A neuroanatomical evaluation of cholinergic, catecholaminergic, serotonergic and orexinergic neural systems in mammals pertaining to the phylogenetic affinities of the Chiroptera

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PhD Thesis

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2015
DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

[Signature]

Tanya Calvey 30 April, 2015.
For my mom, Shayne Kathleen Calvey.

At the core of my being.

I love you, appreciate you and miss you more with every day that goes by.
Publications arising from this thesis:


Presentations arising from this thesis:


• The above paper was presented as a poster at the biennial SONA Congress held in Rabat, Morocco, June 2013
Abstract:

One of the few remaining mysteries in mammalian phylogeny is the issue of Chiropteran phylogeny. In order to further investigate the diphyletic hypothesis that states that Megachiroptera evolved from primate-like gliders and that Microchiroptera evolved from insectivores, the cholinergic, catecholaminergic, serotonergic and orexinergic systems were analyzed in, not only five insectivores (Crocidura cyanea, Crocidura olivieri, Sylvisorex ollula, Paraechinus aethiopicus and Atelerix frontalis) and three prosimian primates (Galagoides demidoff, Perodicticus potto and Lemur catta), but in species from other orders of interest including the Afrotheria (Potamogale velox, Amblysomus hottentotus and Petrodromus tetradactylus), Lagomorpha (Lepus capensis) and Scandentia (Tupaia belangeri). Brains of the mammals were coronally sectioned and immunohistochemically stained with antibodies against cholineacetyltransferase, tyrosine hydroxylase, serotonin and orexin-A. The presence or absence of 93 nuclei within these neuromodulatory systems was entered into modern cladistics software for analysis of the 13 studied species, as well as an additional 40 previously studied mammals. The majority of nuclei revealed in the current study were similar among the species investigated and to mammals generally, but certain differences in the nuclear complement highlighted potential phylogenetic interrelationships. The Afrotherian, A. hottentotus, presented unusual cholinergic interneurons in the cerebral cortex, hippocampus, olfactory bulb and amygdala, and exhibited an unusual foreshortening of the brain, such that a major mesencephalic flexure in the brainstem was evident. The Afrotherian, P. tetradactylus, lacked the catecholaminergic A15d nucleus as in a previously studied member of Macroscelididae. The three Insectivoran shrews lacked the cholinergic parabigeminal and Edinger-Westphal nuclei, had a mediodorsal arch of the cholinergic laterodorsal tegmental nucleus, lacked the catecholaminergic A4 and A15d nuclei and presented an incipient ventral division of the substantia nigra which is identical to previously studied Microchiroptera. All three prosimians presented a central compact division of catecholaminergic locus coeruleus (A6c) surrounded by a shell of less densely packed (A6d) tyrosine hydroxylase immunopositive neurons. This combination of compact and diffuse divisions of the locus coeruleus complex is only found in primates and Megachiropterans of all the mammalian species studied to date. T. belangeri of the Scandentia contained ChAT+ neurons within the nucleus of the trapezoid body as well as the superior olivary nuclear complex, which has not been described in any mammal studied to date. L. capensis of the Lagomorpha presented
the rodent specific rostral dorsal midline medullary nucleus (C3), while *T. belangeri* was lacking both the ventral and dorsal divisions of the anterior hypothalamic group (A15v and A15d), and both species were lacking the primate/Megachiropteran specific compact portion of the locus coeruleus. Our neuroanatomical analysis suggests a phylogenetic relationship between the Soricidae (shrews) and the Microchiroptera, supports the phylogenetic grouping of primates with Megachiropterans, confirms previous molecular evidence of the relationship between lagomorphs and rodents within the super-order Glires, and suggests that primates are phylogenetically closer to Megachiroptera than to any members of the Euarchontoglires. The cladistic analysis confirmed the neuroanatomical analysis with the most parsimonious tree placing Megachiroptera into the Euarchontoglires as a sister group to primates and the Microchiroptera next to Soricidae within the Laurasiatheria.
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CHAPTER ONE: Introduction and chapter outline

1.1 Introduction

The order Chiroptera is divided into two sub-orders, Microchiroptera (microbats or insectivorous bats) and Megachiroptera (megabats, fruit bats or flying foxes). These two groups of bats are commonly thought to share the same common ancestor, being a monophyletic mammalian order; however, as early as Linnaeus (1758), it was noticed that perhaps Microchiroptera and Megachiroptera should not be grouped together. Based on anatomical similarities, Linnaeus suggested that Megachiroptera should be grouped with Primates. This potential phylogenetic relationship between Megachiroptera and Primates was largely ignored until Smith and Madkour (1980) described similarities in the structure of the glans penis of primates and Megachiroptera that are not found in other eutherian mammals. Pettigrew (1986) examined the neuroanatomy of the retinotectal pathway in both Megachiroptera and Microchiroptera and found that Megachiroptera have an advanced retinotectal pathway with a vertical hemidecussation, a feature previously found only in Primates, whereas this is not seen in Microchiroptera. This was the beginning of the ‘Flying Primate’ hypothesis suggesting a diphyletic origin of the Chiropteran order and implying that mammalian flight evolved twice. Using neurological and morphological traits, Pettigrew et al. (1989) suggested that Megachiropterans evolved from the acknowledged sister group to primates, the Dermopterans (flying lemurs). Thus, the megachiropterans are not suggested to be literally ‘flying primates’, but are thought to have been derived from the gliding sister group to Primates, the Dermopterans, an acknowledged sister group to Primates (Baker et al., 1991; Nowak, 1999; Eizirik et al., 2004).

In order to understand the similarities between Megachiropteran and Primate morphology, in addition to Primates, the close relatives of the Primates, and the various Primate lineages, termed the Euarchontoglires (the Scandentia - the tree shrews, the Lagomorphs - the rabbits and hares, and the rodents) must be studied in detail (Asher et al., 2009; Murphy et al., 2001b). Traditionally, Primates have been split into Prosimians and Simians (anthropoids). Prosimian Primates are the earliest branch of the Primates and, in the Chiropteran diphyletic scenario, should possess a central nervous system with the most similarities to the Megachiropterans.
Most global mammalian phylogenies tend to place the Chiropterans as a whole in a grouping with the insectivores, Cetartiodactyls, Perissodactyls and carnivores (Arnason et al., 2002; Asher et al., 2009; Lee and Camens, 2009; Meredith et al., 2011). Of this cluster of the mammalian radiation, the insectivores hold the most promise for a potential sister group to the Microchiropterans. The Insectivora, from which a potential sister group to the Microchiropterans may arise, also has a problematic systematics and is currently considered to be paraphyletic (Symonds, 2005). Stanhope et al. (1998) used molecular analysis on 6 proposed Insectivoran families, as well as an additional 37 taxa, to conclude that golden moles and tenrecs should fall into the Afrotheria clade which includes elephants, sirenians, hyraxes, aardvarks and elephant shrews. The Afrotheria clade as a whole is strongly supported by morphological data such as tooth eruption ages and vertebral variability (Asher et al., 2009) as well as molecular data (Tabuce et al., 2008); however, relationships within the Afrotherian clade are not yet unanimously resolved. Asher et al. (2009) divides the clade into Paenungulata (Proboscidea, Sirenia and Hyracoidea) and Afroinsectiphilia (Tubulidentata, Macroscelidea, Tenrecidae and Chrysochloridae). Thus, in order to solve the problem of Microchiropteran phylogenetic affinities in the diphyletic scenario, the Afroinsectiphilia (Asher et al., 2009) and the various families of the Insectivores (Soricidae, the shrews; Solenodontidae, the solenodons; Talipidae, the moles; and Erinaceidae, the hedgehogs and gymnures; as defined by Symonds, 2005) need to be examined in detail. Molecular evidence has placed three of the Microchiropteran families into Laurasiatheria which includes Soricidae and Erinaceinae (Teeling et al., 2005). Murphy et al. (2001b) acknowledges the relationship between Soricomorpha (shrews and moles) and Chiroptera based on molecular findings. Genetic reconstruction grouped the Microchiropteran family, Rhinolophoidea, with Soricidae (Guo et al., 2013). Shrews, moles and Microchiroptera use echolocation to map their surroundings (Symonds, 2005; Siemers et al., 2009). Crociduran shrews and Microchiropterans can drop their body temperature, displaying a rare process called torpor (Symonds, 2005; Geiser, 2004). It would appear then, that the most likely candidates of the Insectivora lineage that may form a potential sister group to the Microchiropterans are the shrews (Soricidae). This warrants investigation.

Neural structures are useful in assisting in the determination of phylogenetic affinities. Neurological data has led to the solution of phylogenetic puzzles in many different vertebrate classes, most notably in fish (e.g. characid, siluriform and gymnotiform relations, Fink and Fink, 1981). The brain is conservative in terms of its evolution (Galis, 1999; Manger, 2005;
Dell et al., 2010), with changes in the complexity of neural system structure, in terms of the number and complement of discrete and readily identifiable nuclear subdivisions, appearing only to occur during evolutionary events leading to the establishment of a new mammalian order (Manger, 2005). It appears, in general, that all progeny of the established order will retain the same complement of subdivisions of all neural systems, irrespective of the subsequently evolved size of the brain, phenotype or life history of the species comprising the order (Manger, 2005). This means that each mammalian order will exhibit a unique complement of nuclei, or other subdivisions and types of specific neural systems such as cortical areas, that is typical of all its members. This hypothesis has been supported by numerous studies across a range of mammalian species (Da Silva et al., 2005; Dwarika et al., 2007; Moon et al., 2007; Bhagwandin et al., 2008; Gravett et al., 2009; Bux et al., 2010; Dell et al., 2010; Kruger et al., 2010a; Calvey et al., 2013) and the data generated appear to conform to the generally accepted mammalian phylogenies (Asher et al., 2009) to the exception of the Chiroptera (Dell et al., 2010). Studies comparing the two groups of bats to each other and to other mammals, have shown that Megachiroptera possess between 7 to 11 discrete nuclei that are not found in Microchiroptera, and these nuclei align the Megachiroptera to Primates, tree shrews, rabbits and Rodents (Maseko et al., 2007; Dell et al., 2010). The nuclear organization of these same systems in the Microchiroptera closely resembled the Erinaceomorpha and Soricomorpha of Insectivora (Maseko and Manger, 2007; Kruger et al., 2010a) as originally predicted (Pettigrew et al., 1989). The difficulty with these prior studies of Chiroptera affinities within the mammals is the sparseness of the data. Very little data regarding neural systems within a range of Afrotherians, insectivores, Primates, Lagomorphs and tree shrews are available. This gap in the literature means that interpretation of the data related to the question of Chiroptera phylogenetic affinities is not as strong as it might be. This is of major concern, and filling these gaps in our knowledge forms the central pillar of this thesis. The current series of studies has elected to investigate the neural organization of a range of systems that are not directly involved in the production of movement or in the direct sensing of the environment. These systems, the cholinergic, catecholaminergic, serotonergic and orexinergic, are involved in the modulation of neural activity across vast regions of the central nervous system and provide easily identifiable structural data free from behavioural bias.
1.2 Aims

It has been proposed that the Megachiropterans evolved from the acknowledged sister group to the Primates, the Dermopterans, and that Microchiropterans evolved from insectivores (Pettigrew, 1986; Pettigrew et al., 1989). Due to the fact that little neuroanatomical data is available for comparison and that the molecular data is equivocal, the aim of this thesis is to analyse the nuclear organization of four neuromodulatory systems in the proposed sister groups of the Chiroptera to provide data of relevance to this hypothesis.

In chapter three of this thesis, members of Afrotheria that were previously considered insectivores, are investigated in order to establish whether these species, the Afroinsectiphilia show any special neuroanatomical links with the Microchiroptera. This is undertaken through the examination of the brains of the giant otter shrew, Cape golden mole and the four-toed sengi for comparison with data from the Eastern rock elephant shrew (Pieters et al., 2009), rock hyrax (Gravett et al., 2009) and the African elephant (Maseko et al., 2013).

Based on partial examination of the nuclear organization of the neuromodulatory systems examined here, it was found that the complement of nuclei in the brains of the European hedgehog and laboratory shrew was similar to that of the Microchiroptera (Maseko and Manger, 2007; Kruger et al., 2010a). Thus, the aim of the study presented in chapter four is to analyse the neuromodulatory systems of two wild-caught hedgehogs and three wild-caught shrews to compare with the six previously studied Microchiroptera in order to establish any clear neuroanatomical links that would indicate whether the shrews or hedgehogs might be a valid sister group to the Microchiroptera.

The neuroanatomical similarities between Megachiropterans and Primates are well established (Pettigrew, 1986; Pettigrew et al., 1989; Maseko et al., 2007; Dell et al., 2010). Prosimian Primates are the earliest branch of the Primates and, in the Chiropteran diphyletic scenario, should possess a central nervous system with the most similarities to the Megachiropterans. There are also no data regarding the nuclear organization of the four targeted neuromodulatory systems available on prosimian primates. Thus, the aim of chapter five is to analyse these systems in three Prosimian Primates to compare with three previously studied Megachiropterans and six previously studied simian Primates to reveal whether the neuroanatomical links between Megachiropterans and Primates hold under closer and broader scrutiny.
Primates fall into the super-order Euarchontoglires along with the Glires (rabbits, hares and rodents), Scandentia (tree shrews) and Dermoptera (colugos or flying lemurs) (Murphy et al., 2001a,b; Misawa and Janke, 2003). The tree shrews have at times been considered to be Primates, but current thinking places the tree shrews as a second step sister group to the Primates by the Dermopterans. As many studies have been undertaken on the rodents (e.g. Kruger et al., 2012), but few on their closest relatives the Lagomorphs, and that the Glires represent the third step sister group to the Primates, it is of interest to establish the nuclear organisation of the targeted neural systems in these species. In the Chiropteran diphyletic scenario, Megachiroptera would also fall into Euarchontoglires, thus it is important to analyse other members of this super-order for comparison with Primates and Megachiroptera. Hence, the aim of chapter six is to analyse the Cape hare and northern tree shrew, two previously unstudied members of Euarchontoglires, to establish, using neuroanatomical data, the intra-superorder phylogeny.

Chapter seven, the final discussion of the thesis, present a cladistics analysis of the 13 species studied in this thesis along with an additional 41 previously studied mammals using 93 neural characters as the data set. The aim of this chapter is to establish the most parsimonious phylogenetic tree based on the nuclear organization of the four neural systems studied. This provides an analysis relevant to the problem of Chiropteran phylogeny within the mammals.

1.3 Chapter outline:

This thesis is organized into several chapters, with each chapter examining a different cluster of species.

Chapter two: Literature review

This chapter presents a concise summation of past and present research as well as the rational for selecting this topic for my PhD thesis.
Chapter three: Nuclear organisation of some immunohistochemically identifiable neural systems in three Afrotherian species – *Potomogale velox*, *Amblysomus hottentotus* and *Petrodromus tetradactylus*.

The present study describes the organization of the cholinergic, catecholaminergic, and serotonergic neurons in the brains of the giant otter shrew, the Hottentot golden mole and the four-toed sengi, and the orexinergic (hypocretinergic) system in the giant otter shrew and four-toed sengi. The aim of the present study was to investigate the possible differences in the nuclear complement of these neural systems in comparison to previous studies on other Afrotherian species and mammalian species in general. Brains of the golden mole, sengi and giant otter shrew were coronally sectioned and immunohistochemically stained with antibodies against cholineacetyltransferase, tyrosine hydroxylase, serotonin and orexin-A. The majority of nuclei revealed in the current study were similar among the species investigated, to other Afrotherian species, and to mammals generally, but certain differences in the nuclear complement highlighted phylogenetic interrelationships. The golden mole was observed to have cholinergic interneurons in the cerebral cortex, hippocampus, olfactory bulb and amygdala. The four-toed sengi had cholinergic neurons in both colliculi and in the cochlear nucleus, but lacked the catecholaminergic A15d group in the hypothalamus. In both the golden mole and the four-toed sengi, the locus coeruleus (A6d group) was made up of few neurons. The golden mole also exhibited an unusual foreshortening of the brain, such that a major (mesencephalic?) flexure in the brainstem was evident.

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

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 serotoninergic 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.

Chapter five: Organization of cholinergic, catecholaminergic, serotonergic and orexinergic nuclei in three Prosimian Primates: *Galagoides demidoff*, *Perodicticus potto* and *Lemur catta*.

The nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic systems in the brains of three species of prosimian primates is presented. We aimed to investigate the nuclear complement of these neural systems in comparison to those of Simian Primates, Megachiropterans and other mammalian species. The brains were coronally sectioned and immunohistochemically stained with antibodies against choline acetyltransferase, tyrosine hydroxylase, serotonin and orexin-A. The nuclei identified were identical among the Prosimian species investigated and identical to previous reports in Simian Primates. Moreover, a general similarity to all mammals was found, but specific differences in the nuclear complement highlighted potential phylogenetic interrelationships. The central feature of interest was the structure of the locus coeruleus complex in the Primates, where a central compactly packed core (A6c) of tyrosine hydroxylase immunopositive neurons was surrounded by a shell of less densely packed (A6d) tyrosine hydroxylase immunopositive neurons. This combination of compact and diffuse divisions of the locus coeruleus complex is only found in Primates and Megachiropterans of all the mammalian species studied to date. This neural character, along with variances in a range of other neural characters, supports the phylogenetic grouping of Primates with Megachiropterans as a sister group.

The present study describes the organization of the nuclei of the cholinergic, catecholaminergic, serotonergic and orexinergic systems in the brains of two members of Euarchontoglires, *Lepus capensis* and *Tupaia belangeri*. The aim of the present study was to investigate the nuclear complement of these neural systems in comparison to previous studies on Euarchontoglires and generally with other mammalian species. Brains were coronally sectioned and immunohistochemically stained with antibodies against choline acetyltransferase, tyrosine hydroxylase, serotonin and orexin-A. The majority of nuclei revealed in the current study were similar among the species investigated and to mammals generally, but certain differences in the nuclear complement highlighted potential phylogenetic interrelationships within the Euarchontoglires and across mammals. In the cholinergic system, the northern tree shrew the nucleus of the trapezoid body contained neurons immunoreactive to the choline acetyltransferase antibody with some of these neurons extending into the lamellae within the superior olivary nuclear complex. This nucleus and the extension of cholinergic neurons have not been noted in any mammal studied to date. In addition, cholinergic neurons forming the medullary tegmental field were also present in the northern tree shrew. Regarding the catecholaminergic system, the Cape hare presented with the rodent specific rostral dorsal midline medullary nucleus (C3), and the northern tree shrew was lacking both the ventral and dorsal divisions of the anterior hypothalamic group (A15v and A15d). Both species were lacking the Primate/Megachiropteran specific compact portion of the locus coeruleus complex (A6c). The nuclei of the serotonergic and orexinergic systems of both species were similar to those seen across most Eutherian mammals. Our results lend support to the monophyly of the Glires, and more broadly suggest that the Megachiropterans are more closely related to the Primates than are any other members of Euarchontoglires that have been studied.

**Chapter seven: General discussion and cladistic analysis**

This chapter summarizes the implication and significance of the results obtained in the studies comprising this thesis and highlights future directions for research regarding the problem of chiropteran phylogenetic relationships. It achieves this by presenting a cladistic analysis of 93 neural characters over 54 species.
CHAPTER TWO: Literature Review

2.1. Chiropteran phylogeny

The order Chiroptera is divided into two sub-orders, Microchiroptera (Microbats or insectivorous bats) and Megachiroptera (megabats, fruit bats or flying foxes). Microchiroptera are one of the most species rich suborders of mammals consisting of 759 species representing almost 20% of all mammalian species (Nowak, 1994). Megachiroptera are large with body masses ranging from 10 g to 1.5 kg, whereas Microchiroptera are smaller, ranging in body mass from 2 g to 196 g (Nowak, 1994). These two groups of bats are commonly thought to share the same common ancestor, being a monophyletic mammalian order; however, as early as Linnaeus (1758), it was noticed that perhaps Microchiroptera and Megachiroptera should not be grouped together. Based on anatomical similarities, Linnaeus suggested that Megachiroptera should be grouped with Primates. This potential phylogenetic relationship between Megachiroptera and Primates was largely ignored until Smith and Madkour (1980) described similarities in the structure of the glans penis of Primates and Megachiroptera that are not found in other Eutherian mammals. But if Megachiroptera are related to Primates, to which mammalian group are the Microchiroptera related? Chiropteran phylogeny appears to be one of the few remaining unsolved puzzles of mammalian phylogeny.

2.2. The ‘Flying Primate’ hypothesis

Pettigrew (1986) examined the neuroanatomy of the retinotectal pathway in both Megachiroptera and Microchiroptera and found that Megachiroptera have an advanced retinotectal pathway with a vertical hemidecussation, a feature previously found only in Primates, whereas this is not seen in Microchiroptera. This was the beginning of the ‘Flying Primate’ hypothesis suggesting a diphyletic origin of the Chiropteran order and implying that mammalian flight evolved twice. This initial neuroanatomical study was then supported by a larger study (Pettigrew et al., 1989), that examined the neurological traits and wing morphology in Chiroptera. It was found that Megachiroptera and Primates share a variety of complex details in the organization of neural pathways that are not found in any other mammalian group, including Microchiroptera. The morphological features of the forelimbs in
Chiroptera, being related to flight, was thought to suggest a monophyletic origin for this mammalian order, yet measurements of the metacarpals and phalanges of the wings of Microchiropterans and Megachiropterans suggest a paraphyletic origin (Pettigrew et al., 1989). It is possible that the numerous flight-associated musculoskeletal and molecular features may have been derived independently and convergently (Pettigrew and Kirsch, 1998). Pettigrew et al. (1989) suggested that Megachiropterans evolved from the acknowledged sister group to Primates, the Dermopterans (flying lemurs). Thus, the Megachiropterans are not suggested to be literally ‘flying primates’, but are thought to have been derived from the gliding sister group to Primates, the Dermopterans.

Despite a wealth of information arising from studies of Megachiropterans and Microchiropterans suggesting a diphyletic origin of these two groups, there is opposition to this idea, mainly arising from studies of nuclear DNA and mitochondrial RNA (Teeling et al., 2002, 2005; Murphy et al., 2001a; Kirsch et al., 1995; Hutcheon et al., 1998; Adkins and Honeycutt, 1991, 1993; Ammerman and Hillis, 1992; Stanhope et al., 1992, 1996; Knight and Mindell, 1992; Liu and Miyamoto, 1999; Porter et al., 1996). Molecular studies seem to be the last “stronghold” supporting the monophyletic origin of the two groups of bats.

Characters linking Megachiropterans and Microchiropterans are all flight related and since genetic sequence structure must also respond to pressures and processes that may lead to convergent evolution, it is possible that the genetic sequences used to group the Chiroptera have undergone convergent changes in base composition as a result of mutational biases heightened by the intense metabolic demands of flight. Similar genetic convergence has been found to occur between rhinolophoid bats and birds for the same reason (Pettigrew, 1994; Pettigrew and Kirsch, 1998). This idea of convergent evolution of complex traits has also been exposed in two distinct species of squid (Pankey et al., 2014). The bioluminescent organs of Euprymna scolopes and Uroteuthis edulis evolved separately yet Pankey et al. (2014), found that they evolved in a remarkably similar way possibly due to the high metabolic demands of producing light. Both Megachiroptera and Microchiroptera have ‘inordinately high AT bias’ in their genetic material and when the genetic sequence is tested in GC-rich areas of the genome, the molecular link between Microchiroptera and Megachiriptera is weakened (Pettigrew and Kirsch, 1998).

The other problem with the molecular studies is the fact that the genetic and morphological data are not in agreement with each other. This is acknowledged in several
studies in support of Chiropteran monophyly (Adkins and Honeycutt, 1991; Ammerman and Hillis, 1992; Liu and Miyamoto, 1999; Teeling et al., 2000). This problem brings the molecular data into question due to the fact that genetic material codes morphological development and the disagreement between the two data sets further strengthens the argument of genetic sequences having undergone convergent changes in base composition as a result of mutational biases heightened by the intense metabolic demands of flight.

Interestingly, in a DNA study suggesting bat monophyly, the differences between morphological and molecular evidence are acknowledged as well as the fact that the two groups of bats have differing visual systems (Teeling et al., 2000). Megachiropterans have highly developed visual and olfactory senses and systems for foraging and obstacle avoidance, whereas in Microchiropterans the auditory senses and systems are elaborate (Pettigrew, 1989). Primates also have a well-developed visual sense and neural pathways related to vision are shared between Megachiropterans and Primates (Pettigrew, 1986; Pettigrew et al., 1989, 2008; Mindell et al., 1991).

Despite the acknowledged numerous similarities between the Megachiropteran central nervous system and that of the Primates, Megachiropterans have not been proposed to be Primates, rather, they are envisaged to be a branch of the Dermopterans, an acknowledged sister group to Primates (Baker et al., 1991; Nowak, 1999; Eizirik et al., 2004). Thus, in order to understand the similarities between Megachiropterand and Primate morphology, in addition to Primates, the close relatives of the Primates, and the various primate lineages, termed the Euarchontoglires, must be studied in detail. Traditionally, Primates have been split into Prosimians and Simians (anthropoids). Prosimian Primates are the earliest branch of the primates and, in the Chiropteran diphyletic scenario, should possess a central nervous system with the most similarities to the Megachiropterans. Other members of the Euarchontoglires include the Scandentia (the tree shrews), the Lagomorphs (the rabbits and hares), and the Rodents (Asher et al., 2009; Murphy et al., 2001b).

2.3. So to whom are the Microchiropterans related if not Megachiropterans?

For the sake of discussion, if we assume Chiropteran paraphyly, with the Megachiropterans being a derivative of the Dermopteran sister group to Primates, where does this place the Microchiropterans among the mammalian radiation? Most global mammalian
phylogenies tend of place the Chiropterans as a whole in a grouping with the insectivores, Cetartiodactyls, perissodactyls and carnivores (Arnoson et al., 2002; Asher et al., 2009; Lee and Camens, 2009). Of this cluster of the mammalian radiation, the insectivores hold the most promise for a potential sister group to the Microchiropterans. The Insectivora, from which a potential sister group to the Microchiropterans may arise, also has a problematic classification and systematics and is currently considered to be paraphyletic (Symonds, 2005; Jones and Teeling, 2006). The history of the study of the Insectivora order has been plagued by arguments over its composition and true relatedness. The key reason behind the past uncertainties regarding the evolutionary relationships of this order stemmed from the large numbers of primitive Eutherian character states as well as a lack of unifying derived character states. It is really only the individual Insectivoran families that possess derived character states. For a long time, Insectivora was the order into which scientists put species of questionable lineage, or those mammals that were characterized by the lack of distinctive features possessed by other mammals. Hence, it was called the ‘waste-basket’ order containing all the ‘leftovers’ of mammalian classification (Symonds, 2005). Most of the older estimates of Insectivoran phylogeny, based on morphology, assumed Insectivoran monophyly, whereas more recent molecular analysis argues polyphyly. All the molecular-based analyses outlined by Symonds (2005) agree on the fact that the Insectivora is not a legitimate order and that it is, in fact, paraphyletic. Stanhope et al. (1998) used molecular analysis on 6 proposed Insectivoran families, as well as an additional 37 taxa, to conclude that golden moles and tenrecs should fall into the Afrotheria clade which include elephants, sirenians, hyraxes, aardvarks and elephant shrews. The Afrotheria clade as a whole is strongly supported by morphological data such as tooth eruption ages and vertebral variability (Asher et al., 2009) as well as molecular data (Tabuce et al., 2008); however, relationships within the Afrotherian clade are not yet unanimously resolved. Asher et al. (2009) divides the clade into Paenungulata (Proboscidea, Sirenia and Hyracoidea) and Afroinsectiphilia (Tubulidentata, Macroscelidea, Tenrecidae and Chrysochloridae). Thus, in order to solve the problem of Microchiropteran phylogenetic affinities in the diphyletic scenario, the Afroinsectiphilia (Asher et al., 2009) and the various families of the insectivores (Soricidae, the shrews; Solenodontidae, the solenodons; Talipidae, the moles; and Erinaceidae, the hedgehogs and gymnures; as defined by Symonds, 2005) need to be examined in detail.
Molecular evidence has placed three of the Microchiropteran families into Laurasiatheria which includes Soricidae and Erinaceida (Teeling et al., 2005). Murphy et al. (2001b) acknowledges the relationship between Soricomorpha (shrews and moles) and Chiroptera based on molecular findings. Genetic reconstruction grouped the Microchiropteran family, Rhinolophoidea, with Soricidae (Guo et al., 2013). In addition, Microchiroptera and insectivores share two rare behaviours: torpor and echolocation. Mammalian torpor is characterized by substantial reductions of body temperature, metabolic rate and water loss (Geiser, 2004). In addition to reducing energy expenditure and water loss, use of torpor also appears to prolong life span. Torpor has been observed in Microchiropterans living in arid areas in Australia (Geiser, 2004), as well as in shrews belonging to the insectivore family Soricidae (Symonds, 2005). Species from the insectivore families Solenodontidae and Soricidae are also known to echolocate (Symonds, 2005). Siemers et al. (2009) concluded that the high-pitched laryngeal ‘twittering’ in Soricidae shrews parallels that of Microchiroptera echolocation. They suggest that shrews use the echoes for identifying routes through their habitat or for probing habitat type. Even though one genus of Megachiroptera (Rousettus) echolocates, the sounds are not produced by the larynx, as in Microchiroptera and shrews, but by ‘clicking’ the tongue on one side of the mouth and then the other. This ‘clicking’ mechanism of echolocation is said to have evolved independently of the Microchiroptera system and resembles that of cave dwelling birds (Roberts, 1975). It would appear then, that the most likely candidates of the Insectivore lineage that may form a potential sister group to the Microchiropterans are the shrews (Soricidae). The similarities in the rarely occurring behaviours of torpor and laryngeal echolocation support this suggestion and warrant extensive analysis of the neuroanatomy of the insectivores.

2.4. Is brain morphology useful in understanding phylogenetic history?

The brain is a structure that is both plastic and conservative in terms of its evolution and modification of specific character states. This is likely the result of the pleiotrophic effect of the genes involved in the normal development and function of the brain across vertebrates, meaning that a change in morphophysiology brings a high risk as it could lead to a lethal mutation (Galis, 1999). Given this, the use of neural structures is useful in assisting in the determination of phylogenetic affinities. Neurological data has led to the solution of
phylogenetic puzzles in many different vertebrate classes, most notably in fish (e.g. characid, siluriform and gymnotiform relations, Fink and Fink, 1981). Three different phylogenetic reconstruction techniques can be used on the brain data: PAUP (parsimony analysis, Swofford, 2001); MacClade (Maddison and Maddison, 1992); and distance methods such as UPGMA. There are other methods such as Bayesian, maximum likelihood and logdet, but these have been developed specifically for DNA sequence data and have not yet been adapted to deal with morphological data. These techniques permit the discovery of the most parsimonious tree that could have given rise to the array of characters across the sample of taxa. In previous studies, brain data have given highly consistent and parsimonious trees which initiated the debate regarding Chiropteran phylogenetic affinities (Pettigrew, 1986). MacClade has a very useful feature, where characters can be tracked through the tree generated by PAUP and the consequences of removing characters or taxa or changing branching patterns can be tested. All manipulations can be carried out with immediate feedback about their consequences for the generated trees. The distance methods can be included to check for convergence, a calculation that is based upon the fact that four taxa are separated by six distances, with convergence revealed when a direct distance is anomalously short when compared with an indirect measure of the same distance based upon the geometry of the other five. An example of this manipulation is demonstrated in Dell et al. (2010), where the shortest and most parsimonious phylogenetic tree (using 82 neural characters) suggested a diphyletic origin for the Chiroptera and placed Megachiropterans within the Euarchontoglires and placed the Microchiropterans with Insectivora (Dell et al., 2010).

Changes in the complexity of neural system structure, in terms of the number of discrete and readily identifiable nuclear subdivisions, appear to occur during evolutionary events leading to the establishment of a new mammalian order (Manger, 2005). It appears, in general, that all progeny of the established order will retain the same compliment of subdivisions of all the systems, irrespective of the subsequently evolved size of the brain, phenotype or life history of the species comprising the order (Manger, 2005). This means that each mammalian order will exhibit a unique complement of nuclei, or other subdivision types of specific neural systems such as cortical areas, that is typical of all its members. This hypothesis has been supported by numerous studies across a range of mammalian species (Da Silva et al., 2005; Dwarika et al., 2007; Moon et al., 2007; Bhagwandin et al., 2008; Gravett et al., 2009; Bux et al., 2010; Dell et al., 2010; Kruger et al., 2010; Calvey et al., 2013) and the data generated appears to conform, to the generally accepted mammalian phylogenies.
(Asher et al., 2009) to the exception of the Chiropterans (Dell et al., 2010). Thus, if Microchiropterans and Megachiropterans are monophyletic, there should be no specific differences in the number and complement of the subdivisions forming discrete neural systems within their brains. Yet in studies comparing the two groups of bats to each other and to other mammals, Megachiropterans have been found to possess between 7 to 11 discrete nuclei that are not found in Microchiropterans, and these nuclei align the Megachiropterans to Primates, tree shrews, rabbits and Rodents (Maseko et al., 2007; Dell et al., 2010). The nuclear systems of the Microchiropterans studied closely resembled the Erinaceomorpha and Soricomorpha of Insectivora (Maseko and Manger, 2007; Kruger et al., 2010a) as originally predicted (Pettigrew et al., 1989). The difficulty with these prior studies of Chiropteran affinities within the mammals is the sparseness of the data. Very little data regarding neural systems within a range of Afrotherians, insectivores, Primates, Lagomorphs and tree shrews is available. This gap in the literature means that interpretation of the data related to the question of Chiropteran phylogenetic affinities is not as strong as it might be. This is of major concern, and filling these gaps in our knowledge forms the central pillar of this thesis.

2.5. Which neural systems to investigate?

When specifically examining the problem of Chiropteran phylogeny using the organization of the central nervous system, a series of distinct caveats must be put in place first. Both Megachiropterans and Microchiropterans have powered flight. In this sense, any neural systems specifically related to the control of movement and sensation from the wing, must be eliminated from the analysis, as this may bring convergent characteristics into the analysis leading to a potentially false positive monophyletic conclusion. The Megachiropterans are well known to have a well-developed visual system, as well as a substantive olfactory system, in comparison to the often microophthalmic and microsmatic Microchiropterans. Therefore, substantial reliance on characteristics of the visual and olfactory systems may lead to a false positive conclusion supporting the diphyletic hypothesis. Microchiropterans have the distinct ability to perform and interpret laryngeal echolocation. In this sense, neural systems involved in the direct production, reception and interpretation of the echolocation vocalizations must also be excluded from the analysis to prevent a false positive conclusion supporting the diphyletic hypothesis. Thus, when investigating chiropteran phylogenetic relationships, features of the olfactory, visual,
somatomotor and auditory systems should be excluded from the analysis in order to create an analysis free from potential bias. In order to meet this series of stringent requirements, the current series of studies has elected to investigate the neural organization of a range of systems that are not directly involved in the production of movement or in the direct sensing of the environment. These systems, the cholinergic, catecholaminergic, serotonergic and orexinergic, are involved in the modulation of neural activity across vast regions of the central nervous system. Moreover, each of these systems is constituted of a number of distinct nuclei that can be readily identified with immunohistochemical staining techniques, making the identification of specific character states quite reliable. Moreover, these systems are found in all mammals, and indeed all vertebrates, and thus Chiropteran phylogeny can be assessed globally within mammals.

2.5.1. The cholinergic system

The acetylcholine-synthesizing enzyme, choline acetyltransferase, is a reliable marker for the localization of cholinergic neurons in the vertebrate CNS using immunohistochemical methods (e.g. Bhagwandin et al., 2006). The nuclei of the cholinergic system can be divided into five main groups according to their location, connections and morphology; striatal, basal forebrain, diencephalic, pontomesencephalic and cranial motor nerve nuclei (e.g. Woolf, 1991; Calvey et al., 2013). The cholinergic system is thought to be involved in many functions related to the modulation of behaviour, attention, learning and memory, the sleep-wake cycle, generating conscious experiences and controlling REM sleep (Woolf, 1991). The cholinergic system in most mammals is made up of approximately 36 specific nuclei, although the number and complement of these nuclei is known to differ across mammalian species (Maseko et al., 2007; Dell et al., 2010; Calvey et al., 2013). In this sense, the nuclei of the cholinergic system will allow the development of a database of characters that are, at worst, minimally influenced by the specializations of both Megachiroptera and Microchiroptera.

2.5.2. The catecholaminergic system

The catecholaminergic system is involved in the regulation of neuroendocrine functions such as reproduction, growth, lactation and stress. It is also involved in cognitive
functions like motor planning, learning and memory, cognitive flexibility and reward (Smeets and Gonzalez, 2000). Tyrosine hydroxylase is the enzyme responsible for the production of L-DOPA, the precursor of dopamine, noradrenalin and adrenalin. Cells producing this enzyme represent the catecholaminergic system in the brains of vertebrates and form identifiable nuclear complexes that extend from the olfactory bulb to the spinomedullary junction (Smeets and Gonzalez, 2000; Calvey et al., 2013). Tyrosine hydroxylase immunoreactive neurons are found in the neocortex of humans (Benavides-Piccione and De Felipe, 2007) and, although uncommon, have previously been found in the cortex of other species such as the rat (Kosaka et al., 1987; Wachter et al., 2014), hamster (Vincent, 1988), gerbil and rabbit (De Felipe et al., 2007), localising species-specific layers of the neocortex.

All three catecholamines are involved in antinociception as well as sympathetic outflow. Dopamine is also involved in motor control and reward based learning and memory (Smeets and Gonzalez, 2000). Noradrenalin is involved in endocrine control and is the main neurotransmitter of the locus coeruleus complex, which is associated with the control of wakefulness (Siegel, 2004). Both noradrenalin and adrenalin control cardiovascular mechanisms (Smeets and Gonzalez, 2000). Within the catecholaminergic system, approximately 30 nuclei are commonly identified across mammalian species, although the number and complement of nuclei does differ across mammalian species (Manger et al., 2004; Maseko et al., 2007; Dell et al., 2010; Calvey et al., 2013). These nuclei have been given a specific A1-17 and C1-3 terminology (Dahlstrom and Fuxe, 1964), although more recent reviews have called for a change to this terminology (Smeet and Gonzalez, 2000). Despite this call for change, the traditional terminology is quite useful and succinct, especially when one is planning a cladistic analysis. Thus, the nuclei of the catecholaminergic system, like the nuclei of the cholinergic system, will form a database amenable to cladistic analysis, and for the most part, free from bias related to Chiropteran sensorimotor specializations.

2.5.3. The serotonergic system

The neurons producing serotonin, through their projections and post-synaptic action, are involved in the control of mood, eating, sleep and arousal (Tork, 1990). Serotonin is also involved in pain modulation, as well as the ‘fine-tuning’ of locomotor networks (Smeets and Gonzalez, 2000). The neurons forming the nuclei of the serotonergic system are, apart from
one exception in mammals (Manger et al., 2002), all located in the brainstem (midbrain, pons and medulla oblongata). The serotonergic nuclei are normally parcellated into a rostral and caudal cluster, with the rostral cluster being composed of 9 distinct nuclei, while the caudal cluster is composed of 5 distinct nuclei (Tork, 1990; Maseko et al., 2007; Dell et al., 2010; Calvey et al., 2013). Thus, along with the cholinergic and catecholaminergic systems, analysis of the serotonergic system raises the count of potential character states of the central nervous system in mammals, free from Chiropteran sensorimotor specializations, that can be used for unbiased cladistic analysis up to approximately 80 characters (bearing in mind species and order specific variations in nuclear organization of these systems, e.g. Dell et al., 2010; Calvey et al., 2013).

2.5.4. The orexinergic system

Specific neurons with the hypothalamus produce the neuromodulatory peptide orexin-A (De Lecea et al., 1998; Kukkonen et al., 2002). These orexinergic neurons, in most mammals, have been shown to form three distinct clusters in the perifornical and lateral hypothalamic regions, a main cluster (perifornical), a zona incerta cluster (dorsal lateral hypothalamus) and optic tract cluster (ventrolateral hypothalamus) (e.g. Nixon and Smale, 2007). This system is implicated in arousal and the regulation of the sleep/wake cycle, blood pressure, neuroendocrine functions, body temperature regulation, locomotor function and some respiratory functions (Furguson and Samson, 2003; Kirouac et al., 2005; Mintz et al., 2001; Zeitzer et al., 2003; Takakusaki et al., 2005). As with the other systems outlined above, while for the most part these three clusters are similar across mammals, variations in the number and complement of these clusters have been observed (e.g. Kruger et al., 2010b; Dell et al., 2013; Maseko et al., 2013). So while this system only adds a few more characters to the cladistic analysis of Chiropteran affinities, these characters are again, for the most part, not directly involved in Chiropteran sensorimotor specializations.

2.6. Why is understanding Chiropteran affinities important?

Bhagwandin et al. (2006), emphasises the importance of comparative observation and evolutionary interpretation for our understanding of animal models of human mental illness and caution against direct extrapolation from animal model that are not clearly understood.
The noradrenergic, dopaminergic, serotonergic and cholinergic systems consist of nuclei with widely distributed, ascending projections to the neocortex. These neurons play critical roles in regulating cortical function and disturbances in these systems are central to major psychiatric disorders, such as depression, schizophrenia and bipolar disorder (Aston-Jones and Cohen, 2005). The most common animal model used for psychiatric testing are the laboratory rat and mouse models, both Murid rodents which, as neuroanatomical analysis has revealed, have many differences to humans. For example, Murid rodents, including laboratory rats and mice have an unusual locus coeruleus nucleus, which is different to all other Rodents and different to Primates (Kruger et al., 2011). The locus coeruleus neurons are located in the pons, are the principle site for noradrenalin production, and have efferent projections throughout the central nervous system (Aston-Jones and Cohen, 2005). One of the functions of these neurons is arousal (Foote et al., 1980). Arousal plays an important role in mood as it is closely related to sleep, attention, anxiety, stress and motivation (Aston-Jones and Cohen, 2005). Using extracellular recordings, it was found that the neurons of the locus coeruleus in the rat respond differently to sensory stimulation when compared to the same neurons of the squirrel monkey (Foote et al., 2005) which is not surprising considering the known anatomical differences. Perhaps finding a sister group to Primates, that shares far more neuroanatomical and neurophysiological features in common with the Primates, could provide a more accurate model for comparative testing and translational research regarding both normal and abnormal human mental function. A small Megachiroptera species would be convenient in a laboratory setting and may provide results more amenable to translational research than the laboratory Murid Rodents.
Chapter three: Nuclear organisation of some immunohistochemically identifiable neural systems in three Afrotherian species—*Potomogale velox*, *Amblysomus hottentotus* and *Petrodromus tetradactylus*.

3.1. Introduction

The Afrotherian mammalian cohort is comprised of what may be considered to be a very unusual mammalian grouping, one whose relationships have mainly been resolved on molecular rather than morphological grounds (e.g., van Dijk et al., 2001; Arnason et al., 2008; Hallström and Janke, 2008; Prasad et al., 2008; Asher et al., 2010; Dumbacher et al., 2012; McCormack et al., 2012). Within the Afrotheria are species that are fully aquatic (such as manatees and dugongs), extremely large (elephants), fossorial (golden moles), semi-aquatic (otter shrews), insectivorous (aardvarks, elephants shrews and tenrecs) and omnivorous (hyraxes). These species present with a range of body sizes, phenotypes, habitats and life histories, and are considered an ancient radiation of the Eutherian mammals. In this sense, the study of neural systems that are in general quite conservative in their evolution (e.g., Dell et al., 2010) is of interest, as a record of the changes, or indeed lack of changes, may reflect the potential evolutionary plasticity/malleability of the mammalian brain.

The cholinergic, catecholaminergic and serotonergic systems, have been studied previously in two species belonging to the Afrotheria—the rock hyrax, *Procavia capensis* (Gravett et al., 2009) and the eastern rock elephant shrew, *Elephantulus myurus* (Pieters et al., 2010). While for the most part, these systems were similar to those reported in other mammals, several specific differences were noted. In the rock hyrax, the anterior nuclei of the dorsal thalamus were found to contain cholinergic neurons, there were cholinergic parvocellular neurons forming a shell around the typical laterodorsal tegmental and penduclopontine tegmental nuclei, and the locus coeruleus proper was observed to be made up of very few cells (Gravett et al., 2009). In contrast to the rock hyrax, cholinergic neurons were observed in the both superior and inferior colliculi and the cochlear nuclei, and the catecholaminergic anterior hypothalamic group (A15d) was missing from the elephant shrew (Pieters et al., 2010). Observations of the orexinergic neurons in the rock hyrax showed a typically mammalian orexinergic system in this species apart from a dense innervation of the anterior nuclei of the dorsal thalamus (Gravett et al., 2011).
In the present study we extend the observations made on these systems in the Afrotheria by examining, using immunohistochemical means, the cholinergic, catecholaminergic and serotonergic in the brains of the giant otter shrew (*Potomogale velox*), the Hottentot golden mole (*Amblysomus hottentotus*) and the four-toed sengi (*Petrodromus tetradactylus*) and the orexinergic system in the brains of the giant otter shrew and four-toed sengi. The giant otter shrew is a semi-aquatic member of the Tenrecidae that is found in the swamps, streams and forest pools of central Africa. The Hottentot golden mole is a small fossorial golden mole found in the KwaZulu-Natal and Eastern Cape region of South Africa and inhabits a range of ecosystems from temperate to tropical dry forests, dry savanna, through to urban areas and introduced vegetation. The four-toed sengi is a large elephant shrew found in tropical to subtropical moist montane forests as well as moist savanna throughout large areas of sub-Saharan Africa.

As far as the authors are aware, the systems described in the current study have not been examined in any of these three species. In addition to the prior studies of Afrotherian species, the data from the current study provides a broader base for comparison within the Afrotheria and across mammals in general. This may help to elucidate the manner in which systems level changes occur across mammalian brains as features such as brain size, phenotype, life histories, phylogenetic relationships, and time since evolutionary divergence, change, and lead to a better understanding of the partition between the phylogenetic and functional signals they may carry (Manger, 2005).

### 3.2. Materials and Methods

Brains from *Potomogale velox*, *Amblysomus hottentotus* and *Petrodromus tetradactylus* were collected for the present study. Permits were obtained from the relevant wildlife authorities in the Democratic Republic of Congo and South Africa for the capture and euthanasia of the animals from their natural habitat. 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 weight appropriate doses of sodium pentobarbital (200mg sodium pentobarbital/kg, i.p.). In the current study a single *P. velox* (body mass – 540 g; brain mass – 3.46 g; an adult male), two *A. hottentotus* (body masses – 72 g, 86 g; brain masses – 1.3 g, 1.2 g; both adult males), and three *P. tetradactylus* (body masses – 132 g, 138 g, 124 g; brain masses – 3.01 g, 2.80 g, 2.95 g; all
adult males) were used. 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 0.1M PB followed by equilibration in 30% sucrose in 0.1M PB. A one in six series of sections, cut at 50 µm thickness in the coronal plane, was used for Nissl, myelin, choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), serotonin (5HT) and orexin (hypocretin/OxA). An additional golden mole brain was sectioned in the sagittal plane to demonstrate the flexure of the brainstem in this species (Fig. 3.1). 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 in 1% cresyl violet to reveal cell bodies. Myelin sections were first stored in 5% formalin for two weeks at 4°C then mounted on 1.5% gelatine-coated slides and subsequently stained with silver solution to reveal myelin sheaths (Gallyas, 1979).

For the immunohistochemical staining each section was treated with endogenous peroxidase inhibitor (49.2% 0.1M PB: 1.6% of 30% H₂O₂) for 30min and subsequently subjected to three 10min 0.1M PB rinses. Sections were then preincubated for 2 h, at room temperature, in blocking buffer (3% normal goat serum, NGS, for TH, serotonin and orexin sections; 3% normal rabbit serum, NRS, for ChAT sections, 2% bovine serum albumin for all sections and 0.25% Triton-X in 0.1M PB for all sections). This was followed by three 10min rinses in 0.1M PB. The sections were then placed at 4°C under constant gentle shaking in primary antibody solution that contained the appropriate diluted primary antibody in blocking buffer (see above) for 48h. 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 revealed the putative catecholaminergic neurons. Serotonergic neurons were revealed using anti-serotonin (AB938, Millipore, raised in rabbit) at a dilution of 1:10000. Orexinergic neurons were revealed using anti-Orexin A (AB3704, Millipore, raised in rabbit) at a dilution of 1:10000. 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, serotonin and orexin 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 2h at room temperature. This was followed by three
10 min rinses in 0.1M PB, after which sections were incubated for 1h 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% dianaminobenzidine (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 reconstruction without obscuring the immunopositive neurons. Precipitation 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. The controls employed in this experiment included the omission of the primary antibody and the omission of the secondary antibody in selected sections for which no staining was evident.

Sections were examined under a low power stereomicroscope and using a camera lucida, the architectonic borders of the sections were traced following the Nissl and myelin 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. The nomenclature used for the cholinergic system was adopted from Woolf (1991), Limacher et al. (2008), Bhagwandin et al. (2008), Gravett et al. (2009) and Pieters et al. (2010), the catecholaminergic from Hökfelt et al. (1984), Smeets and Gonzalez (2000), Limacher et al. (2008), Bhagwandin et al. (2008), Gravett et al. (2009) and Pieters et al. (2010), the serotonergic from Törk (1990), Limacher et al. (2008), Bhagwandin et al. (2008), Gravett et al. (2009) and Pieters et al. (2010), and the orexinergic from Kruger et al. (2010), Bhagwandin et al. (2011) and Gravett et al. (2011). While we used the standard nomenclature for the catecholaminergic system in this paper, we realise that the neuronal groups revealed with tyrosine hydroxylase immunohistochemistry may not directly correspond with these nuclei as has been described in previous studies by Dahlström and Fuxe (1964), Hökfelt et al. (1976), Meister et al. (1988), Kitahama et al. (1990, 1996), and Ruggiero et al. (1992); however, given the striking similarity of the results of the tyrosine hydroxylase immunohistochemistry to that seen in other mammals, we feel this terminology is appropriate. Clearly further studies in these Afrotherian species with a wider range of antibodies, such as those to phenylethanolamine-N-methyltransferase (PNMT), dopamine-β-hydroxylase (DBH) and aromatic L-amino acid decarboxylase (AADC) would be required to
fully determine the implied homologies ascribed in this study (e.g., Weihe et al., 2006). We address this potential problem with the caveat of putative catecholaminergic neurons where appropriate in the text.

**Abbreviations**

III – oculomotor nucleus

IV – trochlear nucleus

Vmot – motor division of trigeminal nerve nucleus

Vsens – sensory division of trigeminal nerve nucleus

VI – abducens nucleus

VIIId – 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

5n – trigeminal nerve

7n – facial nerve

A1 – caudal ventrolateral medullary tegmental nucleus

A2 – caudal dorsomedial medullary nucleus

A4 – dorsolateral division of locus coeruleus

A5 – fifth arcuate nucleus

A6c – compact portion of locus coeruleus

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
ACN – cholinergic neurons of the amygdala
Amyg – amygdala
AON – anterior olfactory nucleus
AP – area postrema
B9 – supralemniscal serotonergic nucleus
bic – brachium of the inferior colliculus
C1 – rostral ventrolateral medullary tegmental group
C2 – rostral dorsomedial medullary nucleus
C3 – rostral dorsal midline medullary nucleus
C – caudate nucleus
c – cerebral aqueduct
Cb - cerebellum
cc – corpus callosum
CCN – cholinergic neurons of the cerebral cortex
C1 - claustrum
CLi – caudal linear nucleus
CN – deep cerebellar nucleus
Co – cochlear nuclei
CoCN – cholinergic neurons of the cochlear nuclear complex
csc – commissure of the superior colliculi
CVL – caudal ventrolateral serotonergic group
dh – dorsal horn of spinal cord
df – dorsal funiculus of spinal cord
Diag.B – diagonal band of Broca
DRc – dorsal raphe, caudal division
DRd – dorsal raphe, dorsal division
DRif – dorsal raphe, interfascicular division
DRI – dorsal raphe, lateral division
DRp – dorsal raphe, peripheral division
DRv – dorsal raphe, ventral division
DT – dorsal thalamus
EPL – external plexiform layer of olfactory bulb
EW – Edinger-Westphal nucleus
f– fornix
fr – fasciculus retroflexus
GC – central grey matter
GCLi – inner granular cell layer of olfactory bulb
GCLo – outer granular cell layer of olfactory bulb
GL – glomerular layer of olfactory bulb
GP – globus pallidus
Hbc – habenular commissure
Hbl – lateral habenular nucleus
Hbm – medial habenular nucleus
hc – hippocampal commissure
HCN – cholinergic neurons of the hippocampus
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
ICCN – cholinergic neurons of the inferior colliculus
io – inferior olivary nucleus
IP – interpeduncular nucleus
Is.Call/TOL – islands of Calleja and the olfactory tubercle
LDT – laterodorsal tegmental nucleus
LGv – ventral lateral geniculate nucleus
LOT – lateral olfactory tract
LV – lateral ventricle
MB – mammillary bodies
Mc – main cluster of orexinergic neurons
MCL – mitral cell layer of olfactory bulb
mcp – middle cerebellar peduncle
MnR – median raphe nucleus
mlf – medial longitudinal fasciculus
mtf – medullary tegmental field
N.Acc – nucleus accumbens
N.Amb – nucleus ambiguus
N.Bas – nucleus basalis
NEO – cerebral neocortex
OB – olfactory bulb
OC – optic chiasm
OCN – cholinergic neurons of olfactory bulb
ONL – olfactory nerve layer of olfactory bulb
OT – optic tract
OTc – optic tract cluster of orexinergic neurons
OV – olfactory ventricle
P – putamen nucleus
PBg – parabigeminal nucleus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PC</td>
<td>cerebral peduncle</td>
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<tr>
<td>pc</td>
<td>posterior commissure</td>
</tr>
<tr>
<td>PCCN</td>
<td>cholinergic neurons of the piriform cortex</td>
</tr>
<tr>
<td>PIR</td>
<td>piriform cortex</td>
</tr>
<tr>
<td>PPT</td>
<td>pedunculopontine nucleus</td>
</tr>
<tr>
<td>PTa</td>
<td>pretectal area</td>
</tr>
<tr>
<td>py</td>
<td>pyramidal tract</td>
</tr>
<tr>
<td>PVG</td>
<td>periventricular grey matter</td>
</tr>
<tr>
<td>PVL</td>
<td>periventricular layer of olfactory bulb</td>
</tr>
<tr>
<td>pVII</td>
<td>preganglionic motor neurons of the superior salivatory nucleus or facial nerve</td>
</tr>
<tr>
<td>pIX</td>
<td>preganglionic motor neurons of the inferior salivatory nucleus</td>
</tr>
<tr>
<td>R</td>
<td>reticular nucleus of the dorsal thalamus</td>
</tr>
<tr>
<td>Rmc</td>
<td>magnocellular division of red nucleus</td>
</tr>
<tr>
<td>RMg</td>
<td>raphe magnus nucleus</td>
</tr>
<tr>
<td>ROb</td>
<td>raphe obscurus nucleus</td>
</tr>
<tr>
<td>RPa</td>
<td>raphe pallidus nucleus</td>
</tr>
<tr>
<td>RVL</td>
<td>rostral ventrolateral serotonergic group</td>
</tr>
<tr>
<td>S</td>
<td>septal nuclear complex</td>
</tr>
<tr>
<td>SC</td>
<td>superior colliculus</td>
</tr>
<tr>
<td>SCCN</td>
<td>cholinergic neurons of the superior colliculus</td>
</tr>
<tr>
<td>scp</td>
<td>superior cerebellar peduncle</td>
</tr>
<tr>
<td>Sep.L</td>
<td>lateral septal nucleus</td>
</tr>
<tr>
<td>Sep.M</td>
<td>medial septal nucleus</td>
</tr>
<tr>
<td>so</td>
<td>superior olivary nucleus</td>
</tr>
<tr>
<td>spV</td>
<td>spinal trigeminal tract</td>
</tr>
<tr>
<td>vh</td>
<td>ventral horn of spinal cord</td>
</tr>
<tr>
<td>VPO</td>
<td>ventral pontine nucleus</td>
</tr>
<tr>
<td>xscp</td>
<td>decussation of the superior cerebellar peduncle</td>
</tr>
<tr>
<td>zi</td>
<td>zona incerta</td>
</tr>
<tr>
<td>Zic</td>
<td>zona incerta cluster of orexinergic neurons</td>
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Figure 3.1: Photomontage of a Nissl-stained parasagittal section of the brain of the Hottentot golden mole showing the unusual appearance of the diencephalon and brainstem. It appears that a foreshortening and widening of the cranium is related to the large (mesencephalic?) flexure of the subtelencephalic brain, resulting in the pons lying beneath the midbrain, and the inferior colliculus lying above the cerebellum. Scale bar = 1 mm. See list for abbreviations.
3.3. Results

The current study describes and defines the nuclear organization of the cholinergic, catecholaminergic and serotonergic neural systems in three species of Afrotherian mammals (giant otter shrew – *Potomogale velox*, the Hottentot golden mole – *Amblysomus hottentotus*, and the four-toed sengi – *Petrodromus tetradactylus*) and the orexinergic system in the brain of the giant otter shrew and four-toed sengi, species in which these systems have not been previously described. For the most part, the systems investigated exhibited an organization that may be thought of as typically mammalian; however, we did observe many points of departure from this typical organization. One central observation of interest in the current study, and of importance in the interpretation of the organization of the systems studied, was the noticeable folding, or compression, of the brainstem of the golden mole. Within the brain of the golden mole, the axial alignment of the brainstem was foreshortened in such a manner that the pontine region was observed to lie beneath the midbrain, and not caudal to it as seen in most other mammals (Fig. 3.1).

3.3.1. 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 only identified in the Hottentot golden mole (Figs. 3.2, 3.3, 3.4, 3.5). In addition to the cortical cholinergic interneurons, the golden mole also evinced cholinergic interneurons in the olfactory bulb, piriform cortex, amygdala and hippocampus (Fig. 3.5). Interestingly, the four-toed sengi possessed cholinergic interneurons in both superior and inferior colliculi and the cochlear nuclear complex (Fig. 3.4L-P), as described for the eastern rock elephant shrew (Pieters et al., 2010).
3.3.1.1 Cholinergic interneurons in the telencephalon of the golden mole

Previous studies have shown that while cholinergic interneurons are observed in the cerebral cortex of certain species, these are most readily observed in rodents of the Murid family (Bhagwandin et al., 2006; Kruger et al., 2012). Within the Hottentot golden mole, there was an abundance of typically bipolar cholinergic neurons (Fig. 3.3A-K), oriented orthogonal to the pial surface of the cerebral cortex, throughout all layers of the cortex. Similar cholinergic interneurons were also observed throughout the piriform cortex. Interestingly, this species also had cholinergic interneurons in the glomerular, inner granular and periventricular layers of the olfactory bulb, as well as in the CA region of the hippocampus and throughout the amygdala nuclear complex (Fig. 3.5). Similar cholinergic neurons were not observed in the brains of the giant otter shrew or the four-toed sengi (Figs. 3.2, 3.4).

3.3.1.2. 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 three species (Figs. 3.2C-G, 3.3D-G, 3.4E-J). A moderate density of ChAT+ neurons was found within the caudate/putamen and throughout the globus pallidus, but most densely at its borders with the putamen and nucleus basalis. Through the nucleus accumbens a moderate density of ChAT+ neurons was observed, and at the ventral border of this nucleus, they appear to intermingle with the most dorsal cholinergic neurons of the olfactory tubercle. The ChAT+ neurons within the olfactory tubercle and Islands of Calleja were found in the most ventral portion of the anterior telencephalon. Throughout the olfactory tubercle a moderate density of ChAT+ neurons were observed, and within the most ventral portion of this region clusters of ChAT+ neurons were observed to form the Islands of Calleja (Fig. 3.6A, 3.6B).
3.3.1.3. Cholinergic nuclei of the basal forebrain

Cholinergic nuclei that could be identified within the basal forebrain of the three Afrotherian species studied included the medial septal nucleus, the diagonal band of Broca and the nucleus basalis (Figs. 3.2D-I, 3.3E-I, 3.4F-K). The medial septal nucleus exhibited a moderate to high density of ChAT+ neurons and was located within the rostral half of the medial wall of the septal nuclear complex immediately below the rostrum of the corpus callosum. In the Hottentot golden mole, a distinct cluster of ChAT+ neurons was observed in the lateral septal nucleus. These lateral septal cholinergic neurons exhibited a similar morphology to those observed in the medial septal nucleus of this species. The ChAT+ neurons forming the diagonal band of Broca were evidenced as a high density of neurons located in the ventromedial corner of the cerebral hemisphere anterior to the hypothalamus. It was possible to divide this nucleus into both horizontal and vertical limbs, but this was not deemed necessary since it would not add any value to the description. A cluster of moderate to high-density ChAT+ neurons located anterior and ventral to the globus pallidus and caudal to the olfactory tubercle were assigned to the nucleus basalis. The ChAT+ neurons of this nucleus appear to be continuous with those of the globus pallidus in all three species, and interestingly, this nucleus appears to extend quite caudally in all three species, forming a small cluster that finally terminates above the lateral margin of the cerebral peduncle at the level of the mammillary bodies.

3.3.1.4. Diencephalic cholinergic nuclei

In all three species ChAT+ neurons were found in the medial habenular nucleus, as well as the dorsal, lateral and ventral hypothalamic clusters (Figs. 3.2F-G, 3.3G-H, 3.4I-J). The medial habenular nucleus was located in the dorsomedial aspect of the diencephalon adjacent to the third ventricle and the ChAT+ neurons within this nucleus were very densely packed. The three clusters of ChAT+ neurons within the hypothalamus all showed moderate to weak immunoreactivity, but were clearly observed. The dorsal cluster was found in the dorsomedial 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, while the ventral cluster was located in the ventral medial portion of the hypothalamus adjacent to the neurons of the catecholaminergic A12 nucleus (see below).

3.3.1.5. Pontomesencephalic cholinergic nuclei

ChAT+ immunoreactive neurons delineated the parabigeminal nucleus (PBg), and the pedunculopontine (PPT) and laterodorsal (LDT) nuclei in all three species investigated (Figs. 3.2K-L, 3.3K-M, 3.4M-O, 3.6C, 3.6D). The parabigeminal nucleus was located at the very lateral margin of the pontine tegmentum in a location ventral to the inferior colliculus. Within this nucleus the ChAT+ neurons were moderately densely packed, although in both the giant otter shrew and the Hottentot golden mole, this nucleus contained less neurons than the same nucleus in the four-toed sengi. A moderate to high density of ChAT+ neurons forming the PPT were located within the dorsal aspect of the pontine tegmentum surrounding the superior cerebellar peduncle, even in the unusually arranged brainstem of the Hottentot golden mole. Within the periventricular grey matter, caudal to the oculomotor nucleus (inferior to this nucleus in the Hottentot golden mole), a moderate to high density of ChAT+ neurons were designated as those forming the LDT nucleus. The ventrolateral border of the LDT neurons was contiguous with the dorsomedial border of the PPT nucleus, the only reason to separate these two nuclei being the transition from the periventricular grey matter to the pontine tegmentum.

3.3.1.6. Collicular and cochlear cholinergic interneurons of the four-toed sengi

Within the brain of the four-toed sengi ChAT+ neurons were observed in the superior and inferior colliculi, and the cochlear nucleus (Fig. 3.4L-O). These neurons were not observed in either the giant otter shrew or the Hottentot golden mole. These ChAT+ neurons observed in the superior colliculus were found in the superficial layers, in a moderate to low density, were bipolar, with the dendrites oriented orthogonal to the pial surface. A low density of bipolar neurons of similar appearance was observed in the
ventrolateral portion of the inferior colliculus; however, they exhibited no specific
dendritic orientation. A few ChAT+ neurons were observed in the superficial layers of
the cochlear nucleus and these were observed to be a mixture of bipolar and multipolar
types.

3.3.1.7. Cholinergic cranial nerve motor nuclei

These nuclei were found in positions typical of all mammals (Woolf, 1991;
Manger et al., 2002a; Maseko et al., 2007; Dell et al., 2010; Kruger et al, 2010). The
ChAT+ nuclei identified in the three Afrotherian species studied include: the oculomotor
(III), trochlear (IV), motor division of the trigeminal (Vmot), abducens (VI), dorsal and
ventral subdivisions of the facial (VIIid and VIIiv), nucleus ambiguus, dorsal motor vagus
(X), hypoglossal (XII), Edinger–Westphal (EW), medullary tegmental field (mtf) and the
preganglionic motor neurons of the salivatory (pVII) and the glossopharyngeal (pIX)
nerve (Figs. 3.2J-R, 3.3J-R, 3.4L-S). There were certain species specific aspects of these
nuclei including the lack of a medullary tegmental field and a very small trochlear
nucleus in the giant otter shrew, and a small number of neurons that combined form the
oculomotor, trochlear and Edinger-Westphal nuclei in the Hottentot golden mole.

3.3.2. Putative catecholaminergic nuclei

Tyrosine hydroxylase (TH+) immunoreactivity revealed the putatively
catecholaminergic neurons in the brains of the three 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)
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 three species studied were similar to
that seen in many other mammals, but there were some exceptions. The rodent typical C3 nucleus (rostral dorsal midline medullary nucleus) and the Murid rodent/primate/megabat A6c nucleus (compact portion of locus coeruleus) (e.g., Dell et al., 2010; Kruger et al., 2012) were absent in all three species. The A15d nucleus (anterior hypothalamic group, dorsal division) was absent in the four-toed sengi, as seen for the eastern rock elephant shrew (Pieters et al., 2010), and the A6d nucleus (diffuse portion of locus coeruleus) was comparatively small in both the Hottentot golden mole and the four-toed sengi.

3.3.2.1. Olfactory bulb (A16)

The neurons forming the A16 nucleus were observed to be dense clusters of TH+ cells in the glomerular layer of the olfactory bulb in all three species (Figs. 3.2A, 3.3A-C, 3.4A-D). These neurons likely represent the periglomerular dopaminergic neurons, were small in size, and were found in equal density surrounding the entire glomeruli.

3.3.2.2. Diencephalic nuclei (A15 – A11)

In the hypothalamus of all three species TH+ cells formed six distinct nuclei: the dorsal division of the anterior hypothalamic group (A15d – except in the four-toed sengi, where this nucleus was absent), the ventral division of the anterior hypothalamic group (A15v), the rostral periventricular cell group (A14), the zona incerta (A13), the tuberal cell group (A12), and the caudal diencephalic group (A11) (Figs. 3.2F-I, 3.3G-I, 3.4H-K, 3.7A-C). Within the dorsal anterior portion of the hypothalamus of the Hottentot golden mole and giant otter shrew, between the third ventricle and the fornix, a moderate density of TH+ neurons was designated as the A15d nucleus. The TH+ neurons forming the A15v nucleus were located in the ventrolateral portion of the hypothalamus in a moderate density close to the floor of the brain and in the four-toed sengi these neurons appeared to be more numerous than in the other two species examined. A low to moderate density of TH+ neurons forming two columns adjacent to the lateral walls of the third ventricle, were assigned to the A14 nucleus. 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. TH+ neurons assigned to the A12 nucleus were located in the ventromedial portion of the hypothalamus, surrounding and below the floor of the third ventricle in the arcuate nucleus and the immediate vicinity. In the four-toed sengi, these neurons extended into the regions around the mammillary bodies. Lastly, within the hypothalamic grey matter adjacent to the posterior pole of the third ventricle, a moderate density of TH+ neurons formed the A11 nucleus, although the cells were less numerous in the giant otter shrew than in the other species.

3.3.2.3. 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 three species studied (Figs. 3.2I-J, 3.3J-L, 3.4L-N, 3.7D-E). A high density of TH+ neurons, found dorsal and dorsolateral to the interpeduncular nucleus, between this nucleus and the root of the oculomotor nerve (IIIn), 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 dense cluster of TH+ neurons formed the A10c nucleus. Dorsal to A10c, between it and the oculomotor nucleus, was a dense bilateral parasagittal cluster of TH+ neurons that formed the A10d nucleus. The TH+ neurons assigned to the A10dc nuclear complex were found within the periaqueductal grey matter surrounding the ventral half of the cerebral aqueduct.

The substantia nigra nuclear complex was observed in the ventral and lateral portions of the midbrain tegmentum, just dorsal to the cerebral peduncles. 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, scattered TH+ neurons were assigned to the A9v (ventral) nucleus. 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 (III\textsubscript{n}), a dense cluster of TH\textsuperscript{+} neurons formed the A9\textsubscript{m} (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 but relatively numerous, cluster of TH\textsuperscript{+} neurons that formed the A8 nucleus.

3.3.2.4. Rostral rhombencephalon – the locus coeruleus complex, A7 – A4

Within the pontine region of all three species studied a large number of TH\textsuperscript{+} neurons forming the locus coeruleus complex were readily observed. The locus coeruleus complex could be subdivided into five nuclei, these being: the subcoeruleus compact portion (A7\textsubscript{sc}), subcoeruleus diffuse portion (A7\textsubscript{d}), locus coeruleus diffuse portion (A6\textsubscript{d}), fifth arcuate nucleus (A5), and the dorsolateral division of locus coeruleus (A4) (Figs. 3.2L, 3.3K-N, 3.4O-P, 3.7F). Within the dorsal portion of the pontine tegmentum adjacent to the ventrolateral region of the periaqueductal grey matter, a tightly packed cluster of TH\textsuperscript{+} neurons represented the A7 compact portion of the locus coeruleus. 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 A7\textsubscript{sc}, a diffusely organised aggregation of TH\textsuperscript{+} neurons formed the A7\textsubscript{d} nuclear complex. These neurons are located both medially and laterally around the trigeminal motor nucleus (V\textsubscript{mot}). Within the lateral portion of the periventricular grey matter a loosely packed, moderate density of a moderate number of TH\textsuperscript{+} neurons were assigned to the A6\textsubscript{d} nucleus in the giant otter shrew. In the Hottentot golden mole and the four-toed sengi, a lower number of less densely packed neurons were assigned as the A6\textsubscript{d} nucleus in these species. No compact division of the locus coeruleus (the A6\textsubscript{c} division as observed in rodents, e.g., Kruger et al., 2012) was observed in any of the three species examined. In the ventrolateral pontine tegmentum lateral to the superior olivary nucleus and ventrolateral to V\textsubscript{mot} and A7\textsubscript{d}, a small cluster of TH\textsuperscript{+} 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 very dense, but small cluster of TH\textsuperscript{+} neurons represents the A4 nucleus.
3.3.2.5. Medullary nuclei (C1, C2, A1, A2, area postrema)

In the medulla of all three species five putative 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. 3.2M-Q, 3.3N-Q, 3.4P-S). 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 ventrolateral 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 moderately 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.

3.3.3. 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 three species examined exhibited the same complement of serotonergic nuclei in both the rostral and caudal clusters.

3.3.3.1. Rostral cluster

Within the rostral cluster we found evidence for the caudal linear nucleus (CLi), the supramlemniscal nucleus (B9), the median raphe nucleus (MnR) and the dorsal raphe complex formed of six distinct nuclei (Figs. 3.2K-L, 3.3K-M, 3.4M-O, 3.8). The CLi nucleus was the most rostral of the serotonergic nuclei found and the 5HT+ neurons formed a moderate density cluster around the midline immediately dorsal to the interpeduncular nucleus in a location just anterior to the decussation of the superior cerebellar peduncle in all three species. The serotonergic neurons forming the B9 nucleus appeared to be a lateral extension of the most ventral portion of CLi. The 5HT+ B9 neurons were found in a low density caudal to the A9pc (see above) and extended as an arc of neurons into the lateral and ventrolateral portion of the midbrain tegmentum. The median raphe nucleus (MnR) was characterised by two distinct, densely packed 5HT+ neuronal columns on either side of the midline in a para-raphe position. The rostral border of this nucleus was coincident with the level of the decussation of the superior cerebellar peduncle and the caudal border of this nucleus was found at the level of the trigeminal motor nucleus.

Within the dorsal raphe nuclear complex we identified six distinct nuclei in all three 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). 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 three 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. 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. 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 ones found outside the periaqueductal grey matter. The 5HT+ neurons of the DRI 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. As we followed the DRI caudally, where the cerebral aqueduct opened into the fourth ventricle and the DRd, DRv and DRif disappeared, the neurons of the DRI formed an arc across the midline of the dorsal portion of the periventricular grey matter. This caudal arc of the DRI 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., 2002b; Maseko et al., 2007; Dell et al., 2010; Kruger et al., 2012).

3.3.3.2. Caudal cluster

Within the caudal cluster we found evidence for the raphe magnus (RMg), rostral and caudal ventrolateral (RVL and CVL), raphe pallidus (RPa) and raphe obscurus (ROb) nuclei (Figs. 3.2M-P, 3.3M-P, 3.4O-S). The RMg was observed to be two columns of loosely aggregated moderate to large 5HT+ neurons located on either side of the midline from the level of the caudal pole of the trigeminal motor nucleus to the caudal pole of the facial nucleus. Within the left and right ventrolateral medullary tegmentum a distinct anteroposterior column of 5HT+ neurons 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). The RVL began as a lateroventral continuation of 5HT+ neurons from the lower portion of the RMg extending over the pyramidal tracts and consolidating as a distinct column lateral to the inferior olives. The inferior olive
topologically distinguishes left and right RVL, and 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 is reached. Although the RVL and CVL were continuous in the species studied, and indeed several other eutherian mammals previously studied (e.g., Maseko et al., 2007; Moon et al., 2007; Dwarika et al., 2008; Dell et al., 2010; Kruger et al., 2012), we distinguish two components of these ventrolateral columns, as the caudal portions have not been reported in the opossum or the monotremes (Crutcher and Humberston, 1978; Manger et al., 2002b). The 5HT+ neurons forming the RPa nucleus were found in the ventral midline of the medulla associated with the pyramidal tracts. These neurons were for the most part located between the two pyramidal tracts. Two loosely arranged bilateral columns of 5HT+ neurons located either side of the midline from the level of nucleus ambiguus to the spinomedullary junction were classified as the ROB.

3.3.4. Orexinergic (hypocretinergic) nuclei

Orexin-A immunohistochemistry was used to identify orexin-A immunopositive neurons (OrX+) cells in the giant otter shrew and the four-toed sengi. The vast majority of orexin-A immunopositive neurons (OrX+) identified in the brains of these species were localised to the hypothalamus. Within the area where orexinergic neurons were located we could readily divide them into three distinct clusters: a main cluster (Mc), a zona incerta cluster (Zic) and an optic tract cluster (Otc) (Figs. 3.2G-H, 3.4I-K). In both species the main cluster (Mc) was identified as a large group of densely packed OrX+ neuronal cell bodies located lateral to the third ventricle in the perifornical region, with a moderate number of neuronal cell bodies extending medially from this area into the dorsomedial hypothalamus and a larger number extending into the lateral hypothalamic areas (Fig. 3.9). From the main cluster a group of OrX+ neuronal cell bodies extended laterally into the region of the zona incerta (Zic). This cluster had a very low density of OrX+ neurons that were mixed with neurons of the lateral hypothalamic cholinergic nucleus and the A13 nucleus of the catecholaminergic system (see above). The third cluster, the optic tract cluster (Otc) extended ventrolaterally from the main cluster to the
ventrolateral region of the hypothalamus adjacent to the optic tract. This cluster exhibited a moderate density of OrX+ neuronal cell bodies. In both species 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 inferior border of the zona incerta.
Figure 3.2: Serial drawings of coronal sections through one half of the giant otter shrew (*Potomogale velox*) brain from the olfactory bulb through to the spinomedullary junction. **A** is the most rostral section, **R** the most caudal. The outlines of the architectonic regions were drawn using Nissl and myelin 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 1500 µm apart. See list for abbreviations.
Figure 3.3: Serial drawings of coronal sections through one half of the Hottentot golden mole (*Amblysomus hottentotus*) brain from the olfactory bulb through to the spinomedullary junction. A is the most rostral section, R the most caudal. The outlines of the architectonic regions were drawn using Nissl and myelin stains and immunoreactive cells marked on the drawings. Solid black circles depict cholinergic neurons, solid triangles depict catecholaminergic neurons (those immunoreactive for tyrosine hydroxylase), and open squares depict serotonergic neurons. Each circle, triangle or square represents an individual neuron. The figures are approximately 750 µm apart. See list for abbreviations.
Figure 3.4: Serial drawings of coronal sections through one half of the four-toed sengi (Petrodromus tetradactylus) brain from the olfactory bulb through to the spinomedullary junction. A is the most rostral section, S the most caudal. The outlines of the architectonic regions were drawn using Nissl and myelin 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 1200 µm apart. See list for abbreviations.
Figure 3.5: Photomicrographs of sections stained for Nissl substance (A, C, E, G) and immunoreactivity for cholineacetyltransferase (B, D, F, H), showing the location and appearance of cholinergic interneurons in the brain of the Hottentot golden mole. A and B are taken through the olfactory bulb, showing the cholinergic interneurons in the periventricular cell layer. C and D are of the cerebral neocortex showing the cholinergic interneurons in the upper layers of the cortex. E and F are taken through the center of the amygdaloid complex showing the cholinergic interneurons located throughout this nuclear mass. G and H are taken through the hippocampal formation, showing the presence of cholinergic interneurons in the cornu ammonis and dentate gyrus. The scale bar in D = 500 µm and applies to C and D, the scale bar in H = 1000 µm, and applies to A, B, E, F, G and H.
Figure 3.6: Photomicrographs showing neuronal groups immunoreactive for choline acetyltransferase in the olfactory tubercle (TOL) and nucleus accumbens (N.Acc) (A and B) and pontine region (C and D) of the Hottentot golden mole (A and C) and the four-toed sengi (B and D). Note the elaborated appearance of the TOL in the Hottentot golden mole (A) and the four-toed sengi (B) and the standard mammalian appearance and location of the laterodorsal tegmental (LDT), pedunculopontine tegmental (PPT) and parabigeminal (PBg) nuclei in both species (C and D). Scale bar in D = 1000 µm and applies to all.
Figure 3.7: Photomicrographs showing neuronal groups that were immunopositive for tyrosine hydroxylase in the brains of the Afrotherians studied. A. The A14 cell group in the rostral hypothalamus of the giant otter shrew. B. The A15v, A13 and A12 cell groups in the middle hypothalamus of the Hottentot golden mole. C. The A11 groups in the caudal hypothalamus of the giant otter shrew. D. The ventral tegmental A10 complex and substantia nigra A9 complex in the midbrain of the Hottentot golden mole. E. The substantia nigra A9 complex in the midbrain of the four-toed sengi. F. The locus coeruleus complex in the pontine region of the four-toed sengi. Scale bar in E = 1000 µm and applies to B, D and E. Scale bar in F = 500 µm and applies to A, C and F. See list for abbreviations.
Figure 3.8: Photomicrographs showing neuronal groups that were immunopositive for serotonin in the brains of the Afrotherians studied. The dorsal raphe complex is shown for the giant otter shrew (A), the Hottentot golden mole (B) and the four-toed sengi (C). Scale bar in C = 1000 µm and applies to A, B and C. D. The caudal nucleus of the dorsal raphe (DRc) and the median raphe nucleus (MnR) of the giant otter shrew. Scale bar in D = 500 µm. See list for abbreviations. See list for abbreviations.
Figure 3.9: Photomicrographs showing hypothalamic neuronal groups that were immunoreactive for orexin-A in the brain of the giant otter shrew (A) and four-toed sengi (B). Mc – main orexinergic cluster; OT – optic tract; Ote – optic tract orexinergic cluster; Zic – zona incerta orexinergic cluster. Scale bar in B = 1000 µm and applies to both. See list for abbreviations.
3.4. Discussion

The nuclear organization and complement of the neural systems investigated in the current study within the brains of three previously unstudied species of Afrotheria, the giant otter shrew (*Potomogale velox*), the Hottentot golden mole (*Amblysomus hottentotus*), and the four-toed sengi (*Petrodromus tetradactylus*), were, for the most part, similar to that observed in many Eutherian mammals previously described (e.g., Dell et al., 2010). Despite this there were differences of note that may be related either to the phylogenetic history or current life histories of these species. The golden mole exhibited an extensive and divergent distribution of cholinergic interneurons, far beyond that seen in other mammals. The four-toed sengi also exhibited cholinergic neurons in regions of the brain not normally associated with the cholinergic system, but cholinergic neurons were previously observed in these regions in the closely related eastern rock elephant shrew (Pieters et al., 2010). In agreement with previous observations in the eastern rock elephant shrew (Pieters et al., 2010), we could not find evidence for the catecholaminergic dorsal division of the anterior hypothalamic group (A15d) in the four-toed sengi. Lastly, in both the golden mole and the four-toed sengi, the catecholaminergic diffuse division of the locus coeruleus (A6d) was made up of a small number of neurons, similar to that observed in the rock hyrax (Gravett et al., 2009). In contrast to these observations, the giant otter shrew appears to have a nuclear organization of these systems that can be described as very typical of eutherian mammals.

3.4.1 The “hypercholinergic” Hottentot golden mole

While the cholinergic system in the three species studied was mostly similar to that observed in other eutherian mammals, the Hottentot golden mole was observed to have cholinergic interneurons throughout the cerebral neocortex and piriform cortex, the olfactory bulb, amygdala and hippocampus. The existence of cholinergic interneurons in this wide variety of locations has not been previously described. Cholinergic interneurons in the cerebral neocortex have only been observed previously in Murid Rodents, with non-Murid Rodents lacking these neurons (Bhagwandin et al., 2006; Kruger et al., 2012). Studies of the chemical nature of the cholinergic neocortical neurons in *Rattus*...
*norvegicus*, a Murid Rodent, have indicated that these neurons likely co-contain vasoactive intestinal polypeptide (VIP) and gamma-aminobutyric acid (GABA) (Eckenstein and Baughman, 1984; Bayraktar et al., 1997). Thus, these neocortical neurons have multiple transmission lines that may allow them to modulate the cortical microcirculation in relation to these multiple transmission lines (Chedotal et al., 1994). In the case of the golden mole, this may indicate that the microcirculation undergoes a great deal of local control in many brain regions. The question remains as to why this may occur in the golden moles. Our observations show that the olfactory system of the golden mole is likely to be the dominant sense, and this is reflected in the large size of the olfactory bulb and the impressive size and modularization of the olfactory tubercle in comparison to the other species studied herein and previously. The cholinergic neurons found in the olfactory bulb, piriform cortex, and amygdala, all indicate the importance of the modulation of neuronal function and microcirculation in regions of the brain associated with olfaction. While the hippocampus and neocortex are not parts of the brain normally considered to be involved in processing olfactory information, it is likely, given the dominance of the olfactory system in the golden mole, that they are recruited for this purpose, perhaps in the recognition of location in relation to olfactory stimuli for the hippocampus, and in the process of decision making in relation to olfactory stimuli for the cerebral neocortex. While these ideas are obviously speculative, it would be of interest to examine these functional possibilities further in the Hottentot golden mole, and to examine more species of golden moles to determine whether these supernumerary cholinergic neurons are a phylogenetically distinct characteristic of golden moles (the order Chrysochloridae), or whether they are restricted to the single species studied herein.

### 3.4.2 Additional cholinergic neurons in the four-toed sengi

In addition to the supernumerary cholinergic neurons observed in the golden mole, we observed cholinergic neurons in the superior and inferior colliculi and the cochlear nucleus in the four-toed sengi. Similar cholinergic neurons, in the same locations, were previously observed in the eastern rock elephant shrew (Pieters et al., 2010) both species being members of the order Macroscelididae, the elephant shrews or sengis. This similarity in the distribution of cholinergic neurons outside of the classically
defined cholinergic system (Woolf, 1991) indicates a common ancestry for these species, and the evolution of these neural traits during the establishment of the order Macroscelididae (Manger, 2005). Thus, while we can conclude that the cholinergic neurons in these regions of the elephant shrew brain are likely to be a feature common to all elephant shrews, it is still possible that they play an important functional role related to aspects of their life-history. As mentioned above, these cholinergic interneurons are likely to be involved in the modulation of the microcirculation in the brain regions in which they are found. In both species of elephant shrews studied, all three regions of the brain in which the cholinergic interneurons were found are involved in audition. Both species of elephant shrews studied are known to produce species-specific patterns of foot-drumming behaviour in response to agonistic encounters (Faurie et al., 1996; Skliba et al., 2008), with most species of elephant shrews likely to show some form of foot drumming communication (Faurie et al., 1996). The drumming sounds created by the elephant shrews are detectable both via aerial and seismic transmission as a form of sound wave. The modulation of the frequency tuning circuits in the cochlear nuclei and spatial location circuits in the inferior colliculus may enhance the detection and location of the source of the drumming. The cholinergic neurons in the superior colliculus may enhance the visual targeting of the drumming source. Thus, it is possible that the common ancestor of elephant shrews used drumming for conspecific communication, augmented by cholinergic neurons in regions of the brain of importance for the detection and location of these auditory signals. These traits would then have been passed on to all descendants of the common ancestor and we see them in the brains of extant elephant shrews. Further exploration of the occurrence of these neurons across elephant shrew species, with specific reference to the species-specific pattern and behavioural use of foot drumming may provide data of interest to our understanding of the elephant shrews.

3.4.3 Differences in the catecholaminergic systems and phylogenetic relationships

We could not find evidence for the existence of the A15d nucleus in the four-toed sengi. In a previous study of the eastern rock elephant shrew, this nucleus was also absent (Pieters et al., 2010), indicating an order specific absence of this nucleus in the Macroscelididae (Manger, 2005). This particular catecholaminergic nucleus has been
associated with seasonal control of reproduction (Thiery et al., 1989; Beccavin et al., 1998), and while the elephant shrews are seasonal breeders and the males show seasonal recrudescence of the testicles, there are indications that they are spontaneous ovulators and that there may not be a complete absence of sperm in the reduced testicles (Medger et al., 2012). Thus, the release of inhibition of pulsatile luteinizing hormone may allow opportunistic breeding opportunities for these mammals when conducive conditions arise. This process either does not occur in the other species studied or is currently unknown. In addition to the lack of the A15d nucleus, in both the four-toed sengi and the golden mole, the number of neurons forming the diffuse portion of the locus coeruleus (A6d) was small in number. This characteristic is shared by the rock hyrax (Gravett et al., 2009), but not by the giant otter shrew. Thus, it appears that the common ancestor of the Macroscelididae, Chrysochloridae and Hyracoidea may have had this trait. While this concept is supported for the Macroscelididae and Chrysochloridae, to the exclusion of the Tenrecidea, due to the closer relationship of the Macroscelididae and Chrysochloridae, the occurrence of this trait in the Hyracoidea indicates, based on current understandings of the phylogenetic relationships of the Afrotheria (Arnason et al., 2008; Asher et al., 2009), that either this character evolved twice, or was lost in the Tenrecidea. Further comparative work on the anatomy of the locus coeruleus in other members of the Afrotheria will resolve this problem.

3.4.4 The lack of changes in the serotonergic and orexinergic systems

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). The three Afrotherian species investigated in the current study do not negate these previous observations on the organization of the serotonergic system. The orexinergic system also appears to have a quite conservative organization across mammalian species studied to date. As with the two species of Afrotherian studied herein, the giant otter shrew and the four-toed sengi, most mammals have three specific clusters of orexinergic neurons, all located in the hypothalamus – a main cluster, an optic tract cluster and a zona incerta cluster. Three exceptions to this
organization have been reported in the literature. Both hamsters and Microchiropterans appear to lack the optic tract cluster of orexinergic neurons (Mintz et al., 2001; McGranaghan and Piggins, 2001; Korooshi and Klingenspor, 2005; Vidal et al., 2005; Kruger et al., 2010), while the Cetartiodactyla, represented by the giraffe and harbour porpoise, are reported to have an additional cluster of parvocellular orexinergic neurons in the medial hypothalamus (Dell et al., 2012). Thus, for the most part, the organization of the orexinergic system is also quite conservative across mammals.
CHAPTER FOUR: Nuclear organisation of some immunohistochemically identifiable neural systems in five Insectivores – *Crocidura cyanea, Crocidura olivieri, Sylvisorex ollula, Paraechinus aethiopicus and Atelerix frontalis*.

4.1. 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 hedgehog 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.
4.2. 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% H₂O₂) 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 appropriately 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 or 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  
VIIId – 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 – supramammilcal 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
DRI – 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 – mamillary 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

4.3. 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. 4.1), the desert hedgehog and the southern African hedgehog (Fig. 4.2), 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 4.1). The following description applies to all species studied, with species differences noted and highlighted where they occurred.

4.3.1. 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 either not ChAT immunoreactive or absent, in *Crocidura 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.

4.3.1.2. 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. 4.1B – 4.1E, 4.2D – 4.2I). 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 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.

4.3.1.3. 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.
4.1C – 4.1E, 4.2E – 4.2I). 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. 4.3). 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.

4.3.1.4. 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. 4.1E – 4.1F, 4.2G – 4.2K). 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 third ventricle.

4.3.1.5. 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. 4.1I – 4.1K, 4.2M – 4.2O). 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. 4.4). 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. 4.4), 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.

4.3.1.6. 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), abducens nucleus (VI), dorsal and ventral subdivisions of the facial nerve nucleus (VII d and VII v), 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. 4.1I – 4.1P, 4.2L – 4.2U). 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.
4.3.2. 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.

4.3.2.1. 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. 4.1A – 4.1B, 4.2A – 4.2D). 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.

4.3.2.2. 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. 4.1E – 4.1H, 4.2G – 4.2L). 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.

### 4.3.2.3. 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. 4.1G – 4.1J, 4.2K – 4.2M, 4.5). 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. 4.5). 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.

The substantia nigra nuclear complex was observed in the ventral and lateral portions of the midbrain tegmentum, just dorsal to the cerebral peduncles (Fig. 4.5). 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 (III), 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. 4.5D).

4.3.2.4. 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 (A6d), 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. 4.1J – 4.1K, 4.2N – 4.2P, 4.6). 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...
neurons represented the A7 compact portion of the locus subcoeruleus (Fig. 4.6A, 4.6B). 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. 4.6C, 4.6D). 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. 4.6). 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.

4.3.2.5. 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. 4.1L – 4.1O, 4.2Q – 4.2U). 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.

4.3.3. Serotonergic nuclei

The serotonergic nuclei (5HT+) identified in the brains of all five 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.

4.3.3.1. 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. 4.1I – 4.1K, 4.2M –
4.2O, 4.7). 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. 4.7). 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. 4.7). 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 DRI caudally, where the cerebral aqueduct opened into the fourth ventricle and the DRd, DRv and DRif disappeared, the neurons of the DRI formed an arc across the midline of the dorsal portion of the periventricular grey matter. This caudal arc of the DRI 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).

4.3.3.2. 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. 4.1K – 4.1N, 4.2P – 4.2T). 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 RObl. The dendrites of these neurons were oriented parallel to the midline.

4.3.4. 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. 4.1E – 4.1F, 4.2I – 4.2J, 4.8). 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 4.1: 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 and myelin 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 µm apart. See list for abbreviations.
Figure 4.2: 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 and myelin 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 µm apart. See list for abbreviations.
Figure 4.3: 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.
Figure 4.4: 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.
Figure 4.5: Photomicrographs showing the neuronal groups immunoreactive for tyrosine hydroxylase in the ventral tegmental 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.
Figure 4.6: 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.
Figure 4.7: 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.
Figure 4.8: 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.
Table 4.1: 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; Kruger et al., 2010a,b) and Megachiroptera (Maseko et al., 2007; Dell et al., 2010, 2013). Cells with a red background indicate order distinguishing features. Cells with a green background indicate features that align Microchiroptera with shrews to the exception of Megachiropterans and hedgehogs. Cells with a yellow background indicates features that align the Microchiropterans with Soricidae and Erinaceidae, but separates Microchiroptera from Megachiroptera. See list for abbreviations.

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*See list for abbreviations.*
<p>| 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 |
|-----------------------|----------------------------------|----------------------------------|-----------------------------|-------------------|-----------------|-----------------|---------------|----------------|-----------------|------------------|----------------|----------------|-----------------|----------------|----------------|----------------|----------------|----------------|
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| +                     | -                                | +                              | ?                          | +                 | +               | +               | +             | +              | +               | +                | +               | +               | +               | +             | +              | +               | +/ -            | +/ -            |
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| +                     | -                                | -                              | -                          | +                 | +               | +               | +             | +              | +               | +                | +               | +               | +               | +             | +              | +               | +/ -            | +/ -            |
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| 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      |                                  |                                |                            |                   |                 |                 |               |                |                 |                  |                 |                 |                 |               |               |                 |                 |                 |
| 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 | A9n | A9v | A9l | A10 | A10c | A10de | A10d | A11 | A12 | A13 | A14 | A15d | A15v | A16 |
|---|----|----|----|----|---------------|----|----|-------------|-------------|--------------|-------------|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
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The table represents the presence (+) or absence (-) of certain markers in different brain regions.
4.4. 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 4.1) 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 Microchiroptera 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.

4.4.1. 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 Microchiropterans, 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 tegmental 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 microphthamnic 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.

4.4.2. 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 the 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 microphthalmic 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 polyestrous, or seasonally monoestrous, it is difficult to determine how the lack of the A15d nucleus may effect reproductive processes and strategies.

4.4.3. 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.

4.4.4. 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 diphly, 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).
CHAPTER FIVE: Organization of cholinergic, catecholaminergic, serotonergic and orexinergic nuclei in three prosimian primates: *Galagoides demidoff, Perodicticus potto and Lemur catta*.

5.1. Introduction

Primates have traditionally been split into prosimians and simians, with prosimians being the earliest distinct branch of the extant primates, having diverged from simians approximately 64.5 million years ago (Matsui et al., 2009). Prosimians (tarsiers and strepsirrhini) are thought to more closely resemble stem primates due to the retention of several plesiomorphic features (Matsui et al. 2009). For this reason the organization of the various neural systems within the prosimian brain are of interest in understanding the phylogenetic trajectory of primates as a group. To date, no comprehensive mapping of the nuclei of the cholinergic, catecholaminergic, serotonergic or orexinergic systems has been undertaken for any prosimian primates, but data for the pygmy and common marmosets, squirrel monkey, macaque monkey, baboon and human are available (summarized in Maseko et al., 2007; Dell et al., 2010). In this sense, there is a gap in our knowledge of the evolution of the nuclear organization of these systems between primates and other mammals.

In addition to this knowledge gap, our studies of the nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic systems in a range of mammalian species, which are relatively conservative in their evolution (Manger, 2005; Dell et al., 2010), are of interest in providing data of relevance to the “Flying Primate” hypothesis. This hypothesis proposes that Megachiropterans and Microchiropterans are a diphyletic order, rather than a monophyletic order, and are not related to each other, with the Megachiropterans having evolved from the Dermopteran lineage of gliders (also known as the flying lemurs), which are the acknowledged sister group to primates (Pettigrew, 1986; Pettigrew et al., 1989; Meredith et al., 2011). While the “Flying Primate” hypothesis is contentious, there is substantial data coming from studies of the brain that supports this hypothesis (Pettigrew, 1986; Pettigrew et al., 1989; 2008; Manger
et al., 2001; Maseko and Manger, 2007; Maseko et al., 2007; Kruger et al., 2010a,b; Dell et al., 2010, 2013).

Changes in the complexity of neural system structure, in terms of the number and complement of distinct subdivisions, are thought to occur only during the evolutionary events leading to the establishment of a new mammalian order (Manger, 2005). All progeny of the newly established order will then likely retain the same complement of distinct subdivisions of the various systems irrespective of the subsequently evolved size of the brain, phenotype or life history of these progeny (Manger, 2005). While there are some cases that do not adhere directly to this hypothesis, for example the organization of the locus coeruleus in Murid rodents is different to all other rodents (Kruger et al., 2012), this framework for understanding systems level changes in mammalian brain evolution has been supported by a number of studies (see summaries in Dell et al., 2010; Calvey et al., 2013). Given this background, the current study aimed to analyse the nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic systems in three previously unstudied prosimian primate species. This was done for two reasons: (1) to determine whether the prosimian and simian primates species share an identical complement of nuclei of these systems, providing a primate specific nuclear complement of these systems (Manger, 2005); and (2) to determine whether this potential primate specific complement of nuclei share enough similarities with the nuclear complement of these systems found in Megachiropterans (Maseko et al, 2007; Dell et al., 2010) to provide further support from neuroanatomical data to the “Flying Primate” hypothesis.

5.2. Methods and Materials

Brains from two Galagoides demidoff (brain masses of 3.27 and 3.45 g), two Perodicticus potto (brain masses of 12.79 and 14.12 g) and two Lemur catta (brain masses of 24.11 and 26.72 g) were used in the present study. Permits were obtained from the relevant wildlife authority in the Democratic Republic of Congo for G. demidoff and P. potto, as well as from the Copenhagen zoo for L. catta. 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 weight 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 at 4°C. 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 six series of sections, cut at 50 µm thickness in the coronal plane, was made for Nissl, myelin, choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), serotonin (5HT) and orexin-A (hypocretin/OxA) staining. 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 in 1% cresyl violet to reveal cell bodies. Myelin sections were first stored in 5% formalin for two weeks at 4°C then mounted on 1.5% gelatine-coated slides and subsequently stained with silver solution to reveal myelin sheaths (Gallyas, 1979).

For the immunohistochemical staining each section was treated with endogenous peroxidase inhibitor (49.2% methanol:49.2% 0.1M PB: 1.6% of 30% H₂O₂) 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 revealed the putative catecholaminergic neurons. Serotonergic neurons were revealed using anti-serotonin (AB938, Millipore, raised in rabbit) at a dilution of 1:7500. 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 and myelin 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 (Fig. 5.1). 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 orexnergic 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
VIIId – dorsal division of facial nerve nucleus
VIIv – ventral division of facial nerve nucleus
X – dorsal motor vagus nucleus
XII – hypoglossal nucleus
5n – trigeminal nerve
3V – third ventricle
4V – fourth ventricle
A1 – caudal ventrolateral medullary tegmental nucleus
A2 – caudal dorsomedial medullary nucleus
A4 – dorsolateral division of locus coeruleus
A5 – fifth arcuate nucleus
A6c – compact portion of locus coeruleus
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 – amygdaloid body
AON – anterior olfactory nucleus
AP – area postrema
B9 – supramammillary serotonergic nucleus
C – caudate nucleus
C1 – rostral ventrolateral medullary tegmental group
C2 – rostral dorsomedial medullary nucleus
c a – cerebral aqueduct
Cb - cerebellum
cc – corpus callosum
Cl – claustrum
CLi – caudal linear nucleus
CO – cochlear nuclear complex
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
DRI – 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
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
icp – inferior cerebellar peduncle
io – inferior olivary nuclear complex
IP – interpeduncular nucleus
Is.Call/TOL – islands of Calleja/olfactory tubercle
LDT – laterodorsal tegmental nucleus
lfp – longitudinal fasciculus of the pons
L Gn – lateral geniculate nucleus
lot – lateral olfactory tract
LV – lateral ventricle
Mc – main cluster of orexinergic neurons
mcp – middle cerebellar peduncle
mlf – medial longitudinal fasciculus
MnR – median raphe nucleus
N.Acc – nucleus accumbens
N.Amb – nucleus ambiguus
N.Bas – nucleus basalis
NEO - neocortex
OB – olfactory bulb
ON – optic nerve
OT – optic tract
Otc – optic tract cluster of orexinergic neurons
P – putamen nucleus

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

pg – pineal gland

PIR – piriform cortex

PPT – pedunculopontine tegmental nucleus

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

RtTg – reticulotegmental nucleus of the pons

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

Stn – subthalamic nucleus

vfu – ventral funiculus

vh – ventral horn of spinal cord
VPO – ventral pontine nucleus

xscp – decussation of the superior cerebellar peduncle

zi – zona incerta

Zic – zona incerta cluster of orexinergic neurons

5.3. Results

The current study describes and defines the nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic neural systems in three species of prosimian primates (Demidoff’s dwarf bushbaby – Galagoides demidoff, the potto – Perodicticus potto and the ring-tailed lemur – Lemur catta). For the most part, the systems investigated exhibited an organization that may be thought of as typically primate-like, and generally mammalian; however, the structure of the locus coeruleus in the primates appears to be quite different to that observed in most other mammals, although it is very similar to that seen in Megachiropteran bats (Maseko et al., 2007; Dell et al., 2010). As all species investigated showed a near identical pattern of nuclear organization, the following description applies to all three species unless otherwise noted.

5.3.1 Cholinergic nuclei

The cholinergic system is generally subdivided into six main regions containing a cluster of distinct nuclei: cortical cholinergic neurons, 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 cerebral cortex (Fig. 5.1), where no cholinergic interneurons, as observed in some other mammals (e.g. Bhagwandin et al., 2006; Calvey et al., 2013), were observed.
5.3.1.1. 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 three species (Fig. 5.1D – 5.1L). A moderate to high density of ChAT+ interneurons were found throughout the caudate/putamen complex, although the caudate and the putamen nuclei were clearly separated by the internal capsule in all three species. The nucleus accumbens exhibited a low to moderate density of ChAT+ neurons surrounding the ventral border of the anterior commissure directly ventral to the caudate/putamen complex in all three species. These neurons were predominantly bipolar and ovoid in shape, but a few neurons were multipolar. The globus pallidus contained a low density of ChAT+ neurons that were predominantly multipolar and mostly located around the margins of this nucleus. The ChAT+ neurons within the olfactory tubercle and Islands of Calleja were found in the most ventral portion of the cerebral hemisphere (Fig. 5.2C). Throughout the olfactory tubercle a moderate density of ChAT+ neurons were observed, and within the most ventral portion of this region distinct clusters of ChAT+ neurons were observed to form the Islands of Calleja.

5.3.1.2. Cholinergic nuclei of the basal forebrain

Cholinergic nuclei identified within the basal forebrain of the three prosimian species studied included the medial septal nucleus, the diagonal band of Broca and the nucleus basalis (Fig. 5.1F – 5.1H). The ChAT+ neurons representing the medial septal nucleus appeared in the rostral portion of the septum adjacent to the midline. These neurons were bipolar with vertically oriented dendrites. The ChAT+ cells representing the diagonal band of Broca were located ventral to the cells of the medial septal nucleus, in the ventromedial corner of the cerebral hemisphere. No clear dendritic orientation of these bipolar and multipolar neurons was evident. Ventral to the globus pallidus and lateral to the hypothalamus, a moderate to high density of ChAT+ neurons were assigned to the nucleus basalis (Fig. 5.2). These cells were found to intermingle with the most
ventral cells of the globus pallidus and were ovoid in shape and showed no clear dendritic orientation.

5.3.1.3. Diencephalic cholinergic nuclei

Within the prosimian diencephalon, ChAT+ neurons were observed in the medial habenular nucleus, as well as forming three distinct nuclei, dorsal, lateral and ventral, within the hypothalamus (Fig. 5.1I – 5.1L). The ChAT+ neurons of the medial habenular nucleus were small, ovoid in shape and densely packed within this nucleus. The ChAT+ neurons within the hypothalamic nuclei were small in size, sparsely distributed and faintly immunostained, but were readily located. The ChAT+ neurons forming the dorsal hypothalamic cholinergic nucleus were found in the mediodorsal hypothalamus dorsal and medial to the fornix. The lateral hypothalamic cholinergic nucleus appeared to be a lateral extension of the neurons forming the dorsal nucleus, but located lateral to the fornix in the dorsolateral aspect of the hypothalamus. The ventral hypothalamic cholinergic nucleus was found in the ventromedial aspect of the hypothalamus, near, but not within, the arcuate nucleus.

5.3.1.4. Pontomesencephalic cholinergic nuclei

ChAT+ immunoreactive neurons delineated the parabigeminal nucleus (PBg), the pedunculopontine (PPT) and laterodorsal (LDT) tegmental nuclei in all three prosimian species investigated (Fig. 5.1N – 5.1O). The parabigeminal nucleus was located at the very lateral margin of the pontine tegmentum in a location ventral to the posterior pole of the superior colliculus. The ChAT+ neurons were small and densely packed with no clear dendritic orientation. The PPT was a large nucleus with bipolar and multipolar cells found in a moderate density throughout much of the midbrain and rostral pontine tegmentum (Fig. 5.3). This nucleus appeared at the level of the oculomotor nuclei and terminated at the level of the trigeminal motor nucleus. The ChAT+ neurons forming the LDT nucleus were found in the ventrolateral periaqueductal and periventricular grey matter. The most ventrolateral aspect of this nucleus abutted the dorsomedial aspect of the PPT nucleus, with the ventrolateral edge of the gray matter delineating their mutual
border (Fig. 5.3). The ChAT+ immunoreactive neurons of the LDT and PPT showed a very similar morphology, being mostly multipolar and showing no specific dendritic orientation.

5.3.1.5. Cholinergic cranial nerve nuclei.

The ChAT+ immunoreactive neurons forming various cranial nerve nuclei were found in positions typical of all mammals (Woolf, 1991; Maseko et al., 2007; Dell et al., 2010; Kruger et al, 2010a). The ChAT+ nuclei identified in the three prosimian species studied included the oculomotor (III), trochlear (IV), motor division of the trigeminal (Vmot), abducens (VI), dorsal and ventral subdivisions of the facial (VIId and VIIv), nucleus ambiguus, dorsal motor vagus (X), hypoglossal (XII), Edinger–Westphal (EW), the preganglionic motor neurons of the superior salivatory (pVII) and inferior salivatory (pIX) and the ventral horn of the spinal cord (vh) (Fig. 5.1M – 5.1V). The medullary tegmental field (mtf) was absent in all three species. Most nuclei contained large, strongly immunostained ChAT+ motor neurons. The cells in the dorsal motor vagus nucleus, while having a similar morphology were slightly smaller than the neurons observed in the other nuclei, while the neurons within the superior and inferior salivatory nuclei were also smaller and more scattered than seen in the other nuclei.

5.3.2. Putative catecholaminergic nuclei

Tyrosine hydroxylase (TH+) immunoreactivity revealed putative catecholaminergic neurons in the brains of the three 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) 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 three species studied were similar to that seen in many other mammals. The rodent typical C3 nucleus (rostral dorsal midline medullary nucleus) was absent in all three species (e.g., Smeet and Gonzalez, 2000; Dell et al., 2010; Kruger et al, 2012). The primate and Megachiropteran appearance of the locus coeruleus (A6), having a compact and diffuse portion was present in all three species (Dell et al., 2010).

5.3.2.1. The olfactory bulb (A16)

The TH+ neurons forming the A16 nucleus were observed as dense clusters of cells surrounding the inner and lateral aspects of the olfactory glomeruli in all three species (Fig. 5.1A – 5.1D). These neurons likely represent the periglomerular dopaminergic neurons, were small in size, and exhibited dendrites oriented to the edges of the glomeruli. No TH+ neurons were observed outside of the glomerular layer of the olfactory bulb in the prosimian primates.

5.3.2.2. Diencephalic catecholaminergic nuclei (A15-A11)

In the hypothalamus of all three species TH+ neurons formed six distinct nuclei: the dorsal division of the anterior hypothalamic group (A15d), the ventral division of the anterior hypothalamic group (A15v), the rostral periventricular cell group (A14), the zona incerta (A13), the tuberal cell group (A12), and the caudal diencephalic group (A11) (Fig. 5.1I – 5.1K). A15d contained a moderate density of bipolar TH+ neurons lateral to the third ventricle in the dorsal medial portion of the hypothalamus. The TH+ neurons of the A15v nucleus were found medial to the optic tract in the ventrolateral portion of the hypothalamus. These neurons were also small and bipolar and exhibited no specific dendritic orientation. The TH+ neurons forming the A14 nucleus were observed as two distinct columns of moderately dense neurons adjacent to the lateral borders of the third ventricle in the more rostral portion of the hypothalamus. These bipolar neurons exhibited a dorsoventral orientation of their dendrites. The A13 nucleus consisted of a
moderate density of predominantly oval, bipolar cells in the dorsolateral portion of the mid-level of the hypothalamus caudal to the fornix. TH+ neurons representing the A12 nucleus, were found in a moderate to high density lateral and ventral to the ventral portion of the third ventricle in the caudal third of the hypothalamus. At the most caudal level of the hypothalamus, the TH+ neurons forming the A11 nucleus were found caudal to the caudal pole of the third ventricle. In comparison to the other TH+ neurons within the hypothalamus, the A11 neurons were larger and multipolar and exhibited no specific dendritic orientation.

5.3.2.3. Midbrain nuclei (A10-A8)

Tyrosine hydroxylase immunoreactive neurons in the midbrain were found within the ventral tegmental area (the A10 complex, including the A10, A10 central, A10 dorsal, and A10 dorsocaudal nuclei), the substantia nigra (the A9 complex, including the A9 pars compacta, A9 lateral, A9 ventral, and A9 medial nuclei) and the retrorubral nucleus (A8) within the midbrain tegmentum in all three species studied (Fig. 5.1L – 5.1O). In the ventral border of the tegmentum, rostral to and surrounding the interpeduncular nucleus, TH+ neurons representing the A10 nucleus were located. Caudally, these cells were found dorsal and dorsolateral to the interpeduncular nucleus, between it and the root of the oculomotor nerve. Immediately dorsal to the interpeduncular nucleus, a dense cluster of TH+ neurons representing the A10c nucleus was located. Dorsal to the A10c nucleus, and ventral to the oculomotor nucleus, a moderate density of TH+ neurons, forming a distinctive triangular aggregation, represent the A10d nucleus. Within the periaqueductal grey matter, bipolar and multipolar cells representing the A10dc nucleus were found surrounding the ventral aspect of the cerebral aqueduct. The TH+ neurons in all these nuclei were a mixture of bipolar and multipolar types, with ovoid soma, with the dendrites showing no specific orientation.

The substantia nigra nuclear complex was observed in the ventral and lateral portions of the midbrain tegmentum, just dorsal to the cerebral peduncles and ventral to the medial lemniscus. The A9m nucleus appeared as a moderate to high density of
bipolar and multipolar cells located lateral to the A10 nucleus and the root of the oculomotor nerve. The A9pc (pars compacta) appeared to be a lateral extension of the A9m nucleus, but exhibited a slightly higher density of TH+ neurons. The neurons of the A9pc were found as a distinct band lying immediately dorsal to the cerebral peduncle. Throughout the pars reticulata of the substantia nigra, in a position ventral to the A9pc, scattered TH+ neurons were assigned to the A9v (ventral) nucleus. At the lateral edge of A9pc, a loose aggregation of TH+ neurons formed the A9l (lateral) nucleus. The TH+ neurons in all these A9 nuclei were a mixture of bipolar and multipolar types, with ovoid soma, with the dendrites showing no specific orientation except in the A9pc, where the dendrites were oriented in a roughly mediolateral plane. Dorsal to the substantia nigra complex, extending into the caudal aspect of the midbrain tegmentum, numerous TH+ neurons, found in a low to moderate density, represented the A8 nucleus. The morphology of these neurons was similar to that observed in the A10 and A9 nuclear complexes.

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

Within the pontine region of all three species studied a large number of TH+ neurons forming the locus coeruleus complex were readily observed. These nuclei represent the noradrenergic component of the catecholaminergic system (Smeets and Gonzalez, 2000) and could be subdivided into five nuclei: the subcoeruleus compact portion (A7sc), subcoeruleus diffuse portion (A7d), locus coeruleus compact portion (A6c) locus coeruleus diffuse portion (A6d), fifth arcuate nucleus (A5), and the dorsolateral division of locus coeruleus (A4) (Figs. 5.1O – 5.1Q, 5.4). Within the dorsal portion of the pontine tegmentum adjacent to the ventrolateral edge of the periaqueductal grey matter, a tightly packed cluster of TH+ neurons represented the A7 compact portion of the subcoeruleus (A7sc) (Fig. 5.4A). 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 diffuse aggregation of TH+ neurons formed the A7d nuclear complex (Fig. 5.4C). These neurons were 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 tightly packed, moderate to high
density of TH+ neurons were assigned to the A6c nucleus (Fig. 5.4). This tightly packed
cluster of TH+ neurons was surrounded by a band of more loosely packed neurons which
were assigned to the diffuse portion of the locus coerules (A6d) (Fig. 5.4). In the
ventrolateral pontine tegmentum lateral to the superior olivary nucleus and lateral to
Vmot and A7d, a small cluster of TH+ neurons formed the A5 nucleus. These neurons
formed a rough meshlike 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 periventricular grey matter, a dense, but small
cluster of TH+ neurons represented the A4 nucleus.

5.3.2.5. Medullary nuclei (C1, C2, A1, A2, area postrema)

In the medulla of all three 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) (Fig. 5.1R – 5.1U). C1 and C2 represent the adrenergic component of
the catecholaminergic system while A1 and A2 are dopaminergic and noradrenergic
(Smeets and Gonzalez, 2000). Medioventral to the facial nerve nucleus and the nucleus
ambiguus, on the ventrolateral aspect of the medulla, TH+ neurons representing the C1
nucleus appeared in a moderate to high density. These multipolar neurons had dendrites
that formed a mesh-like network in this region around the ascending and descending
fascicules of the medulla. The C2 nucleus was represented by ovoid shaped, bipolar TH+
neurons on the dorsal aspect of the medulla adjacent to the ventral border of the fourth
ventricle. Nucleus ambiguus separated the TH+ neurons of the C1 nucleus from those of
the A1 nucleus, with the A1 nucleus found lateral to nucleus ambiguus, while the C1
nucleus was medial to nucleus ambiguus. The neurons forming the A1 nucleus had a
similar appearance to those of the C1 nucleus. The TH+ neurons assigned to the A2
nucleus were found in the dorsomedial medulla, between the X and XII cranial nerve
nuclei, although some of these neurons were found within the adjacent dorsomedial
medullary tegmentum. The A2 neurons were both bipolar and multipolar in type and
were slightly larger than the neurons with the C2 nucleus. 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.

5.3.3. Serotonergic nuclei

The serotonergic nuclei 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; Calvey et al., 2013). These nuclei were all located within the brainstem and can be divided into a rostral and caudal cluster (Törk, 1990). Both of these clusters contained distinct nuclei, and these were found throughout the brainstem from the level of the decussation of the superior cerebellar peduncle through to the spinomedullary junction. All three species examined exhibited the same complement of serotonergic nuclei in both the rostral and caudal clusters.

5.3.3.1. Rostral cluster

Serotonergic neurons (5HT+) representing the caudal linear nucleus (CLi), the supralemmniscal nucleus (B9), the median raphe nucleus (MnR) and the dorsal raphe complex were found in all three species (Fig. 5.1N – 5.1P). The CLi nucleus was the most rostral of the serotonergic nuclei found and the bipolar 5HT+ neurons formed a cluster of moderate density around the midline immediately dorsal to the interpeduncular nucleus in a location just anterior to the decussation of the superior cerebellar peduncle in all three species. The serotonergic neurons forming the B9 nucleus appeared to be a lateral extension of the most ventral portion of CLi (Fig. 5.5A). The predominantly bipolar 5HT+ B9 neurons were found in a moderate to high density and extended as an arc of neurons into the ventrolateral portion of the midbrain tegmentum with cell density increasing caudally. The median raphe nucleus (MnR) was characterised by two distinct, densely packed 5HT+ neuronal columns on either side of the midline in a para-raphe position, extending from the level of the oculomotor nucleus to the anterior portion of the
pons. The dendrites of mostly bipolar MnR neurons were oriented in a dorsoventral plane. In the ring tailed lemur, the columns of the MnR expanded laterally along the ventral border of the medial longitudinal fasciculus, a feature not observed in the other two species.

Within the dorsal raphe nuclear complex we identified six distinct nuclei in all three species: the dorsal raphe interfascicular nucleus (DRIf), the dorsal raphe ventral nucleus (DRV), the dorsal raphe dorsal nucleus (DRd), the dorsal raphe lateral nucleus (DRI), the dorsal raphe peripheral nucleus (DRp) and the dorsal raphe caudal nucleus (DRc) (Fig. 5.5B). 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 and contained bipolar and multipolar neurons. Two pararaphe columns of 5HT+ neurons located between the bilaterally paired medial longitudinal fasciculi represent the DRIf nucleus in all three species. The DRV was found immediately dorsal to the DRIf, within the periaqueductal grey matter, and was represented by a high density of 5HT+ neurons. Immediately dorsal to DRV, between it and the ventral border of the cerebral aqueduct, a high-density cluster of bipolar 5HT+ neurons was designated as the DRd nucleus. A moderate density of larger, multipolar 5HT+ neurons representing the DRp, were located in the ventrolateral portion of the periaqueductal grey matter. A small number of DRp neurons were found in the adjacent midbrain tegmentum, outside the periaqueductal grey matter, and these were the only neurons of the dorsal raphe complex found external to the central gray matter. The larger, multipolar 5HT+ neurons of the DRI were located dorsolateral to the DRd forming clear aggregations along the edges of the cerebral aqueduct in a low to moderate density. Caudal to DRI, where the cerebral aqueduct opened into the fourth ventricle and the DRd, DRV and DRIf disappeared, the neurons of the DRI formed an arc of 5HT+ neurons across the midline of the dorsal portion of the periventricular grey matter, and this represents the DRc nucleus. The neurons of the DRc evinced a similar morphology to those of the DRI and DRp (Fig. 5.5C).
### 5.3.3.2. Caudal cluster

Within the caudal serotonergic cluster we found evidence for the raphe magnus (RMg), rostral and caudal ventrolateral (RVL and CVL), raphe pallidus (RPa) and raphe obscurus (ROb) nuclei (Fig. 5.1Q – 5.1T). The RMg was observed to be two columns of loosely aggregated moderate to large 5HT+ neurons, with dendrites oriented dorsoventrally, on either side of the midline from the level of the trigeminal motor nucleus to the nucleus ambiguus. Appearing at the same level as RMg within the left and right ventrolateral medullary tegmentum, a distinct anteroposterior column of 5HT+ neurons was found, the rostral and caudal ventrolateral serotonergic columns. The RVL began as a ventrolateral continuation of 5HT+ neurons from the lower portion of the RMg extending over the pyramidal tracts and lateral to the inferior olivary complex. The inferior olivary complex topologically distinguishes left and right RVL, and 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 is reached. The neurons of the RVL and CVL exhibited a similar morphology to those of the RMg. The 5HT+ neurons forming the RPa nucleus were found in the ventral midline of the medulla associated with the pyramidal tracts. These bipolar neurons were for the most part located between the two pyramidal tracts in a tightly packed bundle. Two loosely arranged bilateral columns of large, multipolar 5HT+ neurons located either side of the midline from the level of nucleus ambiguus to the spinomedullary junction were classified as the ROb.

### 5.3.4. Orexinergic (hypocretinergic) nuclei

The vast majority of orexin-A immunopositive neurons (OxA+) identified in the brains of the three prosimian species studied were localised to the hypothalamus and were ovoid in shape and bipolar, exhibiting no clear dendritic orientation (except where noted below). Within the area where orexinergic neurons were located we could readily divide them into three distinct clusters: a main cluster (Mc), a zona incerta cluster (Zic) and an optic tract cluster (Otc) (Figs. 5.1I – 5.1J, 5.6). The main cluster (Mc) was
identified as a large group of moderately densely packed OxA+ neurons located lateral to the third ventricle in the perifornical region of the hypothalamus, with a moderate number of neuronal cell bodies extending medially from this area into the dorsomedial hypothalamus. From the main cluster a group of OxA+ neurons extended laterally into the dorsolateral hypothalamus, with some cells found just outside of the hypothalamus in the region of the zona incerta. This zona incerta cluster (Zic) had a moderate density of OxA+ neurons with dendrites oriented in the mediolateral plane. The third cluster, the optic tract cluster (Otc), extendedventrolaterally from the main cluster to the ventrolateral region of the hypothalamus adjacent to the optic tract. The Otc contained a low to moderate density of OxA+ neurons that showed no specific dendritic orientation.
Figure 5.1: Serial drawings of coronal sections through one half of the potto
(*Perodicticus potto*) brain from the olfactory bulb through to the spinomedullary junction. 
A is the most rostral section, V the most caudal. The outlines of the architectonic regions were drawn using Nissl and myelin 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 1500 µm apart. See list for abbreviations.
Figure 5.2: Photomicrographs showing neurons immunoreactive for choline acetyltransferase in the nucleus basalis (N.Bas) and olfactory tubercle (TOL) in the three species studied: (A) Demidoff’s dwarf bushbaby (Galagoides demidoff); (B) the potto (Perodicticus potto); and (C) the ring-tailed lemur (Lemur catta). Note the similarity in appearance of this region of the brain in all species. Scale bar in C = 1000 μm and applies to all. ac – anterior commissure, P - putamen. In all images, medial is to the left and dorsal to the top.
Figure 5.3: Photomicrographs showing neurons immunoreactive for choline acetyltransferase in the laterodorsal tegmental (LDT) and pedunculopontine (PPT) nuclei in two of the species studied: (A) Demidoff’s dwarf bushbaby (*Galagoides demidoff*); and (B) the ring-tailed lemur (*Lemur catta*). Note the similarity in appearance of this region of the brain in both species. Scale bar in B = 1000 µm and applies to both. 4V – fourth ventricle; ca – cerebral aqueduct. In both images, medial is to the left and dorsal to the top.
Figure 5.4: Photomicrographs showing neurons immunoreactive for tyrosine hydroxylase (A, C) and stained for Nissl substance (B) in the locus coeruleus complex of the three species studied: (A) Demidoff’s dwarf bushbaby (*Galagoides demidoff*); (B) the potto (*Perodicticus potto*); and (C) the ring-tailed lemur (*Lemur catta*). Note the presence of both the compact (A7sc) and diffuse (A7d) portions of the subcoeruleus, and the compact (A6c) and diffuse (A6d) portions of the locus coeruleus. The A6c is only present in primates and Megachiroptera and is readily visible even in Nissl stained sections (B). Scale bar in A = 500 µm, scale bar in C = 1000 µm and applies to B and C. Vmot – motor division of trigeminal nucleus; scp – superior cerebellar peduncle. In all images, medial is to the left and dorsal to the top.
Figure 5.5: Photomicrographs showing neurons immunoreactive for serotonin in the rostral serotonergic cluster in two of the species studied: (A, C) the ring-tailed lemur (*Lemur catta*); and (B) Demidoff’s dwarf bushbaby (*Galagoides demidoff*). (A) The suprameminiscal serotonergic group (B9) in the ventral midbrain tegmentum, located lateral to the interpeduncular nucleus (IP) and ventrolateral to the decussation of the superior cerebellar peduncle (xscp). (B) The most expanded portion of the dorsal raphe nuclear complex showing the lateral (DRl), dorsal (DRd), ventral (DRv), peripheral (DRp) and interfascicular (DRif) divisions. (C) The caudal (DRc) division of the dorsal raphe nuclear complex. Scale bar in C = 1000 µm and applies to all. 4V – fourth ventricle, ca – cerebral aqueduct.
Figure 5.6: Photomicrographs showing neurons immunoreactive for orexin-A in the hypothalamus in two of the species studied: (A) Demidoff’s dwarf bushbaby (*Galagoides demidoff*); and (B) the ring-tailed lemur (*Lemur catta*). Note the similarity of the clustering of the orexinergic neurons into the main cluster (*Mc*) near the fornix (*f*), the zona incerta cluster (*Zic*) in the dorsolateral hypothalamus above the cerebral peduncle (*PC*), and the optic tract cluster (*OTc*), in the ventrolateral hypothalamus near the optic tract (*OT*). Scale bar in A = 1000 µm and applies to both images. In both images, medial is to the left and dorsal to the top. 3V – third ventricle.
Table 5.1: Summary of the nuclei delineated in the current study of Prosimian primates in comparison to similar studies previously undertaken in Microchiroptera, Megachiroptera and Simian primates (data for these species from Dell et al., 2010). Cells with a red background indicate features distinguishing the Microchiroptera from the other species. Cells with a green background indicate features that align the Microchiroptera with the primates to the exclusion of all other mammals.

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5.4. Discussion

The nuclear organization and complement of four immunohistochemically identifiable neural systems within the brains of three previously unstudied species of prosimian primates (Demidoff’s dwarf bushbaby – *Galagoides demidoff*, the potto – *Perodicticus potto* and the ring-tailed lemur – *Lemur catta*) were analysed in this study. For the most part, the organization and complement of nuclei of these systems were similar to that observed in many other Eutherian mammals previously described (e.g. Maseko et al., 2007; Dell et al., 2010), but despite this there were specific differences of note (summarized in Table 5.1) that may be related either to the phylogenetic history or current life histories of these species. The locus coeruleus complex, presenting with both a compact (A6c) and a diffuse (A6d) portion was present in all three prosimian species studied, which is the same as previously observed in simian primates and Megachiropterans (Maseko et al., 2007; Dell et al., 2010). In Microchiropterans, and most other mammals, only the A6d portion of the locus coeruleus is present (Maseko and Manger, 2007; Kruger et al., 2010a). All three prosimians had a large and clearly expressed lateral division of the dorsal raphe, a typical primate feature that has been found in previously studied Megachiropterans (Dell et al., 2010). All three prosimians had the catecholaminergic A4 and A15d nuclei, as well as the optic tract cluster (Otc) of the orexinergic system. These nuclei are present in all previously studied Megachiropterans and absent in all previously studied Microchiropterans (Maseko and Manger, 2007; Kruger et al., 2010a,b). The cholinergic parabigeminal nucleus was present in these prosimians as in Megachiropterans, whereas this nucleus is absent in four out of the six previously studied Microchiroptera (Maseko and Manger, 2007; Kruger et al., 2010a). There was a clear presence of the catecholaminergic ventral division of the substantia nigra nuclear complex in all three prosimian species, but this nucleus is incipient in all previously studied Microchiropterans with one species lacking the nucleus completely (Maseko and Manger, 2007; Kruger et al., 2010a). Overall, the results of the current study, along with those from previous studies support chiropteran diphly and the link between Megachiropterans and primates.
5.4.1. Cholinergic nuclei

The complement of nuclei within the cholinergic system of the three prosimians was identical to that seen in previously studied primates, and very similar to that observed in most other mammals studied to date (e.g. Dell et al., 2010). While there can be significant variation in the complement of cholinergic nuclei in the mammalian brain, it can be said that the complement of cholinergic nuclei in prosimian primates likely reflects that of a generalized Eutherian mammal. In the prosimian primates no cholinergic interneurons were observed in the cerebral cortex, as observed in Murid rodents and the hottentot golden mole (e.g. Bhagwandin et al., 2006; Calvey et al., 2013), or in other regions of the brain such as the olfactory bulb, amygdala, hippocampus, superior and inferior colliculi, or cochlear nuclei as seen in some Afrotherians (Pieters et al., 2010; Calvey et al., 2013). As with most other mammals, the parabigeminal nucleus was present in all three prosimians, but as mentioned, this nucleus was not found in four species of Microchiropterans (Maseko and Manger, 2007; Kruger et al., 2010a). In addition, the prosimian primates did not exhibit parvocellular cholinergic neurons surrounding the pontine cholinergic nuclei (LDT and PPT) as seen in the rock hyrax (Gravett et al., 2009). Thus, the variance in the complement of cholinergic nuclei does, to an extent, distinguishing the primates from several other mammalian lineages, but does not specifically align them with the Megachiropterans; however, it does not argue against this alignment, and the complement of cholinergic nuclei does serve to distinguish the Megachiropterans from the Microchiropterans.

5.4.2. Catecholaminergic nuclei

For the most part, the complement of nuclei identified as belonging to the catecholaminergic system in the prosimians studied was similar to most Eutherian mammals (Dell et al., 2010; Calvey et al., 2013); however, one feature of the catecholaminergic system, the presence of the locus coeruleus compact division (A6c) specifically aligns the primates with the Megachiropterans to the exclusion of all other mammals, including the Microchiropterans (Maseko and Manger, 2007; Kruger et al.,
Thus, in the primates and Megachiropterans, the locus coeruleus has a distinct core (which we term A6c) region of densely packed tyrosine hydroxylase immunoreactive neurons, surrounded by a shell of less densely packed neurons (which we term A6d). In most other mammals, the core region (A6c) is absent, and the neurons of the locus coeruleus are less densely packed (e.g. Dell et al., 2010; Calvey et al., 2013). The one group of possible exceptions to this distinguishing and aligning feature of primates and Megachiropterans are the Murid rodents. In Murid rodents, the A6 region is seen as a very densely packed core of tyrosine hydroxylase immunoreactive neurons (Dahlström and Fuxe, 1964; Kruger et al., 2012); however, in all other rodents previously studied, including Bathyergid mole rats, the greater cane rat, the African porcupine and the Highveld gerbil (Da Silva et al., 2006; Moon et al., 2007; Dwarika et al., 2008; Bhagwandin et al., 2008; Limacher et al., 2008), the A6 region presents as a more loosely packed cluster of tyrosine immunoreactive neurons and has been classified as the diffuse portion of the locus coeruleus complex (A6d). It is therefore likely that the compact appearance of the locus coeruleus in Murid rodents is not a feature shared with primates and Megachiropterans (and therefore lost in all other rodent species) and is a feature specific to the Murid rodents (Kruger et al., 2012). The cholinergic cortical neurons are a second Murid rodent specific feature of the systems investigated (Bhagwandin et al., 2006; Kruger et al., 2012). In this sense, the compact locus coeruleus and the cortical cholinergic interneurons are likely to have evolved specifically within the Murid rodent lineage, and do not affect the concluded alignment of the Megachiropterans with the primates to the exclusion of all other mammals. The appearance of the compact portion of the locus coeruleus is a specific character creating the exclusive Megachiropteran-primate phylogenetic link, although it might be argued that this character could have evolved in both lineages. Studies of other Euarchontoglires, such as hares and rabbits, tree shrews and the colugos (or flying lemurs) will determine whether this feature evolved independently in the Megachiropteran and primate lineages, or whether it was inherited from a common Megachiropteran-primate ancestor. The locus coeruleus is the main site for noradrenalin production in the brain and is involved in arousal as well as the optimization of task performance (Smeets and Gonzalez, 2000; Aston-Jones and Cohen, 2005). To our knowledge, there are no specific behavioural or neural processing
differences in the Megachiropterans and primates compared to other mammals that would explain the presence of this exceptional arrangement of the locus coeruleus, but future studies may reveal differences related to this neural specialization. In addition to the presence of the A6c in the Megachiropterans and primates, the absence of the A4 nucleus, the incipient nature of the A9v and the absence of the A15d nucleus in Microchiropterans (Maseko and Manger, 2007; Kruger et al., 2010a), serve to distinguish the Microchiropterans from both the Megachiropterans and primates, and aligns them with the Soricidae (see previous chapter). Thus, the variance in the nuclear complement of the catecholaminergic system strongly supports the hypothesis of chiropteran diphyly, and moreover, supports the Megachiropteran-primate and Microchiropteran-Soricidae phylogenetic relationships.

5.4.3. 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). Variances in the nuclear complement of the serotonergic system have been observed in monotremes and marsupials, with the presence of hypothalamic serotonergic neurons and lack of the caudal division of the dorsal raphe nuclear complex in the monotremes (Manger et al., 2002c), and the absence of the caudal ventrolateral serotonergic group in marsupials (Patzke et al., 2014). Although the three prosimians possessed all serotonergic nuclei common to primates, and indeed all Eutherian mammals studied to date (Dell et al., 2010; Maseko et al., 2013; Calvey et al., 2013), the presence of a relatively large and clearly expressed lateral division of the dorsal raphe, a feature common to previously studied simians as well as Megachiropterans (Dell et al., 2010) may have some functional effect on the inhibition of REM sleep (Monti, 2011). Thus, only a qualitative enlargement of a single nucleus of the serotonergic system appears to align the Megachiropterans with the primates, but this is a tenuous link that needs to be investigated in more detail, perhaps using quantitative stereological and allometric techniques to determine the strength of this qualitative link.
The orexinergic system also appears to have quite a conservative organization across the mammalian species studied to date, and the organization of the orexinergic clusters in the three prosimian species studied herein appear to follow the organization observed in most Eutherian mammals studied to date (Dell et al., 2013; Calvey et al., 2013). The one main exception of relevance to the problem of chiropteran phylogeny is the absence of the optic tract cluster of orexinergic neurons in the Microchiropterans (Kruger et al., 2010b). This absent feature distinguishes the Microchiropterans from all other mammals, including Megachiropterans (Dell et al., 2013) and supports the hypothesis of chiropteran diphyly. It is presently unclear what specific functional implications the lack of these orexinergic neurons may have on the Microchiropterans.

5.4.4. Primate and Megachiropteran phylogenetic affinities

The present study clearly demonstrates that the organization, number and complement of nuclei belonging to the cholinergic, catecholaminergic, serotonergic and orexinergic systems in the prosimian primates is identical to that seen in the simian primates, including humans. Thus, as proposed previously (Manger, 2005), there is a distinct primate order organization of nuclear belonging these systems. While for the most part these nuclei are commonly found across many mammals (Dell et al., 2010; Calvey et al., 2013), the only species that share the same full complement of nuclei belonging to these systems are the Megachiropterans. Of the 73 neural characters identified in the prosimian and simian primates and the Megachiropterans, only one specific feature links these species to the exclusion of all other mammals (although 5 characters distinguish the Microchiropterans from the Megachiropterans, see Table 5.1). When this neural characteristic (the presence of a compact portion of the locus coeruleus complex, A6c) is added to the extensive suite of neural features listed by Pettigrew et al. (1989) that link the Megachiropterans to primates to the exclusion of the Microchiropterans, as well as all the non-neural features supporting the Megachiropteran-primate phylogenetic link, the data in favour of chiropteran diphyly and the sister-group relationship of Megachiropterans and primates, becomes very extensive. While clearly a great deal more work is required to substantiate this proposed phylogenetic assignation, the Megachiropteran-primate link appears to be more parsimonious with the data that has
been generated than that of chiropteran monophyly.

5.4.5. Why is Chiropteran phylogeny of importance?

It might be said that the relationships of the Chiroptera to other mammals is purely of scientific interest and has no applicability to the real world; however, the extensive similarities of the neuromodulatory systems of Megachiropterans and primates may be of importance for the translation of studies of animal models to the study of human mental function and dysfunction. As a specific example, the locus coeruleus complex is of interest. Murid rodents, the most commonly used mammalian animal model (Manger et al., 2008), have what is likely to be an independently evolved structure of the locus coeruleus, while that of the Megachiropterans and primates is more likely to be the result of shared ancestry. The locus coeruleus complex is involved in many important neural functions, and the possibility that the compact and diffuse divisions of this complex are features specific and unique to the Megachiropterans and primates, to the exclusion of rodents and all other mammals, cannot be ignored. The locus coeruleus optimizes task performance by incorporating cortical mechanisms involved in the evaluation of costs and benefits associated with task performance (Aston-Jones and Cohen, 2005). It also plays a very important role in mediating the sleep/wake cycle and may in fact be a primary factor that differentiates REM sleep from wakefulness (Aston-Jones and Cohen, 2005). This has important implications for human mental health, considering the well-known link between sleep disturbances and psychiatric disorders. For example, 80-90% of people suffering from depression have impaired sleep quality with insomnia increasing the risk for depression, anxiety and substance abuse (Smith and Aston-Jones, 2008). The severity of sleep disturbances correlates with the severity of the psychiatric disorder (Smith and Aston-Jones, 2008). In depression and mania, there is a total increase in the percentage of REM sleep as a function of total sleep time, and it is suggested that REM sleep is disinhibited in depression, narcolepsy and schizophrenia (Smith and Aston-Jones, 2008). The locus coeruleus complex plays a major role in mediating sleep cycles (Siegel, 2004). It is therefore likely that a Megachiropteran would provide a more realistic animal model for the translation of relevant psychiatric testing to
human when compared to the popular rat model. In addition, there is quite a gap between the visual organisation of the primate brain and that of all other mammals, except Megachiropterans. Understanding that phylogenetic gap, which includes complex thalamic lamination and new cortical areas, as well as the hemi-decussated retinotectal pathway, requires a closer intermediate than a rodent, tree shrew or carnivore, a role that could be fulfilled by a megachiropteran sister to primates. While at this stage, the applicability of the Megachiropterans as improved animal models of human mental function and dysfunction over Murid rodents is still speculative, the data being generated in this and other comparative neurological studies, indicate that the Megachiropterans might be a fruitful animal model to be employed in future studies of human mental dysfunction and primate brain evolution. For these, and many other reasons, understanding Chiropteran phylogeny is of more than just scientific interest.

6.1. Introduction

The Euarchontoglire clade is comprised of the rodents, lagomorphs, Scandentia, Dermopterans and primates, a monophyletic clade strongly supported by both morphological and molecular evidence (Murphy et al., 2001a,b; Teeling et al., 2002; Misawa and Janke, 2003; Eizirik et al., 2004; Asher et al., 2009; Meredith et al., 2011). While numerous studies have examined the nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic systems in the brains of rodents and primates (summarized in Dell et al., 2010), to date only partial data of the nuclear organization of these systems in lagomorphs (Blessing et al., 1978) and Scandentia (Murray et al., 1982; Fitzpatrick et al., 1988) have been provided.

In the diphyletic scenario of Chiropteran evolution (Pettigrew, 1986; Pettigrew et al., 1989), the species belonging to the Euarchontoglires are of specific interest in relation to the phylogenetic position of the Megachiropterans. Within the Euarchontoglires, the Dermopterans are the acknowledged sister group to primates, sharing a closer phylogenetic relationship with the primates than the other members of the clade (e.g. Meredith et al., 2011). Pettigrew et al. (1989) proposed that the Megachiropterans evolved from the Dermopteran lineage, and thus evolved the capacity for powered flight from a gliding ancestor, and inherited the numerous neurological synapomorphies reported for Megachiropterans and primates from the common ancestor of Dermopterans and primates. This proposed Dermopteran-Megachiropteran phylogenetic link has support from molecular data, which placed five Megachiropteran genera with Dermopterans (Baker et al., 1991).

As in previous studies, we have chosen to examine the nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic systems, as it has been shown previously that the variation in the number and complement of nuclei within this system is both order specific (Manger, 2005) and the variations reflect, to a substantial
degree, the phylogenetic history of the mammals as a whole (Maseko et al., 2007; Dell et al., 2010; Calvey et al., 2013). Thus, in order to further our comparative studies of these systems in relation to the questions surrounding chiropteran phylogeny, and in this case specifically the phylogenetic placement of the Megachiropteran, we have examined the brains of two Euarchontoglires species for which data is currently not available. In order to obtain a more holistic data set for the Euarchontoglires to expand our comparison, we examined the nuclear organization of the aforementioned systems in a Lagomorph (Cape hare, Lepus capensis) and a Scandentia (northern tree shrew, Tupaia belangeri). The Lagomorphs are the acknowledged sister group to the rodents, while the Scandentia are placed between the rodents and Dermopterans in general phylogenies of the Euarchontoglires (e.g. Meredith et al., 2011). In this sense, the two species examined will provide data of specific relevance to the phylogenetic position of the Megachiroptera in the diphyletic scenario of Chiropteran evolution (Pettigrew, 1986; Pettigrew et al., 1989; 2008).

6.2. Methods and materials

Brains from two Tupaia belangeri (brain masses of 4.39 and 5.00 g) and two Lepus capensis (brain masses of 12.7 and 13.1 g) were used in the present study. Permits were obtained from the relevant wildlife authority in Saudi Arabia and also from the Copenhagen zoo. 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 weight 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 at 4°C. 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 six series of sections, cut at 50 µm thickness in the coronal plane, was made for
Nissl, myelin, choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), serotonin (5HT) and orexin-A (OxA) staining. 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 in 1% cresyl violet to reveal cell bodies. Myelin sections were first stored in 5% formalin for two weeks at 4°C then mounted on 1.5% gelatine-coated slides and subsequently stained with silver solution to reveal myelin sheaths (Gallyas, 1979).

For the immunohistochemical staining each section was treated with endogenous peroxidase inhibitor (49.2% methanol:49.2% 0.1M PB: 1.6% of 30% H₂O₂) 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 appropriately 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 (AB152, Millipore, raised in rabbit) at a dilution of 1:3000 revealed the putative catecholaminergic neurons. Serotonergic neurons were revealed using anti-serotonin (AB938, Millipore, raised in rabbit) at a dilution of 1:7500. 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 or 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. 6.1, 6.2). 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  
VIIId – 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 – supramniscal serotoninergic nucleus
C1 – rostral ventrolateral medullary tegmental group
C2 – rostral dorsomedial medullary nucleus
C3 – rostral dorsal midline 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 serotoninergic 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
DRI – 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
icp – inferior cerebellar peduncle
io – inferior olivary nuclei
IP – interpeduncular nucleus
Is.Call/TOL – island of Calleja/olfactory tubercle
LDT – laterodorsal tegmental nucleus
lfp – longitudinal fasciculus of the pons
lfu – lateral funiculus
LGv – ventral lateral geniculate nucleus
lot – lateral olfactory tract
LV – lateral ventricle
LVe – lateral vestibular nucleus
MB – mammillary bodies
Mc – main cluster of orexinergic neurons
mcp – middle cerebellar peduncle
mlf – medial longitudinal fasciculus
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
PeRt – 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
Tzn – nucleus of the trapezoid body
vfu – ventral funiculus
vh – ventral horn
VPO – ventral pontine nucleus
zi – zona incerta
Zic – zona incerta cluster of orexinergic neurons
6.3. Results

The current study describes and defines the nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic neural systems in the northern tree shrew – *Tupaia belangeri* and the Cape hare – *Lepus capensis*, species in 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 (Figs. 6.1, 6.2); however, we did observe many points of departure from this typical organization that have a strong bearing on the phylogenetic relationships of these species (results summarized in a comparative context in Table 6.1). The following description applies to all species studied, with species differences noted and highlighted where they occurred.

6.3.1. Cholinergic system

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) (Figs. 6.1, 6.2). Choline acetyltransferase immunoreactive neurons (ChAT+) were identified in all these subdivisions, except the cortical interneurons, which were absent in both species. The nuclei forming the cholinergic system in these two 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. For example, the Cape hare lacked the cholinergic neurons of the medullary tegmental field, which were present in the northern tree shrew. Additionally, the northern tree shrew presented with cholinergic neurons in the nucleus of the trapezoid body as well as the superior olivary nuclear complex, which has not been described in any mammal studied to date.
6.3.1.1. 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 both species (Figs. 6.1D – 6.1I, 6.2E – 6.2I). 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 a clear internal capsule forming a distinction between the caudate and putamen nuclei. In both species, the putamen contained a slightly higher density of cholinergic cells when compared to the caudate. A low density of ChAT+ neurons were observed within the globus pallidus of both species, and while the majority of these neurons were found around the lateral edges of this nucleus, some were observed throughout the nucleus. The nucleus accumbens contained a low density of cholinergic cells (Fig. 6.3A) and was distinguished from the striatum by the passage of the anterior commissure. In both 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.

6.3.1.2. Cholinergic nuclei of the basal forebrain

Cholinergic nuclei that could be identified within the basal forebrain of the two species studied included the medial septal nucleus, the diagonal band of Broca and the nucleus basalis (Figs. 6.1F – 6.1H, 6.2E – 6.2H). Cholinergic cells lateral to the midline within the anterior portion of the septal nuclear complex represented the medial septal nucleus in both species. Extending ventral from these cells along the ventromedial borders of the cerebral hemispheres, larger cells of higher density were identified as the diagonal bands of Broca (Fig. 6.3A). The nucleus basalis was located ventral and medial to the globus pallidus and putamen in both species, with neurons that had slightly smaller soma areas than those within the globus pallidus, but were of a similar multipolar type. The density of neurons within the nucleus basalis was moderate to high in both species (Fig. 6.3B).
6.3.1.3. Diencephalic cholinergic nuclei

In both species, ChAT+ neurons were found in the medial habenular nucleus, as well as the dorsal, lateral and ventral hypothalamic clusters (Figs. 6.1H – 6.1I, 6.2H – 6.2I). 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 both 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 third ventricle.

6.3.1.4. Pontomesencephalic cholinergic nuclei

ChAT+ immunoreactive neurons delineated the pedunculopontine tegmental (PPT), laterodorsal tegmental (LDT) and parabigeminal (PBg) nuclei in both species (Figs. 6.1M – 6.1O, 6.2N – 6.2O). 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 superior colliculus. In both species the superior colliculus was very large, but in the northern tree shrew the size of this nucleus distorted the topography of the midbrain and dorsal thalamus quite significantly. In both 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. 6.3C, 6.3D). In both species, the ChAT+ neurons forming the LDT nucleus were found mostly in the ventrolateral periventricular gray matter matter, and the density and number of LDT neurons appeared higher in the Cape hare than the northern tree shrew (Fig. 6.3). 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.

6.3.1.5. Cholinergic cells within the nucleus of the trapezoid body and superior
olivary complex in the northern tree shrew, *Tupaia belangeri*

The nucleus of the trapezoid body, located medial to the superior olivary nuclear
complex, and lateral to the decussating fibres of the trapezoid body, contained neurons
that were clearly immunoreactive to the cholineacetyltransferase antibody used in the
present study (Fig. 6.2P – 6.2Q, 6.3E, 6.3F). While having smaller soma than the motor
neurons of the cranial nerve nuclei, these neurons were multipolar and showed no
specific dendritic orientation. A small number of ChAT+ neurons were found to extend
from the trapezoid nucleus, along the ventromedial border of the superior olivary nuclear
complex, with some of the neurons located dorsal to this border, invested within the small
myelin lamellae that distinguish the subdivisions of the superior olivary nuclear complex.
Similar neurons were not observed in the Cape hare, or indeed in any other mammal
studied to date (Dell et al., 2010; Calvey et al., 2013), making this ChAT+ nucleus a
unique, and potential order distinctive, nucleus of the Scandentia.

6.3.1.6. Cholinergic cranial nerve nuclei

The following cranial nerve motor nuclei and other associated nuclei with ChAT
immunopositive neurons were found in positions typical of all mammals in both species
(Woolf, 1991; Maseko et al., 2007; Dell et al., 2010): the oculomotor nucleus (III),
Edinger-Westphal nucleus (EW), trochlear nucleus (IV), motor division of trigeminal
nerve nucleus (Vmot), abducens 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.
nucleus (pIX) and the ventral horn of the spinal cord (vh) (Figs. 6.1L – 6.1V, 6.2K – 6.2V). These motor nuclei contained predominantly bipolar and multipolar ChAT+ neurons with no predominant dendritic direction. The cholinergic medullary tegmental field (mtf) was absent in the Cape hare, but present in the northern tree shrew (Fig. 6.2S – 6.2V).

6.3.2. Putative catecholaminergic nuclei

Tyrosine hydroxylase (TH+) immunoreactivity revealed the putatively catecholaminergic neurons in the brains of the two 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 (Figs. 6.1, 6.2). These complexes correspond to that seen in other mammals (e.g., Smeets and Gonzalez, 2000; Dell et al., 2010) and could be divided into the olfactory bulb, diencephalic, midbrain, pontine and medullary nuclear clusters. The TH+ nuclei found in the three species studied were similar to that seen in many other mammals, but there were some exceptions. The ventral and dorsal divisions of the anterior hypothalamic group (A15d and A15v) were absent in *T. belangeri*. The rodent typical C3 nucleus (rostral dorsal midline medullary nucleus) was absent in *T. belangeri*, but was present in *L. capensis*. The murid rodent/primate/megachiropteran A6c nucleus (compact portion of locus coeruleus) (e.g., Dell et al., 2010; Kruger et al., 2012) was absent in both species.

6.3.2.1. 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 both species (Figs. 6.1A – 6.1C, 6.2A – 6.2C). 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.
6.3.2.2. Diencephalic nuclei (A15 – A11)

In the hypothalamus of the cape hare TH+ neurons formed six distinct nuclei: the dorsal division of the anterior hypothalamic group (A15d), the ventral division of the anterior hypothalamic group (A15v), the rostral periventricular cell group (A14), the zona incerta (A13), the tuberal cell group (A12), and the caudal diencephalic group (A11) (Fig. 6.1H – 6.1J, 6.2H – 6.2J); however in the northern tree shrews the A15d and A15v were absent (Fig. 6.4A, 6.4B). In the Cape hare, the A15d was found in the dorsal anterior region of the hypothalamus, between the third ventricle and the fornix and contained a high density of TH+ neurons (Fig. 6.4A). In the Cape hare 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 A14 nucleus was represented by small, predominantly bipolar neurons lining the lateral walls of the third ventricle in both species. These cells were of moderate density and extended away from the walls of the ventricle in a dorsolateral direction in the cape hare. In both species the A13 nucleus was found in the dorsolateral hypothalamus and consisted of a moderate density of neurons with dendrites oriented in a roughly mediolateral plane (Fig. 6.4A, 6.4B). In the Cape hare, several of the A13 neurons were observed to extended out of the hypothalamus into the region of the zona incerta. 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 (Fig. 6.4B). Within the hypothalamic grey matter adjacent to the posterior pole of the third ventricle, a low to moderate number of TH+ neurons formed the A11 nucleus.

6.3.2.3. 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 both species (Figs.
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. 6.4C). 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.

The substantia nigra nuclear complex was observed in the ventral and lateral portions of the midbrain tegmentum, just dorsal to the cerebral peduncles. 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 species, but the number of these neurons in the northern tree shrew was lower than in the Cape hare. At the lateral edge of A9pc, a small 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.

6.3.2.4. Rostral rhombencephalon – the locus coeruleus complex, A7 – A4

Within the pontine region of both species 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 (A6d), the fifth arcuate nucleus (A5) and the dorsolateral division of the locus coeruleus (A4) (Figs. 6.1N – 6.1Q, 6.2N – 6.2P). 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 (Figs. 6.4D, 6.4E). 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 (Figs. 6.4D, 6.4E). These neurons were 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 (Figs. 6.4D, 6.4E). No compact division of the locus coeruleus (the A6c division as observed in Murid rodents, primates and Megachiropterans, e.g., Dell et al., 2010; Kruger et al., 2012) was observed in either 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 both species (Fig. 6.4F).

6.3.2.5. Medullary nuclei (C1, C2, C3, A1, A2, area postrema)

In the medulla, six putative catecholaminergic nuclei were observed: the rostral ventrolateral tegmental group (C1), the rostral dorsomedial group (C2), the rostral dorsal midline medullary nucleus (C3) only in L. capensis, the caudal ventrolateral tegmental group (A1), the caudal dorsomedial group (A2), and the area postrema (AP) (Figs. 6.1Q – 6.1V, 6.2Q – 6.2V). 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 (Fig. 6.4G). 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 *L. capensis*, a low density of bipolar, clearly stained neurons represented the C3 nucleus at the dorsal midline of the medulla (Fig. 6.4H). 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 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 moderate number of TH+ neurons represented the A2 nucleus. Some of these A2 neurons were located a small distance into the dorsal caudal medullary tegmentum. In the cape hare, 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; however, in the northern tree shrew, the area postrema did not form a single midline mass, rather, it formed two bilateral masses located approximately 500 µm from the midline.

6.3.3. Serotonergic nuclei

The serotonergic nuclei (5HT+) identified in the brains of both species studied 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 (Figs. 6.1, 6.2). Both species examined exhibited the same complement of serotonergic nuclei in both the rostral and caudal clusters.
6.3.3.1. Rostral 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. 6.1M – 6.1O, 6.2M – 6.2N, 6.5A, 6.5B). The CLi nucleus consisted of a low density of multipolar neurons directly dorsal to the interpeduncular nucleus and anterior to the decussation of the superior cerebellar peduncle in both species. Lateral to CLi in the ventral midbrain tegmentum, a low density of small, multipolar cells represented the B9 nucleus in both species. 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. These neurons had round soma and the dendrites showed no specific orientation.

Within the dorsal raphe nuclear complex we identified six distinct nuclei in both 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). 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 both 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 that 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 to low 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 5HT+ neurons of the DRI 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 DRI caudally, where the cerebral aqueduct opened into the fourth ventricle and the DRd, DRv and DRif disappeared, the neurons of the DRI formed an arc across the midline of the dorsal portion of the periventricular grey matter. This caudal arc of the DRI was classified as the DRc nucleus.

6.3.3.2. Caudal cluster

Within the caudal cluster we found evidence for the raphe magnus nucleus (RMg), the rostral and caudal ventrolateral serotonergic groups (RVL and CVL), the raphe pallidus nucleus (RPa) and the raphe obscurus (ROb) nucleus in both species (Figs. 6.1P – 6.1U, 6.2P – 6.2U). 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 groups (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.

### 6.3.4. Orexinergic (hypocretinergic) nuclei

Orexin-A immunohistochemistry was used to identify orexin-A immunopositive neurons (OxA+) cells in *L. capensis* and *T. belangeri*. The vast majority of OxA+ neurons identified in the brains of both species were localised to the hypothalamus (Figs. 6.1I – 6.1J, 6.2H – 6.2I, 6.5C, 6.5D). Where orexinergic neurons were located we could readily divide them into three distinct clusters: a main cluster (Mc), a zona incerta cluster (Zic) and an optic tract cluster (Otc). 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 hypothalamic and a larger number extending into the lateral hypothalamic areas. 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 optic tract cluster (Otc) was found ventral to the main cluster, in the ventral lateral hypothalamus where it abuts the optic tract. This cluster exhibited a low to moderate density of OxA+ neurons. In both species, 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 6.1: Serial drawings of coronal sections through one half of the Cape hare (*Lepus capensis*) brain from the olfactory bulb through to the spinomedullary junction. A is the most rostral section, V the most caudal. The outlines of the architectonic regions were drawn using Nissl and myelin 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 1800 µm apart. See list for abbreviations.
Figure 6.2: Serial drawings of coronal sections through one half of the northern tree shrew (*Tupaia belangeri*) brain from the olfactory bulb through to the spinomedullary junction. A is the most rostral section, V the most caudal. The outlines of the architectonic regions were drawn using Nissl and myelin 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 1200 µm apart. See list for abbreviations.
Figure 6.3: Photomicrographs showing neuronal groups immunoreactive for choline acetyltransferase in various regions of the brain of the two species studied. (A) The diagonal band of Broca (Diag.B) and the nucleus accumbens (N.Acc) in the basal forebrain of the Cape hare (Lepus capensis). (B) The putamen (P), globus pallidus (GP) and nucleus basalis (N.Bas) in the basal forebrain of the northern tree shrew (Tupaia belangeri). The laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) nuclei in the pontine region of the brains of the Cape hare (C) and the northern tree shrew (D). (E) Nissl stained section through the medulla oblongata of the northern tree shrew at the level of the trapezoid body (Tzb), showing the trapezoid nucleus (Tzn) located medially to the superior olivary nuclear complex (SON). (F) Adjacent cholineacetyltransferase immunostained section to E, showing the presence of immunoreactive neurons, presumably cholinergic, in the trapezoid nucleus and the laterally located ventral division of the facial nerve nucleus (VIIv). The presence of cholineacetyltransferase immunoreactive neurons in the trapezoid nucleus is a unique feature of the northern tree shrew. Scale bar in F = 1000 µm and applies to all. ac – anterior commissure, py – pyramidal tract. In all images, medial is to the left and dorsal to the top.
Figure 6.4: Photomicrographs showing neuronal groups immunoreactive for tyrosine hydroxylase in various regions of the brain of the two species studied. (A) The dorsal hypothalamic region of the Cape hare (*Lepus capensis*) showing the presence of the dorsal division of the anterior hypothalamic catecholaminergic group (A15d) between the fornix (f) and the third ventricle (3V) and the zona incerta catecholaminergic cell group (A13) in the dorsal lateral hypothalamus, dorsal and medial to the nigrostriatal pathway (NSP). (B) A similar region in the northern tree shrew (*Tupaia belangeri*) showing the absence of the A15d nucleus in this region of the hypothalamus. (C) Tyrosine hydroxylase immunopositive neurons in the ventral aspect of the northern tree shrew midbrain showing three standard subdivisions of this region, the ventral tegmental area (A10), as well as the dorsal (A10d) and central (A10c) subdivisions of this region. The locus coeruleus complex of the Cape hare (D) and northern tree shrew (E) show three similar subdivisions, the subcoeruleus compact (A7sc), subcoeruleus diffuse (A7d) and the locus coeruleus diffuse (A6d) divisions located medially to the superior cerebellar peduncle (scp). (F) The dorsolateral division of locus coeruleus complex (A4) in the northern tree shrew. (G) The rostral ventrolateral medullary tegmental group (C1) in the northern tree shrew. (H) The rostral dorsal midline medullary nucleus (C3) in the Cape hare. Scale bar in H = 1000 µm and applies to all. 4V – fourth ventricle, A12 – tuberal cell group, Cb – cerebellum, mlf – medial longitudinal fasciculus, SON – superior olivary nuclear complex. In all images, medial is to the left and dorsal to the top.
Figure 6.5: Photomicrographs showing neuronal groups immunoreactive for serotonin (A, B) and orexin-A (C, D) in various regions of the midbrain (A, B) and hypothalamus (C, D) of the two species studied. In both the Cape hare (Lepus capensis) (A) and the northern tree shrew (Tupaia belangeri) (B), the standard subdivisions of the dorsal raphe nuclear complex were present, including the dorsal (DRd), ventral (DRv), interfascicular (DRif) and peripheral (DRp) subdivisions, along with the median raphe nucleus (MnR). In both the Cape hare (Lepus capensis) (C) and the northern tree shrew (Tupaia belangeri) (D) the pattern of clustering of the orexinergic neurons in the hypothalamus is similar to that seen in most other mammals, and include the main cluster (Mc) in the perifornical region (f - fornix), the zona incerta cluster (Zic) in the dorsolateral hypothalamus, and the optic tract cluster (Otc) in the ventrolateral hypothalamus. Scale bar in D = 1000 µm and applies to all. III – oculomotor nucleus. In all images, medial is to the left and dorsal to the top.
Table 6.1: Summary of the nuclei delineated in the current study of the Cape hare and northern tree shrew in comparison to similar studies previously undertaken in rodents, Megachirotterans and primates (data for some of these species from Dell et al., 2010). Cells with a red background indicate features distinguishing specific orders. Cells with a green background indicate features that align the Megachirotterans with the primates to the exclusion of all other mammals. Cells with a yellow background indicate Murid rodent specific features.

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**Serotonergic**

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| DRI | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
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6.4. Discussion:

The nuclear organization and complement of four immunohistochemically identifiable neural systems within the brains of two previously unstudied species of Euarchontoglires (the Cape hare – *Lepus capensis* and the northern tree shrew – *Tupaia belangeri*) were analyzed in this study. For the most part, the organization and complement of nuclei of these systems were similar to that observed in other Eutherian mammals previously described (e.g., Blessing et al., 1978; Fitzpatrick et al., 1988; Murray et al., 1982; Dell et al., 2010; Calvey et al., 2013); however, there were specific differences of note (summarized in Table 6.1) that may be related either to the phylogenetic history or current life histories of these species. In the cholinergic system, the northern tree shrew contained ChAT+ neurons within the nucleus of the trapezoid body, some of which were found as an extension of this nucleus into the lamellae within the superior olivary nuclear complex. Cholinergic neurons in this nucleus have not been described in any mammal studied to date. The northern tree shrew also presented with the cholinergic medullary tegmental field, which has a variable occurrence across mammals, but is present in monotremes, marsupials, carnivores and rodents (Manger et al., 2002a; Dell et al., 2010; Patzke et al., 2014). The catecholaminergic rostral dorsal midline medullary nucleus (C3), thought to be a rodent specific nucleus (Smeets and Gonzalez, 2000; Kruger et al., 2012), was present in the Cape hare, the northern tree shrew was lacking both the ventral and dorsal divisions of the anterior hypothalamic group (A15v and A15d), and both the Cape hare and the northern tree shrew were lacking the primate/Megachiropteran specific compact portion of the locus coeruleus (A6c). This analysis extends our comparison of these systems across species and, assuming Chiropteran diphyley, supports the suggestion that the Megachiropterans are more closely related to the primates than are any other members of Euarchontoglires studied to date.

6.4.1. Cholinergic nuclei

In the broadest sense, the nuclear organization and complement of the cholinergic system in the two species studied was very similar to all other mammals studied to date.
(Dell et al., 2010; Calvey et al., 2013). While the Cape hare did not present with any specific unusual cholinergic nuclei, the northern tree shrew had cholinergic neurons within the nucleus of the trapezoid body extending into the lamellae of the superior olivary nuclear complex, and in the medullary tegmental field. Cholinergic neurons in the medullary tegmental field are a feature of the cholinergic system of monotremes, marsupials, carnivores and rodents (Manger et al., 2002a; Dell et al., 2010; Patzke et al., 2014). The neurons of the medullary tegmentum appear to be involved in the promotion of REM sleep, as destruction of these neurons leads to a suppression of REM, and in particular, the destruction of the cholinergic neurons in this region of the brain led to increased nuchal muscle tone and head movements during sleep in the cat (Holmes and Jones, 1994). Thus, it might be predicted that all mammals possessing the cholinergic neurons of the medullary tegmental field have an increased amount of REM sleep, which is true for the monotremes (up to 30% of total recording time, Siegel et al., 1998), ferret (24% of total recording time, Jha et al., 2006), cat (24% of total sleep time, Ursin, 1968) and tree shrew (17% of total sleep time, Tupaia glis, Berger and Walker, 1972). It would be of interest to follow this potential link between these cholinergic medullary tegmental neurons and REM sleep further.

In addition, the neurons within the nucleus of the trapezoid body were immunoreactive to the choline acetyltransferase antibody. Trapezoid nucleus cholinergic neurons have not been described in any mammals previously studied (Dell et al., 2010; Calvey et al., 2013), and represent a feature specific to this animal, or perhaps the order Scandentia (Manger, 2005). Moreover an extension of these neurons into the lamellae of the superior olivary nuclear complex indicates that these neurons are likely to interact with, or modulate the activity of, the superior olivary nuclear complex. Both the trapezoid body and the superior olivary nuclei are involved in auditory localization, with the cells of the medial trapezoid body minimizing both signal noise and synaptic delay (Wu and Kelly, 1993) and the cholinergic system is involved in awareness (Woolf, 1991). It is thus reasonable to think that the northern tree shrew is likely to be very much aware of surrounding sounds and their origin, which would be useful in detecting potential prey as well as potential predators.
6.4.2. Catecholaminergic nuclei

For the most part, the complement of nuclei identified as belonging to the catecholaminergic system in the species studied was similar to most eutherian mammals (Dell et al., 2010; Calvey et al., 2013); however, variances were observed in the complement of medullary, midbrain and hypothalamic catecholaminergic nuclei. We found evidence for the presence of the adrenergic rostral dorsal midline medullary nucleus (C3) in the Cape hare. This nucleus has only been found in rodents previously (Smeets and Gonzalez, 2000; Kruger et al., 2012) and serves as a character supporting the established relationship between lagomorphs and rodents within the super order Glires (Murphy et al., 2001a,b; Misawa and Janke, 2003; Meredith et al., 2011). Both the northern tree shrew and the Cape hare lacked the compact portion of locus coeruleus (A6c). This portion of the locus coeruleus is found together with the diffuse portion of the locus coeruleus in primates and Megachiropterans (Dell et al., 2010; previous chapter). While Murid rodents appear to have a compact portion of the locus coeruleus, this is likely to be independently evolved in the rodent (see previous chapter), and the Murid rodents lack the diffuse portion of the locus coeruleus (Kruger et al., 2012). Thus, the finding of a lack of the A6c in both the Cape hare and northern tree shrew argues for a closer relationship of the megachiroptera with primates amongst the Euarchontoglires, if one assumes Chiropteran diphyly and accepts the placement of the Megachiropterans within the Euarchontoglires. The northern tree shrew also lacked both the ventral and dorsal divisions of the anterior hypothalamic group (A15v and A15d), which were also absent in a previously studied tree shrew (Tupaia glis) (Murray et al., 1982). This may well be a feature common to Scandentia and warrants further investigation of other members of Scandentia.

The functional implications of these variances in the complement of catecholaminergic nuclei are not readily explicable, but indications of their added or lost function can be speculated upon. The absence of the dorsal and ventral divisions of the anterior hypothalamic group (the A15 nuclei) in the tree shrews may be related to differences in the control of pituitary hormone secretion and hence reproduction, as these
nuclei send direct projections to the pituitary gland in other mammals (Smeets and Gonzalez, 2000). As tree shrews appear to be aseasonal breeders, or experience a long breeding season (Conaway and Sorenson, 1966; Sorenson and Conaway, 1968), the lack of the A15 nuclei may allow for almost continuous breeding to occur by not specifically controlling the hormonal secretions of the pituitary gland via dopaminergic input. The locus coeruleus complex is the main site for noradrenalin production in the brain and is involved in arousal as well as the optimization of task performance (Smeets and Gonzalez, 2000; Aston-Jones and Cohen, 2005); however, it is unclear what the lack of the A6c nucleus, or indeed the presence of this nucleus, will effect in terms of behavioural outcomes or neural processing. Lastly, the C3 nucleus, which known to be adrenergic in rodents (Smeets and Gonzalez, 2000), has had no specific function assigned beyond those generally ascribed to the other adrenergic nuclei in the medulla.

6.4.3. 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 (Steinbusch, 1981; Dell et al., 2010; Calvey et al., 2013). The two Euarchontoglire species investigated exhibited a complement of serotonergic nuclei that are in accord with previous observations on the nuclear organization of the serotonergic system. 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 two Euarchontoglire species studied herein appear to follow the organization observed in most Eutherian mammals studied to date (Dell et al., 2013; Calvey et al., 2013), to the exception of the Microchiropterans (Kruger et al., 2010b).

6.4.4. Phylogenetic affinities within the Euarchontoglires

Of the 79 neural characters described and discussed in this study, 69 characters (i.e., 91%) have been found in all species of Euarchontoglires studied to date (see Table
6.1), including the Megachiropterans if one accepts both Chiropteran diphyle and the placement of the Megachiropterans within the Euarchontoglires. In this latter scenario, it is of importance to determine, if Megachiropterans are within the Euarchontoglires, what phylogenetic position within this clade they hold. Pettigrew et al. (1989) suggested that the Megachiropterans are a derivative of the Dermopteran lineage. In standard phylogenies of the Euarchontoglires, the Dermopterans are identified as the sister group to primates (Meredith et al., 2011). Thus, as the neural systems examined here have not been studied in any Dermopteran, according to Pettigrew et al. (1989) our analysis should reveal that the Megachiropterans are most closely aligned to the primates within the Euarchontoglires. The one character that links the Megachiropterans to the primates, to the exclusion of all other Euarchontoglires, is the compact portion of the locus coeruleus (A6c). The A6c nucleus is not found in any other Euarchontoglires, including both species studied herein. Moreover, the presence of the C3 nucleus aligns the Cape hare with the rodents in the Glires, and distances the Glires from the Scandentia, Megachiropterans and primates. Lastly, the presence of cholinergic neurons in the trapezoid body and the absence of the A15 nuclei, present as order specific features of the Scandentia, isolating them from the Glires, Megachiropterans and primates. Thus, of all the species within the Euarchontoglires, in the Chiropteran diphyletic scenario the Megachiropterans clearly share the largest complement of homologous nuclei of the neural systems studied with primates. The information that is sorely needed to support and extend this conclusion is a full examination of the nuclear organization of these systems in at least one species of Dermopteran.
CHAPTER SEVEN: General discussion and cladistic analysis

7.1. Introduction

This thesis presented data on the nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic neural systems from a number of previously unstudied mammalian species. These included members from the Afrotheria (the giant otter shrew - *Potomogale velox*, the four-toed sengi - *Petrodromus tetradactylus* and the Hottentot golden mole - *Amblysomus hottentotus*), Insectivora (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*), prosimians (Demidoff’s dwarf bush baby – *Galagoides demidoff*, the potto – *Perodicticus potto* and the ring-tailed lemur – *Lemur catta*) and Euarchontoglires (the northern tree shrew – *Tupaia belangeri* and the Cape hare – *Lepus capensis*). Data on the presence, absence, or incipient nature of the nuclei belonging to the cholinergic, catecholaminergic, serotonergic and orexinergic neural systems were determined using routine immunohistochemical staining and qualitative analysis. For the most part, the nuclear organization of these systems was similar to that of other eutherian mammals previously studied, and between the species studied. This observation underlines the conservative evolution of neural systems in the brain, yet it is exactly this conservative nature of the evolutionary process that makes the brain so useful for studying phylogenetic questions – when changes in brain structure do occur, they tend to match major evolutionary events (Manger, 2005).

While differences in the structure of these neural systems do occur which aids in phylogenetic analysis (see below), it is important to point out that around 49% of the nuclei are conserved across all mammalian taxa, except for the serotonergic system where 80% of the nuclei are common features of mammalian brains. In the cholinergic system, 20 out of the 41 nuclei identified (49%) are conserved within the 54 taxa studied here. Of the 33 catecholaminergic nuclei, 16 are common features in all mammalian brains studied (48%). Eighty percent of serotonergic nuclei are common features within the 54 mammals analysed to date, and two out of the four orexinergic clusters are
common features in all mammals studied. Thus, of the neural characters identified in the current study, roughly 50% of them are variable across mammals and can be used to reconstruct mammalian phylogenetic history and specifically address the issue of chiropteran phylogeny.

Within the species studied, and more broadly across mammals studied with the same techniques, several noticeable differences begin to emerge and four specific examples are given here. The golden mole is a mammal that can be considered to be hypercholinergic, with cholinergic neurons in the cerebral cortex, the hippocampus, olfactory bulb and the amygdala (Calvey et al., 2013). Cholinergic neurons have previously been found in rodents (Bhagwandin et al., 2006; Kruger et al., 2012) yet the presence of cholinergic neurons in the hippocampus, olfactory bulb and amygdala is a novel feature not found in any animal brains studied to date. The northern tree shrew had cholinergic neurons within the nucleus of the trapezoid body, which is a novel feature not found in other mammals studied to date. The northern tree shrew also lacked both the dorsal and ventral divisions of the anterior hypothalamic group (A15d and A15v), a nuclear absence also reported for the common tree shrew (Murray et al., 1982), a seemingly order specific deficit. The four-toed sengi and a previously studied elephant shrew (Pieters et al., 2010) both lacked the dorsal division of the anterior hypothalamic group (A15d), a Macroscelidea specific deficit (Calvey et al., 2013).

7.2 Cladistic analysis

The following phylogenetic reconstruction techniques can be used on the brain data: PAUP (parsimony analysis, Swoford, 2001) and MacClade (Maddison and Maddison, 1992). These two techniques form the basis of the present analysis in order to answer the problem of chiropteran phylogeny and place the two groups into mammalian phylogeny as a whole. These techniques permit the discovery of the most parsimonious tree that could have given rise to the array of characters across the sample of taxa. In previous studies, brain data have given highly consistent and parsimonious trees which initiated the debate regarding Chiropteran phylogenetic affinities (Pettigrew, 1986).
MacClade has a useful feature, where characters can be tracked through the tree generated by PAUP and the consequences of removing characters or taxa or changing branching patterns can be tested. An example of this manipulation is demonstrated in Dell et al. (2010), where the shortest and most parsimonious phylogenetic tree (using 82 neural characters in 39 species) suggested a diphyletic origin for the Chiroptera and placed Megachiropterans within the Euarchontoglires and placed the Microchiropterans with Insectivora (Dell et al., 2010). The present study adds 15 species and 10 additional neural characters in order to generate a cladogram representing the phylogeny of Afrotheria, Insectivora, Microchiroptera, Megachiroptera, primates, Euarchontoglires and other mammals to compare with a generally accepted mammalian phylogeny that proposes Chiropteran monophyly and places Chiroptera as a sister group to the ungulates (Cetartiodactyls and Perissodactyls) (Meredith et al., 2011).

A cladistic analysis of the data regarding nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic systems generated in this study and several previous studies was performed using the commercially available MacClade program (http://macclade.org/macclade.html). In our tables (Tables 7.1, 7.2, 7.3) presenting all the data used, the nuclei have been noted as present (+), absent (-), or incipient (+/-). For scoring of these nuclei in the MacClade program, the data was entered as present (2), absent (0), incipient (1). The classification into these three grouping was based on a comparative qualitative assessment of the expression of the nuclei across all species examined. The data was assembled from the table presented in Dell et al. (2010), the current series of studies and from Kruger et al. (2012) and Maseko et al. (2013).

Using MacClade we ran a series of phylogenetic analyses based on the nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic systems across a range of mammalian species (see Tables 7.1, 7.2 and 7.3 for the data used in this analysis and Fig. 7.1 for the generated phylogenetic trees). This analysis included 54 taxa and 93 neural characters. Our first step was to allow the program to create the shortest and most parsimonious tree without additional interpretation. The result of this initial analysis (Fig. 7.1) demonstrated a diphyletic origin for the Chiroptera based on these
characters, and placed the Megachiroptera as a sister group to primates and the Microchiroptera as a sister group to the Soricidae. The length of this tree was 79 (the minimal number of possible steps for parsimony), with a consistency index of 0.54, a retention index of 0.77, and a rescaled consistency index of 0.42. It is of importance to note that the other relationships formed in this analysis conform strongly to previously published mammalian phylogenies (Meredith et al., 2011) and differ mostly in the placement and internal relationships of the Chiroptera.

Following this initial analysis we tested the strength of the tree by manipulating the phylogenetic relationships (using tools available in MacClade) to adhere strictly to a recently published paper on mammalian phylogeny (Meredith et al., 2011), including Chiropteran monophyly as the major change. Following these manipulations the treelength was 89, the consistency index 0.48, the retention index 0.70, and the rescaled consistency index 0.34.

Thus, by adhering to the phylogeny presented by Meredith et al., (2011), the tree length increased substantially, indicating that the number of steps, changes, or substitutions of characters needed to obtain the proposed phylogeny increased (from 79 to 89, 10 additional steps). The consistency index decreased (from 0.54 to 0.48). A consistency index of 1 equals perfect congruency in the data, anything less than 1 indicates a level of homoplasy in some characters. The self-generated tree, indicating bat diphly provides the highest consistency index, and thus the least need for recourse to homoplasy as an explanation of the data (in this case the sister grouping of Megachiropterans and primates within Euarchontoglires). The retention index decreased from the self-generated tree (from 0.77 to 0.70). This index is a second characteristic of the derived trees, whereby a retention index of 1 equals full parsimony, a retention index of 0 being total homoplasy. The self-generated Chiropteran diphly tree again reduces the need to resort to extensive homoplasy to explain the data. Lastly, the rescaled consistency index also decreased (from 0.42 to 0.34) underlining the same features as described above, this being that assuming Chiropteran monophyly increases the need to use homoplasy as a solution to the longer phylogenetic trees.
7.3 General Discussion

The current series of studies, including the cladistic analysis, provides strong support for the concept of Chiropteran diphyly. Moreover, these studies support the initial hypotheses of Pettigrew et al. (1989) indicating the sister group relationships between Megachiropterans and primates, and Microchiropterans and Soricidae. Other than Chiropteran diphyly, the data conforms strongly to currently accepted mammalian phylogenies (Meredith et al., 2011). In this sense, it indicates that our analyses are of use in understanding mammalian phylogeny.

In addition to the data presented in the current series of studies, there is a vast array of neural, morphological and molecular data in support of Chiropteran diphyly. Firstly, Megachiropterans have highly developed visual and olfactory senses and systems for foraging and obstacle avoidance, whereas in Microchiropterans the auditory senses and systems are elaborate (Pettigrew, 1989). Primates also have a well-developed visual sense and neural pathways related to vision are shared between Megachiropterans and primates (Pettigrew, 1986; Pettigrew et al., 1989, 2008; Mindell et al., 1991). Pettigrew (1986) examined the neuroanatomy of the retinotectal pathway in both Megachiroptera and Microchiroptera and found that Megachiroptera have an advanced retinotectal pathway with a vertical hemidecussation, a feature previously found only in primates, whereas this is not seen in Microchiroptera. Smith and Madkour (1980) also described similarities in the structure of the glans penis of primates and Megachiroptera that are not found in other Eutherian mammals. Measurements of the metacarpals and phalanges of the wings of Microchiropterans and Megachiropterans suggest a paraphyletic origin (Pettigrew et al., 1989). Molecular evidence has placed three of the Microchiropteran families into Laurasiatheria which includes Soricidae and Erinaceinae (Teeling et al., 2005). Genetic reconstruction grouped the Microchiropteran family, Rhinolophoidea, with Soricidae (Guo et al., 2013). Murphy et al. (2001b) acknowledges the relationship between Soricomorpha (shrews and moles) and Chiroptera based on molecular findings. Shrews and moles also echolocate like Microchiropterans do (Symonds, 2005). Mouchaty et al., (2000) placed moles as the sister group to Chiroptera based on molecular evidence.
Given this multiplicity of evidence, not only in the brain, but across the entire body, it is unusual that Chiropteran monophyly remains the dominate phylogenetic conclusion.

7.4 Future Directions

While the current series of studies have filled several gaps in our knowledge regarding Chiropteran evolution, and provided a lot of data regarding a set of specific neural systems in a range of mammals, there is always more questions and work to be done to fully resolve the issues at hand. In terms of the systems used in the current study, it would be important to add species from other insectivore families (such as the Talpidae and Solenodons), the Xenarthra (which are not represented at all in this data), the Perissodactyls (again not represented), and perhaps most importantly the Dermopterans (although they are a difficult species to obtain for scientific work). It would also be of use to examine a range of other neural and non-neural features to develop a greater set of characters than can provide a stronger cladistic analysis. Lastly, and likely the most important point for understanding Chiropteran phylogeny, the concept that DNA, like all other biological entities, is subject to the varying forces that shape evolutionary change, is very important. It is more than just theoretical that errors in phylogenies can arise from ignoring the potential for convergent evolution in the DNA molecule. In the case of the Chiropterans, this error appears to be the result of convergence in the structure of the DNA molecule related to the need of a high metabolism to sustain powered flight.
Figure 7.1: Phylogenetic trees produced by MacClade using 93 neural characters from 54 different mammalian species. The current study contributed 13 of the 54 species and the remaining species were obtained from Dell et al. (2010), Kruger et al. (2012), Maseko et al. (2013) and Meredith et al. (2011). The tree on the left was automatically produced by MacClade from the data. Note that this automatically produced tree, most parsimonious tree with the data, indicates Chiropteran diphyly and aligns the Megachiropterans with the primates and the Microchiropterans with the Soricidae. The tree on the right is a forced tree in MacClade adhering strictly to the tree provided by Meredith et al. (2011). This tree has Chiropteran monophyly as one of its central tenets, but note that the degree of parsimony of this tree is significantly worse than the tree with Chiropteran diphyly.
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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2008/35/01

APPLICANT: Dr P Manger

SCHOOL: School of Anatomical Science

DEPARTMENT:

LOCATION:

PROJECT TITLE: Comparative neuroanatomy of vertebrate species

Number and Species

1-6 various species

Approval was given for the use of animals for the project described above at an AESC meeting held on 20080527. This approval remains valid until 20100527

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Submit an M/E and if time permits an application for the animal species requested. CAS vet approval required prior to any experimentation on a new species.

Signed:  
(Chairperson, AESC)  Date: 07/07/2008

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed:  
(Registered Veterinarian)  Date: 7 July 2008

cc: Supervisor:
    Director: CAS

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ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO.  2012/53/01

APPLICANT:    P Manger

DEPARTMENT:   Anatomical Sciences

PROJECT TITLE: Comparative Neuroanatomy of Vertebrate Species - establishing a world-class brain bank at Wits

Number and Species

Approved: Various species of vertebrates, to be detailed in M&Es as they become available

Approval was given for to the use of animals for the project described above at an AESC meeting held on 27 November 2012. This approval remains valid until 30 November 2014.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application.

Signed: [Signature] (Chairperson, AESC)  Date: 5/12/12

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: [Signature] (Registered Veterinarian)  Date: 5/12/12

cc: Supervisor
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Nuclear organisation of some immunohistochemically identifiable neural systems in three Afrotherian species—Potomagale velox, Amblysomus hottonotus and Petrodromus tetractylus

Tanya Calvey a, Nina Patzke a, Consolate Kaswera b, Emmanuel Gilissen c,d,e, Nigel C. Bennett f,g, Paul R. Manger a,*

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A R T I C L E   I N F O

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A B S T R A C T

The present study describes the organisation of the cholinergic, catecholaminergic, and serotonergic neurons in the brains of the giant otter shrew, the Hottentot golden mole and the four-toed sengi. The aim of the present study was to investigate the possible differences in the neural complement of these neural systems in comparison to previous studies on other Afrotherian species and mammalian species in general. Brains of the golden mole, sengi and giant otter shrew were coronally sectioned and immunohistochemically stained with antibodies against cholineacetyltransferase, tyrosine hydroxylase, serotonin and orexin-A.

Abbreviations: III, oculomotor nucleus; IV, trochlear nucleus; Vm, motor division of trigeminal nerve nucleus; Vssn, sensory division of trigeminal nerve nucleus; VI, abducens nucleus; VIth, dorsal division of facial nerve nucleus; VII, ventral division of facial nerve nucleus; X, dorsal motor vagus nucleus; XII, hypoglossal nucleus; 3V, third ventricle; 4V, fourth ventricle; 5n, trigeminal nerve; 7n, facial nerve; A1, caudal ventrolateral medullary tegmental nucleus; A2, caudal dorsomedial medullary nucleus; A4, dorsolateral division of locus coeruleus; A5, fifth accumbent nucleus; A6d, diffuse portion of locus coeruleus; A7, nucleus subcoeruleus, diffuse portion; A7sc, nucleus subcoeruleus, compact portion; A8, retrorubral nucleus; A9, substantia nigra, lateral; A9m, substantia nigra, medial; A9p, substantia nigra, pars compacta; A9n, 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, dorsal diencephalic group; A12, tuberal cell group; A13, zona incerta cell group; A14, rostral periventricular nucleus; A15d, anterior hypothalamic group; A15s, anterior hypothalamic group, ventral division; A16, catecholaminergic neurons of the olfactory bulb; ac, anterior commissure; ACN, cholinergic neurons of the amygdala; Amyg, amygdala; AON, anterior olfactory nucleus; AP, area postrema; B9, supramammillary serotonergic nucleus; bic, brachium of the inferior colliculus; C1, rostral ventrolateral medullary tegmental nucleus; C2, rostral dorsomedial medullary nucleus; C3, rostral dorsal midline medullary nucleus; C4, caudate nucleus; ca, cerebral aqueduct; Ch, cerebellum; cc, corpus callosum; CCMN, cholinergic neurons of the cerebral cortex; Cl, claustrum; CLI, caudal linear nucleus; CN, deep cerebellar nucleus; CoCn, cholinergic neurons of the cochlear nuclear complex; csc, commissure of the superior colliculi; CVL, caudal ventrolateral serotonergic group; dh, dorsal horn of spinal cord; df, dorsal funiculus of spinal cord; Dg, dorsal, diagonal band of Broca; DR, dorsal raphe, caudal division; DRd, dorsal raphe, dorsal division; DRf, dorsal raphe, interfascicular division; DRl, dorsal raphe, lateral division; DRp, dorsal raphe, peripheral division; DRv, dorsal raphe, ventral division; DT, dorsal thalamus; EPM, external plexiform layer of olfactory bulb; EW, Edinger–Westphal nucleus; f, fornix; fr, fasciculus retroflexus; GC, central grey matter; GCLl, inner granular cell layer of olfactory bulb; GCLO, outer granular cell layer of olfactory bulb; GL, glomerular layer of olfactory bulb; GP, globus pallidus; Hbc, habenular commissure; Hbl, lateral habenular nucleus; Hbm, medial habenular nucleus; hcr, hippocampal commissure; HCN, cholinergic neurons of the hippocampus; HIP, hippocampus; Hyp, hypothalamus; Hyp.d, dorsal hypothalamic cholinergic nucleus; Hyp.l, lateral hypothalamic cholinergic nucleus; Hyp.p, ventral hypothalamic cholinergic nucleus; IC, inferior colliculus; ic, internal capsule; ICCN, cholinergic neurons of the inferior colliculi; io, inferior olivary nucleus; IP, interpeduncular nucleus; Is.CaLi/ToL, islands of Calleja and the olfactory tubercle; LDT, latero dorsal tegmental nucleus; LGc, ventral lateral geniculate nucleus; LOT, lateral olfactory tract; LV, lateral ventricle; ML, mammillary bodies; MC, main cluster of orexinergic neurons; MCL, mitral cell layer of olfactory bulb; mpC, middle cerebellar peduncle; MrN, median raphe nucleus; mlf, medial longitudinal fasciculus; mtf, medial tegmental field; NACC, nucleus accumbens; NAMb, nucleus ambiguus; NBas, nucleus basalis; NEd, cerebral neocortex; OB, olfactory bulb; OC, optic chiasm; OCN, cholinergic neurons of olfactory bulb; ONL, olfactory nerve layer of olfactory bulb; OT, optic tract; OtF, optic tract cluster of orexinergic neurons; OV, olfactory ventricle; P, putamen nucleus; PBlg, parabigeminal nucleus; PC, cerebral peduncle; pc, posterior commissure; PCCN, cholinergic neurons of the piriform cortex; PIR, piriform cortex; PPT, pedunculopontine nucleus; PTA, pretectal area; py, pyramidal tract; PVC, pterventricular grey matter; PVL, periventricular layer of olfactory bulb; pVPL, preganglionic motor neurons of the superior salivatory nucleus or facial nerve; pX, preganglionic motor neurons of the inferior salivatory nucleus or facial nerve; R, reticular nucleus of the dorsal thalamus; RmC, magnocellular division of red nucleus; RMg, magnum raphe nucleus; ROB, raphe obscurus nucleus; RPA, raphe pallidus nucleus; RVL, rostral ventrolateral serotonergic group; S, septal nucleus complex; SC, superior colliculus; SCCN, cholinergic neurons of the superior colliculus; scp, superior cerebellar peduncle; Sep.L, lateral septal nucleus; Sep.M, medial septal nucleus; so, superior olivary nucleus; spV, spinal trigeminal tract; vh, ventral horn of spinal cord; VFO, ventral funiculus of spinal cord; xscp, decussation of the superior cerebellar peduncle; zl, zona incerta; Zsc, zona incerta cluster of orexinergic neurons.

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1. Introduction

The Afrotherian mammalian cohort comprise what may be considered to be a very unusual mammalian grouping, one whose relationships have mainly been resolved on molecular rather than morphological grounds (e.g., van Dijk et al., 2001; Arnason et al., 2008; Hallström and Janke, 2008; Prasad et al., 2008; Asher et al., 2010; Dumbacher et al., 2012; McCormack et al., 2012). Within the Afrotheria are species that are fully aquatic (such as manatees and dugongs), extremely large (elephants), fossorial (golden moles), semi-aquatic (otter shrews), insectivorous (aardvarks, elephants shrews and tenrecs) and omnivorous (hyraxes). These species present with a range of body sizes, phenotypes, habitats and life histories, and are considered an ancient radiation of the Eutherian mammals. In this sense, the study of neural systems that are in general quite conservative in their evolution (e.g., Dell et al., 2010) is of interest, as a record of the changes, or indeed lack of changes, may reflect the potential evolutionary plasticity/malleability of the mammalian brain.

The cholinergic, catecholaminergic and serotonergic systems have been studied previously in two species belonging to the Afrotheria – the rock hyrax, Procavia capensis (Gravett et al., 2009) and the eastern rock elephant shrew, Elephantulus myurus (Pieters et al., 2010). While for the most part, these systems were similar to those reported in other mammals, several specific differences were noted. In the rock hyrax, the anterior nuclei of the dorsal thalamus were found to contain cholinergic neurons, there were cholinergic parvocellular neurons forming a shell around the typical laterodorsal and pericentral nuclei of the lateral geniculate nucleus, and the locus coeruleus proper was observed to be made up of very few cells (Gravett et al., 2009). In contrast to the rock hyrax, cholinergic neurons were observed in both superior and inferior colliculi and the cochlear nuclei, and the catecholaminergic anterior hypothalamic group (A15d) was missing from the elephant shrew (Pieters et al., 2010). Observations of the orexinergic neurons in the rock hyrax showed a typically mammalian orexinergic system in this species apart from a dense innervation of the anterior nucleus of the dorsal thalamus (Gravett et al., 2011).

In the present study we extend the observations made on these systems in the Afrotheria by examining, using immunohistochemical means, the cholinergic, catecholaminergic and serotonergic in the brains of the giant otter shrew (Potomogale velox), the Hottentot golden mole (Amblysomus hottentotus) and the four-toed sengi (Petrodromus tetradactylus) and the orexinergic system in the brains of the giant otter shrew and four-toed sengi. The giant otter shrew is a semi-aquatic member of the Tenrecidae that is found in the swamps, streams and forest pools of central Africa. The Hottentot golden mole is a small fossorial golden mole found in the KwaZulu-Natal and Eastern Cape region of South Africa and inhabits a range of ecosystems from temperate to tropical dry forests, dry savanna, through to urban areas and introduced vegetation. The four-toed sengi is a large elephant shrew found in tropical to subtropical moist montane forests as well as moist savanna throughout large areas of sub-Saharan Africa.

As far as the authors are aware, the systems described in the current study have not been examined in any of these three species. In addition to the prior studies of Afrotherian species, the data from the current study provides a broader base for comparison within the Afrotheria and across mammals in general. This may help to elucidate the manner in which systems level changes occur across mammalian brains as features such as brain size, phenotype, life histories, phylogenetic relationships, and time since evolutionary divergence, change, and lead to a better understanding of the partition between the phylogenetic and functional signals they may carry (Manger, 2005).

2. Materials and methods

Brains from P. velox, A. hottentotus and P. tetradactylus were collected for the present study. Permits were obtained from the relevant wildlife authorities in the Democratic Republic of Congo and South Africa for the capture and euthanasia of the animals from their natural habitat. All animals were handled according to the guidelines of the University of the Witwatersrand Animal Ethics Committee. Each animal was weighed, anaesthetised and subsequently euthanized with weight appropriate doses of sodium pentobarbital (200 mg sodium pentobarbital/kg, i.p.). In the current study a single P. velox (body mass = 540 g; brain mass = 3.46 g; an adult male), two A. hottentotus (body masses = 72 g, 86 g; brain masses = 1.3 g, 1.2 g; both adult males), and three P. tetradactylus (body masses = 132 g, 138 g, 124 g; brain masses = 3.01 g, 2.80 g, 2.95 g; all adult males) were used. Upon cessation of respiration the animals were perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M 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 0.1 M PB. A one in six series of sections, cut at 50 μm thickness in the coronal plane, was used for Nissl, myelin, choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), serotonin (5HT) and orexin (hypocretin/OX-A). An additional golden mole brain was sectioned in the sagittal plane to demonstrate the flexure of the brainstem in this species (Fig. 1). 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 in 1% cresyl violet to reveal cell bodies. Myelin sections were first stored in 5% formalin for two weeks at 4 °C then mounted on 1.5% gelatine-coated slides and subsequently stained with silver solution to reveal myelin sheaths (Gallyas, 1979).

For the immunohistochimical staining each section was treated with endogenous peroxidase inhibitor (49.2% 0.1 M PB 40.23% Methanol: 16% of 30% H2O2) for 30 min and subsequently subjected to three 10 min 0.1 M PB rinses. Sections were then incubated for 2 h. at room temperature, in blocking buffer (3% normal goat serum, NGS, for TH, serotonin and orexin sections; 3% normal rabbit serum, NRS, for ChAT sections, 2% bovine serum albumin for all sections and 0.25% Triton-X in 0.1 M PB for all sections). This was followed by three 10 min rinses in 0.1 M PB. The sections were then placed at 4°C under constant gentle shaking in primary antibody solution that contained the appropriate diluted primary antibody in blocking buffer (see above) for 48 h. Anti-choline acetyltransferase (AB1144P, 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 revealed the putative catecholaminergic neurons. Serotoninergic neurons were revealed using anti-serotonin (AB9038, Millipore, raised in rabbit) at a dilution of 1:10,000. 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 0.1 M 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, serotonin and orexin 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 0.1 M PB) for 2 h at room temperature. This was followed by three 10 min rinses in 0.1 M 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 0.1 M PB. Sections were then placed in a solution of 0.05% diaminobenzidine (DAB) in 0.1 M 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, which was continued until such time as the background staining was at a level that would assist reconstruction without obscuring the immunopositive neurons. Precipitation was arrested by placing sections in 0.1 M 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 reimmersed with Permount. The controls employed in this experiment included the omission of the primary antibody and the omission of the secondary antibody in selected sections for which no staining was evident.

Sections were examined under a low power stereomicroscope and using a camera lucida, the architectonic borders of the sections were traced following the Nissl and myelin 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. The nomenclature used for the cholinergic system was adopted from Woolf (1991), Limacher et al. (2008), Bhagwandien et al. (2008), Gravett et al. (2009) and Pieters et al. (2010), the catecholaminergic (Hökfelt et al. 1984, Sousse and González 2000, Limacher et al. 2008), Bhagwandien et al. (2008), Gravett et al. (2009) and Pieters et al. (2010), the serotonergic from Törk (1990), Limacher et al. (2008), Bhagwandien et al. (2008), Gravett et al. (2009) and Pieters et al. (2010), and the orexinergic from Kruger et al. (2010), Bhagwandien et al. (2011) and Gravett et al. (2011). While we use the standard nomenclature for the catecholaminergic system in this paper, we realise that the neuronal groups revealed with tyrosine hydroxylase immunohistchemistry may not directly correspond with these nuclei as has been described in previous studies by Dahlström and Fuxe (1964), Hökfelt et al. (1976), Meister et al. (1988), Kitahama et al. (1990, 1996), and Ruggiero et al. (1992); however, given the striking similarity of the results of the tyrosine hydroxylase immunohistochromistry to that seen in other mammals, we feel this terminology is appropriate. Clearly further studies in these Afrotherian species with a wider range of antibodies, such as those to phenylethanolamine-N-methyltransferase (PNMT), dopamine-β-hydroxylase (DBH) and aromatic -amino acid decarboxylase (AADC) would be required to fully address these anatomical questions in this study (e.g. Weibe et al. 2006). We address this potential problem with the caveat of putative catecholaminergic neurons where appropriate in the text.

3. Results

The current study describes and defines the nuclear organisation of the cholinergic, catecholaminergic and serotonergic neural systems in three species of Afrotherian mammals (giant otter shrew – P. velo, the Hottentot golden mole – A. hottentotus, and the four-toed sengi – P. tetradactylus) and the orexinergic system in the brain of the giant otter shrew and four-toed sengi, species in which these systems have not been previously described. For the most part, the systems investigated exhibited an organisation that may be thought of as typically mammalian; however, we did observe many points of departure from this typical organisation. One central observation of interest in the current study, and of importance in the interpretation of the organisation of the systems studied, was the noticeable folding, or compression, of the brainstem of the golden mole. Within the brain of the golden mole, the axial alignment of the brainstem was foreshortened in such a manner that the pontine region was observed to lie beneath the midbrain, and not caudal to it as seen in most other mammals (Fig. 1).

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 only identified in the Hottentot golden mole (Figs. 2–5). In addition to the cortical cholinergic interneurons, the golden mole also evinced cholinergic interneurons in the olfactory bulb, piriform cortex, amygdala and hippocampus (Fig. 5). Interestingly, the four-toed sengi possessed cholinergic interneurons in both superior and inferior colliculi and the cochlear nuclear complex (Fig. 4L–P), as described for the eastern rock elephant shrew (Pieters et al., 2010).

3.1.1. Cholinergic interneurons in the telencephalon of the golden mole

Previous studies have shown that while cholinergic interneurons are observed in the cerebral cortex of certain species, these are most readily observed in rodents of the Murid family (Bhagwandien et al., 2006; Kruger et al., 2012). Within the Hottentot golden mole, there was an abundance of typically bipolar cholinergic neurons (Fig. 3A–K), oriented orthogonal to the pial surface of the cerebral cortex, throughout all layers of the cortex. Similar cholinergic interneurons were also observed throughout the piriform cortex. Interestingly, this species also had cholinergic interneurons in the glomerular, inner granular and periventricular layers of the olfactory bulb, as well as in the CA region of the hippocampus and throughout the amygdala nuclear complex (Fig. 5). Similar cholinergic neurons were not observed in the brains of the giant otter shrew or the four-toed sengi (Figs. 2 and 4).

3.1.2. 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 three species (Figs. 2C–G, 3D–G and 4E–J). A moderate density of ChAT+ neurons was found within the caudate/putamen and throughout the globus pallidus, but were found most densely at its borders with the putamen and nucleus basalis. Through the nucleus accumbens a moderate density of ChAT+ neurons was observed, and at the ventral border of this nucleus, they appear to intermingle with the most dorsal cholinergic neurons of the olfactory tubercle. The ChAT+ neurons within the olfactory tubercle and Islands of Calleja were found in the most ventral portion of the anterior telencephalon. Throughout the olfactory tubercle, ChAT+ neurons with a moderate density were observed, and within the most ventral portion of this region clusters of ChAT+ neurons were observed to form the Islands of Calleja (Fig. 6A and B).

3.1.3. Cholinergic nuclei of the basal forebrain

Cholinergic nuclei that could be identified within the basal forebrain of the three Afrotherian species studied included the medial septal nucleus, the diagonal band of Broca and the nucleus basalis (Figs. 2D–I, 3E–I and 4F–K). The medial septal nucleus
Fig. 2. Serial drawings of coronal sections through one half of the giant otter shrew (Potomogale velox) brain from the olfactory bulb through to the spinomedullary junction. A is the most rostral section, R the most caudal. The outlines of the architectonic regions were drawn using Nissl and myelin 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 1500 μm apart. See list for abbreviations.
exhibited a moderate to high density of ChAT+ neurons and was located within the rostral half of the medial wall of the septal nuclear complex immediately below the rostrum of the corpus callosum. In the Hottentot golden mole, a distinct cluster of ChAT+ neurons was observed in the lateral septal nucleus. These lateral septal cholinergic neurons exhibited a similar morphology to those observed in the medial septal nucleus of this species. The ChAT+ neurons forming the diagonal band of Broca were evidenced as a high density of neurons located in the ventromedial corner of the cerebral hemisphere anterior to the hypothalamus. It was possible to divide this nucleus into both horizontal and vertical limbs, but this was not deemed necessary since it would not add any value to the description. A cluster of moderate to high-density ChAT+ neurons located anterior and ventral to the globus pallidus and caudal to the olfactory tubercle were assigned to the nucleus basalis. The ChAT+ neurons of this nucleus appear to be continuous with those of the globus pallidus in all three species, and interestingly, this nucleus appears to extend quite caudally in all three species, forming a small cluster that finally terminates above the lateral margin of the cerebral peduncle at the level of the mammillary bodies.
3.1.4. Diencephalic cholinergic nuclei

In all three species ChAT+ neurons were found in the medial habenular nucleus, as well as the dorsal, lateral and ventral hypothalamic clusters (Figs. 2F, G, 3G, H and 4I, J). The medial habenular nucleus was located in the dorsomedial aspect of the diencephalon adjacent to the third ventricle and the ChAT+ neurons within this nucleus were very densely packed. The three clusters of ChAT+ neurons within the hypothalamus all showed moderate to weak immunoreactivity, but were clearly observed. The dorsal cluster was found in the dorsomedial 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,

Fig. 3. Serial drawings of coronal sections through one half of the Hottentot golden mole (Amblysomus hottentotus) brain from the olfactory bulb through to the spinomedullary junction. A is the most rostral section, R the most caudal. The outlines of the architectonic regions were drawn using Nissl and myelin stains and immunoreactive cells marked on the drawings. Solid black circles depict cholinergic neurons, solid triangles depict catecholaminergic neurons (those immunoreactive for tyrosine hydroxylase), and open squares depict serotonergic neurons. Each circle, triangle or square represents an individual neuron. The figures are approximately 750 μm apart. See list for abbreviations.
while the ventral cluster was located in the ventral medial portion of the hypothalamus adjacent to the neurons of the catecholaminergic A12 nucleus (see below).

3.1.5. Pontomesencephalic cholinergic nuclei

ChAT+ immunoreactive neurons delineated the parabigeminal nucleus (PBg), and the pedunculopontine (PPT) and laterodorsal (LDT) nuclei in all three species were investigated (Figs. 2K, L, 3K–M, 4M–O and 6C, D). The parabigeminal nucleus was located at the very lateral margin of the pontine tegmentum in a location ventral to the inferior colliculus. Within this nucleus the ChAT+ neurons were moderately densely packed, although in both the giant otter shrew and the Hottentot golden mole, this nucleus contained less neurons than the same nucleus in the four-toed sengi. ChAT+ neurons forming the PPT with a moderate to high density were located within the dorsal aspect of the pontine tegmentum surrounding the superior cerebellar peduncle, even in the unusually arranged brainstem of the Hottentot golden mole. Within the periventricular grey matter, caudal to the oculomotor nucleus (inferior to this nucleus in the Hottentot golden mole), ChAT+ neurons with a moderate to high density were designated as those forming the LDT nucleus. The ventrolateral border of the LDT neurons was contiguous with the dorsomedial border of the PPT nucleus, the only reason to separate these two nuclei being the transition from the periventricular grey matter to the pontine tegmentum.

3.1.6. Collicular and cochlear cholinergic interneurons of the four-toed sengi

Within the brain of the four-toed sengi ChAT+ neurons were observed in the superior and inferior colliculi, and the cochlear nucleus (Fig. 4L–O). These neurons were not observed in either the giant otter shrew or the Hottentot golden mole. These ChAT+ neurons observed in the superior colliculus were found in the superficial layers, in a moderate to low density, were bipolar, with the dendrites oriented orthogonal to the pial surface. A low density of bipolar neurons of similar appearance was observed in the ventrolateral portion of the inferior colliculus; however, they exhibited no specific dendritic orientation. A few ChAT+ neurons were observed in the superficial layers of the cochlear nucleus and these were observed to be a mixture of bipolar and multipolar types.

3.1.7. Cholinergic cranial nerve motor nuclei

These nuclei were found in positions typical of all mammals (Woolf, 1991; Manger et al., 2002a; Maseko et al., 2007; Dell et al., 2010; Kruger et al., 2010). The ChAT+ nuclei identified in the three Afrotherian species studied include: the oculomotor (III), trochlear (IV), motor division of the trigeminal (Vmot), abducens (VI), dorsal and ventral subdivisions of the facial (VII and VIIv), nucleus ambiguous, dorsal motor vagus (X), hypoglossal (XII), Edinger–Westphal (EW), medullary terminal field (mtf) and the preganglionic motor nuclei of the salivatory (pVII) and the glossopharyngeal (pIX) nerves (Figs. 2J–R, 3J–R and 4L–S). There were certain species-specific aspects of these nuclei including the lack of a medullary terminal field and a very small trochlear nucleus in the giant otter shrew, and a small number of neurons that combined form the oculomotor, trochlear and Edinger–Westphal nuclei in the Hottentot golden mole.

3.2. Putative catecholaminergic nuclei

Tyrosine hydroxylase (TH+) immunoreactivity revealed the putatively catecholaminergic neurons in the brains of the three
Fig. 4. Serial drawings of coronal sections through one half of the four-toed sengi (Petrodromus tetradactylus) brain from the olfactory bulb through to the spinomedullary junction. A is the most rostral section, S the most caudal. The outlines of the architectonic regions were drawn using Nissl and myelin 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 1200 μm apart. See list for abbreviations.
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 González, 2000) 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., Smeets and González, 2000) were observed. The TH+ nuclei found in the three species studied were similar to that seen in many other mammals.
Fig. 5. Photomicrographs of sections stained for Nissl substance (A, C, E, G) and immunoreactivity for cholineacetyltransferase (B, D, F, H), showing the location and appearance of cholinergic interneurons in the brain of the Hottentot golden mole. A and B are taken through the olfactory bulb, showing the cholinergic interneurons in the periventricular cell layer. C and D are of the cerebral neocortex showing the cholinergic interneurons in the upper layers of the cortex. E and F are taken through the centre of the amygdaloid complex showing the cholinergic interneurons located throughout this nuclear mass. G and H are taken through the hippocampal formation, showing the presence of cholinergic interneurons in the cornu ammonis and dentate gyrus. The scale bar in D = 500 μm and applies to C and D, the scale bar in H = 1000 μm, and applies to A, B, E, F, G and H.
but there were some exceptions. The rodent typical C3 nucleus (rostral dorsal midline medullary nucleus) and the Murid rodent/primate/megabat A6c nucleus (compact portion of locus coeruleus) (e.g., Dell et al., 2010; Kruger et al., 2012) were absent in all three species. The A15d nucleus (anterior hypothalamic group, dorsal division) was absent in the four-toed sengi, as seen for the eastern rock elephant shrew (Pieters et al., 2010), and the A6d nucleus (diffuse portion of locus coeruleus) was comparatively small in both the Hottentot golden mole and the four-toed sengi.

3.2.1. Olfactory bulb (A16)

The neurons forming the A16 nucleus were observed to be dense clusters of TH+ cells in the glomerular layer of the olfactory bulb in all three species (Figs. 2A, 3A–C and A–D). These neurons likely represent the periglomerular dopaminergic neurons, were small in size, and were found in equal density surrounding the entire glomeruli.

3.2.2. Diencephalic nuclei (A15 – A11)

In the hypothalamus of all three species TH+ cells formed six distinct nuclei: the dorsal division of the anterior hypothalamic group (A15d – except in the four-toed sengi, where this nucleus was absent), the ventral division of the anterior hypothalamic group (A15v), the rostral periventricular cell group (A14), the zona incerta (A13), the tuberal cell group (A12), and the caudal diencephalic group (A11) (Figs. 2F–I, 3G–I, 4H–K and 7A–C). Within the dorsal anterior portion of the hypothalamus of the Hottentot golden mole and giant otter shrew, between the third ventricle and the fornix, TH+ neurons with a moderate density were designated as the A15d nucleus. The TH+ neurons forming the A15v nucleus were located in the ventrolateral portion of the hypothalamus in a moderate density close to the floor of the brain and in the four-toed sengi these neurons appeared to be more numerous than in the other two species examined. TH+ neurons, with a low to moderate density forming two columns adjacent to the lateral walls of the third ventricle, were assigned to the A14 nucleus. Within the dorsolateral aspect of the hypothalamus, lateral to the fornix and intermingling with the neurons forming the zona incerta of the ventral thalamus, were TH+ neurons with a moderate density forming to the A13 nucleus. TH+ neurons assigned to the A12 nucleus were located in the ventromedial portion of the hypothalamus, surrounding and below the floor of the third ventricle in the arcuate nucleus and the immediate vicinity. In the four-toed sengi, these neurons extended into the regions around the mammillary bodies. Lastly, within the

Fig. 6. Photomicrographs showing neuronal groups immunoreactive for choline acetyltransferase in the olfactory tubercle (TOL) and nucleus accumbens (N.Acc) (A and B) and pontine region (C and D) of the Hottentot golden mole (A and C) and the four-toed sengi (B and D). Note the elaborated appearance of the TOL in the Hottentot golden mole (A) and the four-toed sengi (B) and the standard mammalian appearance and location of the laterodorsal tegmental (LDT), pedunculopontine tegmental (PPT) and parabigeminal (PBg) nuclei in both species (C and D). Scale bar in D = 1000 μm and applies to all.
hypothalamic grey matter adjacent to the posterior pole of the third ventricle, a moderate density of TH+ neurons formed the A11 nucleus, although the cells were less numerous in the giant otter shrew than in the other species.

3.2.3. 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 retrolubral nucleus (A8) within the midbrain tegmentum in all three species studied (Figs. 2L, J, 3L–4L, N and 7D, E). TH+ neurons with a high density, found dorsal and dorsolateral to the interpeduncular nucleus, between this nucleus and the root of the oculomotor nerve (Illn), were 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 dense cluster of TH+ neurons formed the A10c nucleus. Dorsal to A10c, between it and the oculomotor nucleus, was a dense bilateral parasagittal cluster of TH+ neurons that formed the A10d nucleus. The TH+ neurons assigned to the A10dc nuclear complex were found within the periaqueductal grey matter surrounding the ventral half of the cerebral aqueduct.

The substantia nigra proper complex was observed in the ventral and lateral portions of the midbrain tegmentum, just dorsal to the cerebral peduncles. 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, scattered TH+ neurons were assigned to the A9v (ventral) nucleus. 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 (Illn), 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 but relatively numerous, cluster of TH+ neurons that formed the A8 nucleus.

3.2.4. Rostral rhombencephalon – the locus coeruleus complex, A7 – A4

Within the pontine region of all three species studied a large number of TH+ neurons forming the locus coeruleus complex were readily observed. The locus coeruleus complex could be subdivided into five nuclei, these being: the subcoeruleus compact portion (A7sc), subcoeruleus diffuse portion (A7d), locus coeruleus compact portion (A6c), fifth arcuate nucleus (A5), and the dorsolateral division of locus coeruleus (A4) (Figs. 2L, 3K–N, 4O, P and 7F). Within the dorsal portion of the pontine tegmentum adjacent to the ventrolateral region of the periaqueductal grey matter, a tightly packed cluster of TH+ neurons represented the A7 compact portion of the locus coeruleus. 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. These neurons are located both medially and laterally around the trigeminal motor nucleus (Vmot). 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 in the giant otter shrew. In the Hottentot golden mole and the four-toed sengeri, a lower number of less densely packed neurons were assigned as the A6d nucleus in these species. No compact division of the locus coeruleus (the A6c division as observed in rodents, e.g., Kruger et al., 2012) was observed in any of the three species examined. In the ventrolateral pontine tegmentum lateral to the superior oliveary 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 very dense, but small cluster of TH+ neurons represent the A4 nucleus.

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

In the medulla of all three species five putative 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. 2M–Q, 3N–Q and 4P–S). 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 ventrolateral 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 moderately dense cluster of TH+ neurons were 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, were a single large, densely packed, cluster of intensely stained TH+ neurons, the area postrema.

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 three species examined exhibited the same complement of serotonergic nuclei in both the rostral and caudal clusters.

3.3.1. Rostral cluster

Within the rostral cluster we found evidence for the caudal linear nucleus (Cl), the supraplenalms nucleus (B9), the median raphe nucleus (Mrn) and the dorsal raphe complex formed of six distinct nuclei (Figs. 2K–L, 3K–M, 4M–O and 8). The Cl nucleus was the most rostral of the serotonergic nuclei found and the 5HT+ neurons formed a moderate density cluster around the midline immediately dorsal to the interpeduncular nucleus in a location just anterior to the decussation of the superior cerebellar peduncle in all three species. The serotonergic neurons forming the B9 nucleus appeared to be a lateral extension of the most ventral portion of Cl. The 5HT+ B9 neurons were found in a low density caudal to the A9pc (see above) and extended as an arc of neurons into the lateral and ventrolateral portion of the midbrain tegmentum. The median raphe nucleus (Mrn) was characterised by two distinct, densely packed 5HT+ neuronal columns on either side of the midline in a para-raphe position. The rostral border of this nucleus was coincident with the level of the decussation of the superior cerebellar peduncle and the caudal border of this nucleus was found at the level of the trigeminal motor nucleus.

Within the dorsal raphe nuclear complex we identified six distinct nuclei in all three species: the dorsal raphe interfascicular nucleus (Drif), the dorsal raphe ventral nucleus (Drv), the dorsal...
raphy dorsal nucleus (DRd), the dorsal raphe lateral nucleus (DRI), the dorsal raphe peripheral nucleus (DRp) and the dorsal raphe caudal nucleus (DRc). 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 parapalae columns of 5HT+ cells located between the bilaterally paired medial longitudinal fasciculi represent the DRI nucleus in all three species. The DRv was found immediately dorsal to the DRI and just caudal to the oculomotor nuclei. The DRv exhibited a high density of 5HT+ neurons. Immediately dorsal to DRv and ventral to the inferior border of the cerebral aqueduct a high-density cluster of 5HT+ neurons were designated as the DRd nucleus. 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 ones found outside the periaqueductal grey matter. The 5HT+ neurons of the DRI 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. As we followed the DRI caudally, where the cerebral aqueduct opened into the fourth ventricle and the DRd, DRv and DRI disappeared, the neurons of the DRI formed an arc across the midline of the dorsal portion of the periventricular grey matter. This caudal arc of the DRI 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., 2002b; Maseko et al., 2007; Dell et al., 2010; Kruger et al., 2012).

3.3.2. Caudal cluster

Within the caudal cluster we found evidence for the raphe magnus (RMg), rostral and caudal ventrolateral (RVL and CVL), raphe pallidus (RPa) and raphe obscurus (ROb) nuclei (Figs. 2M–P, 3M–P and 40–S). The RMg was observed to be two columns of loosely aggregated moderate to large 5HT+ neurons located on either side of the midline from the level of the caudal pole of the trigeminal motor nucleus to the caudal pole of the facial nucleus. Within the left and right ventrolateral medullary tegmentum a distinct anteroposterior column of 5HT+ neurons 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; Dwarska et al., 2008). The RVL began as a lateroventral continuation of 5HT+ neurons from the lower portion of the RMg extending over the pyramidal tracts and consolidating as a distinct column lateral to the inferior olives. The inferior olive
topologically distinguishes left and right RVL, and 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 is reached. Although the RVL and CVL were continuous in the species studied, and indeed several other eutherian mammals previously studied (e.g., Maseko et al., 2007; Moon et al., 2007; Dwarika et al., 2008; Dell et al., 2010; Kruger et al., 2012), we distinguish two components of these ventrolateral columns, as the caudal portions have not been reported in the opossum or the monotremes (Crutchler and Humberson, 1978; Manger et al., 2002b). The SHT+ neurons forming the RPa nucleus were found in the ventral midline of the medulla associated with the pyramidal tracts. These neurons were for the most part located between the two pyramidal tracts. Two loosely arranged bilateral columns of SHT+ neurons located either side of the midline from the level of nucleus ambiguous to the spinomedullary junction were classified as the ROb.

3.4. Orexinergic (hypocretinergic) nuclei

Orexin-A immunohistochemistry was used to identify orexin-A immunopositive neurons (Orx+) in the giant otter shrew and the four-toed sengi. The vast majority of orexin-A immunopositive neurons (Orx+) identified in the brains of these species were localised to the hypothalamus. Within the area where orexinergic neurons were located we could readily divide them into three distinct clusters: a main cluster (Mc), a zona incerta cluster (Zic) and an optic tract cluster (Otc) (Figs. 2G, H and 4I–K). In both species the main cluster (Mc) was identified as a large group of densely packed Orx+ neuronal cell bodies located lateral to the third ventricle in the perifornical region, with a moderate number of neuronal cell bodies extending mediially from this area into the dorsomedial hypothalamus and a larger number extending into the lateral hypothalamic areas (Fig. 9). From the main cluster a group of Orx+ neuronal cell bodies extended laterally into the region of the zona incerta (Zic). This cluster had a very low density of Orx+ neurons that were mixed with neurons of the lateral hypothalamic cholinergic nucleus and the A13 nucleus of the catecholaminergic system (see above). The third cluster, the optic tract cluster (Otc) extended ventrolaterally from the main cluster to the ventrolateral region of the hypothalamus adjacent to the optic tract. This cluster exhibited a moderate density of Orx+ neuronal cell bodies. In both species 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 inferior border of the zona incerta.

4. Discussion

The nuclear organisation and complement of the neural systems investigated in the current study within the brains of three previously unstudied species of Afrotheria, the giant otter shrew (P. velox), the Hottentot golden mole (A. hottentotus), and the four-toed sengi (P. tetradactylus), were, for the most part, similar to that observed in many eutherian mammals previously described (e.g., Dell et al., 2010). Despite this there were differences of note that may be related either to the phylogenetic history or current life histories of these species. The golden mole exhibited an extensive and divergent distribution of cholinergic interneurons, far beyond that seen in other mammals. The four-toed sengi also exhibited cholinergic neurons in regions of the brain not normally associated with the cholinergic system, but cholinergic neurons were previously observed in these regions in the closely related eastern rock elephant shrew (Pieters et al., 2010). In agreement with previous observations in the eastern rock elephant shrew (Pieters et al., 2010), we could not find evidence for the catecholaminergic dorsal division of the anterior hypothalamic group (A15d) in the four-toed sengi. Lastly, in both the golden mole and the four-toed sengi, the catecholaminergic diffuse division of the locus coeruleus (A6d) was made up of a small number of neurons, similar to that observed in the rock hyrax (Gravett et al., 2009). In contrast to these observations, the giant otter shrew appears to have a nuclear organisation of these systems that can be described as very typical of eutherian mammals.

4.1. The “hypercholinergic” Hottentot golden mole

While the cholinergic system in the three species studied was mostly similar to that observed in other eutherian mammals, the Hottentot golden mole was observed to have cholinergic interneurons throughout the cerebral neocortex and piriform cortex, the olfactory bulb, amygdala and hippocampus. The existence of cholinergic interneurons in this wide variety of locations has not been previously described. Cholinergic interneurons in the cerebral neocortex have only been observed previously in Murid rodents, with non-Murid rodents lacking these neurons (Bhagwandin et al., 2006; Kruger et al., 2012). Studies of the chemical nature of the cholinergic neocortical neurons in Rattus norvegicus, a Murid rodent, have indicated that these neurons likely co-contain
vascular intestinal polypeptide (VIP) and gamma-aminobutyric acid (GABA) (Eckenstein and Baughman, 1984; Bayraktar et al., 1997). Thus, these neocortical neurons have multiple transmission lines that may allow them to modulate the cortical microcirculation in relation to these multiple transmission lines (Chedotal et al., 1994). In the case of the golden mole, this may indicate that the microcirculation undergoes a great deal of local control in many brain regions. The question remains as to why this may occur in the golden moles. Our observations show that the olfactory system of the golden mole is likely to be the dominant sense, and this is reflected in the large size of the olfactory bulb and the impressive size and modularisation of the olfactory tubercle in comparison to the other species studied herein and previously. The cholinergic neurons found in the olfactory bulb, piriform cortex, and amygdala, all indicate the importance of the modulation of neuronal function and microcirculation in regions of the brain associated with olfaction. While the hippocampus and neocortex are not parts of the brain normally considered to be involved in processing olfactory information, it is likely, given the dominance of the olfactory system in the golden mole, that they are recruited for this purpose, perhaps in the recognition of location in relation to olfactory stimuli for the hippocampus, and in the process of decision making in relation to olfactory stimuli for the cerebral neocortex. While these ideas are obviously speculative, it would be of interest to examine these functional possibilities further in the Hottentot golden mole, and to examine more species of golden moles to determine whether these supernumerary cholinergic neurons are a phylogenetically distinct characteristic of golden moles (the order Chrysochloridae), or whether they are restricted to the single species studied herein.

4.2. Additional cholinergic neurons in the four-toed sengi

In addition to the supernumerary cholinergic neurons observed in the golden mole, we observed cholinergic neurons in the superior and inferior colliculi and the cochlear nucleus in the four-toed sengi. Similar cholinergic neurons, in the same locations, were previously observed in the eastern rock elephant shrew (Pieters et al., 2010) both species being members of the order Macroscelididae, the elephant shrews or sengis. This similarity in the distribution of cholinergic neurons outside of the classically defined cholinergic system (Woolf, 1991) indicates a common ancestry for these species, and the evolution of these neural traits during the establishment of the order Macroscelididae (Manger, 2005). Thus, while we can conclude that the cholinergic neurons in these regions of the elephant shrew brain are likely to be a feature common to all elephant shrews, it is still possible that they play an important functional role related to aspects of their life-history. As mentioned above, these cholinergic interneurons are likely to be involved in the modulation of the microcirculation in the brain regions in which they are found. In both species of elephant shrews studied, all three regions of the brain in which the cholinergic interneurons were found are involved in audition. Both species of elephant shrews studied are known to produce species-specific patterns of foot-drumming behaviour in response to agonistic encounters (Faurie et al., 1996; Skliha et al., 2008), with most species of elephant shrews likely to show some form of foot drumming communication (Faurie et al., 1996). The drumming sounds created by the elephant shrews are detectable both via aerial and seismic transmission as a form of sound wave. The modulation of the frequency tuning circuits in the cochlear nuclei and spatial location circuits in the inferior colliculus may enhance the detection and location of the source of the drumming. The cholinergic neurons in the superior colliculus may enhance the visual targeting of the drumming source. Thus, it is possible that the common ancestor of elephant shrews used drumming for conspecific communication, augmented by cholinergic neurons in regions of the brain of importance for the detection and location of these auditory signals. These traits would then have been passed on to all descendants of the common ancestor and we see them in the brains of extant elephant shrews. Further exploration of the occurrence of these neurons across elephant shrew species, with specific reference to the species-specific pattern and behavioural use of foot drumming may provide data of interest to our understanding of the elephant shrews.

4.3. Differences in the catecholaminergic systems and phylogenetic relationships

We could not find evidence for the existence of the A15d nucleus in the four-toed sengi. In a previous study of the eastern rock elephant shrew, this nucleus was also absent (Pieters et al., 2010), indicating an order specific absence of this nucleus in the Macroscelididae (Manger, 2005). This particular catecholaminergic nucleus has been associated with seasonal control of reproduction (Thiery et al., 1988; Beccevin et al., 1998), and while the elephant shrews are seasonal breeders and the males show seasonal recrudescence of the testicles, there are indications that they are spontaneous ovulators and that there may not be a complete absence of sperm in the reduced testicles (Medger et al., 2012). Thus, the release of inhibition of pulsatile luteinizing hormone may allow opportunistic breeding opportunities for these mammals when conducive conditions arise. This process either does not occur in the other species studied or is currently unknown. In addition to the lack of the A15d nucleus, in both the four-toed sengi and the golden mole, the number of neurons forming the diffuse portion of the locus coeruleus (A6d) was small in number. This characteristic is shared by the rock hyrax (Gravett et al., 2009), but not by the giant otter shrew. Thus, it appears that the common ancestor of the Macroscelididae, Chrysochloridae and Hyracoidea may have had this trait. While this concept is supported for the Macroscelididae and Chrysochloridae, to the exclusion of the Tenrecidea, due to the closer relationship of the Macroscelididae and Chrysochloridae, the occurrence of this trait in the Hyaroida indicates, based on current understandings of the phylogenetic relationships of the Afrotheria (Arnason et al., 2008; Asher et al., 2009), that either this character evolved twice, or was lost in the Tenrecidea. Further comparative work in the anatomy of the locus coeruleus in other members of the Afrotheria will resolve this problem.

4.4. The lack of changes in the serotonergic and orexinergic systems

Previous studies of the nuclear organisation of the serotonergic system across mammals have shown that the organisation 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). The three Afrotherian species investigated in the current study do not negate these previous observations on the organisation of the serotonergic system. The orexinergic system also appears to have a quite conservative organisation across mammalian species studied to date. As with the two species of Afrotherian studied herein, the giant otter shrew and the four-toed sengi, most mammals have three specific clusters of orexinergic neurons, all located in the hypothalamus – a main cluster, an optic tract cluster and a zona incerta cluster. Three exceptions to this organisation have been reported in the literature. Both hamsters and microchiropterans appear to lack the optic tract cluster of orexinergic neurons (Mintz et al., 2001; McGranaghan and Piggins, 2001; Khorooshi and Klingenspor, 2005; Vidal et al., 2005; Kruger et al., 2010), while the cetartiodactyla, represented by the giraffe and harbour porpoise,
are reported to have an additional cluster of parvocellular orexinergic neurons in the medial hypothalamus (Dell et al., 2012). Thus, for the most part, the organisation of the orexinergic system is also quite conservative across mammals.

Ethical statement

The Afrotherian species used in the present study were caught from wild populations in the Democratic Republic of the Congo and South Africa under permission and supervision from the appropriate wildlife authorities. All animals were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee, which parallel those of the NIH for the care and use of animals in scientific experimentation.

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