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IMMUNOHISTOLOGICAL CHARACTERIZATION OF STRIATAL AND AMYGDALAR STRUCTURES IN THE TELENCEPHALON OF THE FIRE-BELLIED TOAD *BOMBINA ORIENTALIS*

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Abstract—The subpallium of the fire-bellied toad *Bombina orientalis* was studied by means of enzyme-histological detection of NADPH-diaphorase and immunohistological demonstration of aspartate, GABA, calretinin, choline-acetyl transferase, Leu- and Met-enkephalin, neuropeptide Y, 5-hydroxy-tryptamine (serotonin), somatostatin, substance P and tyrosine-hydroxylase. As in other vertebrates, the striato-pallidum is characterized by GABA-, substance P- and enkephalin-immunoreactivity. Neurons and fibers differing in immunoreactivity are arranged in layers. Choline-acetyl transferase-immunoreactive neurons were found in a position corresponding to the mammalian cholinergic cell-group (Ch4-group), which therefore may be homologous to the nucleus basalis of Meynert. Within the amygdaloid complex, the cortical and lateral (vomeronasal) nuclei are similar in calretinin-, GABA-, NADPH-diaphorase-, enkephalin, substance P- and neuropeptide Y-(immuno)histology. The medial and central amygdaloid nuclei reveal a dense peptidergic innervation, and the medial amygdala additionally exhibits serotonergic fibers and cell bodies staining for neuropeptides and tyrosine-hydroxylase. Differences between *Bombina* and other anuran species exist, such as the absence of cholinergic neurons in the striatum. Our findings corroborate the view based on recent studies on the hodology and cytoarchitecture of the anuran telencephalon that the anuran ventral telencephalon contains most of the structures found in the mammalian brain. This concerns a septal region, a dorsal and ventral striato-pallidum including a nucleus accumbens and an amygdaloid complex consisting of a central, cortical and vomeronasal amygdala. The only major difference appears to concern the lack of a basolateral amygdala. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: amphibians, limbic system, amygdala, striatum, pallidum, immunohistological staining.

In recent studies, efforts were undertaken to elucidate the structural organization of the amphibian subpallium. In the classical studies by Northcutt and colleagues (Northcutt and Kicliter, 1980; Wilczynski and Northcutt, 1983a,b) on the organization of the amphibian telencephalon, the existence of structures essential for emotional learning and affective-emotional control of behavior, i.e. of a central and (baso)lateral amygdala as well as a bed nucleus of the stria terminalis (BNST), was not discussed. Neither a dorsal pallidum, which is critically involved in selection and preparation of "voluntary" actions (Alexander and Crutcher, 1990; Graybiel et al., 1994) was mentioned, nor a ventral pallidum, which plays an essential role in motivational states (Everitt et al., 1999; Cardinal et al., 2002), including the control of feeding behavior (Stratford et al., 1999; Reynolds and Berridge, 2001; Da Silva et al., 2003). On the basis of histological and immunohistological data in the frog *Rana perezi*, Marín and co-workers (1998) gave a new interpretation of the amphibian subpallium. They defined an anterior amygdala in the striato-pallial transition area (SPTA), a dorsal and ventral pallidum situated in the caudal ventromedial subpallium roughly corresponding to the former medial amygdala, and a BNST starting rostrally in the former ventral lateral septum and extending caudally into the commissural part of the medial amygdala in the sense of Northcutt and Kicliter (1980). Adopting the view of Bruce and Neary (1995) they placed the central amygdala in the caudal striatum sensu Northcutt and Kicliter and defined a (baso)lateral amygdala corresponding to the former ventral lateral pallium. In a subsequent study by Moreno and González (2003), the "lateral" amygdala sensu Northcutt and Kicliter was now called "medial amygdala" and considered homologous to the mammalian medial amygdala despite its lateral position in the amphibian telencephalon, fitting it to the mammalian situation by nomenclature.

The location of the anuran central amygdala in the caudal striatum is problematic, because there is hodological and immunocytological evidence for a rostral striatum proper and a dorsal pallidum in the caudal ventrolateral subpallium (Wilczynski and Northcutt, 1983b; Medina and Reiner, 1995; Reiner et al., 1998; Endepols et al., 2004). It is similarly unclear, whether a BNST as defined by Marín et al. (1998) exists. In mammals, the divisions of the BNST closely resemble the central and medial amygdala, respectively, both in terms of immunohistochemistry and connectivity, while in amphibians, hodological data are missing.

Recently, gene-expression data gave impressive evidence for a very similar development of telencephalic regions in different groups of vertebrates including amphibians (Smith Fernandez et al., 1998; Puelles et al., 2000; González et al., 2002; Brox et al., 2003, 2004; Moreno et

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Abbreviations: ABC, avidin–biotin–horseradish peroxidase complex; BNST, bed nucleus of the stria terminalis; ChAT, choline-acetyl transferase; -ir, immunoreactive; NPY, neuropeptide; PAP, peroxidase–antiperoxidase; PBS, phosphate-buffered saline; SP, substance P; SPTA, striato-pallial transition area; TBS, Tris-buffered saline; TH, tyrosine-hydroxylase; 5-HT, serotonin, 5-hydroxy-tryptamine.

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al., 2004). At early embryonic stages, genetic markers outline pallial and subpallial boundaries as well as medial and lateral subpallial areas (i.e. the medial and lateral ganglionic eminence). As a consequence of cell migration (not well studied in amphibians, however) and progressive change of gene expression patterns, specific boundaries become indistinct or disappear and, therefore, do not fully answer the question about the origin and functional anatomy of neuronal structures in adult animals. Embryological fate-mapping studies and further morphological, cytological, and functional investigations are needed.

A recent study by Roth et al. (2004) tried to contribute to the clarification of the organization of the amphibian subpallium by studying the morphology and projection pattern of telencephalic neurons in the fire-bellied toad *Bombina orientalis* by means of anterograde and retrograde biocytin labeling combined with intracellular biocytin labeling under *in vitro* conditions. The present study adds to these hodological data the (immuno)-histology of the telencephalon in *Bombina orientalis*. The fire-bellied toad was chosen for this study, because this animal is well suited for various behavioral studies including fear-conditioning and context learning. *Bombina* easily adapts to handling and is reliably motivated in experimental situations (A. Heidorn, S. Mühlenbrock-Lenter and G. Roth, unpublished observations). The knowledge of the structural organization of the telencephalon in *Bombina*, enables us to locate the activation of telencephalic limbic structures during emotional learning, for example in combining fear condition experiments with immunohistological demonstration of immediate early genes.

EXPERIMENTAL PROCEDURES

For the experiments, 60 specimens of the fire-bellied toad *Bombina orientalis*, 23 females, 23 males and 14 with undetermined sex, were used. The animals were taken from breeding colonies at our institutes in Cologne (Institute of Zoology, Germany) and Bremen (Brain Research Institute, Germany). Animals were deeply anesthetized in 0.5% or 2% tricaine methanesulfonate (Sigma, St. Louis, MO, USA), perfused transcardially with 40 ml modified ice-cold oxygenated Ringer's solution consisting of Na^+ 100 mM, K^+ 2 mM, Mg^{2+} 0.5 mM, Ca^{2+} 2 mM, Cl^- 82 mM, HCO_3^- 25 mM, glucose 11 mM, buffered to a final pH of 7.3 through continuous perfusion of 95% O_2+5 % CO_2 (Straka and Dieringer, 1993). Alternatively, calcium was substituted by magnesium, and in some GABA-immunohistological preparations, 75 mM NaCl was substituted by an equivalent amount of sucrose in order to block neuronal activity and transmission. Fixation of the brain tissue was achieved by transcardial perfusion or (after opening the brain case) by immersion using a cold phosphate-buffered fixative containing 4% paraformaldehyde or a mixture of paraformaldehyde and glutaraldehyde (for details see Table 1). Brains were removed from the skull by a ventral approach and postfixated for 2–16 h. For cryoprotection, brains were equilibrated in increasing 10, 20 and 30% sucrose-phosphate buffer. Two series of transverse sections were cut which were processed for two different (immuno-) histological procedures. In order to allow complete penetration of antibodies, a thickness of 20 μm was chosen using a cryostat (Leica Microsystems or Reichert-Jung, Nussloch, Germany) and the smallest thickness that was possible to cut (25 μm) using a vibratome (Leica Microsystems). Every tenth section was collected for control experiments. Sections were stained for aspartate, GABA, calretinin, choline-acetyl transferase (ChAT), Leu-and Met-enkephalin, NADPH-diaphorase, neuropeptide Y (NPY), 5-hydroxy-tryptamine (serotonin, 5-HT), somatostatin, substance P (SP) and tyrosine-hydroxylase (TH). All antibodies were obtained commercially, some were affinity-purified (anti-ChAT, anti-GABA) and tested for specificity and cross-reaction (for details see Table 1).

For immuno-histochemical procedures, sections were rinsed in 0.1 M phosphate-buffered saline (PBS) and, in the case of 5-HT preparations, pretreated with 0.1 M $NaBH_4$ for 10 min. During incubation periods sections were placed in gently agitated humid chambers. Unspecific binding sites were saturated in 1.5% normal serum of the animal in which the secondary antibody was raised in (for detail see Table 1). In the case of TH-procedure, sections were saturated in 3% bovine serum albumin (BSA, Sigma) for 1 h in addition. The primary and secondary antibodies were diluted in PBS containing 0.5% of the normal serum and 0.1–0.5% of Triton X-100 or DMSO. The primary antibody was incubated for 14–72 h at 4 °C or at room temperature overnight. Sections were rinsed three times for 10 min in buffer and the secondary antibody was allowed to bind for 60–90 min at room temperature. After rinsing, the binding sites were visualized by means of an avidin–biotin–horseradish peroxidase complex (ABC-Vector Kit, Vector Laboratories, Burlingame, CA, USA) using diaminobenzidine (DAB; Sigma) as chromogen with heavy-metal intensification (Adams, 1981). Alternatively, the peroxidase–antiperoxidase reaction (PAP, Dako, Glostrup, Denmark) was used. Sections were rinsed in buffer, dehydrated in ethanol, cleared in xylene and mounted in Corbit (Hecht, Kiel-Hassee, Germany) or Eukitt (Kindler, Freiburg, Germany). In control experiments, either the primary or the secondary antibody was omitted.

For NADPHd histology, sections were rinsed in 0.05 M Tris-buffered saline (TBS, pH 8.0) and incubated in a TBS-buffered solution containing 0.3 mM nitroblue tetrazolium diluted in 1 ml DMSO, 30 mM L-malic acid, 0.8% Triton X-100 and 1.2 mM β-NADPH for 45–60 min at 37 °C. Sections were rinsed and mounted in glycerol-gelatin. For control-experiments, the substrate NADPH was omitted.

Analysis of immunohistological staining

For the reconstruction of the anatomy of the telencephalon of *Bombina orientalis*, 15 μm thick transverse paraffin sections were made and counterstained with Klüver-Barrera (n=1). Eight sections from different telencephalic levels (see Figs. 1, 3, 6) were reconstructed and served as a basis for the analysis of immunohistochemical staining. For analysis, 77 series of sections were selected and labeled neurons and fibers were reconstructed by hand with the aid of a camera lucida. An overview of the immunohistological experiments gives Table 2. Reconstructions of sections showing maximum staining results were transferred into a chart of eight levels of the reconstructed brain (L1–L8, Figs. 1, 3, 6). Levels of the rostral telencephalon were chosen at a relative wide distance of 300–360 μm because nuclei in this region are elongated. Because nuclei at the level of the lamina terminalis are smaller we added additional levels. For preparing a chart, a single immunoreactive neuron was labeled using a small circle and for clarity, in the case of high densities of stained neurons, neuron number was estimated and every 10 neurons were charted using a large circle. The relative density of labeled fibers was estimated and coded using four different gray scales. Sections were scanned with digital cameras (AxioCam HR, Zeiss, and Coolpix 4500, Nikon) at a resolution of 3900×3090 pixels and 2272×1520 pixels, respectively.

RESULTS

Nomenclature of anatomical structures

In the following, the description of the anuran telencephalon is based on the nomenclature used by Endepols et al.