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AN ASSESSMENT OF PHYLOGENETIC ORIGIN IN *Chiroptera* USING THE NEUROMODULATORY SYSTEM

by:

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A dissertation submitted to the faculty of science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science

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DECLARATION

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

Signature of candidate:

day of 2007

ABSTRACT

The current study documents the findings from immunohistochemical examination of the brains of microbats and megabats (Chiroptera) using antibodies for cholineacetyltransferase (cholinergic neurons), tyrosine hydroxylase (dopaminergic, adrenergic and noradrenergic neurons), and serotonin (serotonergic neurons). The objective of the study was firstly to describe the anatomical organization and morphology of the neuromodulatory systems (nuclear complement) in both microbats and megabats, as there is no literature on these systems in the brains of chiropterans. Secondly, we aimed to investigate whether or not there are differences in these systems between the two suborders of chiroptera in hopes to shed some light on the phylogeny of the two, which is a controversial subject. The two groups were found to possess clear differences in their respective neuromodulatory nuclear complements. The differences observed between the two groups include a dorsal division of the locus coeruleus (A6d), which was absent in microbats but present in megabats, also the absence of an A4 in microbats but clear presence in megabats, and the parabigeminal (PBg) nucleus that was absent in microbats but clearly visible in megabats. The microbats were found to possess a complement that appeared similar to that of insectivores; whereas megabats had a complement resembling that of primates, carnivores and rodents. The differences found between the two groups suggest a diphyletic origin for the two groups.

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In memory of my grandparents,
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who will forever be loved and missed

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1. INTRODUCTION

The flying mammals are classified under the order Chiroptera, which has been split into two suborders: Megachiroptera/megabats and Microchiroptera/microbats. The two groups are classified together because of morphological similarities, particularly that of the musculoskeletal structure of the flying apparatus (Pettigrew *et al.*, 1989). Megabats are indigenous to the Old World, large bodied and vegetarian (feed on fruit, nectar and flowers), while microbats are very diversely spread around the world and are mainly insectivorous (Pettigrew *et al.*, 1989). But do these two suborders really belong together as a monophyletic group? Do they share a more recent common ancestor with each other than with other species? A startling discovery was made when the neural pathway between the retina and the superior colliculus in the two animals was examined. Pettigrew (1986) found that the pathway in megabats is identical to that found in primates, which was thought to be unique to primates; additionally, the microbats showed a pathway similar to that of non-primate vertebrates. Pettigrew (1986) took this result to indicate that the order Chiroptera was not a real designation, and that the megabats are most likely to be a sister group to primates – not even closely related to the microbats.In a list of contrasting attributes of megabats and microbats with specific primate features, megabats appeared to share more in common with primates (29 of the 33 listed attributes), while microbats only shared one feature (Pettigrew *et al.*, 1989). Despite the many differences that have been documented between the two suborders, scholars continue to believe that the two groups belong in the same order. This is based on the fundamental belief that mammalian flight only evolved once, making the two groups monophyletic (Jones *et al.*, 2002). One such researcher is Van Valen (1979), who claimed that gross observation alone indicates common derivation for bat wings. Pettigrew and co-workers (1989) examined the wings in both groups, designating indices to the bone structure and therefore quantifying the wings. They found that megabats and microbats show consistent differences in the indices, even when they compared megabats and microbats of the same body weight and lifestyle, or megabats of lower body weight than microbats, thereby eliminating the possibility that the differences in body weight or

differences in lifestyle were the main reason for the metrical differences they observed in the wings.

The separation of megabats and microbats into different orders would imply a diphyletic origin, i.e. that mammalian flight evolved twice (Pettigrew *et al.*, 1989). This has been widely rejected due firstly to the morphological similarity of the musculoskeletal structure of the wings, and secondly the absence of a recognizable sister group for either of the suborders (Pettigrew *et al.*, 1989). The perception of monophyly in the bats seems more and more of a bias, as more evidence is continually uncovered that points toward a diphyletic origin for the megabats and microbats, despite opposing views being forwarded by molecular phylogenetic studies (e.g. Teeling *et al.,* 2005).
Previous work has also shown many similarities between the megabats and primates, indicating primates being a possible sister group for the megabats (Pettigrew *et al.*, 1989). It is therefore crucial that other aspects of megabats and microbats are researched in order to properly, or at least to a significant extent, illuminate the relationship between the two groups and possibly even find a recognizable sister group for either of the two, if indeed they exist. The present study is aimed at obtaining a data set that may shed light on the relationships between the megabats, microbats, and other mammalian orders, with primates and insectivores being specifically targeted as potential sister groups of megabats and microbats, respectively.

Given that the similarities between mega- and microbats are probably superficial, and therefore the conclusions drawn based on these similarities may be biased, this problem is in need of an objective way of analyzing whether these two groups belong to the same order. Brain organization often follows the same basic plan in groups of animals that share common ancestry (Manger, 2005; Butler and Hodos, 1996). This indicates that other parts of brain organization should be studied, i.e. in addition to the morphological study of retinotectal pathways by Pettigrew *et al.* (1989).

The present work focuses on the neuromodulatory systems of microbats, megabats, and other mammals including insectivores and primates, with the aim of studying the location, parcellation, and basic structure of these systems so as to make an objective decision based on the generated evidence about the most likely phylogenetic relationship of the megabats and microbats. Manger (2005) found that the same nuclear

parcellations of the neuromodulatory systems are seen in all species of the same mammalian order, regardless of the differences in brain size, phenotype, or life history of the animals. This therefore indicates that different nuclear compartments of the neuromodulatory systems may be found in different orders. Therefore if megabats and microbats show different nuclear complements of the neuromodulatory systems, it may be concluded that they belong to different orders. Moreover, if megabats show the same nuclear complement as primates, they may belong to the same order as primates; and if microbats show the same complement as insectivores, they may belong to the same order as insectivores. The neuromodulatory systems represent a good model to search for the answers regarding the question of chiropteran phylogeny, as they are, for the most part, independent of the influence of echolocation and skeleto-muscular morphological adaptations that may have resulted due to flight and hunting mechanisms. Also, this study made use of a megabat that is closest to the microbat studied with regards to lifestyle. The megabat used belongs to the only genus of megabats that echolocates (Baron et al., 1996a), therefore eliminating the chance that differences found could be due to echolocation or lack thereof.

Neuromodulatory systems have been studied in detail in an effort to understand their role in the central nervous system, and are crucial systems to understand in the organism, as they control a large range of functions in the central nervous system. No study of the neuromodulatory systems in chiroptera have as yet been undertaken; we therefore do not have specific data on the location and morphology of these systems in chiroptera; however, data is available for other mammals. This data (mainly rat, cat, monkey, baboon and human) is used herein to describe the potential location and morphology of neuromodulatory nuclei in bats. The neuromodulatory systems are formed by different nuclei, which are identified based on their location in the brain, their morphology, their connections (Woolf, 1991), and the chemicals they release to convey different messages, i.e. neuromodulators, also called neurotransmitters. The neurons forming these nuclei produce and secrete different neurochemicals to achieve different functions, and are named according to the chemicals they release. The neurons can either be monoaminergic or cholinergic. The monoaminergic neurons are further divided into catecholaminergic (consisting of neurons producing the chemicals adrenalin,

noradrenalin, or dopamine) and indolaminergic (consisting of neurons producing serotonin), while the cholinergic neurons release acetylcholine as the functional neurochemical.

1.1. Monoaminergic neurons

Monoaminergic neurons modulate the function of widespread regions throughout the brain (for review see Tork, 1990; Smeets and Gonzalez, 2000). This is by controlling the level of activity in certain brain functions, i.e. they increase or decrease neuronal discharge rates. Most of the monoaminergic cell bodies are located within the brainstem, and the highly branched axons contribute terminal boutons to most brain areas. Dopaminergic neurons are associated with the modulation of movement, and attention and learning, reward and punishment, exhibiting both excitatory and inhibitory effects depending on the site of action. The nuclear groups are called the *ventral tegmental area* and *substantia nigra*, these being the largest of the dopaminergic nuclear complexes and are located in the midbrain. Adrenalin and noradrenalin are associated with the control of alertness and wakefulness, exhibiting inhibitory effects. The nuclear complex releasing noradrenalin is called the *locus coeruleus* and is found in the pons. Neurons producing adrenalin are mostly located in the medulla. Serotonin has been implicated in the regulation of mood, the control of eating, sleep and arousal, and the regulation of pain. Its neurons are also implicated in the control of dreaming. Its nuclei are called *raphe nuclei* due to their location adjacent to the midline (a pararaphe position) throughout the brainstem.

1.2. Cholinergic neurons

These neurons are involved in learning and memory, controlling the stage of sleep during which dreaming occurs (REM sleep) (Woolf, 1991), and potentially in generating conscious experiences (Woolf and Hammeroff, 2001). In most mammals studied to date, the cholinergic neurons form a continuous column along the rostro-caudal axis of the central nervous system, from the ventral horn of the spinal cord (cholinergic

motorneurons), through the cranial nerve motor nuclei, pontine reticular nuclei, hypothalamic nuclei, ending at the basal forebrain (Woolf, 1991; Manger *et al*., 2002a). It has been proposed (Manger, 2005; Manger *et al.,* 2002a, b and c, 2003, 2004; DaSilva et al., 2006) that no changes in complexity of the nuclear groups occur in different species of the same order, however distant the age of the most recent common ancestor. This implies that megabats and microbats should show the same level of complexity in the nuclear parcellation of the neuromodulatory systems if they belong to the same order. The same applies to any similarities found between the bats and any of the other orders to be examined in this study.

The objectives of the current study are firstly to study the neuromodulatory system's basic anatomy in the chiropteran brain; and secondly to determine whether the two suborders of chiroptera should be taxonomically grouped together, i.e. whether they have a monophyletic origin or not. The study hypothesises that megabats and microbats will have fundamental differences in the anatomy of the neuromodulatory systems, and that insectivores and primates will be shown to be sister groups to the microbats and megabats respectively.

To test these hypotheses, the study was divided into two subprojects, the first of which was to use immunohistochemical staining to immunolabel the cell clusters in a microbat and the second to repeat the process in a megabat. The experimental and collected data (from previously published studies in other mammalian orders), is then compared in order to create an evolutionary reconstruction and thereby analyze the phylogenetic relationship between megabats and microbats and other mammals (including primates, carnivores, tree shrews, monotremes, insectivores, marsupials, ungulates, cetaceans, rodents and lagomorphs).

2. **MICROBATS**

2.1. INTRODUCTION

Microchiroptera, also known as microbats, are an extremely diverse and successful group of mammals, consisting of over 800 species and forming one suborder of the Chiroptera, the order of flying mammals (Nowack, 1999; Simmons, 2000). The microbats occupy a variety of niches worldwide, have small bodies (less than 100g) and have a variety of diets but are mainly insectivorous (Nowack, 1999; Adams, 2000). Microbats are largely dependent on echolocation to navigate and acquire food, in contrast to the highly visual megabats (e.g. Suga, 1989; Pettigrew et al, 1989; Vater, 2000). While major works have been undertaken on the structural anatomy of the brain of numerous microbats species (Baron et al., 1996a,b,c; reviewed by Reep and Bhatnagar, 2000) and a great deal of research undertaken on the neurobiology of the echolocation system (e.g. Suga, 1989; Moss and Sinha, 2003; Covey, 2005), work on other aspects of the brain are not so numerous (e.g. Pettigrew et al., 1989; Manger et al., 2001; Wise et al., 1986; Hof et al., 1999). No reports detailing neuromodulatory systems within the microbat brain have yet been furnished.

There are several neuromodulatory systems in the brains of vertebrates, many of which can be identified using immunohistochemical techniques. In the present study the cholinergic, catecholaminergic and serotonergic systems of the microbat brain are revealed. The cholinergic system has been found to be involved in learning and memory, potentially generating conscious experiences, and controlling the stage of sleep at which dreaming occurs (e.g. Woolf, 1991; Woolf and Hammeroff, 2001; Siegel, 2006). This system innervates the entire central nervous system via several nuclei and projections (Woolf, 1991). The catecholaminergic system can be further divided into three subtypes, including dopaminergic, noradrenergic and adrenergic systems. The dopaminergic part of the catecholaminergic system has been found to play an important role in some neuroendocrine regulations, e.g. reproduction, growth, lactation and stress (Tillet and Kitahama, 1998). For example, the dopaminergic nuclei in the hypothalamus of the pig, are implicated to be producing “hypothalamic neurohormones”, influencing the endocrine

secretions, and ultimately affecting functions such as reproduction and lactation (Leshin *et al.*, 1995). Dopaminergic neurons that are located at the substantia nigra and ventral tegmental area are thought to be involved in a range of cognitive functions such as motor planning, learning memory, cognitive flexibility and counteracting hyperthermia (Previc, 1999). Within the pontine region, the locus coeruleus complex is found consisting of neurons that produce noradrenalin, whose function is known to be associated with the control of wakefulness (Siegel, 2006) and the control of muscle tone (Pompeiano, 2001). The medullary catecholaminergic neurons are adrenergic in nature and are known to be essential for cardiorespiratory regulation (Törk, 1990; Chalmers and Pilowsky, 1991; Ellenberger and Feldman, 1994). This system is usually divided into the classical nuclear grouping of A1-A17/C1-C3 (Dahlström and Fuxe, 1964; Hokfelt et al., 1984), but additional nuclei have been located in other brain regions (Smeets and Gonzalez, 2000). The classical nomenclature is used herein for ease of cross-species comparison; however, the anatomical names as described by Smeets and Gonzalez (2000) are also given as appropriate. The serotonergic system, is thought to be involved in the regulation of mood, control of eating, sleep and arousal, and the regulation of pain (Tork, 1990; Jacobs and Azmitia, 1992). In mammals this system is divided into a rostral (projecting to the forebrain) and caudal (projecting to the hindbrain and spinal cord) cluster each containing several nuclei, and the neurons are mostly found adjacent to the midline throughout the brainstem (Tork, 1990), although there is some lateral spreading of neurons into the tegmentum (e.g. Bjarkam et al., 1997).

The neuromodulatory systems that are the object of the present study are not directly involved in the neural processing generating flight or other forms of locomotion, or echolocation; making them ideal to study since any differences or similarities discovered in their organization as compared to other mammals will not be as a result of either of these microbat specializations. While these neuromodulatory systems have clearly changed during the course of evolution, different researchers have forwarded different explanations to account for these changes. For example, Woolf (1991) proposed that a correlation between the somatal area of cholinergic neurons and the overall size of the brain could be found. The serotonergic system on the other hand, is thought to have undergone a "lateralization" in its cellular organization in those mammals with larger

brains (Bjarkam et al., 1997). In an extensive review of the comparative literature, Smeets and Gonzalez (2000) concluded that no specific trend in the evolution of the catecholaminergic system could be discerned.

More recently, Manger (2005) proposed, on the basis of several studies (Manger et al., 2002a,b,c, 2003, 2004) that despite differences in size, phenotype or life history, the same complement of neuromodulatory nuclei exists for mammals that belong to the same phylogenetic order. This means that each mammalian order will exhibit a unique complement of nuclei that is typical of all its members. This proposal has gained support from a recent study of the neuromodulatory systems in the brainstem of the micropthalmic Highveld molerat (Da Silva et al., 2006).

Given these differing evolutionary scenarios concerning the neuromodulatory systems, the contentious phylogeny of the Chiropteran order (e.g. Pettigrew et al., 1989; Simmons, 2000), and the probable independence of the neuromodulatory systems from the specializations exhibited in the two Chiropteran suborders, the present study may reveal a set of characters that has the possibility to shed light on the phylogenetic relationships of the microbats.

2.2. MATERIALS AND METHODS

The brains of three adult female microbats, Schreiber's long fingered bat (*Miniopterus schreibersii*), were used in the current study. The animals were captured from caves in the northern part of Gauteng Province, South Africa under permission and supervision from the Gauteng Nature Conservation Directorate. 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. The microbats were placed under deep barbiturate anaesthesia (Euthanaze, 200mg sodium pentobarbital/ kg, i.p.), and then perfused intracardially upon cessation of respiration. The perfusion was initially done with a rinse of 0.9% saline solution at 4°C, followed by a solution of 4% paraformaldehyde in 0.1M phosphate buffer (PB) (approximately 50 ml of each solution). Brains were then removed from the skull and post-fixed overnight in 4% paraformaldehyde in 0.1M PB, and then allowed to equilibrate in 30% sucrose in PB. Two brains were then frozen and sectioned into serial coronal sections of 50 μm thickness and one in a sagittal plane. A one in four series of stains was made for Nissl, tyrosine hydroxylase (TH), choline acetyltranferase (ChAT) and serotonin. Sections kept for the Nissl series were mounted on 0.5% gel coated glass slides, cleared in a solution of 1:1 chloroform and absolute alcohol, then stained with 1% cresyl violet.

For immunohistochemical staining the sections were first treated for 30 min with an endogenous peroxidase inhibitor (49.2% methanol: 49.2% 0.1PB: 1.6% 30% H_2O_2) followed by three 10 min rinses in 0.1M PB. This was followed by a 2 hour preincubation, at room temperature, in a solution (blocking buffer) containing 3% normal goat serum (NGS, Chemicon), 2% bovine serum albumin (BSA, Sigma), and 0.25% Triton X-100 (Merck) in 0.1M PB. The sections were then placed in a primary antibody solution containing the appropriately diluted antibody in blocking buffer, for 48 hours at 4°C. To reveal cholinergic neurons we used anti-cholineacetyltransferase (AB143, Chemicon) at a dilution of 1:1500. To reveal catecholaminergic neurons we used anti-tyrosine hydroxylase (TH) (AB151, Chemicon) at a dilution of 1:6000. To reveal serotonergic neurons we used anti-serotonin (AB938, Chemicon) at a dilution of 1:7500.

This step was followed by three 10 min rinses in 0.1M PB, after which the sections were incubated in a secondary antibody for two hours. The secondary antibody solution contained a 1:500 dilution of biotinylated anti-rabbit IgG (BA-100 Vector), in blocking buffer containing 3% NGS, and 2% BSA in 0.1M PB. After three 10 min rinses in 0.1M PB, the sections were incubated for 1 hour in AB solution (Vector Labs), and again rinsed. The sections were then treated in a solution of 0.05% diaminobenzidine (DAB) in 0.1M PB for 5 minutes, following which 3µl of 30% H_2O_2 was added to the solution in which each section was immersed. Development was monitored visually and checked under a low power stereomicroscope. This was allowed to continue until the background staining was at a level at which it could assist reconstruction without obscuring the immunopositive neurons. Development was then arrested by placing the sections in 0.1M PB, and then rinsed twice more in the same solution.

Sections were mounted on glass slides coated with 0.5% gel and left to dry overnight. They were then dehydrated in a graded series of alcohols, cleared in xylene, and coverslipped with Depex. Two controls were employed in the immunochemistry, including the omission of the primary antibody, and omission of the secondary antibody. The sections were observed with a low power stereomicroscope, and the architectonic borders of the sections traced according to the Nissl stained sections using a camera lucida. The immunostained sections were then matched to the drawings and the immunopositive neurons marked. The drawings were then scanned and redrawn using the Canvas 8 drawing program. The architectonic nomenclature employed in this study was for the most part adopted from the atlas of a microchiropteran provided by Baron *et al*. (1996a). The nomenclature used for the cholinergic system was adopted from Woolf, (1991) and Manger et al. (2002a), the catecholaminergic system from Hokfelt et al. (1984), Smeets and Gonzalez (2000), and Manger et al. (2002b), and for the serotonergic system from Tork, (1990), Bjarkam et al. (1997), and Manger et al. (2002c).

2.2.1. Abbreviations

III – oculomotor nucleus
IV – trochlear nucleus
Vmot – motor nucleus of trigeminal nerve
VI – abducens nucleus
VIId – facial nerve nucleus, dorsal
VIIn – facial nerve
VIIv – facial nerve nucleus, ventral
VIIIn – vestibulocochlear nerve
X – dorsal motor vagus nucleus
XII – hypoglossal nucleus
3V – third ventricle
4V – fourth ventricle
A1 – caudal ventrolateral tegmental nucleus
A2 – caudal dorsomedial nucleus
A5 – fifth arcuate nucleus
A6v – ventral division of locus coeruleus
A8 – retrorubral nucleus
A7d – nucleus subcoeruleus, dorsal division
A7v – nucleus subcoeruleus, ventral division
A9l – substantia nigra, lateral
A9m – substantia nigra, medial
A9pc – substantia nigra, pars compacta
A10 – ventral tegmental area
A10c – ventral tegmental area, central
A11 – caudal diencephalic group
A12 – tuberal cell group
A13 – zona incerta
A14 – rostral periventricular nucleus
A16 – catecholaminergic neurons of the olfactory bulb
ac – anterior commissure
Amyg - amygdala
AN – anterior thalamic nuclei
AP – area postrema
AVCO – anteroventral cochlear nucleus
B9 – supralemniscal nucleus
BC – brachium conjuctivum
C – caudate nucleus
C1 – rostral ventrolateral tegmental group
C2 – rostral dorsomedial nucleus
ca – cerebral aqueduct
Cb - cerebellum
cc – corpus callosum
cic – commissure of the inferior colliculi
CGM – medial geniculate nucleus

Cl – claustrum
CLi - caudal linear nucleus
C/P – caudate and putamen nuclei
csc – commissure of the superior colliculus
CVL – caudal ventrolateral serotonergic group
dec BC – decussation of brachium conjuctivum
Diag. B – diagonal band of Broca
DLL –dorsal nucleus of lateral lemnsicus
DR – dorsal raphe
DRc – dorsal raphe nucleus, caudal division
DRd – dorsal raphe nucleus, dorsal division
DRif – dorsal raphe nucleus, interfascicular division
DRl – dorsal raphe nucleus, lateral division
DRv – dorsal raphe nucleus, ventral division
DRvm – dorsal raphe nucleus, ventromedial division
ERctx – entorhinal cortex
f - fornix
FCE – fasciculus of the external cuneate nucleus
fr - fasciculus retroflexus
FR – reticular formation
FRP – pontine reticular formation
FRTM – mesencephalic tegmental reticular formation
GC – central periaqueductal grey matter
GP – globus pallidus
GLD – dorsal lateral geniculate nucleus
Hbm – habenular nucleus, medial
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
ICN – intermediate cerebellar nucleus
ILL –intermediate nucleus of the lateral lemniscus
Inf –infundibular nucleus
IO – inferior olive
IP – interpeduncular nucleus
Is.Call/TOL – islands of Calleja and olfactory tubercule
LCN – lateral cerebellar nucleus
LD –dorsal lateral thalamic nucleus
LDT - laterodorsal tegmental nucleus
LV – lateral ventricle
MCN – medial cerebellar nucleus
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
OLS – superior olive
P – putamen nucleus
PB - parabrachial nucleus
PC – cerebral peduncle
pc – posterior commissure
Po – posterior thalamic nucleus
PPT – pedunculopontine tegmental nucleus
PRP – hypoglossal prepositus nucleus
PRPI – prepiriform region
PVCO – posteroventral cochlear nucleus
py – pyramidal tract
R – thalamic reticular nucleus
RED – dorsal reticular nucleus
REL – lateral reticular nucleus
REV – ventral 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 nuclei
S.Acc – shell of nucleus accumbens
SC – superior colliculus
Sep.M – medial septal nucleus
TOL – olfactory tubercle
tri – internal olfactory tract
TRS – trigeminal spinal nerve nucleus
TSO – solitary tract nucleus
vh – ventral horn
VS – superior vestibular nucleus
VL – lateral vestibular nucleus
VLL – ventral nucleus of lateral lemniscus
VM – medial vestibular nucleus
VP – ventral posterior thalamic nuclei
VPO – ventral pons
ZI – zona incerta

2.3. RESULTS

The distribution and morphology of many immunohistochemically reactive neuronal groups belonging to the cholinergic, catecholaminergic and serotonergic systems were found differentially distributed throughout the brain of the microbat studied. Although cell bodies were the most heavily immunolabelled parts of the neurons, it was possible to see axons and dendrites of many neuronal clusters. The axonal projections were sometimes very strongly immunolabelled, such as the fasciculus retroflexus and the exiting nerves of the cranial nerve nuclei. The cholinergic system was in general found to be very similar to other mammals, in that it was divisible into five groups, including striatal interneurons, basal forebrain, diencephalic, pontine, and cranial nerve nuclei (Woolf, 1991). The catecholaminergic system in microbats also follows the typical mammalian subdivisions, which include the olfactory bulb, diencephalon, midbrain, rostral rhombencephalon and caudal rhombencephalon (Smeets and Gonzalez, 2000). The serotonergic nuclei, as is typical for all mammals, were located mainly at the midline of the brainstem and could be divided into a rostral and caudal cluster (Törk, 1990; Jacobs and Azmitia, 1992). Microbats showed considerable lateralization of serotonergic neurons, which is considered a feature of mammals with complex brain organization (Bjarkam et al., 1997).

2.3.1. **Cholinergic nuclei**

As previously documented in a range of mammalian species (Woolf, 1991; Manger et al., 2002a), there are several subdivisions of the cholinergic system. In the present examination of the microbat, we were able to identify cholinergic neurons using ChAT immunohistochemistry in the striatal region, basal forebrain, diencephalon, pontomesencephalon and cranial nerve motor nuclei. We were unable to find evidence for cholinergic neurons in the cerebral cortex, a feature varying across mammals (Bhagwandin et al., 2006), or for the parabigeminal nucleus and cholinergic neurons in the medullary tegmentum as described in other species (Woolf, 1991; Manger et al., 2002a).

2.3.1.1. Cholinergic Striatal Interneurons

The cholinergic striatal interneurons in the microbat are largely similar to those found typically in all mammals. These are divided into three subgroups, including the nucleus accumbens, islands of Calleja and olfactory tubercle, and the caudate-putamen complex (Woolf, 1991).

2.3.1.1.1. *Nucleus Accumbens (N.Acc)*

This nucleus is found in a typically mammalian position, which is the ventral rostral aspect of the cerebral hemispheres, inferior to the caudate-putamen (Fig.1C-F). The nucleus extends 1 to 1.5 mm mediolaterally and 1 to 1.5mm dorsoventrally at its largest point and ends at the level of the anterior commissure. It exhibits a shell that also contains ChAT immunoreactive (ChAT+) neurons. The ChAT+ neurons are ovoid in shape, bipolar, show no specific dendritic orientation, and are found in a low density throughout the nucleus and shell of the accumbens nucleus (Fig. 1C).

2.3.1.1.2. *Caudate-Putamen Complex(C/P)*

This nucleus is located in a position similar to other mammals, which is lateral and ventral to the lateral ventricle (Figs. 1D-J, 2B). It begins at a location just anterior to the anterior horn of the lateral ventricle as a large cell mass 1 to 1.5mm wide (mediolateral) and 2 to 2.5 mm high (dorsoventral), and continues caudally in that location, tapering off to finish at the level of the habenular nuclei. No clear division was observed between the caudate and putamen, except quite caudally where a reasonably robust internal capsule was observed. The complex contains a low density of ChAT+ neurons throughout its extent and these cells are ovoid in shape, bipolar, and show no particular orientation of the dendrites. Caudally, within the globus pallidus, occasional ChAT+ neurons were observed.

2.3.1.1.3. *Islands of Calleja and Olfactory Tubercle (TOL)*

Located in the ventral-most part of the forebrain, these neurons form a continuous band in a medial to lateral direction along the floor of the forebrain, from the level of the anterior pole of the lateral ventricle to the level of the anterior commisure (Fig. 1E and

F). The ChAT+ neurons forming the islands of Calleja and the TOL neurons mixed within this band. The neuronal density was found to be higher than that of nucleus accumbens and the caudate/putamen. Neurons were found varying in type between bipolar and multipolar, but ovoid in shape regardless of polarity, and exhibit no specific dendritic orientation.

2.3.1.2. Cholinergic Basal Forebrain Nuclei

The ChAT+ neurons in the basal forebrain of the microbat studied conform to the typical pattern reported for all mammals, in that they are divisible into the diagonal band of Broca, medial septal nucleus and the nucleus basalis. The three nuclei are described below.

2.3.1.2.1. *Diagonal band of Broca (Diag.B)*

The ChAT+ neurons forming this nucleus were evidenced as a dense cluster of neurons found at the anterior ventromedial corner of the cerebral hemisphere (Fig. 1E-F). This nucleus could be divided into horizontal and vertical limbs, as described for other species (Woolf, 1991), but this was not really appropriate as the neurons formed a continuous cluster around the corner of the hemisphere and distinct regions were not observable. These neurons formed an almost continuous link between the ChAT+ neurons of the olfactory tubercle and those of the medial septal nucleus (see below), but were distinct in terms of their density and morphology from these adjacent nuclei. The neurons were a mixture of multipolar and bipolar, ovoid in shape, and the dendrites were oriented parallel to the edge of the cerebral hemisphere.

2.3.1.2.2. *Medial Septal Nucleus (MSN)*

This nucleus was found adjacent to the ventral half of the midline of the forebrain, below the rostrum of the corpus callosum (Figs. 1D and E, 2A). This nucleus, like the nucleus accumbens, ends at the anterior most border of the anterior commissure. This nucleus contains a low density of ChAT+ neurons that are ovoid in shape and bipolar, with the dendrites oriented dorsoventrally.

2.3.1.2.3 *Nucleus Basalis (N.Bas)*

This nucleus is located ventral to the anterior commisure, lateral to the hypothalamus between the hypothalamus and the piriform cortex, posterior to the nucleus accumbens and the olfactory tubercle, and just anterior to the amygdala (Fig. 1G). ChAT+ neurons are found throughout this nucleus, but in a low density. The neurons, like those of the olfactory tubercle, are ovoid in shape, with a mixture of bipolar and multipolar types and the dendrites showed no specific orientation.

2.3.1.3 Diencephalic Cholinergic Nuclei

The ChAT + neurons in the diencephalon of the microbat studied were divisible into two groups, the medial habenular nucleus and the hypothalamic cluster, which is the case in most mammals (Tago et al., 1987; Woolf, 1991).

2.3.1.3.1. *Medial Habenular nucleus*

This nucleus is composed of very densely packed small, round ChAT+ neurons and lies in the dorsal midline portion of the diencephalon, adjacent to the third ventricle (Fig. 1I and J). The axonal projections of this nucleus, the fasciculus retroflexus, was also strongly ChAT immunoreactive and was seen to end in a complex network in the interpeduncular nucleus (IP).

2.3.1.3.2. *Hypothalamic Clusters*

Adjacent to the third ventricular wall in the anterior dorsal aspect of the hypothalamus, a moderately dense cluster of medium sized, round, multipolar ChAT+ neurons was observed (Fig. 1I). This cluster of neurons was seen to extend from the midline to the lateral edge of the hypothalamus, and probably represents both the dorsal and lateral divisions of the hypothalamic cholinergic neurons seen in other mammals (Tago et al., 1987). A second cluster of ChAT+ neurons with similar morphology was observed in the very ventral portion of the hypothalamus. These multipolar neurons exhibited no specific dendritic orientation.

2.3.1.4. Pontine Cholinergic Nuclei

The pontine region in the microbat studied housed two nuclei with neurons that are ChAT immunoreactive. In some other mammals this region houses three cholinergic nuclei (Woolf, 1991). In the microbat these are the laterodorsal tegmental nucleus (LDT) and the pedunculopontine nucleus (PPT) that are found in a location typical of that seen in all other mammals studied (Woolf, 1991; Manger et a l., 2002a). No evidence of the parabigeminal nucleus was found in the microbat.

2.3.1.4.1. *Laterodorsal tegmental nucleus (LDT)*

This cluster of ChAT+ neurons is found within the lateral and ventral periaqueductal gray (PAG) matter of the pons, at the same level and medial to, the fifth motor nucleus (Vmot) (Fig. 1S and T, 2C). It contains a moderate to high density of neurons, which are circular to irregular in shape, and bipolar to multipolar in type. The dendrites show a weak mediolateral orientation.

2.3.1.4.2. *Pedunculopontine tegmental nucleus (PPN)*

The ChAT+ neurons forming this nucleus are found in the laterodorsal portion of the pontine tegmentum, starting from the level of the fourth cranial nerve nucleus (CN IV) and the decussation of the brachium conjuctivum (decBC, or the superior cerebellar peduncle), and extending caudally to end at the level of the fifth motor nucleus (Fig. 1P-T, 2C). This nucleus consists of a moderate density of bipolar and multipolar neurons that are circular to irregular in shape with a dendritic orientation that is weakly mediolateral. The most lateral neurons in this nucleus are seen surrounding the brachium conjunctivum (BC).

2.3.1.5. Cranial Nerve Nuclei (CN)

In the microbat studied we found evidence for several ChAT immunoreactive neuronal clusters that correspond to the cranial nerve nuclei generally revealed with this technique in other mammals (Woolf, 1991). We found evidence for the third (III, oculomotor), fourth (IV, trochlear), fifth motor (Vmot, motor division of the trigeminal), sixth (VI, abducens), seventh dorsal (VIId, dorsal division of the facial), seventh ventral

(VIIv, ventral division of the facial), nucleus ambiguus (N.Amb), tenth (X, dorsal motor vagus), and twelfth (XII, hypoglossal) nuclei. These nuclei are located in regions typical to other mammals and evince the typical motoneuron morphology. We did not find evidence for tegmental ChAT+ neurons that are distinct from these aforementioned nuclei as seen in some other mammals studied (Woolf, 1991; Manger et al., 2002a).

2.3.2. **Catecholaminergic nuclei**

Tyrosine hydroxylase immunohistochemistry revealed several clusters of positively stained neurons (TH+) forming nuclei throughout the CNS of the microbat. These were found from the olfactory bulb through to the caudal medulla oblongata. We have used the nomenclature initially proposed by Dahlstrom and Fuxe (1964) and elaborated by Hokfelt et al (1984). In some mammals, clusters of TH+ neurons have been found in locations outside of these routinely identified regions; however, in the microbat we could find no evidence for these clusters of neurons as variously reported in other mammalian and vertebrate species (Smeets and Gonzalez, 2000).

2.3.2.1. A16 – olfactory bulb

These neurons are found in and around the glomerular layer of the olfactory bulb, a position where they are found in all mammals (Smeet and Gonzalez, 2000; Manger et al., 2002b) (Fig. 1A and B). The somata are ovoid to triangular in shape, small and multipolar, with the dendrites oriented to surround the glomeruli. The region has a moderate to high density of TH+ neurons.

2.3.2.2. Hypothalamic nuclei (A11-A14)

The hypothalamic catecholaminergic nuclei are generally subdivided in mammals into the A11, A12, A13, A14 and A15 groups, with the A15 group having dorsal and ventral subdivisions (Smeets and Gonzalez, 2000). The microbat hypothalamic groups largely conformed to this pattern but lacked an A15 group.

2.3.2.2.1. *A14 – Rostral Periventricular Cell Group*

A number of TH+ neurons were seen adjacent to the wall of the third ventricle, forming a bilateral dorsoventral column of cells stretching from the roof of the ventricle almost to its floor (Fig. 1G-I, 3A). These neurons were first identified at the level of the anterior commisure and were seen in this location caudally to the level of the infundibulum. This moderately expressed population of neurons were ovoid in shape and bipolar, with a dorsoventral orientation of dendrites.

2.3.2.2.2. *A13 – Zona incerta*

TH+ neurons found posteriorly, dorsally and laterally in the hypothalamus, adjacent to the zona incerta of the ventral thalamus, were identified as the A13 subdivison of the microbat diencephalon (Fig. 1(K)). This nucleus formed a thin medio-lateral band in this location and has a low density of neurons. These neurons are ovoid in shape and bipolar, with the dendrites oriented in a mediolateral direction.

2.3.2.2.3. *A12 – Tuberal cell group*

This nucleus of TH+ neurons is found on the ventral medial floor of the hypothalamus, anterior to and around the infundibulum (Figs. 1I and J, 3A). The neurons of this nucleus are found close to the wall of the third ventricle and the floor of the hypothalamus. The neurons are ovoid in shape and bipolar, are of moderate to low density, with their dendrites oriented parallel either to the wall of the third ventricle or the floor of the hypothalamus, depending on their location.

2.3.2.2.4. *A11 – Caudal diencephalic group*

TH+ neurons found posteriorly in the hypothalamus in a bilateral, dorsoventral column parallel to but slightly away from the wall of the third ventricle, at the level of the posterior commisure, were assigned to the A11 group (Fig. 1L). This nucleus exhibits a low density of bipolar, ovoid shaped neurons, with a dendritic orientation that is a mixture of dorsoventral and mediolateral.

2.3.2.3. Midbrain Catecholaminergic Nuclei (A8-A10)

This midbrain group of catecholaminergic nuclei consists of A8, A9 and A10 groups in the microbat studied, and are located in a typically mammalian position. The A9 and A10 groups are further subdivided, and are reported to be dopaminergic (Smeets and Gonzalez, 2000). The A10 group showed all the subdivisions reported for other mammals except for the A10 dorsal (A10d) and A10 dorsal caudal (A10dc) subdivisions, The A9 group was lacking in the A9 ventral (pars reticulata) subdivision typically seen for mammals.

2.3.2.3.1. A10 – Ventral Tegmental Area

The A10 cluster of TH+ neurons in the microbat were readily divisible into two subdivisions, which include A10 and A10c (central) (Fig. 1M and O). The A10 subdivision is found medial to the exiting oculomotor nerve, just dorsal and lateral to the interpeduncular nucleus. A10 has a high density of cells that are circular to ovoid and bipolar, and whose dendrites are oriented mediolaterally. A10c is located just dorsal to the A10 group at the midline, at a level just anterior to the interpeduncular nucleus. It contains a high density of cells that are circular to ovoid and bipolar with dendrites that are oriented mediodorsally to ventrolaterally.

2.3.2.3.2. *A9 – Substantia nigra*

This nucleus was readily divisible, in the microbat studied, into pars compacta (A9pc), pars lateralis (A9l) and pars medialis (A9m) subdivisions. No evidence for the A9 ventral (A9v, pars reticulata) subdivision, as seen in other mammals (Smeets and Gonzalez, 2000), was found. This cluster of TH+ neurons was seen in the ventral part of the midbrain tegmentum, for the most part immediately dorsal to the cerebral peduncle, extending as a horizontally oriented band from the medially located A10 subdivision to the edge of the midbrain tegmentum (Fig. 1L-O, 3C). The major portion of this cluster was made up of the A9pc, the neurons of which are ovoid in shape, mostly bipolar, with a dendritic orientation that is mediolateral. The A9pc subdivision was found immediately above the cerebral peduncle. Medial to A9pc, and lateral to A10, was the A9m subdivision, which was seen in a position dorsomedial to the medial edge of the cerebral

peduncle. A9pc and A9m both have a high density of TH+ neurons. A9m cells have the same morphology as those of A9pc; but their dendrites are oriented in a dorsomedial to ventrolateral direction. The A9l subdivision was found at the lateral most edge of the A9pc subdivision, in a ventral lateral location within the midbrain tegmentum. A9l cells are ovoid in shape and bipolar to multipolar in form and there was no clear orientation to the dendrites. A9l exhibited a low density of neurons.

2.3.2.3.3. A8 - Retrorubral

TH+ neurons, found in the ventral midbrain tegmentum, dorsal to the A9pc and caudal to the magnocellular division of the red nucleus, were assigned to the A8 catecholaminergic nucleus (Fig. 1O). This nucleus was composed of a small number of neurons that were not densely packed. The neurons observed were ovoid and pisiform in shape, and were bipolar with a weak mediolateral orientation of their dendrites.

2.3.2.4. Rostral Rhombencephalon (Locus Coeruleus Complex, A5-A7)

Within the pons of the microbat, several TH+ neurons forming the locus coeruleus complex were observed. These neurons could be readily divided into several divisions that include the A5, A6v (locus coeruleus ventral), A6α (locus coeruleus alpha), A7d (subcoeruleus dorsal) and A7v (subcoeruleus ventral) divisions. No evidence for an A6d (locus coeruleus dorsal) normally found in the dorsal lateral periventricular gray matter or an A4 division normally found in the dorsal medial periventricular gray matter (Smeets and Gonzalez, 2000) could be found in the microbat.

2.3.2.4.1. A5 – Fifth Arcuate Nucleus

This nucleus is represented by a small number of TH+ neurons found in the ventrolateral pontine tegmentum, lateral to the superior olivary nucleus and the ventral subdivision of the facial nucleus (CN VIIv) (Fig. 1T). The neurons are ovoid in shape and bipolar, forming a loose dorsoventrally oriented column. The dendrites of these neurons are oriented in a dorsomedial to ventrolateral direction.

2.3.2.4.2. *A6v – Locus coeruleus ventral*

The TH+ neurons of this nucleus are found intermingled with those of the ChAT+ neurons of the lateral dorsal tegmental nucleus, in the lateral ventral portion of the periaqueductal and periventricular gray matter of the pons (Figs. 1S and T, 3D). There is a high density of neurons that are ovoid in shape and bipolar exhibiting a rough mediolateral orientation of the dendrites.

2.3.2.4.3. A6α - Locus Coeruleus alpha

TH+ neurons immediately adjacent and ventral lateral to A6v, but just within the dorsal tegmentum of the pons, external to the margin of the periventricular gray matter, were assigned to the A6α subdivision of the locus coeruleus complex (Fig. 3D). This subdivision consists of moderate to high density of round to ovoid shaped, bipolar neurons that exhibit no clear orientation of the dendrites.

2.3.2.4.4. *A7d and A7v – Subcoeruleus dorsal and ventral*

The neurons of these two subdivisions form a continuous column running in a dorsomedial to ventrolateral orientation in the lateral tegmentum of the pons in a location medial to the brachium conjuctivum, ventral to the A6α subdivision and dorsal to the A5 subdivision (Fig. 1S and T). The neurons have the same morphology as those of A6α, but the density is lower and the dendrites are oriented in a dorsomedial to ventrolateral direction. The neurons of the A7v subdivision were fewer in number and lower in density than those of the A7d subdivision.

2.3.2.5. Caudal Rhombencephalon, (C1, C2, A1, A2, Area postrema)

This group is divided into several nuclei and those found in the microbat include C1, C2, A1, A2, and Area Postrema. In the microbat studied, no evidence for the previously reported C3 and A3 subdivisions (Dahlstrom and Fuxe, 1964; Hokfelt et al., 1984) was found using tyrosine hydroxylase immunohistochemistry.

2.3.2.5.1. *C1 – Rostral ventrolateral tegmental group*

The TH+ neurons assigned to this nucleus exhibit a similar column-like appearance as seen in A5, and are found in the rostral ventrolateral medullary tegmentum (Fig. 1V and W). This nucleus was located in the region immediately medial and inferior to nucleus ambiguus, and lateral to the inferior olive. The neurons are ovoid in shape and bipolar, with the dendrites oriented in the same roughly dorsoventral direction as the column.

2.3.2.5.2. *C2 – Rostral dorsomedial group*

The nucleus was seen to be composed of a small cluster of TH+ neurons located in the dorsal medial aspect of the medulla, between the prepositus hypoglossal nucleus and the floor of the fourth ventricle, at the same rostrocaudal level as C1 (Fig. 1U-W). The cells are ovoid in shape and bipolar with their dendrites oriented parallel to the floor of the fourth ventricle.

2.3.2.5.3 *A1 - Caudal ventrolateral tegmental group*

This nucleus of TH+ neurons forms a column that is continuous with that of C1, is similar in appearance, but is found between the most caudal pole of nucleus ambiguus to the spinal cord (Fig. 1X and Y). This column of ovoid bipolar neurons is oriented in a dorsomedial to ventrolateral direction, and the dendrites were seen to be oriented in the same plane.

2.3.2.5.4. *A2 - Caudal dorsomedial group*

The TH+ neurons forming this nucleus were located between CN X and CN XII, and extending somewhat laterally into the dorsal most medullary tegmentum (Fig. 1X). There were very few neurons in this nucleus and they exhibited a low density, were ovoid in shape and bipolar. The dendrites were oriented in a dorsomedial to ventrolateral plane.

2.3.2.5.5 Area Postrema

Area postrema was found located in the dorsal caudal medulla, immediately dorsal to CN XII and medial to CN X at the midline (Fig. 1Y). This nucleus was densely

packed with ovoid shaped, bipolar, TH+ neurons. Due to the density of packing within this small nucleus it was difficult to identify any specific orientation of the dendrites.

2.3.3. **Serotonergic Neurons**

In the brainstem of the microbat studied numerous neurons immunoreactive to the serotonin antibody used were found. In mammals it is typically reported that there is a rostral and a caudal cluster of serotonergic nuclei, with neurons often lying close to the midline (Bjarkam et al., 1997). Terminology employed in this description is derived from reviews by Tork, (1990) and Jacobs and Azmitia (1992), and from recent papers by Bjarkam et al. (1997) and Ferguson et al. (1999). In the microbat studied we found evidence of multiple serotonergic nuclei in the brainstem in regions that correspond to the typically described rostral and caudal clusters; however, we did not find any evidence of hypothalamic serotonergic neurons as reported for the monotremes (Manger et al., 2002c).

2.3.3.1. Rostral Cluster

This cluster of serotonergic immunopositive neurons were readily subdivided into all the nuclei normally identified in this region of the mammalian brainstem (Bjarkam et al., 1997; Manger et al., 2002c). We were able to identify the following nuclei: caudal linear (Cli), supralemniscal (B9), median raphe (MnR), and the dorsal raphe (DR). The dorsal raphe could be further parcellated according to the location and morphology of the neurons into interfascicular, caudal, ventral, dorsal, lateral, and ventromedial subdivisions.

2.3.3.1.1. *Caudal Linear (CLi)*

The neurons comprising this nucleus were located in and around the midline between the decussation of the brachium conjuctivum (BC) and the interpeduncular nucleus (Fig. 1O and P). The neurons were ovoid in shape and bipolar, with their dendrites oriented in a mediolateral direction. The nucleus exhibited a moderate density of neurons.

2.3.3.1.2. *B9 (Supralemniscal nucleus)*

The neurons classified as belonging to this nucleus appear to form a lateral extension of the CLi, into the ventral tegmentum of the midbrain (Fig. 1P). This nucleus was located lateral to the interpeduncular nucleus and dorsal to the lemniscal pathways. The neurons were ovoid in shape and bipolar with the dendrites oriented mediolaterally.

2.3.3.1.3. *Median Raphe (MnR)*

This nucleus was defined as a region of high serotonergic immunopositive neuronal density forming two distinct columns on either side of the midline at the mid-dorsoventral level of the midbrain tegmentum (Figs. 1P-Q, 3B). The MnR was found immediately caudal to the decussation of the BC, at the same coronal level as CN IV, and extended caudally to the anterior level of the Vmot. The neurons were round in shape, and it was difficult to see the dendrites due to the high density of immunoreactive neurons.

2.3.3.1.4. Dorsal Raphe(DR)

The neurons of the dorsal raphe were found from a level immediately caudal to CNN IV to the anterior-most level of Vmot, and is located for the most part within the periaqueductal and periventricular gray matter. These serotonergic immunopositive neurons can be parcellated into the interfascicular (DRif), ventral (DRv), dorsal (DRd), lateral (DRl), caudal (DRc) and ventromedial (DRvm) nuclei (Figs. 1Q-T, 3A). A dense cluster of serotonergic immunopositive neurons located between the two medial longitudinal fasciculi (MLF) is a subdivision that is readily classified as the interfascicular portion of the dorsal raphe (DRif). The neurons are ovoid in shape and bipolar with the dendrites oriented dorsoventrally. The ventral division of the dorsal raphe (DRv) was seen to be a dense cluster of serotonergic immunopositive neurons found immediately dorsal to the DRIF and immediately caudal to the CN IV, in the ventral middle portion of the periaqueductal grey matter. The neurons were circular to ovoid in shape, but it was difficult to observe the dendritic orientation due to the high density of neurons. The dorsal division of the dorsal raphe (DRd) is found immediately

superior the DRv in and around the ventral midline of the periaqueductal grey matter, but it doesn't reach the level of the cerebral aqueduct, falling short of this level by about 250μm. The neurons of the DRd are bipolar in shape and ovoid, with their dendrites not clearly oriented in any specific direction. The lateral group of dorsal raphe (DRL) is found dorsal, lateral and caudal to the DRv and DRd, in the periventricular gray matter and consists of a moderately dense cluster of immunopositive neurons, adjacent to the wall of the cerebral aqueduct and the floor of the fourth ventricle. The neurons are a mixture of multipolar, triagonal and ovoid shapes. The dendrites are seen to run roughly parallel to the ventricular wall. Extending caudally from the DRl, into the periventricular grey matter, medial to the locus coeruleus and LDT, was a low to moderate density of serotonergic immunoreactive neurons that correspond to the caudal (DRc) subdivision of the dorsal raphe. These neurons exhibited the same morphology as those of the DRl. The ventromedial subdivision of the dorsal raphe (DRvm) was located in the ventrolateral portion of the periventricular grey matter extending into the dorsolateral pontine tegmentum, anterior to the locus coeruleus (A6v), lateral dorsal tegmental nucleus (LDT) and the brachium conjunctivum (BC). The neurons are a mixture of bipolar ovoid and multipolar triagonal shapes and showed no specific dendritic orientation.

2.3.3.2. Caudal cluster

The serotonergic neurons forming this cluster were all located between the fifth motor trigeminal nucleus (Vmot) and the spinal cord and could be readily subdivided into five nuclei, including raphe magnus (RMg), raphe pallidus (RPa), rostral and caudal ventrolateral nuclei (RVL and CVL) and raphe obscurus (ROb). All these divisions have homologues in other eutherian mammals (Tork, 1990; Bjarkam et al., 1997).

2.3.3.2.1. *Raphe Magnus (RMg)*

This is the most rostrally located of the nuclei of the caudal cluster and it consists of two columns of neurons on either side of the midline, in a pararaphe position, extending from dorsal to ventral through the rostral medulla. The columns are first seen at the anterior most pole of Vmot and end at a level adjacent to the posterior pole of the facial nerve nuclei (Fig. 1S). The neurons comprising this nucleus are not numerous, are

ovoid in shape and bipolar, with the dendrites oriented dorsoventrally parallel to the midline.

2.3.3.2.2. *Raphe Pallidus (RPa)*

The serotonergic neurons making up the RPa were found in the ventral midline in and around the pyramidal tracts and found for the most part between the two inferior olives (Figs. 1T-Y, 3C). The serotonergic cells constituting the RPa were ovoid in shape and bipolar. The dendrites of the neurons of the RPa were oriented dorsoventrally. The density and number of neurons were high in comparison to the other serotonergic nuclei of the caudal cluster.

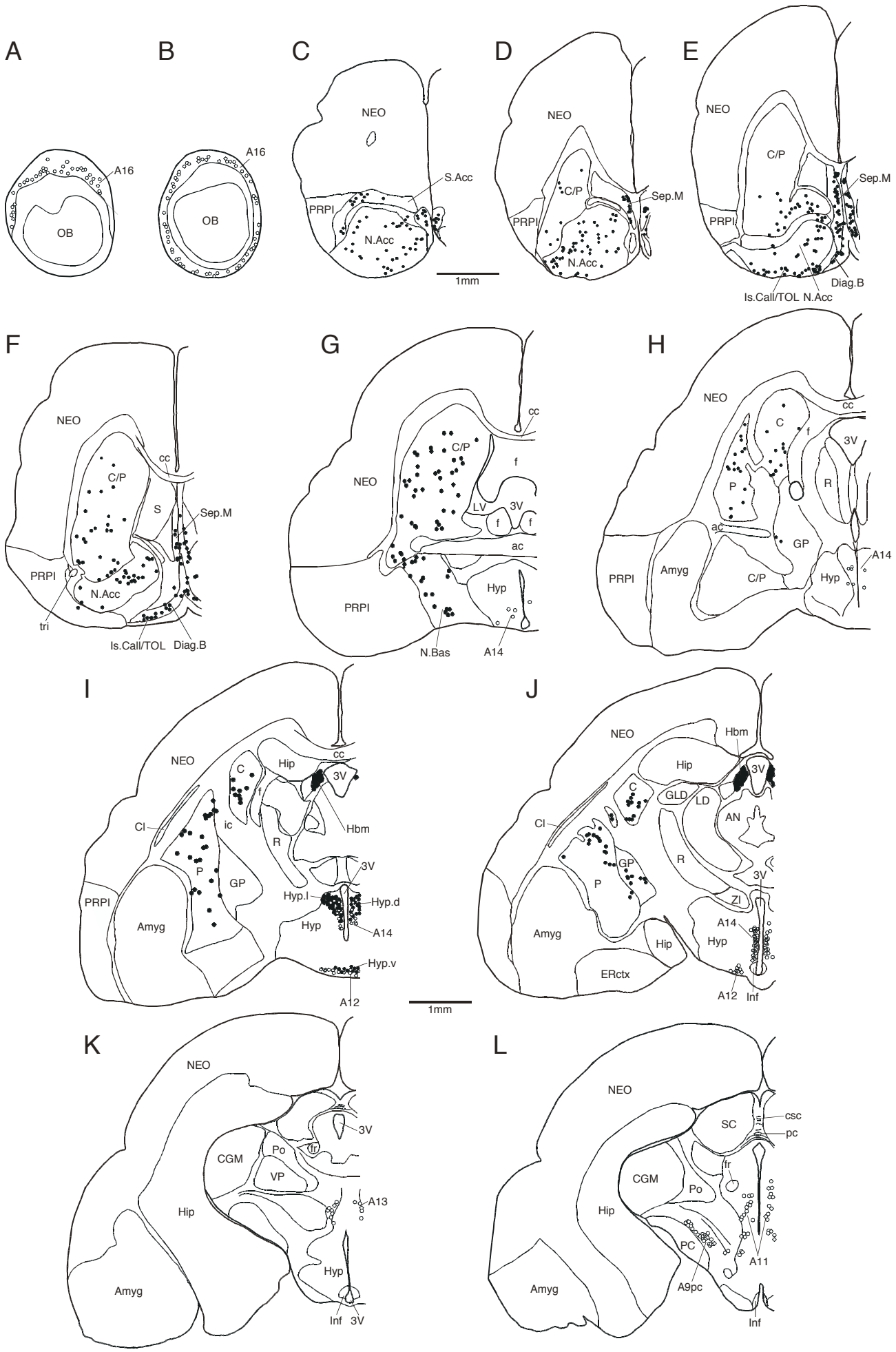
2.3.3.2.3. *Rostral and Caudal Ventrolateral Cell columns (RVL, CVL)*

The serotonergic neurons found in the ventrolateral medulla, from the level of the facial nerve nucleus to the cervical spinal cord were classified under these two nuclei. These serotonergic immunopositive neurons are found lateral to the inferior olivary nuclei, and in a ventral location within the medullary tegmentum. The RVL and CVL form a single column running rostro-caudally in this location, but the neuronal density decreases caudally. The RVL is found from the level of the facial nerve nucleus to the trapezoid body (Figs. 1V and W, 3D), and the CVL from the trapezoid body to the cervical spinal cord (Fig. 1X-Y). The neurons that form this ventral lateral serotonergic column are ovoid in shape and bipolar with mediolaterally oriented dendrites. The density and number of neurons were high in comparison to the other serotonergic nuclei of the caudal cluster except for the RPa, which exhibited a similar density.

2.3.3.2.4. *Raphe Obscurus (ROb)*

The neurons comprising ROb were found in the midline, forming pararaphe columns, in a position dorsal to the RPa. These two columns of neurons were seen to extend from the caudal level of the facial nucleus (CN VII) to the spinal cord. This nucleus was composed of fusiform shaped neuronal somata that were bipolar with dendrites oriented dorsoventrally (Figs. 1V-Y, 3C).

Figure 1. Serial drawings of coronal sections through one half of the microbat brain, from the olfactory bulbs through to the medulla. The outlines of the architectonic regions were drawn using nissl and myelin stains and immunoreactive cells marked on the drawings. Closed black circles depict cholinergic neurons, open circles depict catecholaminergic neurons (those immunoreactive for tyrosine hydroxylase) and open squares depict serotonergic neurons. The figures are approximately 500 μm apart. See list for abbreviations.


A
A16
OB
B
A16
OB
C
NEO
S.Acc
PRPI
N.Acc
1mm
D
NEO
C/P
Sep.M
PRPI
N.Acc
E
NEO
C/P
Sep.M
PRPI
Diag.B
Is.Call/TOL
N.Acc
F
NEO
cc
C/P
S
Sep.M
PRPI
N.Acc
tri
Is.Call/TOL
Diag.B
G
cc
C/P
f
NEO
LV
3V
f
f
ac
Hyp
PRPI
N.Bas
A14
H
NEO
cc
C
f
3V
P
R
ac
GP
A14
PRPI
Amyg
C/P
Hyp
I
NEO
cc
C
Hip
3V
f
Cl
ic
Hbm
R
3V
P
GP
Hyp.l
Hyp.d
PRPI
Amyg
Hyp
A14
Hyp.v
A12
J
NEO
Hbm
Hip
3V
C
GLD
LD
AN
Cl
GP
R
P
3V
ZI
A14
Amyg
Hip
Hyp
ERctx
A12
Inf
1mm
K
NEO
3V
fr
CGM
Po
VP
A13
Hip
Hyp
Amyg
Inf
3V
L
NEO
SC
csc
pc
fr
CGM
Po
Hip
PC
A11
A9pc
Amyg
Inf


M
NEO
SC
GC
ca
FRTM
fr
Po
Hip
A9l
PC
A9pc
A9m/A10
A10c
N
NEO
SC
GC
ca
Hip
FRTM
Rmc
A10c
PC
A9m
A10
O
NEO
SC
GC
ca
Hip
III
FTRM
A10c
A10
CLi
PC
IP
A8
A9m
1mm
P
SC
GC
NEO
ca
IV
FRP
MnR
dec BC
CLi
PPT
IP
B9
VPO
Q
cic
SC
IC
GC
ca
DRp
DRd
DRv
DLL
PPT
MLF
DRif
BC
FRP
MnR
ILL
VLL
VPO
OLS
py
R
IC
DRp
A6d
4V
DRl
DRv
DRif
PPT
DLL
FRP
MnR
ILL
VLL
VPO
VIIIn
OLS
S
IC
LDT
A6d
DRp
Cb
PPT
DRc
BC
DRif
MLF
A7d
DLL
FRP
RMg
VLL
AVCO
OLS
py
1mm
T
Cb
BC
A6d
DRc
4V
PPT
TRS
A7d
Vmot
VIIn
AVCO
OLS
A5
PVCO
py
VIIv
RPa
U
LCN
Cb
PB
VS
4V
C2
VI
TRS
FR
AVCO
PVCO
VIId
VIIv
RPa
V
Cb
LCN
ICN
MCN
VL
C2
VM
PRP
TRS
FR
VIId
ROb
VIIv
C1
RVL
IO
RPa
W
C2
VM
X
VL
PRP
TRS
ROb
N.Amb
C1
RVL
RPa
X
Cb
X
A2
XII
VL
TRS
RED
ROb
REV
A1
N.Amb
RPa
REL
CVL
IO
Y
FCE
VL
VM
TSO
X
XII
AP
TRS
RED
ROb
N.Amb
REV
A1
REL
CVL
RPa

Figure 2. Photomicrographs showing neuronal groups that are immunoreactive for acetylcholineesterase in the forebrain and pons of the microbat brain. (A) Basal forebrain, with the medial septal nucleus (**S**) located at a mediodorsal position and the diagonal band of Broca (**Diag.B**) located at the medial ventral corner of the cerebral hemisphere; (B) striatal cholinergic interneurons; (C) lateral dorsal temgemtal nucleus (**LDT**) dorsally and the pedunculopontine nucleus (**PPN**) ventrolaterally; (D) facial nerve nucleus, dorsal (**VIId**) and ventral (**VIIv**) subdivisions. The scale bar in D = 250 μm and applies to all.

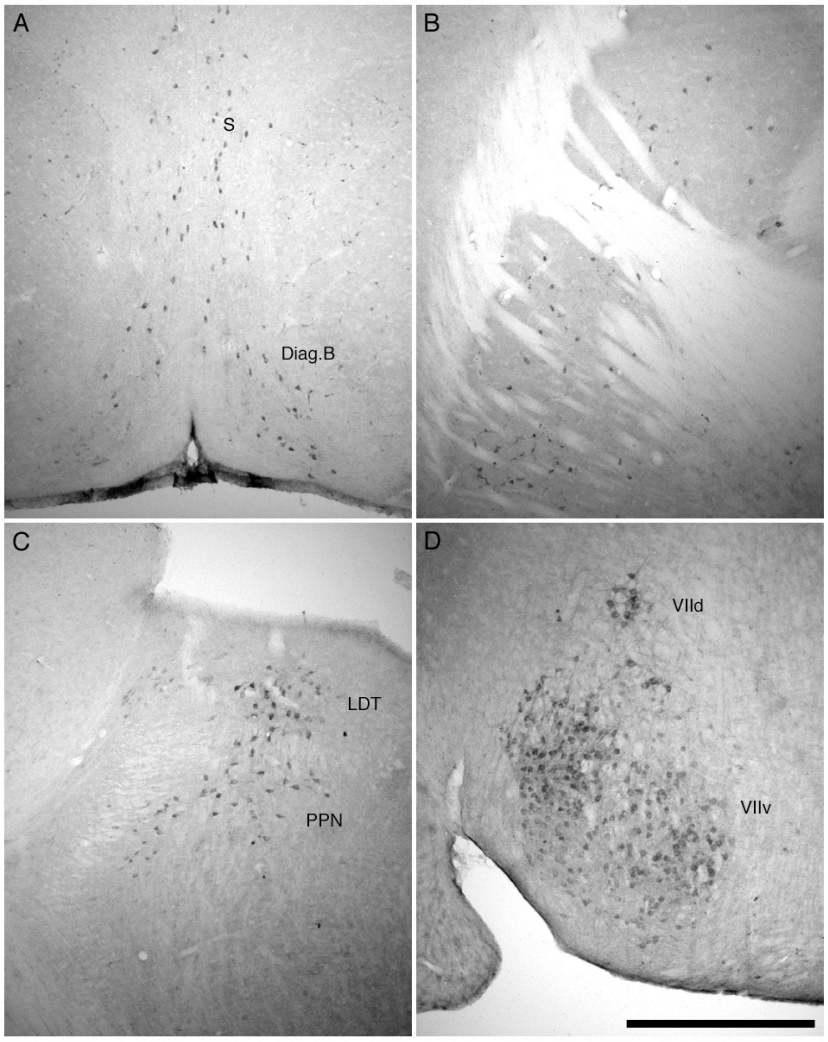

A
S
Diag.B
B
C
LDT
PPN
D
VIId
VIIv

Figure 3. Photomicrographs showing neuronal groups that are immunoreactive for tyrosine hydroxylase in the diencephalon, midbrain and pons. (A) **A14** neurons are seen adjacent to the third ventricle and **A12** neurons are seen ventrally and adjacent to the infundibulum; (B) A10 central (**A10c**) more dorsally, and **A10** just dorsal to the interpeduncular nucleus (**IP**); (C) substantia nigra, pars compacta (A9pc) is seen just dorsal to the cerebral peduncle (PC); (D) locus coeruleus, alpha (**A6α**) and dorsal (**A6v**) subdivisions, and subcoeruleus dorsal (**A7d**). The scale bar in D = 250 μm and applies to all.

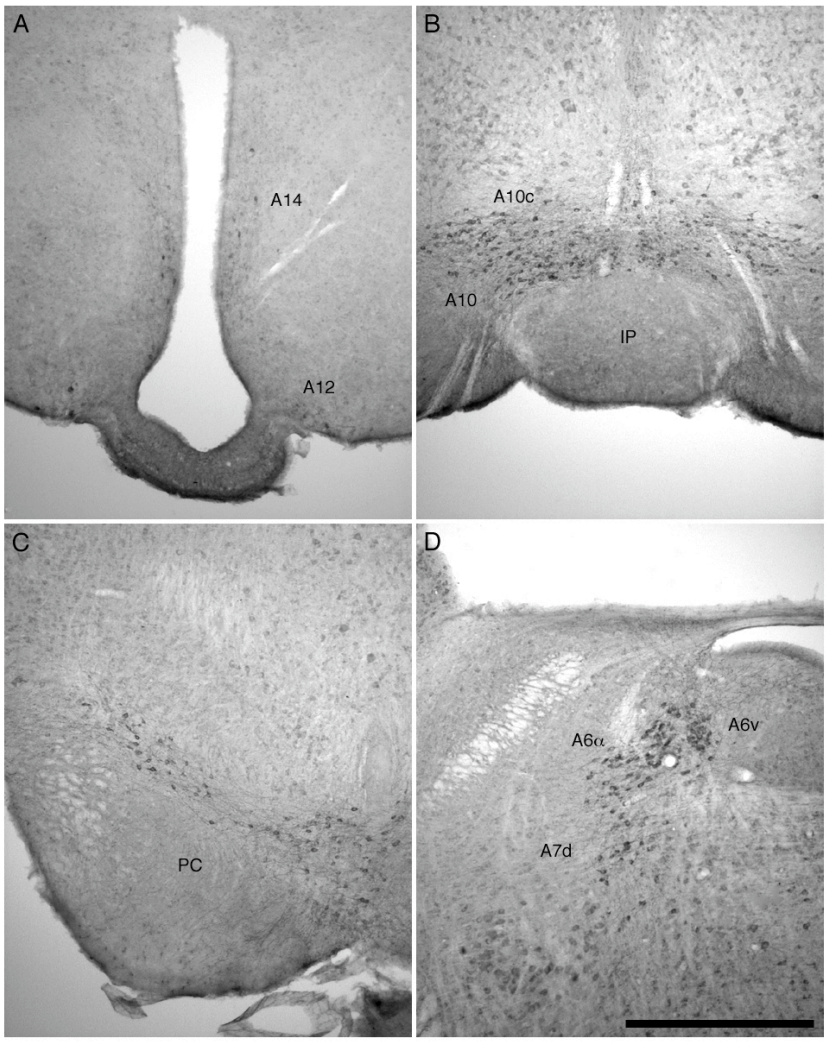
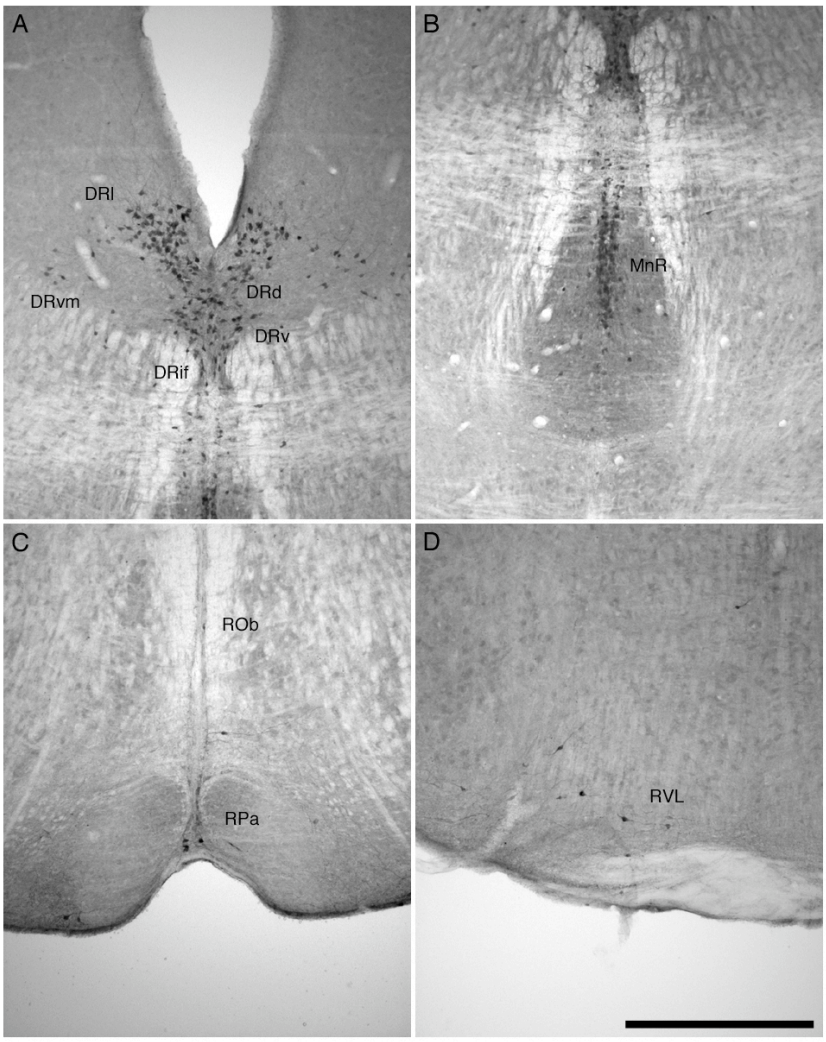

A
A14
A12
B
A10c
A10
IP
C
PC
D
A6α
A6v
A7d

Figure 4. Photomicrographs showing neuronal groups that are immunoreactive for serotonin in the pons and medulla. (A) Dorsal raphe nucleus, showing the subdivisions dorsal, ventral, lateral, ventromedial, and interfascicular (**DRd**, **DRv**, **DRl**, **DRvm**, and **DRif**, respectively); (B) median raphe nucleus (**MnR**); (C) raphe obscurus (**ROb**) at the midline and raphe pallidus (**RPa**) at the ventral midline; (D) rostral ventrolateral serotonergic cell group (**RVL**). The scale bar in D = 250 μm and applies to all.


A
DRl
DRvm
DRd
DRv
DRif
B
MnR
C
ROb
RPa
D
RVL

2.4. **DISCUSSION**

The current investigation of the microbat neuromodulatory systems revealed many similarities between the microbat studied and other mammals as would be expected from previous reports in more commonly investigated species (Smeets and Gonzalez, 2000; Tork, 1990, Woolf, 1991). However, several specific differences that may be of interest in understanding the evolution of these systems in mammals and the phylogenetic relationships of the microbats emerged. When the systems examined are compared with that reported in other mammalian species, the microbat showed more in common with the laboratory shrew and hedgehog, members of the Insectivora (Symonds, 2005), than the rodents, carnivores and primates.

2.4.1. Cholinergic system

In the current study several cholinergic neurons were found aggregated into discrete nuclei within the dorsal striatopallidal complex and basal forebrain, of the microbat. All the nuclei found in these regions within the microbat have direct homologues in other mammals (Woolf, 1991; Manger et al., 2002a). Within the diencephalon microbats were found to have a medial habenular nucleus and dorsal, lateral and ventral hypothalamic ChAT+ neuronal group as described in all other mammals (Tago et al., 1987), except monotremes, which lack cholinergic neurons in the hypothalamus (Manger et al., 2002a).

As for all mammals previously investigated, the microbat had ChAT+ neuronal clusters that represent the laterodorsal tegmental (LDT) and pedunculopontine (PPN) nuclei (Woolf, 1991; Manger et al., 2002a); but they lack a ChAT+ parabigeminal (PBg) nucleus, which is also not seen in monotremes and the laboratory shrews (Karasawa et al., 2003; Manger et al., 2002a). A distinct ChAT+ parabigeminal nucleus is found in the rodents, carnivores, tree shrews and primates (Henderson and Sherriff, 1991; Mesulam et al., 1989; Murray et al., 1982). In terms of the cranial nerve nuclei, the microbats are in most respects similar to other mammals, but they lack ChAT immunoreactivity in the Edinger-Westphal nucleus and within the medullary tegmental field of the caudal ventromedial medulla. The cholinergic immunoreactive neurons of the Edinger-Westphal nucleus have not been located in studies of the monotremes and laboratory shrews (Karasawa et al., 2003; Manger et al., 2002a), but are clearly ChAT immunoreactive in rodents, carnivores

and primates (Armstrong et al., 1983; Mizukawa et al., 1986; Reiner and Vincent, 1987; Satoh and Fibiger, 1985). The medullary tegmental field, ChAT immunoreactive neurons are not seen in primates (Kus et al., 2003), but have been reported in carnivores, rodents and monotremes (Henderson and Sherriff, 1991; Manger et al., 2002a).

The current observations made on the cholinergic system of the microbat appear to align it most closely in the overall complement of nuclei with that of laboratory shrews, a representative of the Insectivora (Symonds, 2005). The lack of parabigeminal and Edinger-Westphal nuclei in particular separate the microbats from the rodents, carnivores and primates, and aligns them with observations made in insectivores and monotremes.

2.4.2. Catecholaminergic system

Catecholaminergic neurons were found in the stratum glomerulosum of the olfactory bulbs (A16) of the microbat studied, which is the case in all mammals investigated (Smeets and Gonzalez, 2000), except the odontocete cetaceans, which have no olfactory bulbs (Ridgway, 1990). Within the diencephalon of the microbat examined, there was no clear evidence for both A15 ventral and A15 dorsal subdivisions as originally described for rodents (Dahlstrom and Fuxe, 1964; Hokfelt et al., 1984). No other mammal investigated lacks both of these subdivisions, except *Tupaia glis* (tree shrew), which also lacks an A14 nucleus (Murray et al., 1982). The A15d group has been found to be absent in the hedgehog (Michaloudi and Papadopoulos, 1996) and artiodactyls (Tillet and Kitahama, 1998), while the A15v group is absent in the rabbit (Blessing et al., 1978).

In the midbrain of the microbat, the tyrosine hydroxylase immunoreactive groups defined as A8 (retrorubral), A9 (substantia nigra) and A10 (ventral tegmental area) were found; however, no evidence of a clear A9 ventral (pars reticulata), A10 dorsal, or A10 dorsal caudal was found. According to previous reports, the A9 ventral subdivision of the substantia nigra is lacking in carnivores (Tafti et al., 1997), and although reported in the hedgehog, the existence of this group is uncertain since it is represented by a very small number of neurons (Michaloudi and Papadopulos, 1996). The A9 ventral subdivision has been seen in monotremes, opossum, artiodactyls, dolphin, rodents, tree shrews and primates (Crutcher and Humbertson, 1978; Dahlstrom and Fuxe, 1964; Hokfelt et al., 1984; Manger et al., 2002b, 2004; Murray

et al., 1982; Østegaard et al., 1992; Tillet and Kitahama, 1998). On the other hand, the two subdivisions of the A10 complex that are missing in microbats, namely the A10d and A10dc, are present in all mammals investigated to date (Smeets and Gonzalez, 2000; Manger et al., 2002b), making the absence of these nuclei a feature unique to the microbats.

As in all other mammals, the microbat locus coeruleus complex contains A5, A6 ventral, A6α, A7 dorsal and A7 ventral subdivisions, but lacks the A4 and A6 dorsal groups, as do monotremes, opossums, hedgehogs, sheep, pigs, bottlenose dolphins, carnivores and rodents (Armstrong et al., 1982; Crutcher and Humbertson, 1978; Manger et al., 2002b, 2003; Manger et al., 2004; Michaloudi and Papadopulos, 1996; Tillet and Kitahama, 1998; Wiklund et al., 1981). A6 dorsal and A4 groups have been described in rabbits, tree shrews and primates (Blessing et al., 1978; Murray et al., 1982; Tillet and Kitahama, 1998). As in all mammals examined to date, the medullary catecholaminergic nuclei of microbats include the A1, A2, C1, C2 and area postrema (AP) nuclei. However, the microbats lack the C3 group, which has only been reported to be present in rodents (Dahlstrom and Fuxe, 1964; Hokfelt et al., 1984; Howe et al., 2000; Smeets and Gonzalez, 2000).

Overall, the complement of catecholaminergic neuronal groups in the microbat resembles most closely that seen in the hedgehog. Certain features that are absent, such as the A15 group, and A9v group are also absent in other mammals, such as *Tupaia* and carnivores, but these similarities of absence appear to be more coincidental than diagnostic. The lack of an A4 and A6d group in the microbat does clearly distinguish them from the rabbit, *Tupaia* and primates, aligning them with other mammalian species. But the overall complement of catecholaminergic nuclei is specific to the microbat, and not shared with the species from other mammalian orders that have been studied (Manger, 2005).

2.4.3. Serotonergic system

The complement of nuclear subdivisions of the serotonergic system within the microbat was seen to be identical to that seen in all eutherian mammals studied to date (e.g. Tork, 1990; Bjarkam et al., 1997). This complement, which includes several nuclei and nuclear subdivisions within a rostral and a caudal cluster was also the same as that observed in the wallaby (Ferguson et al., 1999). However, this complement is different to that seen in the opossum (Crutcher and Humbertson, 1978) and

monotremes (Manger et al., 2002c), both of which lack the caudal ventrolateral serotonergic cluster. Additionally, in the monotremes there are serotonergic neurons in the hypothalamus that are not found in other mammalian species (Manger et al., 2002c). In terms of the serotonergic system the microbats are aligned with eutherian mammals as a whole, but distinguished clearly from the monotremes and the opossum, but not the wallaby.

Although many similarities are found in microbats to other mammals, the entire complement of identified nuclei in the microbats serves to distinguish them from all other mammalian species investigated to date. As this is the first study of a member of the Chiropteran order, it might be expected that the currently described complement of nuclei for the neuromodulatory systems investigated herein will be found in all species of the Chiroptera investigated (Manger, 2005). However, given the questions regarding monophyly and diphyly of the Chiroptera (Pettigrew et al., 1989), it might be predicted that the megabats will demonstrate significant differences in the complement of nuclei of these systems in comparison to the microbat. This possibility is supported by previous observations showing significant differences in the neuroanatomy of the two chiropteran suborders (e.g. Pettigrew, 1986; Pettigrew et al., 1989; Manger et al., 2001). In terms of the overall nuclear complement of the cholinergic, catecholaminergic and serotonergic systems of the microbat, they most closely resembles the complements of these nuclei seen in representatives of the Erinaceomorpha and Soricomorpha, which are both part of the more generalized, but controversial mammalian order Insectivora (Symonds, 2005). It is possible then to view the microbats as closely related to these members of the Insectivora, a situation seen in current mammalian phylogenies (Arnason et al., 2002). In relation to other mammals in which these nuclei have been described, the sheep and pig show the greatest resemblance, followed by the carnivores , again resembling the relationships described for current mammalian phylogenetic relationships (Arnason et al., 2002). These phylogenies and the current study all serve to distance the relationship between microbats and *Tupaia* and primates.

3. MEGABATS

3.1. INTRODUCTION

Megachiroptera, or megabats, a subgroup of the order of flying mammals, Chiroptera, formed by over 160 species, are large bodied (from 100 g to 5 kg) bats that are indigenous to the old world, and are mainly vegetarian (Nowack, 1999). These bats are highly visual (e.g. Pettigrew et al., 1989; Rosa, 1999; Manger and Rosa, 2002) and have a well-developed olfactory system (Baron et al., 1996a,b,c). Megabats do not echolocate, like the Microbats, but there is one notable exception, the Egyptian Rousette (*Rousettus aegyptaicus*) which is the species used in this study.

Several studies have examined the brain of the megabats, but of most interest it was found that the projection in the retinotectal pathway of megabats is identical to that of primates (Pettigrew, 1986). This feature has only been found in the megabats and primates, and is considered a unique primate feature (Pettigrew, 1986; Pettigrew et al., 1989). This initial observation and the related studies (Pettigrew et al., 1989; Pettigrew and Kirsch, 1998; Kirsch and Pettigrew, 1998; Hutcheon et al., 1998) have sparked a controversy regarding the phylogeny of microbats and megabats, since the implication is that megabats, sharing a suite of unique primate features (Pettigrew et al., 1989), are phylogenetically more closely related to primates than to microbats, which are commonly known as their sister group. This suggests a diphyletic origin of the Chiropteran order ultimately implying that mammalian flight evolved not once, as commonly believed, but twice. However, this diphyletic origin of Chiroptera is not supported by all workers and has been the subject of vigorous debate and study (for review see Simmons, 2000).

The current study aims to provide a description of three neuromodulatory systems of *Rousettus aegyptiacus*, the cholinergic, catecholaminergic and serotonergic systems. These systems project widely throughout the brain and are involved in a range of CNS functions (for review see Woolf, 1991; Smeets and Gonzalez, 2000; Tork, 1991; Jacobs and Azmitia, 1992) including the sleep-wake cycle (e.g. Webster and Jones, 1988; Siegel et al., 1996; Siegel, 2006; Fornal and Jacobs, 1988), cognition (e.g. Bartus et al., 1982), reproduction (e.g. Tillet, 1994), movement (e.g. Brooks and Piccini, 2006), and reward and punishment (e.g. Hyman et al., 2006) amongst many

others. The interesting aspect of the functional attributes of these systems is that none of these systems are specifically related to the ultimate generation of echolocation or flight, or sensory perception, although they may play a modulatory role not dissimilar to that seen for locomotion and sensation in other mammals.

A recent hypothesis forwarded regarding these systems (Manger, 2005) has indicated that changes in the nuclear complements of these systems are likely only to occur at the origin of a new mammalian order, and that all members of an order will exhibit the same complement of homologous nuclei. Manger (2005) has proposed that this is a constraining feature of brain evolution and will occur irrespective of the natural history, size of the brain, or phenotype of the species. This proposal is supported by a series of studies on monotremes (Manger et al., 2002a,b,c), cetaceans (Manger et al., 2003, 2004), and rodents (Da Silva et al., 2006). This proposal is not reconcilable with earlier concepts of the evolution of these systems (Smeets and Gonzalez, 2000; Woolf, 1991; Bjarkam et al., 1998).

The object of the present study, the Egyptian Rousette, is a megachiropteran, and is the only species of this suborder that echolocates (Nowack, 1999). Moreover, it shares its roosting site with several microbat species (Nowack, 1999), and ultimately exhibits many similarities in its natural history and phenotype with microchiropterans. Given these ecological and phenotypical similarities, the independence of the systems under study from species specific neural specializations, the contentious phylogeny of the Chiroptera, and the potential evolutionary patterns of the neuromodulatory systems, the present study aims to add a suite of characters that may be of significance in our understanding of Chiropteran affinities and evolution.

3.2. MATERIALS AND METHODS

The brains of three adult female megabats, the Egyptian Rousette (*Rousettus aegyptiacus*), were used in the current study. The specimens were obtained from a cave adjacent to Legalametse Nature Reserve in Limpopo Province, South Africa, under the permission and supervision of the Limpopo Provincial Nature Conservation Directorate. 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. The megabats were placed under deep barbiturate anaesthesia (Euthanaze, 200mg sodium pentobarbital/ kg, i.p.), and then perfused intracardially upon cessation of respiration. The perfusion was initially done with a rinse of 0.9% saline solution at 4° C, followed by a solution of 4% paraformaldehyde in 0.1M phosphate buffer (PB) (approximately 1 l/kg of each solution). Brains were then removed from the skull and post-fixed overnight in 4% paraformaldehyde in 0.1M PB, and then allowed to equilibrate in 30% sucrose in PB. Two brains were then frozen and sectioned into serial coronal sections of 50 μm thickness and one in a sagittal plane. A one in five series of stains was made for Nissl, myelin, tyrosine hydroxylase (TH), cholineacetyltransferase (ChAT) and serotonin. Sections kept for the Nissl series were mounted on 0.5% gel coated glass slides, cleared in a solution of 1:1 chloroform and absolute alcohol, then stained with 1% cresyl violet. Sections kept for the myelin series were stored for two weeks in a 5% formalin solution, mounted on 1% gel coated slides, and then stained with a modified Gallyas silver stain (Gallyas, 1979).

For immunohistochemical staining the sections were first treated for 30 min with an endogenous peroxidase inhibitor (49.2% methanol: 49.2% 0.1PB: 1.6% 30% H_2O_2) followed by three 10 min rinses in 0.1M PB. This was followed by a 2 hour preincubation, at room temperature, in a solution (blocking buffer) containing 3% normal goat serum (NGS, Chemicon), 2% bovine serum albumin (BSA, Sigma), and 0.25% Triton X-100 (Merck) in 0.1M PB. The sections were then placed in a primary antibody solution containing the appropriately diluted antibody in blocking buffer, for 48 hours at 4°C. To reveal cholinergic neurons we used anti-cholineacetyltransferase (AB143, Chemicon) at a dilution of 1:1500. To reveal catecholaminergic neurons we used anti-tyrosine hydroxylase (TH) (AB151, Chemicon) at a dilution of 1:6000. To

reveal serotonergic neurons we used anti-serotonin (AB 938, Chemicon) at a dilution of 1:7500. This step was followed by three 10 min rinses in 0.1M PB, after which the sections were incubated in a secondary antibody solution for two hours. This solution contained a 1:500 dilution of biotinylated anti-rabbit IgG (BA-1000, Vector Labs), in blocking buffer containing 3% NGS, and 2% BSA in 0.1M PB. After three 10 min rinses in 0.1M PB, the sections were incubated for 1 hour in AB solution (Vector Labs), and again rinsed. The sections were then treated in a solution of 0.05% diaminobenzidine (DAB) in 0.1M PB for 5 minutes, following which 3µl of 30% H_2O_2 was added to the solution in which each section was immersed. Development was monitored visually and checked under a low power stereomicroscope. This was allowed to continue until the background staining was at a level at which it could assist reconstruction without obscuring the immunopositive neurons. Development was then arrested by placing the sections in 0.1M PB, and then rinsed twice more in the same solution.

Sections were mounted on glass slides coated with 0.5% gel and left to dry overnight. They were then dehydrated in a graded series of alcohols, cleared in xylene, and coverslipped with Depex. Two controls were employed in the immunochemistry, including the omission of the primary antibody, and omission of the secondary antibody. The sections were observed with a low power stereomicroscope, and the architectonic borders of the sections traced according to the Nissl stained sections using a Camera Lucida. The immunostained sections were then matched to the drawings and the immunopositive neurons were marked. The drawings were then scanned and redrawn using the Canvas 8 drawing program. The architectonic nomenclature employed in this study was for the most part adopted from the published brain atlas of an Egyptian Rousette provided by Schneider (1966). The nomenclature used for the cholinergic system was adopted from Woolf, (1991) and Manger et al. (2002a), the catecholaminergic system from Hokfelt et al. (1984), Smeets and Gonzalez (2000), and Manger et al. (2002b), and for the serotonergic system from Tork, (1990), Bjarkam et al. (1997), and Manger et al. (2002c).

3.2.1. Abbreviations

III – oculomotor nucleus
IV – trochlear nucleus
Vmot – motor nucleus of trigeminal nerve
VI – abducens nucleus
VIId – facial nerve nucleus, dorsal
VIIv – facial nerve nucleus, ventral
X – dorsal motor vagus nucleus
XII – hypoglossal nucleus
3V – third ventricle
A1 – caudal ventrolateral tegmental nucleus
A2 – caudal dorsomedial nucleus
A5 – fifth arcuate nucleus
A6α – alpha/tegmental division of locus coeruleus
A6d – dorsal division of locus coeruleus
A6v – ventral division of locus coeruleus
A8 – retrorubral nucleus
A7d – nucleus subcoeruleus, dorsal division
A7v – nucleus subcoeruleus, ventral division
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
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
AP – area postrema
AVCO – anteroventral cochlear nucleus
B9 – supralemniscal nucleus
BC – brachium conjuctivum
C – caudate nucleus
C1 – rostral ventrolateral tegmental group
C2 – rostral dorsomedial nucleus
ca – cerebral aqueduct
cc – corpus callosum
CGM – medial geniculate nucleus
CLi - caudal linear nucleus
CN – cerebellar nuclei
CVL – caudal ventrolateral serotonergic group

DCO – dorsal cochlear nucleus
Diag. B – diagonal band of Broca
DR – dorsal raphe
DRc – dorsal raphe nucleus, caudal division
DRd – dorsal raphe nucleus, dorsal division
DRif – dorsal raphe nucleus, interfascicular division
DRl – dorsal raphe nucleus, lateral division
DRv – dorsal raphe nucleus, ventral division
DRvm – dorsal raphe nucleus, ventromedial division
DT – dorsal thalamus
ERctx – entorhinal cortex
EW – Edinger-Westphal nucleus
f - fornix
FCE – fasciculus of the external cuneate nucleus
FCM – fasciculus of the medial cuneate nucleus
FGR – fasciculus of the gracile nucleus
fr - fasciculus retroflexus
FRP – pontine reticular formation
FRTM – mesencephalic tegmental reticular formation
GC – central periaqueductal grey matter
GP – globus pallidus
GLD – dorsal lateral geniculate nucleus
Hbl – habenular nucleus, lateral
Hbm – habenular nucleus, medial
Hip – hippocampus
Hyp.d – dorsal hypothalamic cholinergic nucleus
Hyp.l – lateral hypothalamic cholinergic nucleus
Hyp.v – ventral hypothalamic cholinergic nucleus
IC – inferior colliculus
ic – internal capsule
IP – interpeduncular nucleus
Is.Call/TOL – islands of Calleja and olfactory tubercule
LDT - laterodorsal tegmental nucleus
LV – lateral ventricle
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
OLS – superior olive
OT – optic tract
pVII – preganglionic motor neurons of the salivatory nerve
pIX – preganglionic motor neurons of the glossopharyngeal nerve
P – putamen nucleus
PBg – parabigeminal nucleus
PC – cerebral peduncle
Pg – pineal gland
Pir – piriform cortex

PPT – pedunculopontine tegmental nucleus
PRPI – prepiriform region
PVCO – posteroventral cochlear nucleus
py – pyramidal tract
R – thalamic reticular nucleus
REL – lateral reticular nucleus
REV – ventral reticular nucleus
RMg – raphe magnus nucleus
ROb – raphe obscurus nucleus
RPa – raphe pallidus nucleus
RVL – rostral ventrolateral serotonergic group
SC – superior colliculus
Sep.M – medial septal nucleus
TOL – olfactory tubercle
tri – internal olfactory tract
trl – lateral olfactory tract
TRS – trigeminal spinal nerve nucleus
vh – ventral horn
VL – lateral vestibular nucleus
VPO – ventral pons
ZI – zona incerta

3.3. RESULTS

Megabats were found to comply largely with the known patterns of mammals with respect to the immunohistochemically identifiable nuclei (cholinergic, catecholaminergic, and serotonergic) studied here. The cholinergic system was, as is the case for most mammals, divisible into several groups including striatal interneurons, basal forebrain, diencephalic, pontine, medullary tegmental field and cranial nerve nuclei. Megabats did show some differences in the organization of their neuromodulatory nuclei; for example, a parabigeminal nucleus was observed containing ChAT+ neurons that were large and intensely stained, but this nucleus was found to be absent in microbats (Chapter 2). The striatum is clearly demarcated into a caudate nucleus and a putamen by an internal capsule but this is not the case in microbats (Chapter 2). With respect to catecholaminergic nuclei, there existed the typical pattern found in most mammals (Smeets and Gonzalez, 2000). In addition, the A4, the dorsal division of the locus coeruleus (A6d) and the A15 groups were present, and these were found to be absent in microbats (Chapter 2). The serotonergic nuclei found were consistent with other eutherian mammals, including the microbats.

3.3.1. Cholinergic Neurons

The mammalian cholinergic system is typically divided into a striatal group, basal forebrain group, diencephalic group, pontine group, and the cranial nerve nuclei (Woolf, 1991; Manger et al., 2002a). This is congruent with the manner in which the cholinergic nuclei are divided in the megabat brain. Each of the groups above is further divisible into subgroups that extend from the anterior horn of the lateral ventricles to the spinomedullary junction.

3.3.1.1. Striatal Cholinergic Interneurons

In all mammals, as is found in megabats, these neurons can be classified into three nuclei that include the nucleus accumbens, caudate/putamen and globus pallidus, and the olfactory tubercle and islands of Calleja (Woolf, 1991).

3.3.1.1.1. *Nucleus Accumbens (N.Acc)*

The anterior border of this nucleus is found at the level of the anterior horn of the lateral ventricle, caudal to the level of the anterior commisure, ventral to the dorsal

striatopallidal complex, and dorsal to the olfactory tract (Fig. 5C-E). This position is typical of mammals (Woolf, 1991). The nucleus has a moderate density of ChAT+ neurons, which are a mixture of ovoid and triangular shapes. The cells are bipolar and multipolar and the dendrites show no specific orientation.

3.3.1.1.2. *Caudate/Putamen (C/P) and Globus Pallidus (GP)*

In the megabat studied, this nucleus is divisible into a clearly defined caudate and putamen, demarcated by the presence of a strongly coalesced internal capsule. The anterior border of this nuclear complex begin rostrally in the cerebral hemisphere, lateral to the lateral ventricle, and tapers caudally to end at the mid-diencephalic level (Fig. 5C-H). It contains a moderate to low density of pisiform multipolar ChAT + neurons that exhibit no specific dendritic orientation. Some ChAT+ neurons are present within the globus pallidus. The overall level of neuropil staining within the globus pallidus is less than that seen in the caudate and putamen.

3.3.1.1.3. *Islands of Calleja and Olfactory Tubercle (TOL)*

This cluster of ChAT+ neurons was found on the floor of the cerebral hemisphere, ventral to the nucleus accumbens, and rostral to the anterior commisure (Fig. 5D-F). It contained a moderate density of ChAT+ neurons that have a morphology similar to those of medial septal nucleus and diagonal band of Broca (see below). The dendrites of these neurons were oriented parallel to the floor of the brain.

3.3.1.2. Cholinergic Nuclei of the Basal Forebrain

Within the basal forebrain of mammals, three groups of cholinergic cells have been identified [Woolf, 1991]. These include the medial septal nucleus, the vertical and horizontal diagonal band of Broca nucleus, and the nucleus basalis. All three of these nuclei were readily identified in the brain of the megabat studied.

3.3.1.2.1. *Medial Septal Nucleus (MSN)*

This nucleus is found in the rostral lower half of the medial wall of the cerebral hemisphere, between the rostrum of the corpus callosum and floor of the cerebral hemisphere, medial to the dorsal striatopallidal complex and rostro-dorsal to the hypothalamus, ending at the level of the anterior commisure. A moderate density of ChAT+ neurons was seen throughout the nucleus (Figs. 5D-F, 2A). These ChAT+

neurons are a mixture of ovoid and triangular shapes and are a mixture of bipolar and multipolar types, whose dendrites show a dorsoventral orientation.

3.3.1.2.2. *Diagonal Band of Broca (Diag.B)*

This nucleus was located on the ventromedial corner of the cerebral hemisphere, anterior to the hypothalamus, and contained a high density of ChAT+ neurons that possess a similar morphology to the cells of medial septal nucleus (see above) (Figs. 5D and E, Fig. 6A). The dendrites of these neurons were oriented parallel to the outer edge of the brain (horizontally or vertically depending on neuronal location). This nucleus could have been divided into both vertical and horizontal limbs, however the continuity of the neurons did not reveal any specific demarcation that justified such a division.

3.3.1.2.3. *Nucleus Basalis (N.Bas)*

This nucleus is located ventral to the anterior commisure, lateral to the hypothalamus between the hypothalamus and the piriform cortex, posterior to the nucleus accumbens and the olfactory tubercle, and just anterior to the amygdala (Fig. 5G), a position similar to that seen in all mammals (Woolf, 1991). ChAT+ neurons are found throughout this nucleus, but in a low to moderate density. The neurons were mostly triangular in shape, with a mixture of bipolar and multipolar types. The dendrites showed no specific orientation.

3.3.1.3. Diencephalic Cholinergic Neurons

Within the diencephalon the cholinergic immunopositive neuronal clusters are divisible into two nuclear groups, namely, the medial habenular nucleus and the hypothalamic nuclei. The hypothalamic neurons are organized into three nuclei in megabats, similar to rats, laboratory shrews, cats, common marmosets, macaque monkeys and humans (Everitt et al., 1988; Henderson and Sherriff, 1991; Karasawa et al., 2003; Tago et al., 1987; Vincent and Reiner, 1987), but not in microbats (Chapter 6) or monotremes (Manger et al., 2002a).

3.3.1.3.1. *Medial habenular nucleus*

This nucleus is found in the dorsal medial aspect the diencephalon (Fig. 5I), next to the third ventricle, which is the typical location of this nucleus in mammals. A

very dense population of cholinergic immunoreactive neurons is found within this nucleus. The neurons are small and round, with intensely labelled axons that form the fasciculus retroflexus, which is also visible with ChAT immunoreactivity throughout its trajectory to the interpeduncular nucleus.

3.3.1.3.2. *Hypothalamic dorsal group*

Located in a position similar to other mammals, this nucleus is found adjacent to the wall of the third ventricle, mid-dorsal in the hypothalamus, at the middle anteroposterior level of the hypothalamus (Fig. 5G and H). It contains a low density of cells, which are circular to ovoid and bipolar with a weak mediolateral orientation of dendrites.

3.3.1.3.3. *Hypothalamic lateral group*

This nucleus is found in the dorsal lateral aspect of the hypothalamus, intermingled with the catecholaminergic A13 nucleus near the zona incerta (see below) (Fig. 5H). The cells are ovoid and bipolar with a clear mediolateral orientation of dendrites. Within the region that neurons are found, they show a moderate to high density.

3.3.1.3.4. *Hypothalamic ventral group*

This group is found in the ventromedial aspect of the hypothalamus, adjacent to the wall of the third ventricle and the floor of the brain (Fig. 5H and I). It is located caudally in the hypothalamus, just anterior to the mammillary bodies. It contains a high density of neurons, which are ovoid and bipolar, with their dendrites oriented parallel to the floor of the brain and wall of the third ventricle.

3.3.1.4. Pontine Cholinergic Nuclei

The pontomesencephalic cholinergic group of neurons are usually divided into three groups, the pendunculopontine tegmental nucleus (PPT), the laterodorsal tegmental nucleus (LDT), and the parabigeminal nucleus (Woolf, 1991). All three of these nuclei were observed in the megabat.

3.3.1.4.1. *Parabigeminal nucleus (PBg)*

This strongly ChAT+ nucleus is found at the lateral wall of the midbrain tegmentum, ventral to the inferior colliculus, at the same rostro-caudal level as the oculomotor nucleus. A very high density of neurons is evident within this nucleus, these neurons being small and circular with dendrites that were difficult to see due to the high density (Figs. 5L, 6D).

3.3.1.4.2. *Pedunculopontine tegemental nucleus (PPT)*

The neurons forming this nucleus are found dorsally within the pontine tegmentum, surrounding the superior cerebellar peduncle (SP) or brachium conjuctivum (BC) (Figs. 5L-N, 6C). This nucleus extends from the level the fourth cranial nerve nucleus to the fifth motor nucleus, contains a moderate density of neurons, that are medium sized, irregular shaped and multipolar. There is no obvious dendritic orientation.

3.3.1.4.3. *Laterodorsal Tegmental nucleus (LDT)*

Located in the lateral ventral periventricular grey matter in the pons, some of the neurons of this nucleus intermingle with the neurons of the ventral division of the locus coeruleus (A6v see below) (Figs. 5N, 6B). The nucleus consists of a high density of ChAT+ neurons that are mostly bipolar, with a dorsomedial to ventrolateral orientation of the dendrites. The neuronal bodies are larger than those of the pedunculopontine tegmental nucleus, but smaller than the motor neurons of the oculomotor nucleus.

3.3.1.5. Cholinergic Cranial Nerve Nuclei

These nuclei are located in a position typically found in mammals, all containing large multipolar motor neurons (Woolf, 1991). The nuclei include the Edinger-Westphal nucleus, third (CN III, oculomotor), fourth (CN IV, trochlear), fifth motor (Vmot, motor trigeminal), sixth (CN VI, abducens), seventh dorsal and ventral (CN VIId and CN VIIv, facial), ambiguus (N.Amb.), tenth (CN X, dorsal motor vagus), and twelfth (CN XII, hypoglossal) nerve nuclei. In addition to these nuclei, ChAT immunoreactivity was found for the preganglionic motor neurons of the salivatory (pVII) and glossopharyngeal (pIX) nerves. These were located dorsal to the dorsal division of the facial nucleus and the nucleus ambiguus, and anterior to the anterior poles of the hypoglossal and dorsal motor vagal nuclei. The ChAT+ neurons

of these two regions exhibited a morphology similar to that seen for the cranial nerve nuclei, in that they were large and multipolar.

3.3.2. Catecholaminergic Neurons

Tyrosine hydroxylase immunoreactive neurons (TH+) formed nuclei that were found in a range of locations rostrocaudally, throughout the length of the megabat brain extending from the olfactory bulbs to the caudal portion of the medulla. The positions were typical of that seen in other mammals, and divisible into several clusters including the olfactory bulb, hypothalamic , midbrain , pontine and medullary nuclei. For simplicity, the nuclei are referred to using the nomenclature from Dahlström and Fuxe (1964) and Hokfelt et al (1984). No catecholaminergic nuclei outside the bounds of the classically defined nuclei, as occasionally referred to for mammals and other vertebrates (Smeets and Gonzalez, 2000), were found.

3.3.2.1. Olfactory bulb neurons – A16

This cluster of small triangular, multipolar, catecholaminergic neurons is located throughout the stratum granulosum (Fig. 5A and B). The dendrites are oriented around the glomeruli. This appearance and location is typical for mammals (Smeets and Gonzalez, 2000).

3.3.2.2. Hypothalamic Nuclei

A series of catecholaminergic nuclei were found extending from the dorsal anterior aspect of the hypothalamus to the caudal part of the hypothalamus. Several nuclei are found within this region, including the A15 dorsal, A15 ventral, A14 (rostral periventricular group), A13 (zona incerta), A12 (tuberal) and A11 (caudal diencephalic) nuclei.

3.3.2.2.1. *A15 dorsal (A15d)*

This nucleus, consisting only of a few TH+ neurons, was found in the dorsal anterior aspect of the hypothalamus, immediately ventral to the anterior commisure (Fig. 5F and G). This location is similar to that seen in rodents (Hokfelt et al., 1984). The cells are small, ovoid and bipolar and are found as a low-density population. The dendrites are oriented mediolaterally, parallel to the lower edge of the anterior commisure.

3.3.2.2.2. *A15 ventral (A15v)*

The TH+ neurons assigned to this nucleus were located in the ventral lateral portion of the hypothalamus at the level of the optic chiasm (Fig. 5G). The cells are ovoid and bipolar with a dendritic orientation that is roughly mediolateral, parallel to the floor of the brain.

3.3.2.2.3. *A14 (rostral periventricular group)*

The neurons forming this nucleus were found at the midline of the hypothalamus, adjacent to the walls of the third ventricle forming a dorsoventral column very close to the ventricular wall (Figs. 5G, 7A). The cells are not particularly numerous, and are bipolar and ovoid with the dendrites oriented dorsoventrally.

3.3.2.2.4. *A13 (zona incerta)*

TH+ neurons extending from medial to lateral in the dorsal aspect of the hypothalamus, adjacent to the zona incerta, were assigned to this nucleus (Figs. 5H, 7A). This nucleus was composed of a reasonably numerous mixture of bipolar and multipolar cells, which were mostly ovoid in shape with their dendrites oriented mediolaterally.

3.3.2.2.5. *A12 (Tuberal cell group)*

This cluster of TH+ neurons was found in the ventral medial hypothalamus between the inferior margin of the third ventricle and the optic chiasm in the region of the arcuate nucleus and infundibulum (Fig. 5F-H). The cells were ovoid in shape and bipolar, with the dendrites oriented towards the wall of the third ventricle and the floor of the brain.

3.3.2.2.6. *A11 (caudal diencephalic)*

This nucleus was found in the very caudal part of the hypothalamus, surrounding the caudal and inferior aspects of the third ventricle (Fig. 5I and J). These ovoid and triangular shaped neurons evinced both bipolar and multipolar forms, with their dendrites not oriented in any specific direction.

3.3.2.3. Midbrain Catecholaminergic Nuclei

The midbrain contains several clusters of catecholaminergic neurons, which when divided according to location, are termed the A10 (ventral tegemental area), A9 (substantia nigra) and A8 (retrorubral) nuclei. This regional subdivision is the case in all mammals (Kitahama *et al.*, 1994). These midbrain catecholaminergic nuclei extend from the level of the CN III to the level of the exit of the oculomotor nerve. The A10 and A9 nuclei are further subdivided according to location.

3.3.2.3.1. *A10 Ventral Tegmental Area Nuclei (VTA)*

This nuclear cluster, also known as the ventral tegmental area (VTA), could be readily subdivided into A10, A10 central, A10 dorsal and A10 dorsocaudal nuclei in the megabat studied.

3.3.2.3.2. *A10*

The TH+ neurons making up this nucleus were found in a position dorsolateral to the interpeduncular nucleus, between the interpeduncular nucleus and the exit of the oculomotor nerve (Fig. 5J and K). The neurons were ovoid in shape and bipolar in nature with a dorsomedial to ventrolateral dendritic orientation.

3.3.2.3.3. *A10 central (A10c)*

The neurons comprising this nucleus were found at the midline immediately dorsal and anterior to the interpeduncular nucleus (Fig. 5K). The neurons were ovoid in shape and bipolar with mediolaterally oriented dendrites.

3.3.2.3.4. *A10 dorsal (A10d)*

The dorsal portion of the VTA was evinced as a cluster of TH+ neurons located around the midline immediately ventral to CN III and dorsal to the A10c nucleus (Fig. 5K). The cells were ovoid in shape and bipolar in nature, with dorsoventrally oriented dendrites.

3.3.2.3.5. *A10 dorsocaudal (A10dc)*

This nucleus was found within the periaqueductal gray (GC), between the cerebral aqueduct and the oculomotor nucleus and close to the walls of the cerebral aqueduct (Figs. 5L and M, 7B). In this location, the TH+ neurons, while sparsely distributed were relatively numerous. The neurons were bipolar to multipolar in

nature, and ovoid to triangular in shape, with their dendrites oriented parallel to the wall of the cerebral aqueduct.

3.3.2.3.6. *A9 Nuclei (substantia nigra)*

In the megabat studied we could readily subdivide this neuronal cluster into pars compacta, ventral, lateral and medial nuclei. This pattern of subdivisions is directly comparable with that seen in many other mammalian species (Kitahama et al., 1994).

3.3.2.3.7. *A9 pars compacta (A9pc)*

This nucleus consists of a high-density mediolateral band of TH+ neurons running immediately dorsal to the cerebral peduncle (Fig. 5J and K). The neurons comprising this nucleus were ovoid in shape and bipolar, with a mediolateral orientation of dendrites.

3.3.2.3.8. *A9 ventral (A9v)*

The TH+ neurons assigned to this nucleus were found ventral to the A9pc, intermingled with fibres that form the cerebral peduncle (Fig. 5J and K). The cell density is substantially less than that seen in A9pc. The neurons were multipolar and stellate shaped, with a dorsomedial to ventrolateral orientation of dendrites.

3.3.2.3.9. *A9 lateral (A9l)*

This nucleus was found at the extreme lateral end of A9pc, on the ventral lateral edge of the midbrain tegmentum (Figs. 5K, 7C). The cells were triangular in shape and multipolar, and show no regular dendritic orientation. A substantial number of neurons could be assigned to this nucleus.

3.3.2.3.10 *A9 medial (A9m)*

The TH+ neurons that could be assigned to this nucleus were found lateral to the A10 nucleus and the exiting oculomotor nerve, but medial to the neurons that coalesce to form the A9pc in a region that can be described as mediodorsal to the medial boundary of the cerebral peduncle (Fig. 5K). This nucleus had a high density of ovoid shaped neurons that were bipolar. No distinct orientation of dendrites emanating from these neurons could be discerned.

3.3.2.3.11. *A8 (Retrorubral nucleus)*

This TH+ neurons making up this nucleus were found in the midbrain tegmentum, dorsal to A9pc, and dorsal, lateral and caudal to the magnocellular division of the red nucleus (Figs. 5L, 7C). These multipolar neurons were of low to moderate density, and showed a mixture of shapes with no specific dendritic orientation.

3.3.2.4. Pontine Catecholaminergic Nuclei

The catecholaminergic nuclei found in the pons of most mammals is also known as the locus coeruleus complex (LC) (Smeets and Gonzalez, 2000). These nuclei can be subdivided into A7 dorsal, A7 ventral, A6 dorsal, A6 ventral, A6 alpha, A5 and A4 nuclei. Many of these divisions are present in mammals, although the overall complement does differ between species (Kitahama *et al*., 1994; Manger et al., 2003).

3.3.2.4.1. *A7dorsal (subcoeruleus dorsal) (A7d)*

The TH+ neurons found in the dorsal pontine tegmentum, anterior and dorsal to the trigeminal motor nucleus, and medial to the brachium conjunctivum, were assigned to this nucleus (Fig. 5N). The cells are ovoid in shape and bipolar, with the dendrites oriented mediodorsal to ventrolateral.

3.3.2.4.2. *A7ventral (subcoeruleus ventral) (A7v)*

This nucleus is located in a position ventral, lateral and anterolateral to the trigeminal motor nucleus in the ventral lateral pontine tegmentum. The TH+ neurons assigned to this nucleus were not as numerous as that seen in the A7d nucleus, and appeared to form a ventral continuation of A7d (Fig. 5N). The neurons were similar in morphology to those of A7d.

3.3.2.4.3. *A6 ventral (locus coeruleus ventral) (A6v)*

This nucleus is found in the ventral lateral pontine periventricular gray matter, in a location very similar to the cholinergic lateral dorsal tegemental nucleus (Fig. 5N). This nucleus consisted of a moderate density of TH+ neurons that were evenly,

but not particularly densely packed throughout its extent. The neurons were ovoid in shape and bipolar with the dendrites oriented in dorsomedial to ventrolateral manner.

3.3.2.4.4. *A6 alpha (locus coeruleus alpha) (A6α)*

This tightly packed cluster of TH+ neurons was found adjacent to the A6v, but within the pontine tegmentum in a position immediately dorsal to the A7d nucleus (Fig. 5N). It appears to represent a continuation of the A6v into the tegmentum. The neuronal morphology was similar to that of the A6v neurons.

3.3.2.4.5. *A6 dorsal (locus coeruleus dorsal) (A6d)*

The TH+ neurons assigned to this nucleus were found in the lateral-most pontine periventricular gray matter, bordering the dorsal pontine tegmentum (Figs. 5N, 7D). This nucleus is comprised of a very dense cluster of TH+ neurons that were ovoid in shape, bipolar, with a mediolateral orientation of the dendrites.

3.3.2.4.6. *A5 (fifth arcuate nucleus)*

The TH+ neurons comprising this nucleus were found in the ventrolateral pontine tegmentum, lateral to the superior olivary nucleus (OLS) and the facial nucleus. The neurons formed a small column that projected from the edge of the pons into the tegmentum (Fig. 5N-P). The neurons evinced an irregular shape and multipolar morphology, with dendrites that are oriented parallel to the lateral and dorsal borders of the OLS and the lateral border of the facial nucleus.

3.3.2.4.7. *A4*

This nucleus was found in the dorsomedial periventricular gray, adjacent to the ventricular wall, and immediately ventral to the superior cerebellar peduncle (Figs. 5O, 7E). It is formed of a tightly packed cluster of TH+ neurons that were ovoid in shape and bipolar with a mediolateral orientation of the dendrites.

3.3.2.5. Medullary Catecholaminergic Nuclei

The medullary catecholaminergic neuronal groups are traditionally divided into C1, C2, A1, A2, C3 and area postrema nuclei (Hokfelt et al., 1984), all of which were found in the megabat, except the C3 group which appears to be restricted to rodents (Smeets and Gonzalez, 2000). The existence of an A3 group was reported in

rats by Dahlström and Fuxe (1964), but has not been recorded in later studies of the rat, or the present one in the megabat, and its existence is therefore considered questionable (Smeets and Gonzalez, 2000). In the megabat, these nuclei were all located within the medulla, from the level of the nucleus ambiguus to the very caudal level of the medulla at the spino-medullary junction.

3.3.2.5.1. *C1 (Rostral ventrolateral tegmental group)*

The TH+ neurons belonging to this nucleus were found within the ventrolateral medullary tegmentum between the levels of the nucleus ambiguus and the hypoglossal nucleus extending into the tegmentum from the ventrolateral edge of the medulla (Fig. 5Q). The cellular morphology is similar to that of the catecholaminergic neurons of the A5 nucleus, with a rough dorsomedial to ventrolateral orientation of dendrites.

3.3.2.5.2. *C2 (Rostral dorsomedial group)*

This cluster of TH+ neurons was found dorsal to the motor vagus nucleus between it and the floor of the fourth ventricle (Fig. 5P and Q). Within the bounds of this nucleus the neurons were moderately packed. The neurons were ovoid in shape and bipolar with mediolaterally oriented dendrites.

3.3.2.5.3. *A1 (Caudal ventrolateral tegmental group)*

The TH+ neurons found in the ventrolateral caudal medullary tegmentum, lateral to the lateral reticular nucleus, were assigned to this nucleus (Fig. 5R). This nucleus appears to be a caudal continuation of the C1 nucleus, but is located more dorsally and laterally than the C1 neuronal column. The cells were of the same morphology as those of C1.

3.3.2.5.4. *A2 (Caudal dorsomedial group)*

This nucleus was found between the dorsal motor vagus and hypoglossal nuclei, extending into the dorsal caudal medullary tegmentum (Fig. 5Q and R). There were not a great number of neurons in this nucleus, and they were sparsely packed. The TH+ neurons were ovoid in shape and bipolar, with mediolaterally oriented dendrites.

3.3.2.5.5. *Area postrema (AP)*

The area postrema was found in the very caudal part of the dorsal medulla, dorsal to the dorsal motor vagus and hypoglossal nuclei (Fig. 5P). It was very densely packed with very small TH+ round neurons that show no particular orientation of dendrites.

3.3.3. **Serotonergic Neurons**

The serotonergic nuclei are located along the rostrocaudal extent of the pons and medulla in the midline and immediately adjacent tegmental areas. The serotonergic system in mammals is readily divisible into two groups, the rostral cluster and the caudal cluster (Tork, 1990; Bjarkam et al., 1998), and this was the case for the megabat studied. Each of these clusters were further divisible into several nuclei, which are described below.

3.3.3.1. Rostral Cluster

This cluster is normally subdivided into several nuclei that are located in different regions, including the caudal linear, supralemniscal, and median raphe nuclei, and the dorsal raphe group. The dorsal raphe group is again divided into several nuclei.

3.3.3.1.1. *Caudal Linear nucleus (CLi)*

This cluster of serotonergic immunopositive neurons forms a central column around the midline anterior and inferior to the decussation of the BC, dorsal and slightly lateral to the interpeduncular nucleus (Fig. 5K and L). It consists of small, circular, bipolar neurons, with dendrites oriented mediolaterally.

3.3.3.1.2. *B9 (Supralemniscal)*

This group of neurons was found in the ventral lateral midbrain tegmentum, dorsal to the lemniscal pathways. It is an arc of serotonin immunopositive neurons, which sweeps out to the lateral aspect of the tegmentum, seemingly a lateral continuation of the caudal linear nucleus (Figs. 5L and M, 8B). It was made up of ovoid shaped, mostly bipolar, with some multipolar, neurons that had mediolaterally oriented dendrites.

3.3.3.1.3. *Median Raphe (MnR)*

The neurons that formed this nucleus were seen as a dense column of cells on either side of the midline (pararaphe position), beginning just caudal to the decussation of the brachium conjunctivum, ventral to the oculomotor and trochlear nuclei, and continuous caudally to the anterior-most level of the trigeminal motor nucleus (Fig. 5L and M). The neurons were round and ovoid in shape, and bipolar, with mostly dorsoventrally oriented dendrites.

3.3.3.1.4. *Dorsal Raphe (DR)*

The dorsal raphe group is divided into several distinct nuclei, all located within the periaqueductal and periventricular grey matter beginning immediately caudal to the oculomotor and trochlear nuclei, and ending at the anterior-most level of trigeminal motor nucleus.

3.3.3.1.5. *Dorsal Raphe interfascicular (DRif)*

This nucleus was located between the two medial longitudinal fasciculi, and was seen to be comprised of a high density of serotonergic immunopositive neurons (Fig. 5M). The neurons were ovoid in shape and bipolar with dorsoventrally oriented dendrites.

3.3.3.1.6. *Dorsal Raphe ventral (DRv)*

This nucleus was found in the ventromedial portion of the periaqueductal gray matter, immediately dorsal to the DRif (Figs. 5M, 8A). This highly dense cluster of mixed ovoid and circular shape neurons, were mostly bipolar, and showed no specific dendritic orientation.

3.3.3.1.7. *Dorsal Raphe dorsal (DRd)*

This nucleus was located just dorsal to the DRv, within the periaqueductal gray matter, but not quiet reaching the ventral aspect of the cerebral aqueduct (Figs. 5L and M, 8A). The cell morphology was similar to DRv cells.

3.3.3.1.8. *Dorsal Raphe lateral (DRl)*

The serotonergic immunopositive neurons assigned to this nucleus were found dorsal and lateral to the DRv and DRd, within the periaqeductal gray matter (Figs. 5M, 8A). The neurons were of moderate to low density, and were all located

within 300μm of the wall of the cerebral aqueduct. The neurons were large and multipolar with no clear dendritic orientation.

3.3.3.1.9. *Dorsal Raphe ventromedial (DRvm)*

This serotonergic nucleus was found in the ventrolateral part of the periaqueductal gray matter, lateral to the DRd and DRv (Fig. 5M, 8A). The neurons were multipolar and low in number. Some neurons were found within the adjacent tegmentum outside the periaqueductal gray matter. These were the only serotonergic neurons of the dorsal raphe group to be located outside of the periaqueductal gray matter.

3.3.3.1.10. *Dorsal Raphe caudal (DRc)*

At the most caudal extent of the dorsal raphe, a small number of serotonergic positive neurons were seen within the periventricular gray matter, and appeared to be a caudal continuation of the neurons forming the DRl nucleus (Fig. 5N). These neurons exhibited a similar morphology to those of the DRl.

3.3.3.2. Caudal Cluster

The caudal serotonergic cluster consists of five nuclei, the raphe magnus, raphe pallidus, rostral ventrolateral, caudal ventrolateral and raphe obscurus nuclei (Tork, 1990), which are all located adjacent to the midline of the brain, except the rostral and caudal ventrolateral nuclei.

3.3.3.2.1. *Raphe Magnus (RMg)*

This group of serotonergic neurons was found in the midline from the level of the trigeminal motor nucleus to the caudal level of the facial nucleus (Fig. 5N). Forming two columns either side of the midline, the neurons were ovoid in shape and bipolar, with a dorsoventral orientation of the dendrites.

3.3.3.2.2. *Raphe Pallidus (RPa)*

This nucleus was found at the ventral-most midline of the medulla, intimately associated with the pyramidal tracts (Figs. 5N-R, 8A). The neurons were ovoid in shape and bipolar, with dendrites oriented parallel to the nearest edge of the pyramidal

tracts. These neurons were seen to extend from the level of the trigeminal motor nucleus through to the spino-medullary junction.

3.3.3.2.3 *Rostral ventrolateral (RVL)*

Lateral to the inferior olives, in the ventrolateral medullary tegmemtum, a column of serotonergic immunreactive neurons were found to extend from the anterior-most level of the trigeminal motor nucleus to the trapezoid body (Figs. 5N-P, 8D). These neurons were ovoid in shape, bipolar for the most part, and had dendrites oriented parallel to the floor of the medulla. These neurons were most dense rostrally, and steadily declined in number caudally.

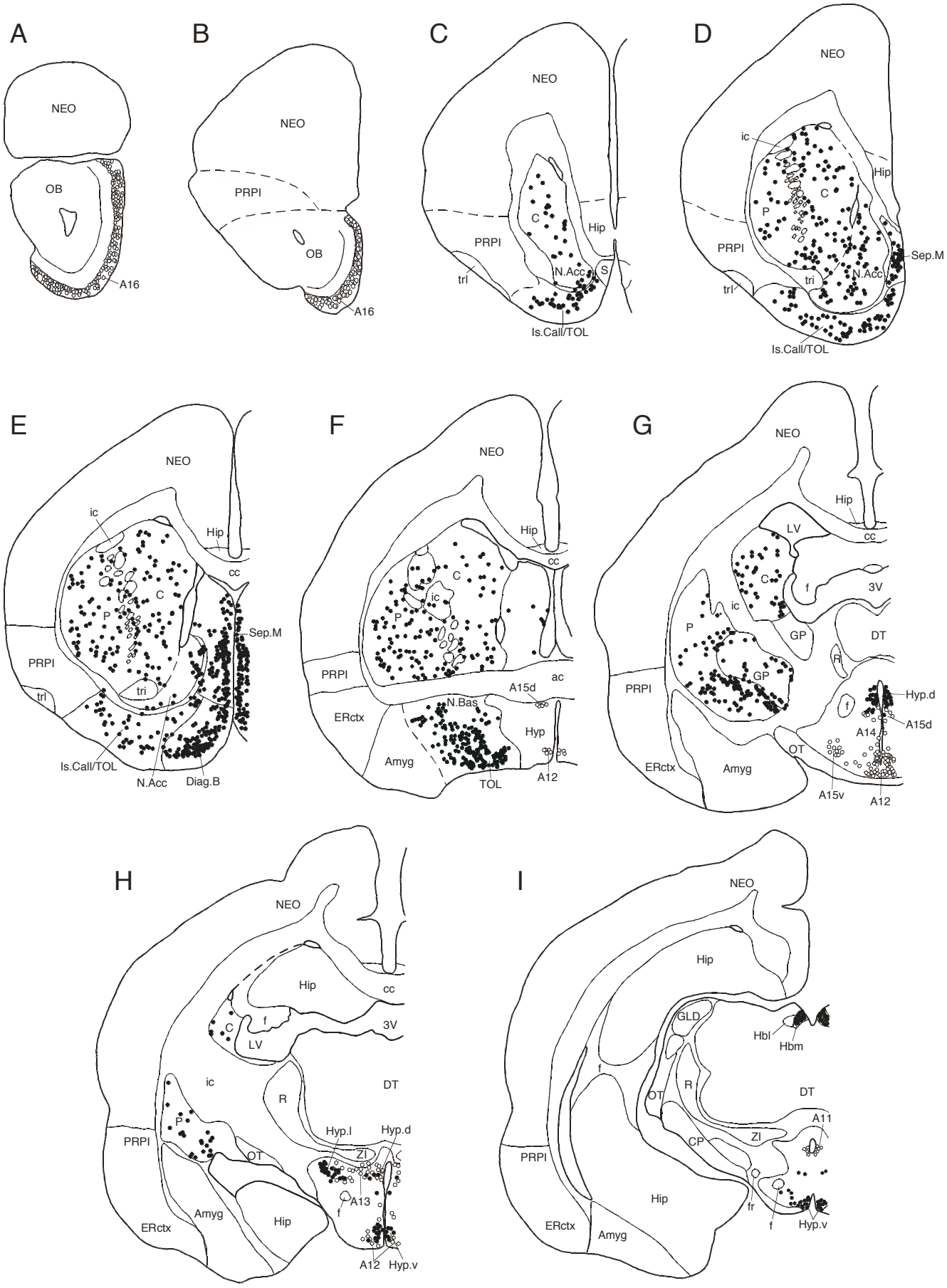
3.3.3.2.4. *Caudal ventrolateral (CVL)*

This nucleus is a caudal continuation of the RVL, and is located in the ventral lateral tegmentum of the medulla, caudal to the trapezoid body, and continuing to the spinomeduallary junction (Fig. 5Q and R). The neurons exhibited the same morphology as those of the RVL, and the number of neurons steadily decreased caudally.

3.3.3.2.5. *Raphe Obscurus (ROb)*

The serotonergic neurons assigned to this nucleus were located adjacent to the midline but were very few in number. This nucleus was first observed at the level of nucleus ambiguus and was seen to continue to the spinomedullary junction (Figs. 5P-R, 8C). The neurons were ovoid in shape and bipolar, with the dendrites oriented in a dorsoventral direction.

Figure 5. Serial drawings of coronal sections through one half of the megabat brain, from the olfactory bulbs through to the medulla. The outlines of the architectonic regions were drawn using nissl and myelin stains and immunoreactive cells marked on the drawings. Closed black circles depict cholinergic neurons, open circles depict catecholaminergic neurons (those immunoreactive for tyrosine hydroxylase) and open squares depict serotonergic neurons. The figures are approximately 750 μm apart. See list for abbreviations.


A
NEO
OB
A16
B
NEO
PRPI
OB
A16
C
NEO
C
Hip
PRPI
N.Acc
S
trl
Is.Call/TOL
D
NEO
ic
Hip
C
P
PRPI
Sep.M
tri
N.Acc
trl
Is.Call/TOL
E
NEO
ic
Hip
cc
C
P
Sep.M
PRPI
tri
trl
Is.Call/TOL
N.Acc
Diag.B
F
NEO
Hip
cc
C
ic
P
PRPI
A15d
ac
N.Bas
ERctx
Hyp
Amyg
TOL
A12
G
NEO
Hip
LV
cc
C
f
3V
ic
P
GP
DT
R
GP
PRPI
Hyp.d
f
A15d
A14
OT
ERctx
Amyg
A15v
A12
H
NEO
Hip
cc
C
f
3V
LV
ic
DT
R
P
Hyp.l
Hyp.d
PRPI
OT
ZI
A13
f
Amyg
ERctx
Hip
A12
Hyp.v
I
NEO
Hip
GLD
Hbl
Hbm
f
OT
R
DT
A11
CP
ZI
PRPI
Hip
fr
ERctx
Amyg
f
Hyp.v

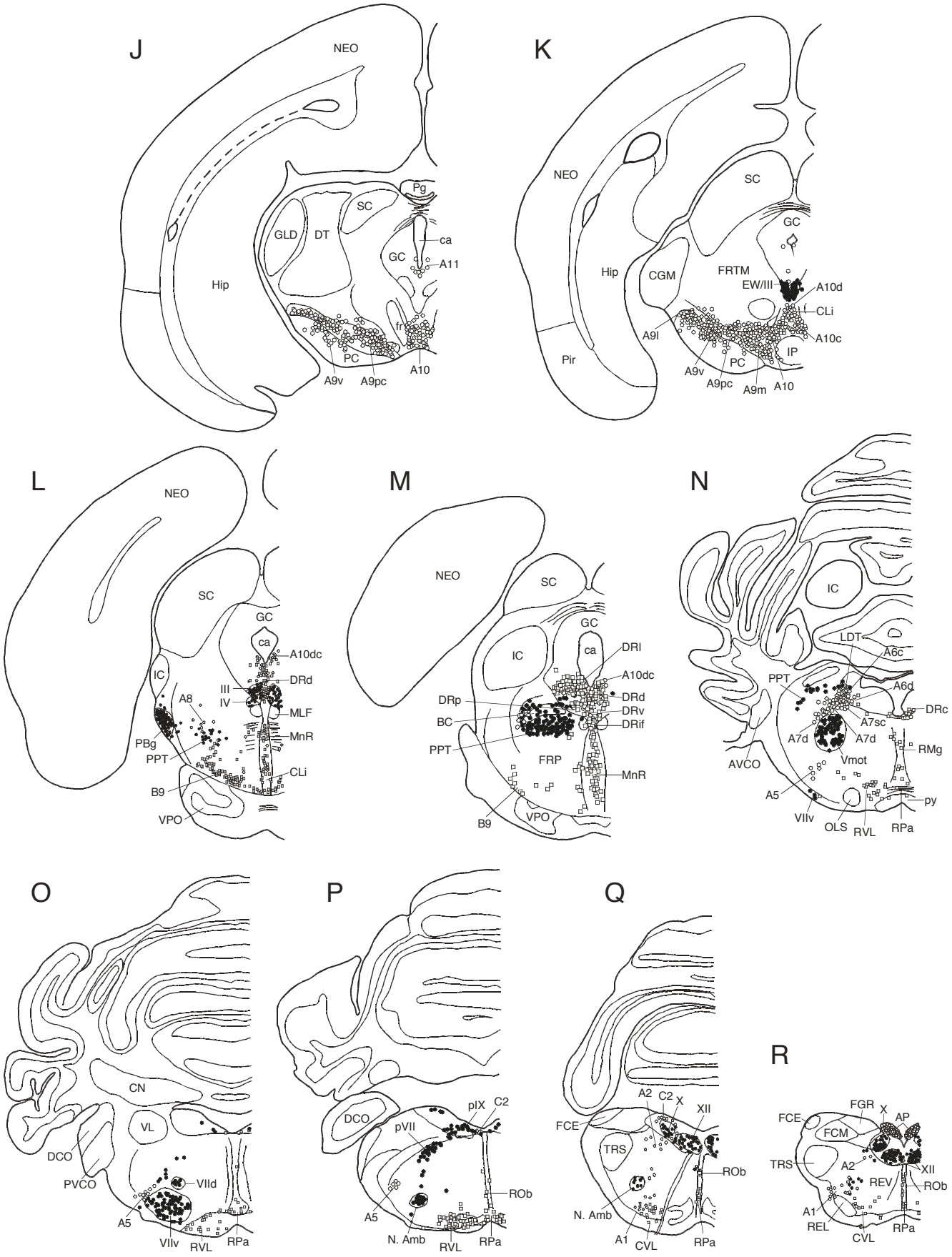

J
NEO
Pg
SC
GLD
DT
ca
GC
A11
Hip
fr
PC
A10
A9v
A9pc
K
NEO
SC
GC
Hip
CGM
FRTM
EW/III
A10d
CLi
A9l
A10c
IP
Pir
PC
A9v
A9pc
A9m
A10
L
NEO
SC
GC
ca
A10dc
IC
III
DRd
A8
IV
MLF
MnR
PBg
PPT
CLi
B9
VPO
M
NEO
SC
GC
IC
ca
DRl
A10dc
DRd
DRp
DRv
BC
DRif
PPT
FRP
MnR
B9
VPO
N
IC
LDT
A6c
PPT
A6d
DRc
A7sc
A7d
A7d
RMg
Vmot
AVCO
A5
py
VIIv
OLS
RVL
RPa
O
CN
VL
DCO
PVCO
VIId
A5
VIIv
RVL
RPa
P
DCO
pIX
C2
pVII
ROb
A5
N. Amb
RVL
RPa
Q
A2
C2
X
XII
FCE
TRS
ROb
N. Amb
A1
CVL
RPa
R
FGR
X
AP
FCE
FCM
TRS
A2
XII
REV
ROb
A1
REL
RPa
CVL

Figure 6. Photomicrographs showing neuronal groups that are immunoreactive for acetylcholinesterase in the forebrain and pons of the megabat brain. (A) Basal forebrain, with the medial septal nucleus (**S**) located at a mediodorsal position and the diagonal band of Broca (**Diag.B**) located around the medial ventral corner of the cerebral hemisphere; (B) laterodorsal tegmental nucleus (LDT); (C) pedunculopontine nucleus (**PPN**) and ventral subdivision of the facial nerve nucleus (**VIIv**); (D) parabigeminal nucleus (PBg); (E) abducens nerve nucleus (VI). The scale bar in E = 250 μm and applies to all.

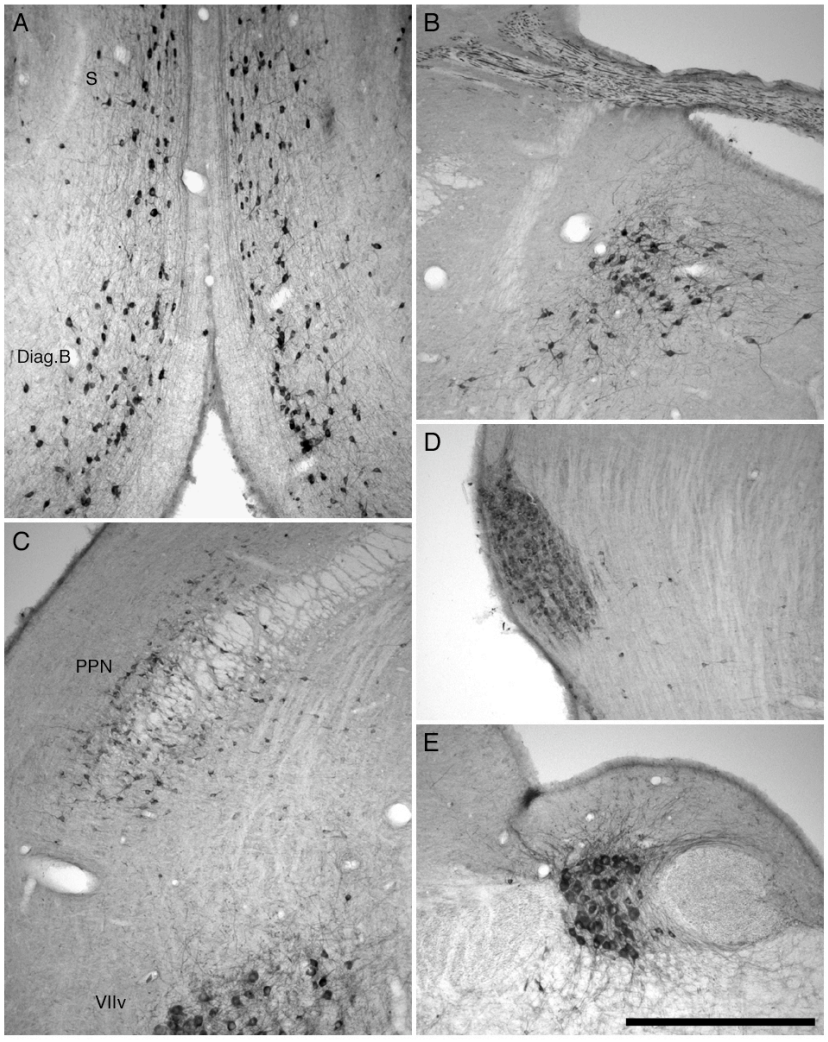

A
S
Diag.B
B
C
PPN
VIIv
D
E

Figure 7. Photomicrographs showing neuronal groups that are immunopositive for tyrosine hydroxylase in the diencephalon, midbrain and pons of the megabat brain. (A) **A13** and **A14**; (B) A10 dorsocaudal (A10dc); (C) substantia nigra, lateral (**A9l**) and retrorubral field (**A8**) medially; (D) locus coeruleus, dorsal (A6d); (E) A4. The scale bar in E = 250 μm and applies to all.

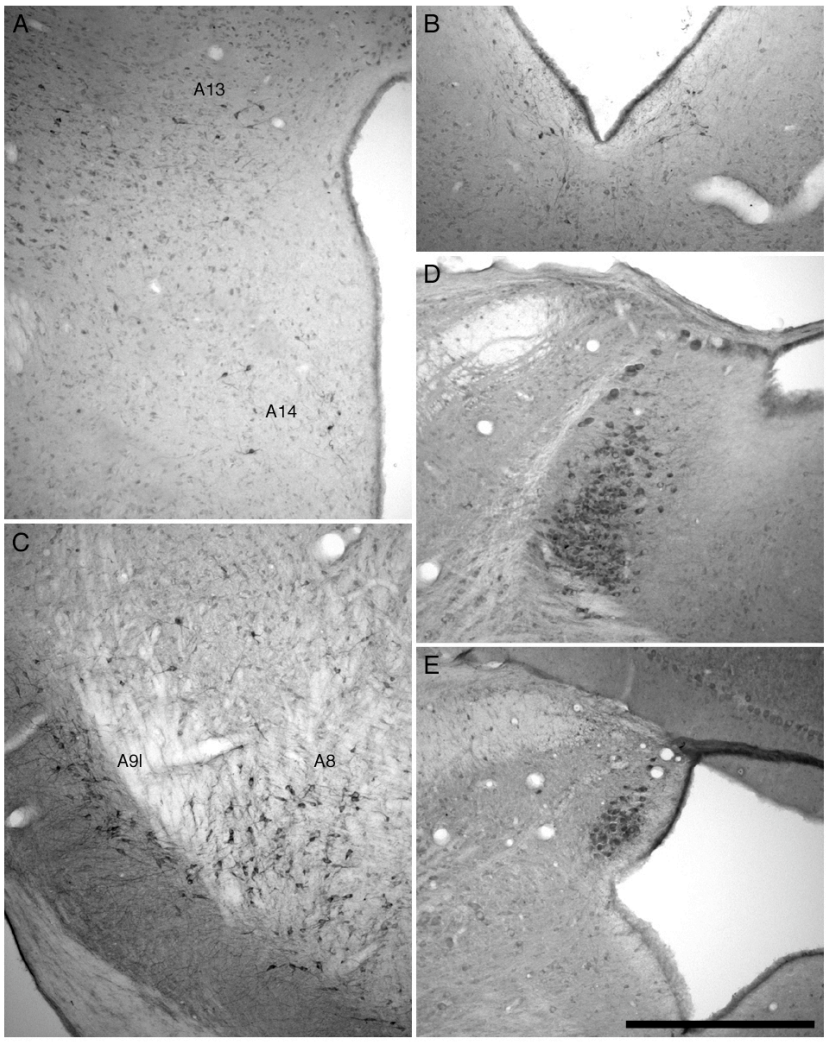
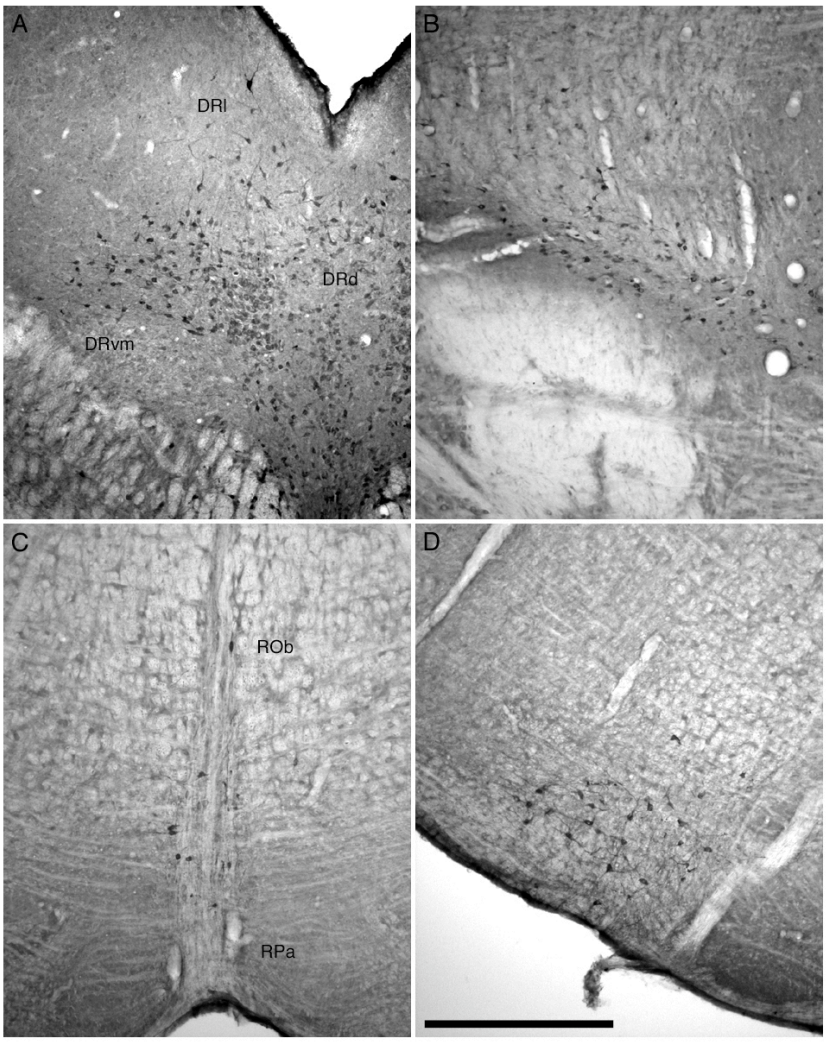

A
A13
A14
B
C
A9l
A8
D
E

Figure 8. Photomicrographs showing neuronal groups that are immunopositive for serotonin in the pons and medulla of the megabat brain. (A) Dorsal raphe nucleus, showing the lateral (**DRl**), dorsal (**DRd**) and ventromedial (**DRvm**) subdivisions; (B) B9, or supralemniscal group; (C) raphe obsucurus (**ROb**) and raphe pallidus (**RPa**) ventrally; (D) rostral ventrolateral group (RVL). The scale bar in D = 250 μm and applies to all.


A
DRl
DRd
DRvm
B
C
ROb
RPa
D

3.4. **DISCUSSION**

In general, the megabat studied exhibited many similarities to the other mammals that have been studied with regards to the cholinergic, catecholaminergic and serotonergic systems (Smeets and Gonzalez, 2000; Tork, 1990, Woolf, 1991). Certain similarities and differences were interesting to compare across the mammals and specifically to the microbats (see Chapter 2). The complement of nuclei that emerged from observation of the megabat is in certain ways very different to that found microbats. In the megabat brain, the catecholaminergic nuclei including the A4, A6d, A9v, A10d, A10dc, A15d and A15v; and the cholinergic nuclei including the parabigeminal nucleus, Edinger-Westphal nucleus, and the preganglionic motor neurons of the salivatory and glossopharygeal nerves, were all present in the megabat but could not be found in the microbat brain (Chapter 2). With regards to the serotonergic system, the megabat and microbat studied were identical in their nuclear complement. While there is a list of 11 differences, it should also be pointed out that there are 57 nuclei that are homologous between these two species. However, these 57 homologous nuclei are found in almost every mammal studied to date, thus rendering their heuristic value for comparison void. Moreover, many of the nuclei that are seen in the megabat but are absent in the microbat, are present in rabbits, carnivores, tree shrews and primates. The differences in nuclear complements of these systems are supportive of a diphyletic origin of Chiroptera due to the manner in which the nuclear complexity of these systems appear to change in the course of evolution (Manger, 2005;Da Silva et al., 2006).

3.4.1. Cholinergic system

The cholinergic nuclei found in the region of the dorsal striatopallidal complex and the basal forebrain of the megabat brain are similar to that seen in other mammals previously studied (Woolf, 1991), including the microbat studied herein (Chapter 2). Within the diencephalon of the megabat studied, three hypothalamic subdivisions were found, including a lateral (lHyp), ventral (vHyp) and dorsal (dHyp) hypothalamic group, along with the medial habenular nucleus (Hbm) of the epithalamus. These three hypothalamic nuclei have been reported for rats, laboratory shrews, cats, primates and the microbat (Karasawa et al., 2003; Satoh et al., 1983; Tago et al., 1987; Vincent and Reiner, 1987; Chapter 2); however, no hypothalamic cholinergic neuronal groups are present in monotremes (Manger, 2002a).

Within the pontine region of the megabat, the laterodorsal tegmental area (LDT), pedunculopontine (PPN) and parabigeminal (PBg) nuclei were observed. The LDT and PPN have been found in all mammals studied to date, including the microbats (Chapter 2); however, the PBg has been reported only in rats, highveld molerats, cats, ferrets, tree shrews and primates (Da Silva et al., 2006; Henderson, 1986; Kimura et al., 1981; Mesulam et al., 1989; Murray et al., 1982; Reiner and Vincent, 1987; Henderson, 1987). This nucleus has not been found in studies of the monotremes, laboratory shrews or the microbats (Karasawa et al., 2003; Manger, 2002a; Chapter 2). A basic similarity in the location and identity of the cranial nerve nuclei was found in the megabat in comparison to all other mammals studied previously (Woolf, 1991). The megabats evinced a clearly ChAT immunoreactive Edinger-Westphal nucleus, which is also seen in the rat, cat, ferret and primates (Armstrong et al., 1983; Kimura et al., 1981; Lavoie and Parent, 1994; Mesulam et al., 1989; Mizukawa et al., 1985; Reiner and Vincent, 1987; Satoh et al., 1983; Henderson, 1987); however, this nucleus has not been observed in monotremes, laboratory shrews or the microbats (Karasawa et al., 2003; Manger, 2002a; Chapter 2). We also found ChAT immunoreactive neurons that represent the preganglionic motor neurons of the salivatory and glossopharyngeal nerves in the megabat. Similar neurons have also been reported in the macaque monkey, baboon, human, rat, cat and ferret (Armstrong et al., 1983; Henderson, 1987; Mesulam et al, 1984; Mizukawa et al., 1986; Satoh and Fibiger, 1985; Shiromani et al., 1988), but not in the monotremes or microbats (Manger, 2002a; Chapter 2). A medullary tegmental field was not found in the megabat. This loose aggregation of cholinergic neurons in the medullary tegmentum has been reported for monotremes, rats, cats and ferrets (Armstrong et al., 1982; Armstrong et al., 1983; Henderson, 1987; Manger, 2002a; Shiromani et al., 1988), but it is absent in the microbat and primates (Kus et al., 2003; Chapter 2). The general complement of cholinergic nuclei found for the megabat exhibits many similarities to that seen in primates, carnivores and rodents, and several dissimilarities to that seen in the monotremes, microbat and the laboratory shrew. Features that are absent in the microbat, but which are seen in the megabat creating the impression of this phylogenetic alignment include the parabigeminal and Edinger-Westphal nuclei, as well as the preganglionic motor neurons of the salivatory and glossopharyngeal nerves. Thus, if the complement of cholinergic nuclei is indicative of phylogenetic

separation, as indicated by Manger (2005), then the present observations support separate evolutionary origins of the two chiropteran suborders.

3.4.2. Catecholaminergic nuclei

The megabat showed a clear A16 catecholaminegic cell group in the stratum granulosum of the olfactory bulbs. This group has been found in all the mammals studied to date (Smeets and Gonzalez, 2000) including the microbat (Chapter 2), making this neuronal group a common feature of the mammalian catecholaminergic system. In the diencephalon of the megabat the A15 dorsal, A15 ventral, A14, A13, A12 and A11 catecholaminergic neuronal groups were all readily observable. All of these diencephalic nuclei have been found in primates, cats, rabbits, rodents, pigs, sheep and monotremes (Cheung and Sladek, 1975; Leshin et al., 1995; Manger, 2002b; Tillet and Kitahama, 1998, Blessing et al., 1978; Smeet and González, 2000). In contrast, the tree shrew, *Tupaia*, appears to lack the A14, A15 dorsal and A15 ventral neuronal groups (Murray et al., 1982), and the bottlenose dolphin lacks an A13 group (Manger et al., 2004). Both the microbat and hedgehog lack the A15 dorsal subdivision (Michaloudi and Papadopoulos, 1996; Chapter 2).

Within the midbrain of the megabat we found the A8 (retrorubral), A9 (substantia nigra) and A10 (ventral tegmental area) nuclear groups, of which the A9 and A10 are further subdivided into the A9 lateral, A9 pars compacta, A9 ventral (or pars reticulata) and A9 medial, and A10, A10 central, A10 dorsal and A10 dorsal caudal. All these subdivisions are seen in the primates, tree shrews, rodents, bottlenose dolphins, pigs, sheep, opossum and monotremes (Crutcher and Humbertson, 1978; Da Silva et al., 2006; Felten et al., 1974; Manger, 2002b; Manger et al., 2004; Murray et al., 1982; Østergaard et al., 1992; Tillet and Kitahama, 1998). The carnivores and rabbits lack the A9 ventral subdivision (Blessing et al., 1978; Dormer et al., 1993; Henderson, 1987; Reiner and Vincent, 1987), while in the hedgehog, the distinction of this subdivision of the substantia nigra (A9v) is not particularly clear (Michaloudi and Papadopoulos, 1996). Microbats were found to lack the A9 ventral, A10 dorsal and A10 dorsal caudal subdivisions (Chapter 2), thereby setting it apart as unique amongst the mammals studied to date.

Seven subdivisions of the locus coeruleus complex are seen in the megabats, including the A4, A5, A6 dorsal, A6 ventral, A6 alpha, A7 dorsal and A7 ventral. All seven locus subdivisions are seen in the primates, the tree shrews and rabbits

(Blessing et al., 1978; Murray et al, 1982; Bogerts, 1981; Schofield and Everitt, 1981). Several mammals lack the A6 dorsal and A4 subdivisions of the locus coeruleus complex, and these include the carnivores, rodents, bottlenose dolphins, pigs, sheep, microbats, hedgehogs, opossums and monotremes (Crutcher and Humbertson, 1978; Manger et al., 2002b, 2004; Michaloudi and Papadppoulos, 1995; Tillet and Kitahama, 1998; Chapter 2). The catecholaminergic nuclei of the megabat medulla are very similar to all other mammals studied, being comprised of the groups A1, A2, C1, C2 and area postrema (AP) nuclei, but they lack a C3 group. The C3 group has only been reported for rodents and no other mammals (Hokfelt et al., 1984; Smeets and Gonzalez, 2000). The overall complement of catecholaminergic nuclei within the megabat resembles that seen in primates, rodents and rabbits most closely, and differs substantially from that seen in the microbat.

3.4.3. Serotonergic system

With respect to the various nuclei of the serotonergic system, the megabats appear identical to the other eutharian mammals that have been previously studied (Tork, 1990; Bjarkam et al, 1997). The nuclei of the serotonergic system identified in the megabat are also found in the wallaby (Ferguson et al., 1999). However, the megabat is different to the opossum and the monotremes as these species lack the caudal ventrolateral serotonergic group (Crutcher and Humbertson, 1978; Manger, 2002c), and monotremes also possess hypothalamic serotonergic nuclei (Manger, 2002c). The serotonergic system on the whole is not strongly diagnostic in relation to specific mammalian orders, but does serves to clearly distinguish the monotremes from the remaining mammals.

3.4.4. Contrasts between the megabats and microbats

In the current and previous study of the megabat and microbat brains, 11 specific differences were found in the nuclear complement of the neuromodulatory systems. Certain nuclei appear to be strongly diagnostic in terms of the phylogenetic relationships of the megabats and microbats and their relationship to other mammals. This diagnostic strength is based on the previous observations that the nuclear complement of these systems do not change within an order, irrespective of the brain size, lifestyle or phenotype of the species, but changes in the nuclear complement are observed in the different orders (Manger, 2005). Thus, these differences between the

megabat and microbat support the notion that these species are from different orders, and the similarities in the complements of these systems with other mammals aligns both the megabat and microbat studied with different mammalian orders, which may be reflective of their phylogenetic origin.

In the cholinergic system, the parabigeminal and Edinger-Westphal nuclei, as well as the preganglionic motor neurons of the salivatory and glossopharyngeal nerves are found in the rodents, carnivores, tree shrews and primates and are seen to form part of the megabat cholinergic system. In constrast, none of these features are found in the microbat cholinergic system (Chapter 2), nor in the cholinergic system of the laboratory shrew and monotremes. These specific differences align the megabats phylogenetically with the rodents, carnivores and primates, and aligns the microbats with the insectivores.

In the catecholaminergic system, the existence of A4 and A6 dorsal subdivisions of the locus coeruleus complex in megabats, aligns the megabats with rabbits, tree shrews and primates, to the exclusion of other mammals previously studied. The lack of these divisions aligns the microbats with the ungulates, carnivores, insectivores, marsupials and monotremes. The carnivores and rabbits lack the A9 ventral subdivision of the substantia nigra, while the tree shrew lacks the A15 (both the dorsal and ventral subdivisions) and A14 divisions, which are all present in the megabats, thereby differentiating them from the microbats. The megabat catecholaminergic system is most like that found in primates. The lack of A10d and A10dc in the microbats distinguishes this species from all other mammals, but the other differences align them most closely with the insectivores. The serotonergic system does not appear to be diagnostic in terms of separating eutherian mammalian species into different orders.

The findings of the current study of the megabat and the previous study of the microbat are both supportive of the diphyletic origin of the Chiroptera (Chapter 2), as the complement of nuclear subdivisions can be interpreted to support the notion that the megabats and microbats derive from two different mammalian orders (Manger, 2005). The findings also indicate that the microbat is most closely aligned with representatives of the Insectivora, while suggesting that the megabat is mostly closely aligned with the tree shrew and the various primates that have been studied. It appears reasonable to conclude that the features of the neuromodulatory systems of the megabat resemble that found in the groups most closely related to primates and

support earlier neuroanatomical studies that suggested this sister grouping (Pettigrew, 1986; Pettigrew et al., 1989).

4. OVERALL DISCUSSION

The current study demonstrates significant and phylogenetically relevant differences in the organization of the complements of neuromodulatory systems between the megabats and microbats. Despite this, both the megabat and microbat studied shared many nuclear similarities with other mammals; for example, the presence of cholinergic nuclei with widespread projections in the striatum (figs. 1D-H, 2B, 3.1C-H), the basal forebrain (figs. 1C-G, 2A and B, 5C-F, 6A), and the pons (figs. 1P-S, 2C, 5L-N, 6B-C). The presence of catecholaminergic neurons in the olfactory bulb (figs. 1A and B, 5A and B), substantia nigra (1L-O, 3C, 5J and K, 7C), and other regions contained many homologous nuclei in the two chiropteran species studied. Interestingly, the nuclear complement of the serotonergic system was identical between the two species studied. In fact, when all the studies of the neuromodulatory systems are collated (see Table 1) it becomes clear that a total of fifty nuclei are found to be homologous across all the mammals, including the bats. Of the neuromodulatory nuclei revealed using the immunohistochemical stains in the current study, sixteen of these were found to have a variable, and phylogenetically relevant, occurrence when compared across mammalian species.

When the megabat and microbat are specifically compared, ten nuclei were found to be variable. All the nuclei found in the microbat were common to those found in all eutharian mammals. Moreover, it was also noted that the microbat had the least number of nuclear subdivisions in comparison to the other mammals included in the current study, this being the specific lack of the dorsal and dorsal caudal subdivisions of the ventral tegmental area. When the megabats and microbats are specifically compared, the cholinergic nuclei including the Edinger-Westphal nucleus, parabigeminal nucleus and the preganglionic motor neurons of the salivatory and glossopharangeal nerves, were all present in the megabats but not in the microbats. The catecholaminergic nuclei including the A15 dorsal, A9 ventral, A10 dorsal, A10 dorsal caudal, A6 dorsal and the A4, were also all present in megabats but not in the microbats. The nuclear complement of the serotonergic system was directly comparable between the two species.

Thus, the ten nuclei found varying between the two bat species were all present in the megabats but all absent in the microbat, making the differences between

these two species a case of missing nuclei in one species. Further to this, these nuclei that were present in the megabat but absent in the microbat, were also all present in all the primates previously examined. Additionally, some, but not all, were present in *Tupaia* (Table 1, column T), carnivores (Table 1, columns Q-S), rodents (Table 1, columns L-N) and rabbits (Table 1, column P). The complement of nuclei missing from the microbat was similar to the complement of nuclei not found in the hedgehog and the laboratory shrew (Table 1), with the extra lack of two A10 subdivisions in the microbat.

The phylogenetic origin of bats has been a controversial subject; therefore, many different methodologies have been employed in an effort to elucidate the true phylogenetic history of the megabats and microbats. These methodologies include morphological observations, particularly on the brain, and molecular observation. The conflict lies between the two hypotheses of monophyly and diphyly; the former implying that megabats and microbats share a more recent ancestor with each other than with any other animal, and the latter implies that the two groups do not share a common ancestor and that mammalian flight evolved twice (Pettigrew et al., 1989). Many studies have come to conclusions that support one or the other of these phylogenetic possibilities. Interestingly, while the primates have been suggested to be a sister group to the megabats (Pettigrew et al., 1989), no sister group has been proposed for the microbats.

4.1. The monophyly vs diphyly debate: the Brain *vs* DNA

4.1.1. *Observations on brain morphology supporting diphyly*

As mentioned above, many studies have been conducted in efforts to illuminate the phylogeny of Chiroptera, and several have come to the conclusion that it is diphyletic. The majority of the studies supporting diphyly are based on investigations of the morphology of the brain, the first of which was research on the neural pathway between the retina and the superior colliculus, which showed that megabats share a similar pattern to that which was previously thought to be unique to primates; but microbats were very different in this regard sharing a retinotectal projection that did not differ from other mammals (Cooper and Pettigrew, 1986; Pettigrew, 1986). Rosa and Schmid (1994) confirmed that the retinotectal pathway of the megabat was indeed similar to primates using electrophysiological methods. The

size of the superior colliculus was found to be consistently smaller than the inferior colliculus in microbats and the opposite was found to be true in megabats (Bauchot and Stephan, 1970; Stephan and Pirlot, 1970). In terms of the echolocation, laryngeal sonar was found to be universally present in microbats and universally absent in the megabats (Neuweiler et al., 1980; Novick, 1977). Within the somatosensory cortex a small representation for the hindlimbs was found in microbats, but a consistently larger one is seen for megabats (Wise et al., 1986; Krubitzer and Calford, 1992). In a study of the motor cortex it was found that the corticospinal region had a primitive arrangement in the microbat and an advanced primate-like arrangement in the megabats (Kennedy et al., 1987; Nudo and Masterton, 1985). In an investigation of the organization of the ventroposterior nuclei of the chiropteran dorsal thalamus, Manger et al. (2001), showed clear and phylogenetically relevant differences in the structure of this distinct region in megabats and microbats. These are not all the examples of the differences in the organization of the brain between megabats and microbats, but do represent a range of structural differences among many that indicate that when brain morphology is considered the diphyletic theory of chiropteran origins is the favoured hypothesis.

4.1.2. *Observations on brain morphology supporting monophyly*

Other researchers studying the morphology of the brain have arrived at conclusions that appear to support the monophyletic hypothesis of chiropteran evolution using brain morphological studies. For example, Thiele et al. (1991), reported that no decussation of the retino-tectal pathway was evident in *Rousettus*, therefore contradicting the work of Rosa and Schmid (1992) and implying monophyly for the bats. However, this is addressed in Rosa and Schmid (1994) by pointing out that a technical difference existed between the two studies, since in the study on *Rousettus*, the head was angled horizontally and therefore the visual axis was angled upwards, thus only permitting examination of the inferior visual field, at which the decussation is more diffuse. Another neuroanatomical study appearing to contradict the diphyletic theory is that of Lapointe et al. (1999) in which brain morphometrics and adaptations were examined in order to decide which of the two hypotheses, i.e. monophyly or diphyly, is true. These researchers found evidence that they believe supported bat monophyly and presented this in the form of phylogenetic trees. Although the trees presented do depict bat monophyly, they also depict microbat

polyphyly, along with primate polyphyly. Both these scenarios are largely accepted to be false, therefore the reliability of the conclusions of the study with respect to megabat-microbat monophyly are also questionable.

4.1.3. *Molecular (DNA) observations supporting monophyly*

In addition to research on the brain, many workers have carried out research on the molecular level to address the bat phylogeny problem. Although there's a disagreement between the conclusions of the different studies, the majority point towards the megabat-microbat monophyletic hypothesis. Examples of this work include that of Bailey et al. (1992), Bullejos et al. (2000), Liu et al. (2001), Martin (1999), Porter et al. (1996), Stanhope et al. (1992), and Teeling et al. (2000).

4.1.4. *Molecular (DNA) observations supporting diphyly and other conclusions*

Not all molecular evolutionary studies have led to the conclusion of bat monophyly. Indeed many have reached conclusions other than monophyly in bats, including megabat-microbat diphyly, microbat diphyly, or even microbat paraphyly. The work of McNiff and Allard (1998) and that of Jaworski (1995) provide evidence for a diphyletic origin for the bats. The work of Bailey et al. (1992), Bullejos et al. (2000), Liu et al. (2001), Martin (1999), Porter et al. (1996), Stanhope et al. (1992), and Teeling et al. (2000), all concluded that megabats are monophyletic but associated the megabats with one of four different microbat families or super families to the exclusion of other microbats. These vicarious results imply chiropteran polyphyly, and in some cases, paraphyly within the microbats. While a full analysis of the molecular evidence is beyond the scope of the present study, it is clear that the molecular evidence provided is indeed fraught with many problems and leads to a variety of conclusions, whereas the data of studies of the brain is more clear cut and leads to the diphyletic conclusion.

4.2. <u>Findings of the current study</u>

The findings of the current study, being based on observable and reliably identifiable systems of the brain, point towards there being a diphyletic origin between the megabats and the microbats, as do all other studies of the neuroanatomy. The specific inference made in the present study is based on prior work that has shown that animals that belong to the same phylogenetic order show an identical

complement of the nuclei of the neuromodulatory systems. In the current study, it was found that megabats and microbats are very different with regards to the neuromodulatory system nuclear complements (chapter 2 and chapter 3). Therefore, it is suggested herein that megabats and microbats do not share a more recent common ancestor with each other than they do with any other mammals. Secondly, the comparison of neuromodulatory complements between the two groups of bats and other mammals, showed that megabats are closer to the primates than to any other mammal included in the study. In fact, the nuclear complement of the neuromodulatory systems in the megabat studied was identical to that seen in a range of primates that have been studied previously (see Table 1). This would suggest that the megabat studied was indeed a member of the order primates, as the megabat and the primates shared this complement of nuclear subdivisions to the exclusion of all other mammals. This exclusion includes *Tupaia*, which while having many nuclei in common with the megabat and primates, lacked three distinct hypothalamic catecholaminergic nuclei that were found in megabats and primates. It was also found that the nuclear complement of the neuromodulatory systems in the microbat had the most in common with the nuclear complement of these systems seen in insectivores. Thus, it can be proposed that the potential sister group for the microbats would be one of the insectivore lineages; however, the phylogeny of the insectivores is unclear (Symonds, 2005), thus specifying which of the lineages is the sister group to microbats is difficult. More data on the neuromodulatory systems of the various insectivore lineages is required to determine which resembles the situation seen in microbats most closely.

4.3 SUMMARY

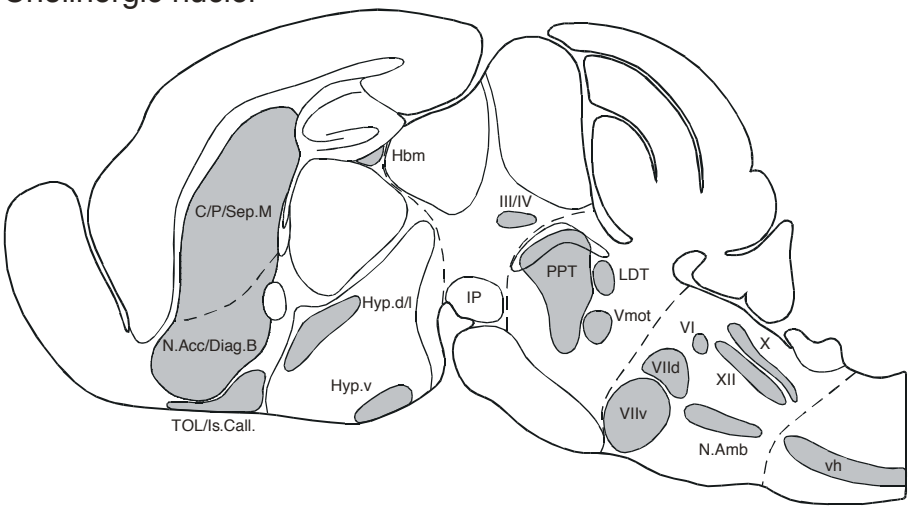
The two most commonly employed methods in the investigation of the bat phylogeny problem include brain morphological studies and molecular or DNA studies, examples of which are provided above. Most of the molecular studies on this subject point towards a monophyletic origin, whereas the work done on morphology, particularly that of the brain, point towards a diphyletic origin.

The diphylogenetic grouping of bats based on morphological studies of the brain also succeeds in placing other mammals in positions on the phylogenetic tree that are widely accepted, e.g. they do not lead to microbat or primate polyphyly, therefore pointing towards their reliability for use as phylogenetic tools. These neural studies

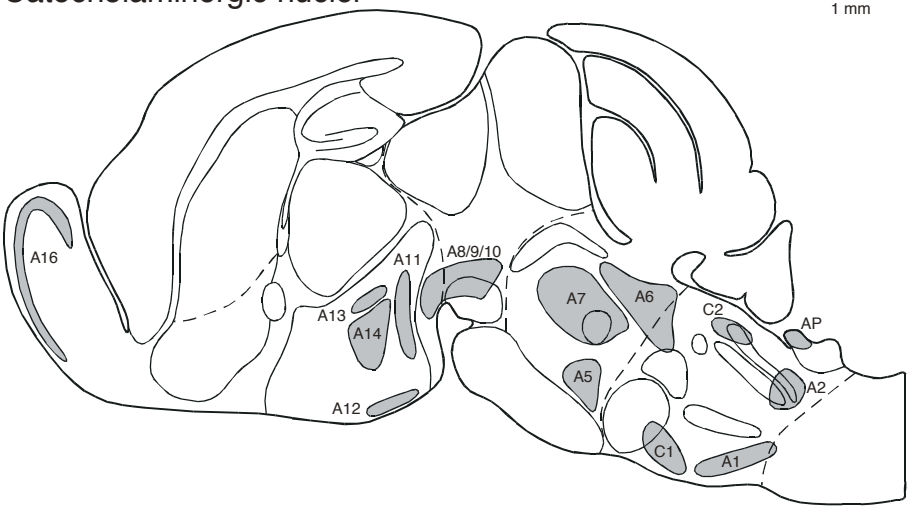
are also widely supported by all other morphological and ecological studies, for example studies on the penis (Smith and Madkour, 1980), skin (Quay, 1962), sperm morphology (Rouse and Robson, 1986) and wings (Pettigrew et al., 1989), all supporting diphyly. Pettigrew et al. (1989) concluded that all the similarities observed between megabats and microbats pertain to flight, and these similarities could simply be a result of phylogenetic constraints in the repertoire of mammalian adaptation, as both groups are mammals that evolved flight. The similarities could therefore be a result of convergent evolution driven by phylogenetic constraints that would face all mammals evolving flight (Pettigrew et al., 1989). Bat phylogeny is still an unresolved problem, but it appears that brain morphology and DNA studies are at odds regarding their phylogeny. Clearly more work is required to unravel this contentious phylogenetic issue, however, the value gained in our understanding of mammalian phylogeny, evolutionary processes, and the plasticity of phenotypic evolution are certainly worth the effort.

Figure 9. Diagrams of sagittal sections through the microbat brain, showing cholinergic, catecholaminergic and serotonergic neurons

Cholinergic nuclei



Catecholaminergic nuclei



Serotonergic nuclei

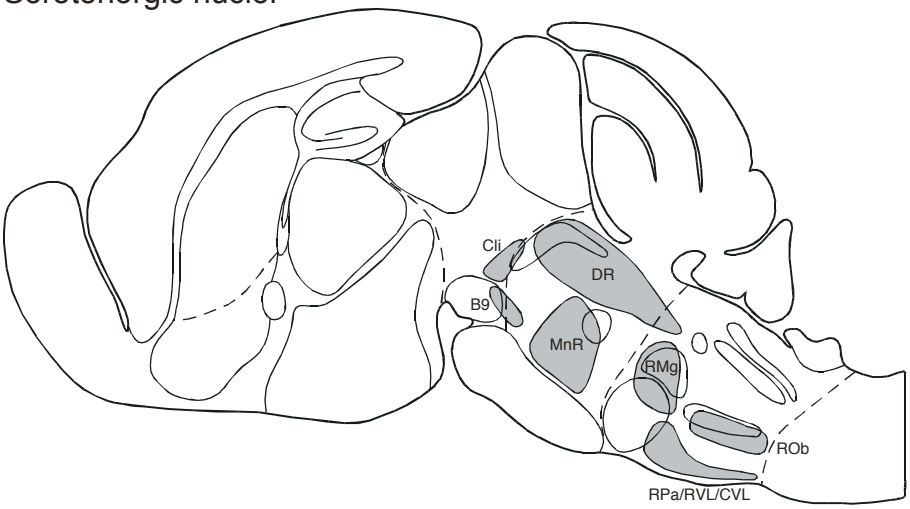
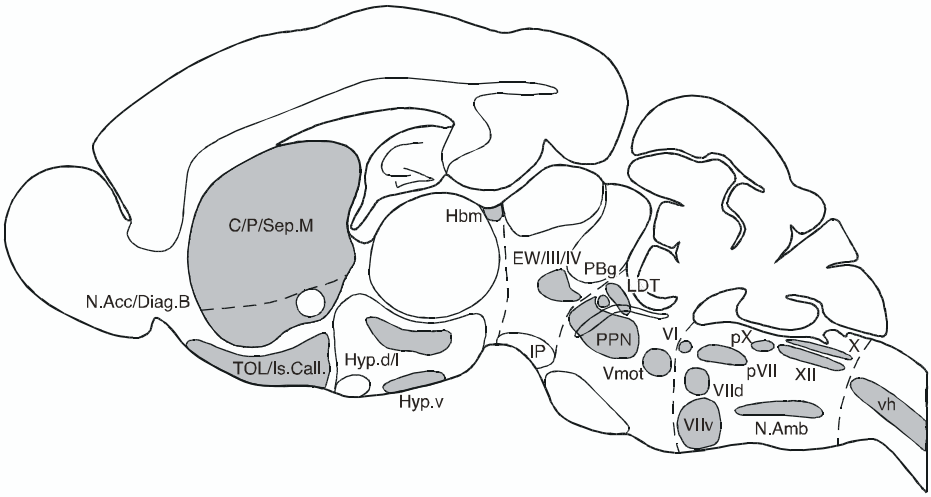


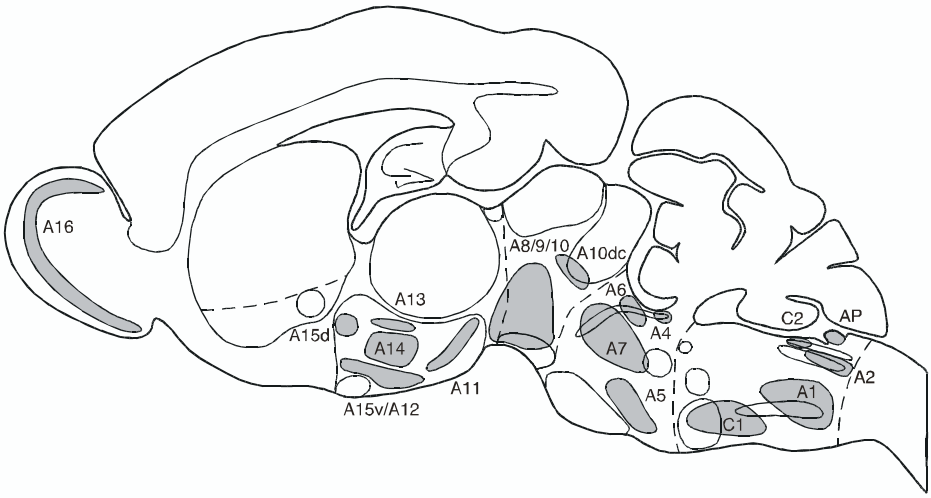
Figure 10. Diagrams of sagittal sections through the megabat brain, showing cholinergic, catecholaminergic and serotonergic neurons

Cholinergic nuclei

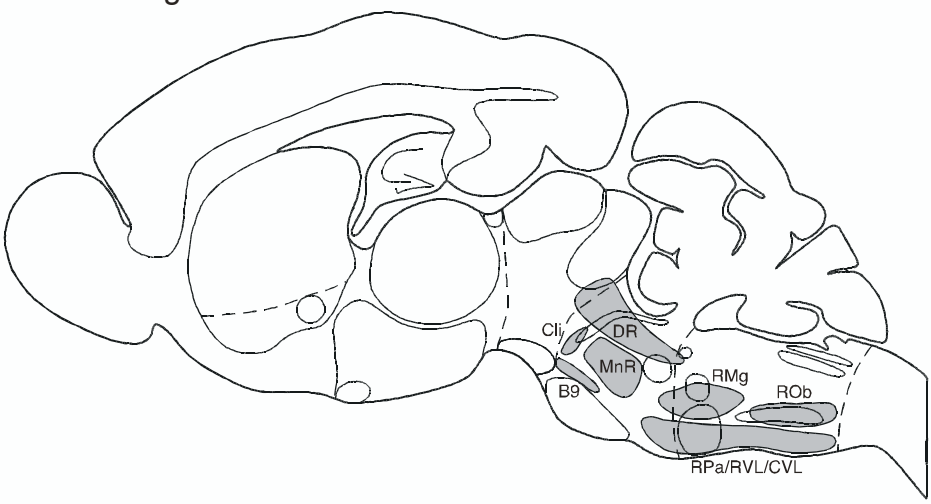


Catecholaminergic nuclei

1 mm



Serotonergic nuclei



Species	*Ornithorhynchus anatinus*	*Tachyglossus aculeatus*	*Didelphis virginiana*	*Macropus eugenii*	*Suncus murinus*	*Erinaceus europaeus*	*Miniopterus schreibersii*	*Sus scrofa*	*Ovis aries*	*Tursiops truncatus*	*Rattus norvegicus*	*Thryonomys swinderianus*	*Tatera brantsii*	*Cryptomys hottentotus*	*Oryctolagus cuniculus*	*Felis cattus*	*Mustela putorious*	*Canis familiaris*	*Tupaia glis*	*Rousettus aegyptiacus*	*Cebuella pygmaea*	*Callithrix jacchus*	*Saimiri sciuerus*	*Macaca sp.*	*Papio papio*	*Homo sapiens*
Common names	Platypus	Echidna	Opossum	Wallaby	Lab shrew	Hedgehog	Schreiber's long fingered bat	Pig	Sheep	Bottlenose dolphin	Rat	Greater Canerat	Highveld gerbil	Highveld molerat	Rabbit	Cat	Ferret	Dog	Tree shrew	Egyptian Rousette	Pygmy Marmoset	Common Marmoset	Squirrel monkey	Macaque monkey	Baboon	Human
Cholinergic																										
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 (PBg)	-	-	?	?	-	?	-	?	?	?	+	?	?	+	?	+	+	?	+	+	?	+	+	+	+	+
PPN (pedunculopontine)	+	+	?	?	+	?	+	?	?	?	+	?	?	+	?	+	+	+	+	+	?	+	+	+	+	+
LDT (laterodorsal tegmental)	+	+	?	?	+	?	+	?	?	?	+	?	?	+	?	+	+	+	+	+	?	+	+	+	+	+
Edinger-Westphal nucleus	-	-	?	?	-	?	-	?	?	?	+	?	?	?	?	+	+	?	?	+	?	+	+	+	+	+

III (oculomotor nucleus)	+	+	?	?	+	?	+	?	?	?	+	?	?	+	?	+	+	?	?	+	?	+	+	+	+	+
IV (trochlear nucleus)	+	+	?	?	+	?	+	?	?	?	+	?	?	+	?	+	+	?	?	+	?	+	+	+	+	+
Vmot (trigeminal)	+	+	?	?	+	?	+	?	?	?	+	?	?	+	?	+	+	?	?	+	?	+	?	+	+	+
VI (abducens nerve nucleus)	+	+	?	?	+	?	+	?	?	?	+	?	?	?	?	+	+	?	?	+	?	+	?	+	+	+
VII dors	+	+	?	?	+	?	+	?	?	?	+	?	?	?	?	+	+	?	?	+	?	+	?	+	+	+
VII vent	+	+	?	?	+	?	+	?	?	?	+	?	?	?	?	+	+	?	?	+	?	+	?	+	+	+
Nucleus Ambiguus	+	+	?	?	+	?	+	?	?	?	+	?	?	?	?	+	+	?	?	+	?	+	?	+	+	+
X (vagus nerve nucleus)	+	+	?	?	+	?	+	?	?	?	+	?	?	?	?	+	+	?	?	+	?	+	?	+	+	+
XII (hypoglossal nucleus)	+	+	?	?	+	?	+	?	?	?	+	?	?	?	?	+	+	?	?	+	?	+	?	+	+	+
spinal cord,ventral horn	+	+	?	?	+	?	+	?	?	?	+	?	?	?	?	+	?	?	?	+	?	+	?	+	+	+
pVII, preganglionic salivatory nucleus	-	-	?	?	?	?	-	?	?	?	+	?	?	?	?	+	+	?	?	+	?	+	?	+	+	+
pIX, pregangionic inferior salivatory nucleus	-	-	?	?	?	?	-	?	?	?	+	?	?	?	?	+	+	?	?	+	?	+	?	+	+	+
medullary tegmental field	+	+	?	?	?	?	-	?	?	?	+	?	?	?	?	+	+	?	?	-	?	-	?	-	-	-

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Catecholaminergic																										
Spinomedullary junction	+	+	?	?	?	?	?	?	?	?	+	+	+	?	?	?	?	?	+	?	?	?	?	?	?	+
medulla	+	+	+	?	?	+	+	+	+	?	+	+	+	?	+	+	?	+	+	+	+	?	+	+	+	+
A2 caudal dorsomedial group	+	+	+	?	?	+	+	+	+	?	+	+	+	?	+	+	?	+	+	+	+	?	+	+	+	+
medulla	+	+	?	?	?	?	+	+	+	?	+	+	+	?	+	+	?	+	+	+	?	?	+	+	+	+
C2 rostral dorsomedial group	+	+	?	?	?	?	+	+	+	?	+	+	+	?	+	+	?	+	+	+	?	?	+	+	?	+
C3	-	-	?	?	?	?	-	-	-	?	+	+	+	?	-	-	?	-	-	-	-	?	-	-	-	-
Area postrema	+	+	?	?	?	?	+	+	+	?	+	+	+	?	+	+	?	+	+	+	+	?	+	+	+	+
A4	-	-	-	?	?	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	?	+	+	+	+
A5	+	+	?	?	?	+	+	+	+	+	+	+	+	?	+	+	+	+	+	+	+	?	+	+	+	+
A6 locus coeruleus diffuse	+	+	+	?	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
A6 locus coeruleus compact	-	-	-	?	?	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	?	+	+	+	+
Subcoerleus compact	+	+	+	?	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
Subcoeruleus diffuse	+	+	+	?	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
A8 retrorubral area	+	+	+	?	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
A9 pars compacta	+	+	+	?	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
A9 medial	+	+	+	?	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+

A9ventral reticulata	+	+	+	?	?	+'/'-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	?	+	+	+	+
A9 lateral or pars lateralis	+	+	+	?	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
(VTA)	+	+	+	?	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
A10c	+	+	+	?	?	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
A10dc	+	+	+	?	?	+	-	+	+	+	+	+	+	+	+	?	+	+	+	+	+	?	+	+	+	+
A10 dorsal	+	+	+	?	?	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
A10 dorso-lateral	-	-	-	?	?	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	?	-	-	-	-
raphe cluster	-	-	-	?	?	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	?	-	-	-	-
periaqueductal gray cluster	-	-	-	?	?	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	?	-	-	-	-
A11 caudal diencephalic	+	+	+	?	?	+	+	+	+	+	+	+	+	?	+	+	?	?	+	+	+	?	+	+	+	+
A12 tuberal cell group	+	+	?	?	?	+	+	+	+	+	+	+	+	?	+	+	?	?	+	+	+	?	+	+	+	+
A13 zona incerta	+	+	?	?	?	+	+	+	+	-	+	+	+	?	+	+	?	?	+	+	+	?	+	+	+	+
A14 rostral periventricular	+	+	?	?	?	+	+	+	+	+	+	+	+	?	+	+	?	?	-	+	+	?	+	+	?	+
A15 dorsal	+	+	?	?	?	-	-	-	-	?	+	+	+	?	+	+	?	?	-	+	?	?	+	+	?	+
A15 ventral	+	+	?	?	?	+	-	+	+	?	+	+	+	?	-	+	?	?	-	+	?	?	+	+	?	+
A16 (olfactory bulb)	+	+	?	?	?	+	+	?	?	-	+	+	+	?	?	+	?	?	?	+	?	?	?	?	?	?

Species	*Ornithorhynchus anatinus*	*Tachyglossus aculeatus*	*Didelphis virginiana*	*Macropus eugenii*	*Suncus murinus*	*Erinaceus europaeus*	*Miniopterus schreibersii*	*Sus scrofa*	*Ovis aries*	*Tursiops truncatus*	*Rattus norvegicus*	*Thryonomys swinderianus*	*Tatera brantsii*	*Cryptomys hottentotus*	*Oryctolagus cuniculus*	*Felis cattus*	*Mustela putorious*	*Canis familiaris*	*Tupaia glis*	*Rousettus aegyptiacus*	*Cebuella pygmaea*	*Callithrix jacchus*	*Saimiri sciuerus*	*Macaca sp.*	*Papio papio*	*Homo sapiens*
Common names	Platypus	Echidna	Opossum	Wallaby	Lab shrew	Hedgehog	Schreiber's long fingered bat	Pig	Sheep	Bottlenose dolphin	Rat	Greater Canerat	Highveld gerbil	Highveld molerat	Rabbit	Cat	Ferret	Dog	Tree shrew	Egyptian Rousette	Pygmy Marmoset	Common Marmoset	Squirrel monkey	Macaque monkey	Baboon	Human
Serotonergic																										
Periventricular organ	+	+	-	-	?	-	-	?	-	?	-	-	-	-	-	-	?	-	?	-	-	?	-	-	?	-
Caudal linear nucleus (CLi)	+	+	+	+	?	+	+	?	+	?	+	+	+	+	+	+	?	+	?	+	+	?	+	+	?	+
Supralemniscal (B9)	+	+	+	+	?	+	+	?	+	?	+	+	+	+	+	+	?	+	?	+	+	?	+	+	?	+
Median raphe nucleus (MnR)	+	+	+	+	?	+	+	?	+	?	+	+	+	+	+	+	?	+	+	+	+	?	+	+	?	+
DR lateral (DRL)	+	+	+	+	?	+	+	?	+	?	+	+	+	+	+	+	?	+	+	+	+	?	+	+	?	+
DR ventral (DRV)	+	+	+	+	?	+	+	?	+	?	+	+	+	+	+	+	?	+	?	+	+	?	+	+	?	+
DR dorsal (DRd)	+	+	+	+	?	+	+	?	+	?	+	+	+	+	+	+	?	+	?	+	+	?	+	+	?	+
DR interfascicular (DRif)	+	+	+	+	?	+	+	?	+	?	+	+	+	+	+	+	?	+	?	+	+	?	+	+	?	+
DR peripheral (DRp)	+	+	+	+	?	+	+	?	+	?	+	+	+	+	+	+	?	+	?	+	+	?	+	+	?	+
DR caudal (B6)	-	-	+	+	?	+	+	?	+	?	+	+	+	+	+	+	?	+	?	+	+	?	+	+	?	+
Raphe magnus (RMg)	+	+	+	+	?	+	+	?	+	?	+	+	+	+	+	+	?	+	?	+	+	?	+	+	?	+
Raphe pallidus (RPa)	+	+	+	+	?	+	+	?	+	?	+	+	+	?	+	+	?	+	?	+	+	?	+	+	?	+
RVL rostral ventrolateral	+	+	+	+	?	+	+	?	+	?	+	+	+	?	+	+	?	+	?	+	+	?	+	+	?	+
CVL caudal ventrolateral	-	-	-	+	?	+	+	?	+	?	+	+	+	?	+	+	?	+	?	+	+	?	+	+	?	+
Raphe obscurus (ROb)	+	+	+	+	?	+	+	?	+	?	+	+	+	?	+	+	?	+	?	+	+	?	+	+	?	+

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