photomicrographs showing the neuronal clusters immunoreactive for orexin-A in the hypothalamus in four of the species studied: (**A**) the African giant shrew (*Crocidura olivieri*); (**B**) the greater forest shrew (*Sylvisorex ollula*); (**C**) the desert hedgehog (*Paraechinus aethiopicus*); and (**D**) the southern African hedgehog (*Atelerix frontalis*). Note the similarity in the organization of the cluster within the hypothalamus across the species. Scale bar in **D** = 1000 µm and applies to all. See list for abbreviations. In all images, medial is to the left and dorsal to the top.

4. Discussion

The nuclear organization and complement of four immunohistochemically identifiable neural systems within the brains of five previously unstudied species of Insectivora (the greater forest shrew *Sylvisorex ollula*, the reddish-grey musk shrew *Crocidura cyanea*, the African giant shrew, *Crocidura olivieri*, the Southern African hedgehog, *Atelerix frontalis* and the desert hedgehog, *Paraechinus aethiopicus*) were presented in this study. For the most part, the organization and complement of nuclei of these systems were similar to that observed in many other typical Eutherian mammals (e.g., Maseko et al., 2007; Dell et al., 2010). Despite this there were specific differences of note (summarized in Table 1) that may be related either to the phylogenetic history or current life histories of these species. Within

the cholinergic system, the parabigeminal nucleus (PBg) and the Edinger-Westphal nucleus (EW) were absent in all three shrew (Soricidae) species. In the catecholaminergic system, the dorsolateral division of the locus coeruleus (A4) was absent in all three shrew species, the dorsal division of the anterior hypothalamic group (A15d) was absent in two of the three shrew species and the ventral pars reticulata of the substantia nigra (A9v) was poorly expressed in all insectivore species. These variations in the number and complement of nuclei in the systems examined support the possibility that the microchiropterans may have a close phylogenetic affinity to the Soricidae and other insectivores, rather than the artiodactyls and perissodactyls as commonly depicted (e.g. Meredith et al., 2011).

The absence of specific nuclei can be explained in two possible ways, which are of importance to understand in terms of discussing the results and specifically potential phylogenetic relationships based on these results. First, the neurons forming these nuclei may be completely absent from the species investigated. Second, the neurons forming these nuclei are present, but are not immunoreactive for the antibodies used (e.g. Bhagwandin et al., 2006). Either of these possibilities may explain the absence of specific nuclei being identified using the immunohistochemical methods employed in the various species studied; however, either explanation is also relevant in terms of phylogenetic relationships. The simple presence/absence of nuclei is a concrete observation that can be readily interpreted without serious doubts (given the accuracy of the observer), but if the variation is a result of the antibodies used, the results may be questionable. For example, Bhagwandin et al. (2006) found that by using different antibodies to cholineacetyltransferase, cholinergic interneurons in the cerebral cortex of rodents this could be found in a broader range of species. Interestingly, the cortical cholinergic interneurons were limited to the Murid rodents and not observed in other rodent species (Bhagwandin et al., 2006; Kruger et al., 2012). This indicates that the structure of the binding site attached to by the antibody used might differ across species, and thus false negatives of the absence of neurons/nuclei might be reported. Despite this, the fact that there is a reflection of the phylogenetic history in the studies of rodent cortical cholinergic neurons (Bhagwandin et al., 2006; Kruger et al., 2012), indicates that the lack of immunostaining of specific neurons, even if they are present, are useful indicators of phylogenetic relationships. Moreover, the presence of nuclei in similar regions of the brain being revealed by the antibodies also strengthens the interpretation of absence. Thus, below we discuss the presence or absence of nuclei with these caveats in mind, but use the terms presence and absence for ease of reading. In some cases, the nuclei are most likely

to be absent, but in other cases the apparent absence may be the result of the antibody used. In order to overcome this problem, the same antibodies were used universally across all species studied.

4.1. Cholinergic nuclei

Overall, the nuclear organization and complement of the cholinergic system in the five insectivorous species studied was similar to all other mammals studied to date (Dell et al., 2010; Calvey et al., 2013). Two notable differences were the absence of the parabigeminal and Edinger-Westphal nuclei in the three shrew (Soricidae) species studied, although these nuclei were both present in the two hedgehog species studied. In four out of the six microchiropterans (Maseko and Manger, 2007; Kruger et al., 2010a), the parabigeminal nucleus is also absent and the Edinger-Westphal nucleus was absent in one microchiropteran (Maseko and Manger, 2007). In all other mammals, the parabigeminal and Edinger-Westphal nuclei are present (Dell et al., 2010; Calvey et al. 2013), even in the microphthalmic mole rats and golden mole (Da Silva et al., 2006; Bhagwandin et al., 2008; Calvey et al., 2013). The laterodorsal tegmental nucleus, while present in all mammals studied to date (Dell et al., 2010), has shown some variance across species (e.g. Gravett et al., 2009) and evinces a very specific appearance in the microchirotperans, where the cholinergic neurons form a dorsomedial arch across the periventricular gray matter to almost reach the midline (Kruger et al., 2010a). In other mammals, this medial expansion of the laterodorsal tegmental nucleus has not been noted (e.g. Calvey et al., 2013). In the current study, all shrews and both hedgehogs displayed this dorsal arch of the laterodorsal tegemental nucleus. Thus, in terms of the cholinergic system, the microchiropterans show some specific similarities to the hedgehogs and shrews, but more so with the shrews.

One of the many similarities between shrews and microchiroptera is the reduced visual system. Both parabigeminal and Edinger-Westphal nuclei are involved in the processing of visual information (Woolf, 1991). For example, the parabigeminal neurons project to the superficial layers of the superior colliculus and to the lateral geniculate nucleus, (Barker et al., 2012). The neurons within the Edinger-Westphal nucleus are parasympathetic pre-ganglionic neurons whose axons pass through the oculomotor nerve to supply the iris sphincter and ciliary muscles that constrict the pupil, accommodate the lens and allow for eye convergence (Woolf, 1991). Thus while the differing characters of the cholinergic system can be used to align the microchiroptera with the shrews, the fact that two of these characters are

related to the visual system is of concern for interpretation – it is possible that the reduction of the visual system in both groups explains the absence of the parabigeminal and Edinger-Westphal nuclei. This concern can be allayed by the presence of these nuclei in truly micropthalmic animals such as the mole rats and golden mole (Da Silva et al., 2006; Bhagwandin et al., 2008; Calvey et al., 2013) and the unusual structure of the laterodorsal tegmental nucleus that aligns the insectivores and microchiropterans.

4.2. Catecholaminergic nuclei

For the most part, the complement of nuclei identified as belonging to the catecholaminergic system in the insectivores studied was common to most eutherian mammals (Dell et al., 2010; Calvey et al., 2013); however, variances were observed in the complement of pontine, midbrain and hypothalamic catecholaminergic nuclei. While present in both species of hedgehog, the dorsolateral division of locus coeruleus (the A4 nucleus) was absent in all three shrews species studied. The A4 nucleus was also absent in all species of microchiropterans that have been studied to date (Maseko and Manger, 2007; Kruger et al, 2010a). This common absence aligns the microchiropterans and the Soricidae to the exclusion of all other closely related mammals studied to date. The ventral division of the substantia nigra nuclear complex (the A9v nucleus within the pars reticulata) was poorly expressed, or incipient, in the three shrew species, but was readily observed in both hedgehogs studied (although an earlier report suggests it is incipient in the European hedgehog, Michaloudi and Papadopoulos, 1996). This nucleus was also poorly expressed in five of the previously studied microchiropterans (Kruger et al., 2010a) and was absent in Schreiber's long fingered bat (Maseko and Manger, 2007). This second variance in the appearance of the catecholaminergic nuclei strengthens to potential alignment of microchiroptera to Soricidae. The third variable feature of the catecholaminergic system that is of relevance was the absence of the dorsal division of the anterior hypothalamic group (the A15d nucleus) in two of the shrew species studied, in the European hedgehog (Michaloudi and Papadopoulos, 1996), but not the hedgehogs studied herein, and the microchiropterans (Maseko and Manger, 2007; Kruger et al., 2010a). This feature again strengthens the potential alignment of microchiroptera to Soricidae specifically, but also potentially more generally to the insectivores. Thus, as with the cholinergic system, the variances in the nuclear complement of the catecholaminergic system, argues towards the phylgenetic alignment of the microchiropterans specifically with the Soricidae, but more generally with the insectivores.

The A4 nucleus is thought to be a dorsal continuation of the noradrenergic neurons of the A6 locus coeruleus complex, and as such, are thought to have a similar function to the A6 noradrenergic neurons (Smeets and Gonzalez, 2000). The absence of this nucleus in the Soricidae and microchiropterans is likely then to have no major functional implications given the presence of clear A6 nuclei in these species, although this remains to be investigated further. The incipient nature of the ventral division of the substantia nigra nuclear complex (the A9v nucleus within the pars reticulata) may also have some functional implications. In other mammals, the A9 complex as a whole appears to play a role in movement control through its projections to the striatum (Smeets and Gonzalez, 2000). The A9v nucleus in particular has been found to project to the superior colliculi and is thought to play a role in reward-orientated saccadic eye movement (Sato and Hikosaka, 2002). Given the reduced visual system in the Soricidae and microchiropterans it is possible that the reduction in cell numbers in the A9v in these species is related to their reduced visual system, but it must also be noted that in the micropthalmic mole rats and golden mole (Da Silva et al., 2006; Bhagwandin et al., 2008; Calvey et al., 2013) the A9v nucleus shows what appears to be a typical number of A9v neurons. Lastly, the absence of the dorsal division of the anterior hypothalamic group (the A15d nucleus) in most of the Soricidae and microchiropterans may be related to differences in the control of pituitary hormone secretion and hence reproduction, as this nucleus sends direct projections to the pituitary gland in other mammals (Smeets and Gonzalez, 2000); however, as species of both Soricidae and microchiropterans can be either seasonally or aseasonally polyestrus, or seasonally monoestrous, it is difficult to determine how the lack of the A15d nucleus may effect reproductive processes and strategies.

4.3. Serotonergic and orexinergic nuclei

Previous studies of the nuclear organization of the serotonergic system across mammals have shown that the organization of this system is very conservative in terms of evolutionary differences, with all Eutherian mammals studied to date showing a similar nuclear complement (Dell et al., 2010). While variances in the nuclear complement of the serotonergic system have been observed in monotremes and marsupials (Manger et al., 2002c; Patzke et al., 2014), the five insectivore species investigated in the current study do not exhibit a different serotonergic nuclear complement to that reported previously for Eutherian mammals. The orexinergic system also appears to have quite a conservative

organization across mammalian species studied to date, and the organization of the orexinergic clusters in the four insectivore species studied herein appear to follow the organization observed in most Eutherian mammals studied to date (Dell et al., 2013; Calvey et al., 2013). Interestingly, the microchiropterans appear to lack the optic tract cluster of orexinergic neurons (Kruger et al., 2010b), being a neuroanatomical feature specific to the microchiropteran order that sets them apart from all other mammals, including megachiroptera who do not lack this orexinergic cluster (Dell et al., 2013). Thus, the organization of the serotonergic and orexinergic systems do not appear to provide variance that can link the microchiropterans to the insectivores, but it does indicate that the microchiropterans are different to all other mammals, including the megachiropterans.

4.4. Insectivores and microchiropteran affinities

In the diphyletic scenario of chiropteran evolution a potential place for the megachiropterans as a branch of the dermopteran lineage, the sister group to primates, has been proposed (Pettigrew et al., 1989); however, a potential sister grouping for the microchiropterans is currently lacking. Previous studies of the systems examined herein for a broader range of insectivores have indicated that the insectivores are a likely potential candidate grouping for the microchiropterans (Maseko and Manger, 2007; Maseko et al., 2007; Kruger et al., 2010a,b; Dell et al., 2010, 2013). This indication is fully supported by this study of five species of insectivores, and more specifically appears to align the microchiropterans with the Soricidae (or shrews). A range of features from both the cholinergic and catecholaminergic systems (discussed above) indicate this phylogenetic alignment, but it is not only features of the brain that suggest this link, but several molecular studies also provide supportive evidence for the proposed microchiropteran-Soricidae sister grouping. Molecular studies have placed three microchiropteran families into Laurasiatheria which includes shrews and hedgehogs (Teeling et al., 2005). Murphy et al. (2001b) acknowledge the relationship between Soricomorpha (shrews and moles) and chiroptera based on molecular findings, and viral evolution studies have grouped the microchiropteran family Rhinolophoidea with the Soricidae (Guo et al., 2013). Thus, the molecular studies are, in a sense, supportive of microchiropteran and megachiropteran diphyly, with Soricidae forming a potential sister group to the microchiropterans. In addition, certain species of shrews and all microchiropterans use echolocation to map their surroundings (Symonds, 2005; Siemers et al., 2009), and species from both groups are known to undergo a process of

torpor (Nagal, 1977; Geiser, 2004; Symonds, 2005). Thus, there is substantial preliminary evidence linking the microchiropterans with the Soricidae, providing a potential phylogenetic niche for the microchiropterans in the diphyletic scenario of chiropteran evolution. While clearly a great deal more work is required to substantiate this proposed phylogenetic assignation, the microchiropteran-Soricidae link appears to make more sense that the microchiropteran-ungulate sister grouping reported in recent studies of mammalian phylogeny (Meredith et al., 2011).

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References

Arnason, U., Adegoke, J.A., Gullberg, A., Harley, E.H., Janke, A., Kullberg, M., 2008. Mitogenomic relationships of placental mammals and molecular estimates of their divergences. Gene 421, 37-51.

Asher, R.J., Maree, S., Bronner, G., Bennett, N.C., Bloomer, P., Czechowski, P., Meyer, M., Hofreiter, M., 2010. A phylogenetic estimate for golden moles (Mammalia, Afrotheria, Chrysochloridae). BMC Evol. Biol. 10, 69.

Barker, R.A., Cicchetti, F., Neal, M.J., 2012. Neuroanatomy and Neuroscience at a glance. Wiley-Blackwell. Fourth edition.

Bhagwandin, A., Fuxe, K., Manger, P.R., 2006. Choline acetyltransferase immunoreactive cortical interneurons do not occur in all rodents: a study of the phylogenetic occurrence of this neural characteristic. J. Chem. Neuroanat. 32, 208-216.

Bhagwandin, A., Fuxe, K., Bennett, N.C., Manger, P.R., 2008. Nuclear organization and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brains of two species of African mole-rat. J. Chem. Neuroanat. 35, 371-387.

Bhagwandin, A., Fuxe, K., Bennett, N.C., Manger, P.R., 2011.Distribution of orexinergic neurons and their terminal networks in the brains of two species of African mole rats. J. Chem. Neuroanat. 41, 32-42.

Calvey, T., Patzke, N., Kaswere, C., Gilissen, E., Bennett, N.C., Manger, P.R., 2013. Nuclear organisation of some immunohistochemically identifiable neural systems in three Afrotherian species –*Potomogale velox*, *Amblysomus hottentotus* and *Petrodromus tetradactylus*. J. Chem. Neuroanat. 50, 48-65.

Dahlström, A., Fuxe, K., 1964. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamine in the cell bodies of brainstem neurons. Acta Physiol. Scand. 62, 1-52.

Da Silva, J.N., Fuxe, K., Manger, P.R., 2006. Nuclear parcellation of certain immunohistochemically identifiable neuronal systems in the midbrain and pons of the Highveld molerat (*Cryptomys hottentotus*). J. Chem. Neuroanat. 31, 37-50.

Dell, L-A., Kruger, J-L., Bhagwandin, A., Jillani, N.E., Pettigrew, J.D., Manger, P.R., 2010. Nuclear organization of cholinergic, putative catecholaminergic and serotonergic systems in the brains of two megachiropteran species. J. Chem. Neuroanat. 40, 177-195.

Dell, L.A., Kruger, J.L., Pettigrew, J.D., Manger, P.R., 2013. Cellular location and major terminal networks of the orexinergic system in the brain of two megachiropterans. J. Chem. Neuroanat. 53, 64-71.

Dinopoulos, A., Michaloudi, H., Karamanlidis, A.N., Antonopoulos, J., Parnavelas, J.G., 1988. Basal forebrain neurons project to the cortical mantle of the European hedgehog (*Erinaceus europaeus*). Neurosci. Lett. 86, 127-132.

Dwarika, S., Maseko, B.C., Ihunwo, A.O., Fuxe, K., Manger, P.R., 2008. Distribution and morphology of putative catecholaminergic and serotonergic neurons in the brain of the greater canerat, *Thryonomys swinderianus*. J. Chem. Neuroanat. 35, 108-122.

Geiser, F., 2004. The role of torpor in the life of Australian arid zone mammals. Aust. Mammal. 26, 125-134.

Gravett, N., Bhagwandin, A., Fuxe, K., Manger, P.R., 2009. Nuclear organization and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brain of the rock hyrax, *Procavia capensis*. J. Chem. Neuroanat. 38, 57-74.

Gravett, N., Bhagwandin, A., Fuxe, K., Manger, P.R., 2011. Distribution of orexin-A immunoreactive neurons and their terminal networks in the brain of the rock hyrax, *Procavia capensis*. J. Chem. Neuroanat. 41, 86-96.

Guo, W.P., Lin, X.D., Wang, W., Tian, J.H.,Cong, M.L., Zhang, H.L., Wang, M.R., Zhou, R.H., Wang, J.B., LI, M.H., Xu, J., Holmes, E.C., Zhang, Y.Z., 2013. Phylogeny and origins of hantaviruses harbored by bats, insectivores, and rodents. PloS Pathog. 9, e1003159.

Hökfelt, T., Martenson, R., Björklund, A., Kleinau, S., Goldstein, M., 1984. Distributional maps of tyrosine-hydroxylase-immunoreactive neurons in the rat brain, in: Björklund, A., Hökfelt, T. (Eds.), Handbook of Chemical Neuroanatomy, vol. 2. Classical Neurotransmitters in the CNS, part 1. Elsevier, Amsterdam, pp. 277–379.

Karasawa, N., Takeuchi, T., Yamada, K., Iwasa, M., Isomura, G., 2003. Choline acetyltransferase positive neurons in the laboratory shrew (*Suncus murinus*) brain: coexistence of ChAT/5-HT (Raphe dorsalis) and ChAT/TH (Locus ceruleus). Acta Histochem. Cytochem. 36, 399-407.

Kruger, J.L., Dell, L.A., Bhagwandin, A., Jillani, N.E., Pettigrew, J.D., Manger, P.R., 2010a. Nuclear organization of cholinergic, putative catecholaminergic and serotonergic systems in the brains of five microchiropteran species. J. Chem. Neuroanat. 40, 210-222.

Kruger, J-L., Dell, L-A., Pettigrew, J.D., Manger, P.R., 2010b. Cellular location and major terminal networks of the orexinergic system in the brains of five microchiropteran species. J. Chem. Neuroanat. 40, 256-262.

Kruger, J-L., Patzke, N., Fuxe, K., Bennett, N.C., Manger, P.R., 2012. Nuclear organization of cholinergic, putative catecholaminergic, serotonergic and orexinergic systems in the brain of the African pygmy mouse (*Mus minutoides*): organizational complexity is preserved in small brains. J. Chem. Neuroanat. 44, 45-56.

Lee, M.S.Y., Camens, A.B., 2009. Strong morphological support for the molecular evolutionary tree of placental mammals. J. Evol. Biol. 22, 2243-2257.

Limacher, A.M., Bhagwandin, A., Fuxe, K., Manger, P.R., 2008. Nuclear organization and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brain of the Cape porcupine (*Hystrix africaeaustralis*): Increased brain size does not lead to increased organizational complexity. J. Chem. Neuroanat. 36, 33-52.

Linnaei, Caroli, 1758. Systema naturae per regna tria naturiae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Tomus I. Editio Decima, Reformata. Holmiae, Impensis direct. Stockholm: Laurentii Salvii.

Manger, P.R., Fahringer, H.M., Pettigrew, J.D., Siegel, J.M., 2002a. Distribution and morphology of cholinergic neurons in the brain of the monotremes as revealed by ChAT immunohistochemistry. Brain Behav. Evol. 60, 275-297.

Manger, P.R., Fahringer, H.M., Pettigrew, J.D., Siegel, J.M., 2002b. Distribution and morphology of serotonergic neurons in the brain of the monotremes. Brain Behav. Evol. 60, 315-332.

Manger, P.R., Fahringer, H.M., Pettigrew, J.D., Siegel, J.M., 2002c. Distribution and morphology of serotonergic neurons in the brain of the monotremes. Brain Behav. Evol. 60, 315-332.

Maseko, B.C., Manger, P.R., 2007. Distribution and morphology of cholinergic, catecholaminergic and serotonergic neurons in the brain of Schreiber's long-fingered bat, *Miniopterus schreibersii*. J. Chem. Neuroanat. 34, 80-94.

Maseko, B.C., Bourne, J.A., Manger, P.R., 2007. Distribution and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brain of the Egyptian Rousette flying fox, *Rousettus aegyptiacus*. J. Chem. Neuroanat. 34, 108-127.

Meredith, R.W., Janecka, J.E., Gatesy, J., Ryder, O.A., Fisher, C.A., Teeling, E.C., Goodbla, A., Eizirik, E., Simao, T.L., Stadler, T., Rabosky, D.L., Honeycutt, R.L., Flynn, J.J., Ingram, C.M., Steiner, C., Williams, T.L., Robinson, T.J., Burk-Herrick, A., Westerman, M., Ayoub, N.A., Springer, M.A., Murphy, W.J., 2011. Impacts of the Cretaceous terrestrial revolution and KPg extinction on mammal diversification. Science 334, 521-524.

Michaloudi, H.C., Papadopoulos, G.C., 1995. Atlas of serotonin-containing cell bodies and fibres in the central nervous system of the hedgehog. J. Brain Res. 1, 77-100.

Michaloudi, H.C., Papadopoulos, G.C., 1996. Noradrenergic and dopaminergic systems in the central nervous system of the hedgehog (*Erinaceus europaeus*). J. Hirnforsch. 37, 319-350.

Moon, D.J., Maseko, B.C., Ihunwo, A., Fuxe, K., Manger, P.R., 2007. Distribution and morphology of catecholaminergic and serotonergic neurons in the brain of the highveld gerbil, *Tatera brantsii*. J. Chem. Neuroanat. 34, 134-144.

Murphy, W., Eizirik, E., O'Brien, S., Madsen, O., Scally, M., Douady, C., Teeling, E., Ryder, O., Stanhope, M., de Jong, W., Springer, M., 2001. Resolution of the early placental mammal radiation using Bayesian phylogenetics. Science. 294, 2348-2351.

Nagal, A., 1977. Torpor in the European white-toothed shrews. Experientia 33, 1455-1456.

Patzke, N., Bertelsen, M.F., Fuxe, K., Manger, P.R., 2014. Nuclear organization of cholinergic, catecholaminergic, sertonergic and orexinergic systems in the brain of the Tasmanian devil (*Sarcophilus harrisii*). J. Chem. Neuroanat. 61-62, 94-106.

Pettigrew, J.D., 1986. Flying primates? Megabats have the advanced pathway from eye to midbrain. Science. 231:1304-1306.

Pettigrew, J.D., Jamieson, B.G.M., Robson, S.K., Hall, L.S., McNally, K.I., Cooper, H.M., 1989. Phylogenetic relations between microbats, megabats and primates (Mammalia: Chiroptera and Primates). Philos. Trans. R. Soc. Lond. 325, 489-559.

Pettigrew, J.D., Maseko, B.C., Manger, P.R., 2008. Primate-like retinotectal decussation in an echolocating megabat, *Rousettus aegyptiacus*. Neuroscience. 153, 226-231.

Pieters, R.P., Gravett, N., Fuxe, K., Manger, P.R., 2010. Nuclear organization of cholinergic, putative catecholaminergic and serotonergic nuclei in the brain of the eastern rock elephant shrew, *Elephantulus myurus*. J. Chem. Neuroanat. 39, 175-188.

Sato, M., Hikosaka, O., 2002. Role of primate substantia nigra pars reticulate in reward-oriented saccadic eye movement. J. Neurosci. 6, 2363-2373.

Siemers, B., Schauermann, G., Turni, H., von Merten, S., 2009. Why do shrews twitter? Communication or simple echo-based orientation. Biol. Lett. 5, 593-596.

Smeets, W.J.A.J., González, A., 2000. Catecholamine systems in the brain of vertebrates: new perspectives through a comparative approach. Brain Res. Rev. 33, 308-379.

Smith, J.D., Madkour, G., 1980. Penial morphology and the question of chiropteran phylogeny. In: Wilson, D.E., Gardner, A.L. (Eds.), Proceedings Fifth International Bat Research Conference. Lubbock: Texas Tech Press, Lubbock, pp. 347-365.

Steinbusch, H.W.M., 1981. Distribution of serotonin-immunoreactivity in the central nervous system of the rat – cell bodies and terminals. Neuroscience. 6, 557-618.

Symonds, M., 2005. Phylogeny and life histories of the Insectivora: controversies and consequences. Biol Rev. 80, 93-128.

Teeling, E.C., Madsen, O., Van den Bussche, R.A., de Jong, W.W., Stanhope, M.J., Springer, M.S., 2002. Microbat paraphyly and the convergent evolution of a key innovation in Old World rhinolophoid microbats. Proc Natl Acad Sci USA 99, 1431-1436.

Teeling, E.C., Springer, M.S., Madsen, O., Bates, P., O'Brien, S.J., Murphy, W.J., 2005. A molecular phylogeny for bats illuminates biogeography and the fossil record. Science 307, 580-584.

Törk, I., 1990. Anatomy of the serotonergic system. Ann. N.Y. Acad. Sci. 600, 9–35.

Woolf, N.J., 1991. Cholinergic systems in mammalian brain and spinal cord. Prog. Neurobiol. 37, 475–524.