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Research report

# Architectonic subdivisions of the amygdalar complex of a primitive marsupial (*Didelphis aurita*)

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**Abstract**

The architecture of the amygdaloid complex of a marsupial, the opossum *Didelphis aurita*, was analyzed using classical stains like Nissl staining and myelin (Gallyas) staining, and enzyme histochemistry for acetylcholinesterase and NADPH-diaphorase. Most of the subdivisions of the amygdaloid complex described in eutherian mammals were identified in the opossum brain. NADPH-diaphorase revealed reactivity in the neuropil of nearly all amygdaloid subdivisions with different intensities, allowing the identification of the medial and lateral subdivisions of the cortical posterior nucleus and the lateral subdivision of the lateral nucleus. The lateral, central, basolateral and basomedial nuclei exhibited acetylcholinesterase positivity, which provided a useful chemoarchitectural criterion for the identification of the anterior basolateral nucleus. Myelin stain allowed the identification of the medial subdivision of the lateral nucleus, and resulted in intense staining of the medial subdivisions of the central nucleus. The medial, posterior, and cortical nuclei, as well as the amygdalopiriform area did not exhibit positivity for myelin staining. On the basis of cyto- and chemoarchitectural criteria, the present study highlights that the opossum amygdaloid complex shares similarities with that of other species, thus supporting the idea that the organization of the amygdala is part of a basic plan conserved through mammalian evolution.


*Keywords:* Comparative neuroanatomy; Amygdala; Opossum; Cytoarchitecture; Histochemistry; Evolution

## 1. Introduction

The eutherian amygdala is known to be a heterogeneous structure, composed by different subdivisions, and involved in generation of appropriate affective behavior in response to sensory stimuli and events in the external world [1,2,20]. The amygdala receives cortical [26] and subcortical [11,20,23,30] afferents from all the sensory systems and projects to different cerebral regions responsible for somatic and autonomic functions [13,21]. The amygdala modulates complex patterns of behaviors, such as defense, ingestion, aggression, reproduction, memory and learning [9,22]. Among the affective behaviors, fear response is the best studied. The amygdala is one of the main neural regions in which injuries result in a reduction of the fear response to a predator [6]. Accordingly, when rats are exposed to their natural predator (the cat), there is an increase in the expression of Fos in the amygdala [7]. In addition, the amygdala is a key structure in Pavlovian conditioning [22], being involved in the so-called emotional memory formation.

In the opossum, Oswaldo-Cruz and Rocha-Miranda [28] provided the first description of the amygdaloid complex in the stereotaxic atlas of this species. This work consisted of an extensive architectonic study on the main nuclear masses of telencephalon, diencephalon, and brain stem. However, the atlas of the opossum brain did not focus on the amygdala, and lateral telencephalic structures, such as the lateral amygdaloid nuclei, were partially cut out from the enlarged micrographs in order to optimize the visualization of diencephalic structures. In addition, amygdaloid subdivisions depicted in this atlas [28] were based mainly on Nissl-stained sections.

More recent anatomical studies of amygdaloid organization in other species took into account other cytoarchitectonic markers such as myelin staining and histochemical activity for acetylcholinesterase (AChE) [3,4] and NADPH-diaphorase

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(NADPH-d) [27]. Current cytoarchitectonic studies use AChE as a useful tool for delineating a number of regions in the central nervous system (e.g. [33]). The histochemical method for NADPH-d reveals the activity of nitric oxide synthase (NOS) in fixed tissue [25]. NADPH-d reactivity can be found in the reactive neuropil, which, by means of standard optical microscopy, cannot be assigned to any particular cellular profile. Also, Na + K + ATPase and cytochrome oxidase usually co-localize with the NADPH-diaphorase reactive neuropil, revealing brain regions that are presumably more metabolically active [15,36]. In addition to the neuropil label, NADPH-d reveals reactive neurons that, in the cerebral cortex, were futher subdivided in Types I and II cells, based on their staining intensity [14,15,24,37]. Type I neurons are strongly labelled cells, with a Golgi-like appearence. Type II neurons have smaller weakly labeled cell bodies and almost no labeled dendrites. Similar to the neocortex, in the amygdala, staining intensity of different NADPH-d neuronal subpopulations varies, and at least two different neuronal populations can be depicted [27].

Using Nissl and myelin staining along with NADPH-d and AChE neuropil reactivity, in our study, we provide a complete description of the opossum amygdaloid complex, including subdivisions that have not been previously considered in the opossum atlas [28], but have been described in other species.

## 2. Materials and methods

This study followed the guidelines of the "Principles of laboratory animal care" (NIH publication No 86–23, revised 1985). In addition, all procedures were approved by the Commission of Animal Care of the Institute of Biophysics Carlos Chagas Filho (IBCCF) from the Federal University of Rio de Janeiro, Brazil. Animals were obtained from the IBCCF animal facility, under the license of the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA, n° 244534).

### 2.1. Animals and perfusion

Nine adult opossums, each weighing from 1.0 to 1.9 kg, were deeply anesthetized with sodium pentobarbital (90 mg/ml per kg i.p.; Knoll, RJ, Brazil). After confirmation of absence of reflexes, a thoracotomy was performed. Heparin was injected into the left ventricle to avoid intravascular coagulation. Perfusion was performed through the aorta with 100 ml of 0.9% saline followed by 4% paraformaldehyde (200–300 ml) diluted in phosphate buffer (PB; 0.1 M pH 7.4). Finally, solutions of 10% and 30% sucrose in PB were used for cryoprotection.

### 2.2. Histological procedures

Brains were subsequently removed from the skull and stored in 30% sucrose-PB. On the next day, the brains were frozen in dry ice. Coronal sections were cut at 50 μm or 60 μm thickness with a cryostat and collected in PB. In each specimen, different series of alternate sections were processed for NADPH-diaphorase (NADPH-d) or acetylcholinesterase (AChE) histochemistry, as well as myelin and Nissl staining, respectively. The section interval in each series ranged from 120 μm to 200 μm, depending on the number of series and the thickness applied to a given hemisphere.

#### 2.2.1. AChE histochemistry

AChE histochemistry followed the protocol described by Gardino et al. [17]. Briefly, the sections were washed three times in acetate buffer, pH 6.0, for 5 min each. They were then incubated overnight at 4 °C in a solution containing 0.5 mg/ml acetylthiocholine iodide (Sigma, St. Louis, MO, USA), 25 mM sodium citrate, 300 μM copper sulfate, and 250 μM potassium ferricianate in 0.1 M of acetate buffer, pH 6.0. After three 5 min-washes, the reaction was developed with 1% ammonium sulfate and placed in a solution of 0.1 M sodium nitrate for 5 min.

#### 2.2.2. NADPH-d histochemistry

We used the "indirect" protocol for NADPH-d histochemistry [14,31,32,35]. Initially, the sections were washed in 0.1 M Tris buffer three times for 5 min each. Later, they were incubated in a solution containing, 0.1% β-NADP (Sigma, St. Louis, MO, USA), 0.03% nitroblue tetrazolium (Sigma), 0.6% malic acid, 1% dimethylsulfoxide, 0.02% manganese chloride, and 1% triton X-100 in 0.1 M Tris–HCl, pH 8.2. After 4–6 h incubation in a light-protected environment at 37 °C, the sections were washed three times with 0.1 M Tris buffer for 5 min each. Finally, the sections were dehydrated, and mounted with Entellan from Merk (BDH, Temecula, CA).

#### 2.2.3. Myelin stain (Gallyas)

A third series of sections was fixed for 1 month in formalin before staining for myelin according to the procedure of Gallyas [16]. These sections were incubated in a mixture of pyridine and acetic acid (2:1) for 30 min, and then rinsed three times in 0.5% acetic acid, 3 min each. This was followed by incubation in a solution of 0.1% ammoniacal silver nitrate, and three rinses in 0.5% acetic acid (3 min each). The sections were then stained in a "developing" solution containing 2.5% sodium carbonate, 0.1% ammonium nitrate, 0.1% silver nitrate, 0.5% tungstosilicic acid and 0.25% formalin, and rinsed several times in deionized water. Contrast was optimized with a 0.5% potassium ferricyanide "bleach" solution. After a new rinse in deionized water, the sections were again immersed in the developing solution followed by new rinses in water and a new bath in "bleach" solution. Finally, the sections were rinsed again, fixed in 0.8% sodium thiosulfate for 5 min, and mounted in a warmed gelatin-alcohol solution onto gelatin-subbed slides.

### 2.3. Data analysis

Section outlines of amygdaloid nuclei were drawn with the aid of a camera lucida coupled to an Axioplan-Zeiss microscope. A Zeiss Axiocam coupled to a Zeiss microscope captured the digital images with 1.6×, 6.3×, 10×, and 20× objectives. Images were adjusted for brightness and contrast using Adobe

Table 1
Classification of neuropil labeling intensity: +++ intense, ++ moderate, + light, − absent as based on densitometric evaluation (see text)

| Nuclei | AChE | Myelin | NADPH-d |
|---|---|---|---|
| LAv | + (0.50 ± 0.05) | − (0.20 ± 0.10) | ++ (0.72 ± 0.10) |
| LAm | + (0.30 ± 0.04) | +++ (0.77 ± 0.10) | ++ (0.56 ± 0.13) |
| LAl | + (0.40 ± 0.04) | − (0.08 ± 0.06) | +++ (0.87 ± 0.10) |
| MEA | − (0.2 ± 0.03) | − (0.08 ± 0.04) | +++ (1.0 ± 0.06) |
| CEAl | ++ (0.70 ± 0.18) | + (0.45 ± 0.01) | +++ (0.79 ± 0.11) |
| CEAm | + (0.4 ± 0.12) | +++ (0.90 ± 0.06) | ++(0.70 ± 0.08) |
| BLAa | +++ (0.90 ± 0.07) | ++ (0.60 ± 0.10) | ++ (0.74 ± 0.09) |
| BLAp | ++ (0.60 ± 0.08) | ++ (0.72 ± 0.10) | ++ (0.74 ± 0.11) |
| BMAa | + (0.41 ± 0.04) | ++ (0.76 ± 0.10) | + (0.34 ± 0.06) |
| BMAp | − (0.20 ± 0.10) | − (0.22 ± 0.16) | + (0.42 ± 0.10) |
| COAa | − (0.19 ± 0.01) | + (0.40 ± 0.13) | + (0.40 ± 0.07) |
| COApm | − (0.16 ± 0.04) | − (0.12 ± 0.03) | +++ (0.80 ± 0.10) |
| COApl | − (0.20 ± 0.04) | − (0.2 ± 0.04) | ++ (0.60 ± 0.10) |
| AAA | − (0.20 ± 0.01) | ++ (0.7 ± 0.10) | ++ (0.72 ± 0.05) |
| PA | − (0.21 ± 0.05) | − (0.2 ± 0.08) | +++ (0.80 ± 0.07) |
| PAA | − (0.10 ± 0.06) | − (0.13 ± 0.08) | + (0.37 ± 0.06) |
| NLOT | − (0.20 ± 0.01) | − (0.23 ± 0.10) | ++ (0.58 ± 0.10) |
| IA | − (0.20 ± 0.03) | ++ (0.65 ± 0.10) | +++ (0.84 ± 0.10) |

The mean pixel gray intensity and standard deviations of each nucleus are shown between parentheses.