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Nuclear organization of cholinergic, putative catecholaminergic and serotonergic systems in the brains of five microchiropteran species

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ABSTRACT

The current study describes, using immunohistochemical methods, the nuclear organization of the cholinergic, catecholaminergic and serotonergic systems within the brains of five microchiropteran species. For the vast majority of nuclei observed, direct homologies are evident in other mammalian species; however, there were several distinctions in the presence or absence of specific nuclei that provide important clues regarding the use of the brain in the analysis of chiropteran phylogenetic affinities. Within the five species studied, three specific differences (presence of a parabigeminal nucleus, dorsal caudal nucleus of the ventral tegmental area and the absence of the substantia nigra ventral) found in two species from two different families (*Cardioderma cor*; Megadermatidae, and *Coleura afra*; Emballonuridae), illustrates the diversity of microchiropteran phylogeny and the usefulness of brain characters in phylogenetic reconstruction. A number of distinct differences separate the microchiropterans from the megachiropterans, supporting the diphyletic hypothesis of chiropteran phylogenetic origins. These differences phylogenetically align the microchiropterans with the heterogenous grouping of insectivores, in contrast to the alignment of megachiropterans with primates. The consistency of the changes and stasis of neural characters with mammalian phylogeny indicate that the investigation of the microchiropterans as a sister group to one of the five orders of insectivores to be a potentially fruitful area of future research.

1. Introduction

The proposed monophyletic order Chiroptera has been divided into two suborders: megachiroptera and microchiroptera; however, Linnaeus originally grouped the megachiropterans with primates. This classification was largely ignored until the finding that primates and megachiropterans share several advanced visual pathway characteristics, in particular the retinotectal pathway, that are not shared by other mammals (Pettigrew, 1986; Pettigrew et al., 1989, 2008). The "flying primate" hypothesis proposes that the megachiropterans, with the dermopterans, form a sister group to the primates, and is based on several derived neural features that are absent in microchiropterans and other mammals (Pettigrew et al., 1989, 2008; Manger et al., 2001; Maseko and Manger, 2007; Maseko

Abbreviations: III, oculomotor nucleus; Vmot, motor division of trigeminal nucleus; VI, abducens nucleus; VIId, facial nerve nucleus, dorsal division; VIIv, facial nerve nucleus, ventral division; A1, caudal ventrolateral medullary tegmental nucleus; A2, caudal dorsomedial medullary nucleus; A4, dorsal medial division of locus coeruleus; A5, fifth arcuate nucleus; A6c, compact portion of locus coeruleus; A6d, diffuse portion of locus coeruleus; A7d, nucleus subcoeruleus, diffuse portion; A7sc, nucleus subcoeruleus, compact portion; A8, retrorubral nucleus; A9l, substantia nigra, lateral; A9m, substantia nigra, medial; A9pc, substantia nigra, pars compacta; A9v, substantia nigra, ventral or 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; AP, area postrema; B9, supralemniscal serotonergic nucleus; C1, rostral ventrolateral medullary tegmental group; C2, rostral dorsomedial medullary nucleus; ca, cerebral aqueduct; CLi, caudal linear nucleus; CVL, caudal ventrolateral serotonergic group; DRc, dorsal raphe nucleus, caudal division; DRd, dorsal raphe nucleus, dorsal division; DRif, dorsal raphe nucleus, interfascicular division; DRl, dorsal raphe nucleus, lateral division; DRp, dorsal raphe nucleus, peripheral division; DRv, dorsal raphe nucleus, ventral division; EW, Edinger–Westphal nucleus; Fr, fasciculus retroflexus; GC, periaqueductal grey matter; IC, inferior colliculus; IP, interpeduncular nucleus; LDT, laterodorsal tegmental nucleus; MnR, median raphe nucleus; PC, cerebral peduncle; pVII, preganglionic motor neurons of the superior salivatory nucleus or facial nerve; pIX, preganglionic motor neurons of the inferior salivatory nucleus; PBg, parabigeminal nucleus; PPT, pedunculopontine nucleus; Rmc, red nucleus, magnocellular division; RMg, raphe magnus nucleus; ROb, raphe obscurus nucleus; RPa, raphe pallidus nucleus; RVL, rostral ventrolateral serotonergic group; SC, superior colliculus; scp, superior cerebellar peduncle.

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et al., 2007). The logical conclusion of this hypothesis is that the Chiropteran order is actually diphyletic and flight evolved twice in mammals. A summary of the evidence on each side of the debate about whether bats are monophyletic or diphyletic is provided in the accompanying paper (Dell et al., 2010).

Most studies that support the diphyletic origin of bats have concentrated on specific neuroanatomical structures in megachiropterans, with little work to date having been done in microchiropterans. Maseko and Manger (2007) and Maseko et al. (2007) undertook investigations into the nuclear organization of the cholinergic, catecholaminergic and serotonergic systems in the microchiropteran *Miniopterus schreibersii*, and the megachiropteran *Rousettus aegyptiacus*, which defined several further differences between the mega- and microchiropterans lending further support for the diphyletic origin of the Chiroptera. In contrast, studies favouring Chiropteran monophyly are usually based on DNA and molecular findings (e.g. Murphy et al., 2001; Teeling et al., 2005; Van Den Bussche and Hoofer, 2004).

Manger (2005) proposed, based on studies of the nuclear organization of the cholinergic, catecholaminergic and serotonergic systems of a range of mammalian species (see also Maseko et al., 2007; Bhagwandin et al., 2008; Limacher et al., 2008; Gravett et al., 2009; Pieters et al., 2010; Bux et al., 2010), that all species within an order will exhibit the same complement of homologous nuclei of these systems. This proposal infers that if mega- and microchiropterans belonged to the same mammalian order, they should have the same nuclear organization of these systems; however this is not the case as shown by Maseko and Manger (2007) and Maseko et al. (2007).

While these previous studies lend support to the diphyletic origin of the Chiropterans, it should be noted that the microchiropterans in particular are one of the most species-rich suborders of mammals, consisting of over 800 species (Nowack, 1999). Thus, before firm conclusions regarding differences between the two suborders can be made, further species should be investigated. The current study describes the nuclear organization of the cholinergic, catecholaminergic and serotonergic systems in the brains of five previously unstudied microchiropteran species from a range of phylogenetically distant families (distant within the microchiropteran suborder). A speculative hypothesis proposed that the shared midbrain binocular circuitry of primates and megachiropterans represented two independent evolutionary events that were each driven by the selective forces of the "small branch niche" (Martin, 1986). It is important to note that the vast majority of the nuclei of the systems under investigation here do not play any direct role in the neural processes related to flight, vision or echolocation and as such the findings cannot be ignored on the basis of sensory or motor specialisations of the Chiroptera, an argument that has been previously levelled at the studies of Chiropteran neuroanatomy that support the diphyletic hypothesis (Martin, 1986; Allman, 1999).

2. Materials and methods

Three brains of each of the following microchiropteran species were used in this study: *Cardioderma cor* (average body mass = 26 g; average brain mass = 670 mg), *Chaerophon pumilus* (average body mass = 5.4 g; average brain mass = 122 mg), *Coleura afra* (average body mass = 11.5 g; average brain mass = 257 mg), *Hipposideros commersoni* (average body mass = 101.9 g; average brain mass = 750 mg) and *Triaenops persicus* (average body mass = 13.7 g; average brain mass = 271 mg). All animals were captured from wild populations in Kenya and were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee, the Kenya National Museums and the Kenyan Wildlife Services. The animals were euthanazed (Euthanaze, 1 ml/kg, i.p.) and upon cessation of respiration, perfused intracardially with an initial rinse of 0.9% saline solution at 4 °C (1 ml/g body mass) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4 °C (1 ml/g body mass). After removal from the skull, each brain was post-fixed overnight in the paraformaldehyde solution and subsequently stored in an anti-freeze solution at −20 °C. Before sectioning, the brains were allowed to equilibrate in 30% sucrose in 0.1 M PB at 4 °C. Each brain was then frozen in crushed dry ice and sectioned into 50 μm thick serial coronal sections on a freezing microtome. A one in four series of stains was made for Nissl substance, choline-acyltransferase (ChAT), tyrosine hydroxylase (TH) and serotonin (5-HT). Sections for Nissl staining were first mounted on 0.5% gelatine coated slides, cleared in a solution of 1:1 absolute alcohol and chloroform and then stained with 1% cresyl violet.

The sections used for immunohistochemical staining were treated for 30 min in an endogenous peroxidase inhibitor (49.2% methanol:49.2% 0.1 M PB:1.6% of 30% hydrogen peroxide) followed by three 10 min rinses in 0.1 M PB. Sections were then pre-incubated for 2 h, at room temperature, in blocking buffer (3% normal rabbit serum, NRS, for ChAT sections or 3% normal goat serum, NGS, for TH and 5-HT sections, 2% bovine serum albumin, BSA, and 0.25% Triton X-100 in 0.1 M PB). Thereafter sections were incubated in the primary antibody solution in blocking buffer for 48 h at 4 °C under gentle agitation. Anti-cholineacetyltransferase (AB144P, Millipore, raised in goat) at a dilution of 1:3000 was used to reveal cholinergic neurons. Anti-tyrosine hydroxylase (AB151, Millipore, raised in rabbit) at a dilution of 1:7500 revealed the catecholaminergic neurons. Serotonergic neurons were revealed using anti-serotonin (AB938, Millipore, raised in rabbit) at a dilution of 1:10,000. This incubation was followed by three 10 min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (1:750 dilution of biotinylated anti-goat IgG, BA 5000, Vector Labs, for ChAT sections or a 1:750 dilution of biotinylated anti-rabbit IgG, BA 1000, Vector Labs, for TH and 5-HT sections, in a blocking buffer containing 3% NGS/NRS and 2% BSA in 0.1 M PB) for 2 h at room temperature. This was followed by three 10 min rinses in 0.1 M PB, after which sections were incubated for 1 h in AB solution (Vector Labs), followed by three 10 min rinses in 0.1 M PB. Sections were then placed in a solution containing 0.05% diaminobenzidine (DAB) in 0.1 M PB for 5 min, followed by the addition of 3 μl of 30% hydrogen peroxide per 0.5 ml of solution. Chromatic precipitation was visually monitored and verified under a low power stereomicroscope. Staining continued until such time as the background stain was at a level that would allow for accurate architectonic reconstruction without obscuring the immunopositive neurons. Development was arrested by placing sections in 0.1 M PB, followed by two more rinses in this solution.

Sections were then mounted on 0.5% gelatine coated glass slides, dried overnight, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Depex. The controls employed in this experiment included the omission of the primary antibody and the omission of the secondary antibody in selected sections. As a further control for the cholinergic immunohistochemistry, we used choline acetyltransferase (AG220, Millipore) at a dilution of 5 μg/ml in the primary antibody solution (see above) as an inhibition assay. This solution was incubated for 3 h at 4 °C prior to being used on the sections. We also reacted adjacent sections that were not inhibited. In the sections where the primary antibody had been inhibited, no staining was evident. Sections were examined under a low power stereomicroscope and using a camera lucida the architectonic borders of the sections were traced following the Nissl stained sections. Sections containing the immunopositive neurons were then matched to the drawings and the neurons were marked. Select drawings were then scanned and redrawn using the Canvas 8 drawing program. Digital photomicrographs were captured using a Zeiss Axioskop and the Axiovision software. No adjustments of pixels, or manipulation of the captured images were undertaken, except for the adjustment of contrast, brightness, and levels using Adobe Photoshop 7.

All architectonic nomenclature was taken from the atlas of a Microchiropteran brain (Baron et al., 1996), while the nomenclature used to describe the immunohistochemically revealed systems was based on Dahlström and Fuxe (1964), Hökfelt et al. (1984), Törk (1990), Woolf (1991), Smeets and González (2000), Manger et al. (2002a,b,c), Maseko and Manger (2007), Maseko et al. (2007), Moon et al. (2007), Dwarika et al. (2008), Limacher et al. (2008), Bhagwandin et al. (2008), Gravett et al. (2009) and Pieters et al. (2010).

3. Results

The nuclear organization of the cholinergic, catecholaminergic and serotonergic neural systems generally followed that found by Maseko and Manger (2007) in their study of *M. schreibersii*, although there were some notable differences between species, specifically in the cholinergic and catecholaminergic systems, and these are explicitly described. The following description applies generally to all the microchiropteran species studied, except where specifically noted.

3.1. Cholinergic nuclei

The cholinergic system is generally subdivided into the cortical cholinergic interneurons, the striatal region, basal forebrain, diencephalon, pontomesencephalon and the cranial nerve nuclei groups (Woolf, 1991). In the five species examined in the current study, we identified cholineacetyltransferase immunoreactive