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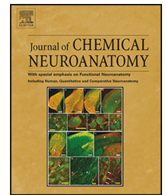

Nuclear organization of cholinergic, catecholaminergic, serotonergic and orexinergic systems in the brain of the Tasmanian devil (*Sarcophilus harrisii*)

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ABSTRACT

This study investigated the nuclear organization of four immunohistochemically identifiable neural systems (cholinergic, catecholaminergic, serotonergic and orexinergic) within the brains of three male Tasmanian devils (*Sarcophilus harrisii*), which had a mean brain mass of 11.6 g. We found that the nuclei generally observed for these systems in other mammalian brains were present in the brain of the Tasmanian devil. Despite this, specific differences in the nuclear organization of the cholinergic, catecholaminergic and serotonergic systems appear to carry a phylogenetic signal. In the cholinergic system, only the dorsal hypothalamic cholinergic nucleus could be observed, while an extra dorsal subdivision of the laterodorsal tegmental nucleus and cholinergic neurons within the gelatinous layer of the caudal spinal trigeminal nucleus were observed. Within the catecholaminergic system the A4 nucleus of the locus coeruleus complex was absent, as was the caudal ventrolateral serotonergic group of the serotonergic system. The organization of the orexinergic system was similar to that seen in many mammals previously studied. Overall, while showing strong similarities to the organization of these

Abbreviations: III, oculomotor nucleus; IV, trochlear nucleus; Vmot, motor division of the trigeminal nerve nucleus; Vs, sensory division of the trigeminal nerve nucleus; VI, abducens nucleus; VIId, dorsal division of facial nerve nucleus; VIIv, ventral division of facial nerve nucleus; X, dorsal motor vagus nucleus; XII, hypoglossal nucleus; 3n, oculomotor nerve; 3V, third ventricle; 4V, fourth ventricle; 7n, facial nerve; 8n, vestibulocochlear nerve; A1, caudal ventrolateral medullary tegmental nucleus; A2, caudal dorsomedial medullary nucleus; A5, fifth arcuate nucleus; A6c, compact portion of locus coeruleus; A7d, nucleus subcoeruleus, diffuse portion; A7sc, nucleus subcoeruleus, compact portion; A8, retrorubral nucleus; A9l, substantia nigra, lateral; A9m, substantia nigra, medial; A9pc, substantia nigra, pars compacta; A9v, substantia nigra, ventral, pars reticulata; A10, ventral tegmental area; A10c, ventral tegmental area, central; A10d, ventral tegmental area, dorsal; A10dc, ventral tegmental area, dorsal caudal; A11, caudal diencephalic group; A12, tuberal cell group; A13, zona incerta cell group; A14, rostral periventricular nucleus; A15d, anterior hypothalamic group, dorsal division; A15v, anterior hypothalamic group, ventral division; A16, catecholaminergic neurons of the olfactory bulb; ac, anterior commissure; Amyg, amygdaloid body; AOB, accessory olfactory bulb; AON, anterior olfactory nucleus; AP, area postrema; arc, arcuate nucleus of the hypothalamus; B9, supralemniscal serotonergic nucleus; C, caudate nucleus; C1, rostral ventrolateral medullary tegmental group; C2, rostral dorsomedial medullary nucleus; ca, cerebral aqueduct; Cb, cerebellum; Chat IN, cholinergic interneurons of the gelatinous layer of the caudal spinal trigeminal nucleus; cic, commissure of the inferior colliculus; Cl, claustrum; CLi, caudal linear nucleus; Cu, cuneate nucleus; DCN, deep cerebellar nuclei; dfu, dorsal funiculus; Diag.B, diagonal band of Broca; DR, dorsal raphe; DRc, dorsal raphe, caudal division; DRd, dorsal raphe, dorsal division; DRif, dorsal raphe, interfascicular division; DRl, dorsal raphe, lateral division; DRp, dorsal raphe, peripheral division; DRv, dorsal raphe, ventral division; DT, dorsal thalamus; ER, entorhinal cortex; EW, Edinger–Westphal nucleus; f, fornix; fr, fasciculus retroflexus; GC, central grey matter; Ge5, gelatinous layer of the caudal spinal trigeminal nucleus; GiCRt, gigantocellular reticular column; GP, globus pallidus; Gr, gracilis nucleus; Hbl, lateral habenular nucleus; Hbm, medial habenular nucleus; Hc, hippocampal commissure; Hip, hippocampus; Hyp, hypothalamus; Hyp.d, dorsal hypothalamic cholinergic nucleus; IC, inferior colliculus; ic, internal capsule; icp, inferior cerebellar peduncle; IGL, intergeniculate leaflet; io, inferior olivary nucleus; IP, interpeduncular nucleus; Is.Call/TOL, islands of calleja/olfactory tubercle; LDT, laterodorsal tegmental nucleus; LDTd, laterodorsal tegmental nucleus, dorsal division; lfp, longitudinal fasciculus of the pons; lfu, lateral funiculus; LGd, dorsal lateral geniculate nucleus; LGv, ventral lateral geniculate nucleus; LOT, lateral olfactory tract; LRt, lateral reticular nucleus; LV, lateral ventricle; Mc, main orexinergic cluster; mcp, middle cerebellar peduncle; mlf, median longitudinal fasciculus; MOB, main olfactory bulb; MnR, medial raphe nucleus; mtf, medullary tegmental field; N.Acc, nucleus accumbens; N.Amb, nucleus ambiguus; N.Bas, nucleus basalis; NEO, neocortex; OT, optic tract; OTc, orexinergic optic tract cluster; OV, olfactory ventricle; P, putamen nucleus; PBg, parabigeminal nucleus; PC, cerebral peduncle; PCRt, parvocellular reticular column; PIR, piriform cortex; PPT, pedunculopontine nucleus; PV, paraventricular thalamic nucleus; py, pyramidal tract; pyx, decussation of the pyramidal tract; pVII, preganglionic motor neurons of the superior salivatory nucleus or facial nerve; pIX, preganglionic motor neurons of the inferior salivatory nucleus; R, thalamic reticular nucleus; Rmc, red nucleus, magnocellular division; Rmg, raphe magnus nucleus; ROb, raphe obscurus nucleus; RPa, raphe pallidus nucleus; RVL, rostral ventrolateral serotonergic group; SC, superior colliculus; scp, superior cerebellar peduncle; Sep.M, medial septal nucleus; SON, supraoptic nucleus; Sp5, spinal trigeminal tract; Sp5c, spinal trigeminal nucleus, caudal part; Stn, subthalamic nucleus; sub, subiculum; vfu, ventral funiculus; vh, ventral horn; VPO, ventral pontine nucleus; zi, zona incerta; Zic, orexinergic zona incerta cluster.

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systems in other mammals, the specific differences observed in the Tasmanian devil reveal either order specific, or class specific, features of these systems. Further studies will reveal the extent of change in the nuclear organization of these systems in marsupials and how these potential changes may affect functionality.

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Introduction

The Tasmanian devil (*Sarcophilus harrisii*), a member of the *Dasyuridae* family, order *Dasyuromorphia*, is a marsupial carnivore currently found only on the Australian island of Tasmania. It is the largest of the dasyurids, reaches a length of 57–65 cm and males can weigh as much as 14 kg, while females weigh around 9 kg (Jones et al., 2008). The Tasmanian devil is a scavenger, feeding upon the carcasses of large mammals such as kangaroos, but it can also catch and eat small possums and pademelons (Ashwell, 2010). The Tasmanian devil is nocturnal, solitary and has a home range of 8 to 20 square kilometres (Ashwell, 2010). Once widespread across Tasmania, the population of wild Tasmanian devils is rapidly declining due to facial tumour disease, a unique and lethal infectious cancer (Keeley et al., 2012).

In the present study we detail the nuclear organization of several specific neural systems (cholinergic, catecholaminergic, serotonergic and orexinergic) within the brain of the Tasmanian devil; systems which have not been previously studied in this species. To date only a few descriptions of the systems examined in the present study are available for the marsupial brain. The catecholaminergic system was described in the North American opossum (*Didelphis virginiana*) (Crutcher and Humbertson, 1978), while the serotonergic system has been described in the North American opossum (Crutcher and Humbertson, 1978; Martin et al., 1985) and the tammar wallaby (*Macropus eugenii*) (Ferguson et al., 1999). The distribution of orexinergic cells has been reported in the Eastern grey kangaroo (*Macropus giganteus*) (Yamamoto et al., 2006). A full characterization of the cholinergic system in the marsupial brain is absent from the literature, but a partial description was provided by Ashwell (2010). While for the most part the nuclear organization of these systems were similar to those reported in placentals, several species-specific differences were observed in prior studies of marsupials. A detailed list of the presence/absence of specific nuclei within these systems for mammals can be found in Dell et al. (2010). The basic similarity of these systems in monotremes, marsupials and placentals indicates that the structure and function of these systems are probably broadly similar in all mammals (Manger et al., 2002a–c; Ashwell, 2010).

The present study provides, for the first time in the same marsupial species, a detailed chemoarchitectural characterization of the cholinergic, catecholaminergic, serotonergic and orexinergic systems in the Tasmanian devil. In addition to the available chemoarchitectural descriptions of the marsupial brain, the data from the current study provide a broader base for comparison within the marsupials and across mammals in general. This may help to further elucidate evolutionary significant differences in the brain between mammalian groups and lead to a better understanding of the phylogenetic and functional signals they carry (Manger, 2005).

Materials and methods

Brains from three male Tasmanian devils (*S. harrisii*) (brain masses of 11.2. 11.4 and 12.2 g, body masses of 9.5, 8.2 and 10.1 kg) were used in this study (Fig. 1). The specimens were obtained from the Copenhagen zoo following the euthanasia of these animals due to veterinary reasons (metastatic squamous cell carcinoma, prolapsed thoracic intervertebral disc, senescence and dilated cardiomyopathy, respectively). No issues concerning the brains of these animals were noted prior to euthanasia, or during specimen preparation and analysis (e.g. Holz and Little, 2005). The harvesting and use of the specimens was approved by the University of the Witwatersrand Animal Ethics Committee (2008/36/1). The animals were anesthetized with sevoflurane delivered by facemask, euthanized (200 mg sodium pentobarbital/kg, i.v.) and, upon cessation of respiration, perfused intracardially with 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), both solutions having a temperature of approximately 4 °C. The brains were removed and post-fixed in 4% paraformaldehyde overnight, equilibrated in 30% sucrose in 0.1 M PB at 4 °C and stored in an antifreeze solution at −20 °C until sectioning. Before sectioning, the tissue was allowed to equilibrate in 30% sucrose in 0.1 M PB at 4 °C. The specimens were frozen in crushed dry ice and sectioned in the frontal plane into 50-μm-thick sections on a sliding microtome.

A one in six series of sections was used for Nissl and myelin staining, and choline-acyltransferase (ChAT), tyrosine hydroxylase (TH), serotonin (5-HT) and orexin-A (hypocretin/OxA) immunohistochemistry. Sections for Nissl staining were first mounted on 0.5% gelatine coated slides, cleared overnight in a solution of 1:1 absolute alcohol and chloroform and then stained with 1% cresyl violet. The series of sections used for myelin staining were refrigerated for two weeks in 5% formalin then mounted on 1% gelatine coated slides and stained with a modified silver stain (Gallyas, 1979).

The sections used for immunohistochemical staining were incubated in a solution containing 1.6% of 30% H_2O_2, 49.2% methanol and 49.2% 0.1 M PB solution, for 30 min to reduce endogenous peroxidase activity, which was followed by three 10 min rinses in 0.1 M PB. To block unspecific binding sites the sections were then pre-incubated for 2 h, at room temperature, in blocking buffer (3% normal goat serum, NGS, for TH, 5HT and OxA; 3% normal rabbit serum, NRS, for ChAT, 2% bovine serum albumin, BSA, and 0.25% Triton-X in 0.1 M PB). Thereafter, the sections were incubated for 48 h at 4 °C in the primary antibody solutions that contained the appropriate diluted primary antibody in blocking buffer under gentle agitation. Goat anti-cholineacetyltransferase antibody (AB144P, Millipore) at a dilution of 1:3000 was used to reveal cholinergic neurons. Rabbit anti-tyrosine hydroxylase antibody (AB151, Millipore) at a dilution of 1:7500 revealed the putative catecholaminergic neurons. Serotonergic neurons were revealed using rabbit anti-serotonin antibody (AB938, Millipore) at a dilution of 1:7500. Orexinergic neurons were revealed using rabbit anti-Orexin-A antibody (AB3704, Millipore) at a dilution of 1:3000. The primary antibody incubation was followed by three 10-min rinses in 0.1 M PB and the sections were then incubated in a secondary antibody solution (1:1000 dilution of biotinylated goat anti-rabbit IgG, BA-1000, Vector Labs, for TH, 5-HT and OxA sections, or a 1:1000 dilution of biotinylated rabbit anti-goat IgG, BA-5000, Vector Labs, for ChAT sections, in a blocking buffer containing 3%NGS/NRS and 2% BSA in 0.1 M PB) for 2 h at room temperature. This was followed by three 10 min rinses in 0.1 M PB, after which sections were incubated for 1 h in an avidin–biotin solution (1:125; 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 3.3 μl of 30% hydrogen peroxide per 1 ml of DAB solution. Chromatic precipitation was visually monitored under a low power stereomicroscope. Staining continued until such time as the background stain was at a level that would allow for accurate architectonic matching to the Nissl and myelin sections without obscuring the immunopositive structures. Development was arrested by placing sections in 0.1 M PB for 10 min, followed by two more rinses in this solution. Sections were then mounted on 0.5% gelatine-coated glass slides, dried overnight, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Depex. To ensure that there was no non-specific staining of the immunohistochemical protocol, we ran tests on sections where we omitted the primary antibody, and sections where we omitted the secondary antibody. In both cases no staining was observed. All sections were examined under low power using a stereomicroscope and the architectonic borders of the sections were traced according to the Nissl and myelin stained sections using a camera lucida. The immunostained sections were then matched to the traced drawings, adjusted slightly for any differential shrinkage of the stained sections and immunopositive neurons were marked. The drawings were then scanned and redrawn using the Canvas 8^{TM} (Deneba) drawing programme. 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.

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