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Cyto- and chemoarchitecture of the amygdala of a monotreme, *Tachyglossus aculeatus* (the short-beaked echidna)

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Abstract

We have examined the cyto- and chemoarchitecture of the temporal and extended amygdala in the brain of a monotreme (the short-beaked echidna *Tachyglossus aculeatus*) using Nissl and myelin staining, enzyme histochemistry for acetylcholine esterase and NADPH diaphorase, immunohistochemistry for calcium binding proteins (parvalbumin, calbindin and calretinin) and tyrosine hydroxylase. While the broad subdivisions of the eutherian temporal amygdala were present in the echidna brain, there were some noticeable differences. No immunoreactivity for parvalbumin or calretinin for somata was found in the temporal amygdala of the echidna. The nucleus of the lateral olfactory tract could not be definitively identified and the medial nucleus of amygdala appeared to be very small in the echidna. Calbindin immunoreactive neurons were most frequently found in the ventrolateral part of the lateral nucleus, intraamygdaloid parts of the bed nucleus of the stria terminalis and the lateral part of the central nucleus. Neurons strongly reactive for NADPH diaphorase with filling of the dendritic tree were found mainly scattered through the cortical, central and lateral subnuclei, while neurons showing only somata reactivity for NADPH diaphorase were concentrated in the basomedial and basolateral subnuclei. Most of the components of the extended amygdala of eutherians could also be identified in the echidna. Volumetric analysis indicated that the temporal amygdala in both the platypus and echidna is small compared to the same structure in both insectivores and primates, with the central and medial components of the temporal amygdala being particularly small.

Keywords: Calbindin; Parvalbumin; NADPH diaphorase; Tyrosine hydroxylase

1. Introduction

The prototherian mammal radiation is thought to have separated from the therian radiation shortly after the origin of mammals in the mid to late Triassic (Griffiths, 1968, 1978; Clemens, 1979; Musser, 2003). This means that monotreme and therian brain evolution may have followed independent paths for over 150 million years, with highly encephalized and gyrencephalic groups appearing among both the monotreme (e.g. the short-beaked echidna, *Tachyglossus aculeatus* belonging to the family Tachyglossidae) and placental lineages (e.g. primates and cetaceans). A comparison of the monotreme brain with that of more familiar eutherian brains provides clues to the organization of the ancestral mammalian brain. Brain regions or nuclei with clear structural similarities in monotremes and eutherians were probably present in the brain of the common ancestor of the two groups. Dissimilarities may indicate features that developed subsequent to the separation of the two lineages. This basis for comparison with ancestral mammalian brains has been established and demonstrated in previous neuroanatomical studies of the monotremes (Krubitzer et al., 1995; Manger et al., 2002a, 2002b, 2002c).

The eutherian amygdala is known to be a heterogeneous structure, which has been shown to be involved in the modulation of neuroendocrine functions, visceral effector mechanisms and complex patterns of integrated behaviours, such as defence, ingestion, aggression, reproduction, memory and learning (De Olmos, 2004). This role is exerted through a vast network of connections with other brain regions.

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While Hines (1929) has commented in passing that the amygdala of the platypus is broadly similar to that in eutherian brains and several subdivisions of the platypus amygdala have been identified and illustrated for the platypus (Butler et al., 2002), there has never been a detailed study of the cyto- and chemoarchitecture of the amygdala of any of the living monotremes using modern enzyme- and immunohistochemical techniques. In the present study, we have used Nissl and myelin staining in conjunction with enzyme histochemistry (acetylcholine esterase—AChE and NADPH diaphorase—NADPH-d) and immunohistochemistry (parvalbumin—Pv, calbindin—Cb, calretinin—Cr, tyrosine hydroxylase—TH) to identify component nuclei of the amygdala of the echidna and to establish homologies between amygdaloid nuclei in the monotreme and eutherian brain. We have also performed volumetric analysis of the amygdala in two monotremes (platypus and echidna) in order to determine whether this structure shows comparable expansion to that seen in highly encephalized and gyrencephalic eutherians like the primates.

2. Materials and methods

2.1. Animals

The original research reported herein was performed under guidelines established by the National Health and Medical Research Council of Australia. The experiments outlined in this study were approved by the Animal Care and Ethics Committee of the University Of New South Wales and the four echidnas used were obtained from the wild under license from the New South Wales National Parks and Wildlife Service. The short-beaked echidnas (*T. aculeatus)* used in this study were obtained from Mudgee and Tumut in western and southern rural New South Wales, respectively, and had been used for a previous study of the cerebral cortex (Hassiotis et al., 2004). The platypus was a juvenile female which had been brought injured to the native animal veterinary clinic at Taronga Zoological Park, Sydney and died during the course of treatment.

Four echidnas were used for the present study. Three of these echidnas were captured live and anaesthetized by an intramuscular injection of pentobarbitone sodium (60 mg/kg) and transcardially perfused with normal saline at 4 °C for 15 min followed by 4% paraformaldehyde in 0.1 M phosphate buffer (4 °C, pH 7.4) for 40 min. One echidna used for cytoarchitecture was a roadkill victim, which was cooled to 4 °C within an hour of death and immersion fixed in 4% paraformaldehyde. The brain of the platypus was removed at postmortem and fixed by immersion for 24 h in the same fixative used for the echidnas.

Coronal sections through the temporal amygdala from a diprotodontid metatherian, the tammar wallaby (*Macropus eugenii*), rat, cat and marmoset (*Callithrix jacchus*) were also available for comparison with the monotreme material and have been illustrated in the present paper. All of these brains were processed in an identical fashion to the monotreme material.

2.2. Tissue processing

All brains were cryoprotected in 25% sucrose and cut using a cryostat into 40 μm or 50 μm thick coronal sections. These sections were arranged into 8 or 10 series with each section within a series separated by a regular interval (i.e. 320 μm, 400 μm or 500 μm depending on the animal). Each series was stained for either Nissl substance (using cresyl violet), myelin (using a modified Heidenhain technique described by Hutchins and Weber, 1983), acetylcholine esterase (AChE, by the technique used by Paxinos and Watson, 1986, as modified from Koelle and Friedenwald, 1949 and Lewis, 1961), NADPH diaphorase (Carrive and Paxinos, 1994) or immunohistochemistry (Pv, Cb, Cr, TH). Immunohistochemistry was performed on free-floating sections in order to maximize penetration of the tissue by reagents. The monoclonal antibodies for Cb (diluted 1/8000) and Pv (diluted 1/64,000) were obtained from *Swiss Antibodies*. The anti-Cb antibody and anti-Pv had both been raised in mouse. The monoclonal antibody against Cb specifically stains the ^{45}Ca-binding spot of calbindin D-28k in tetrapods (Celio, 1990) and does not cross react with Cr or other known calcium-binding proteins. The polyclonal antiserum against Cr (*Swiss Antibodies*) does not cross-react with Cb in a wide range of mammals from mouse to human (Schwaller et al., 1993). The monoclonal antibody against TH (*INCSTAR Corporation*, diluted 1/8000) is effective in a wide range of species because it recognizes an epitope in the mid-portion of the TH molecule where extensive species homology exists. It does not cross-react with mammalian dopamine-β-hydroxylase, phenylethanolamine-*N*-methyltransferase or tryptophan hydroxylase using Western blot methods (*INCSTAR* product information sheet). This antibody has also been used in a previous study of catecholaminergic cells in monotreme brains (Manger et al., 2002b). All primary antibodies were diluted in PBA (0.1 M phosphate buffer, pH 7.4, with 0.1% bovine serum albumin, 2% normal horse serum and 0.2% Triton-X 100). Primary incubation was for 2 days at 4 °C on a rotating table. The biotinylated secondary antibody was applied for 2 h at room temperature, at a dilution of 1/200 in PBA (anti-mouse or anti-rabbit IgG, *Jackson ImmunoResearch Laboratories, Inc*.). To reveal labelling, peroxidase-conjugated extravidin (*Sigma*) was applied with Nickel enhancement. Extravidin was diluted 1/500 and applied for 2.5 h at room temperature. Finally, sections were reacted with 0.5 mg/ml 3, 3′ diaminobenzidine (DAB) in 0.04% nickel ammonium sulphate, with 1 μl of glucose oxidase per ml of final solution. Negative controls for both enzyme and immunohistochemistry were performed by omitting the substrate in enzyme histochemical sections and omitting the primary antibody in immunohistochemical reactions.