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Galanin: Presence and distribution in the brain and pituitary of *Rhinella arenarum* (Amphibia: Anura) during development

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Abstract

The immunohistochemical distribution of galanin (Gal) in the brain and pituitary of *Rhinella arenarum* was studied during development. Gal-immunoreactivity was first observed in the brain just after hatching in anterior preoptic area, infundibular area, median eminence and pars distalis of the pituitary as well as in the olfactory epithelium. At the beginning of prometamorphosis new Gal-immunoreactive (ir) cells were observed in the olfactory nerve and bulb. Later in prometamorphosis new Gal-ir cells were observed in the telencephalon, suprachiasmatic nucleus, rostral rhombencephalon and in the pars nervosa of the pituitary. The most numerous accumulations of Gal-ir neurons throughout the larval development were observed in the ventral hypothalamus where numerous Gal-ir cells of cerebrospinal fluid-contacting type were found. During metamorphic climax and soon after we did not detect Gal-ir neurons in the pallium, medial or pretecal dorsal thalamus.

In the median eminence and pars distalis of the pituitary many Gal-ir fibers were found during development indicating that Gal may play a role in the modulation of hypophyseal secretion. Furthermore, the distribution of Gal-ir elements observed throughout larvae development indicates that galaninergic system maturation continues until sexual maturity.

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Keywords: *Bufo arenarum*; Neuropeptide; Development; Nervous system; Pituitary

1. Introduction

Galanin (Gal) is a 29-amino acid peptide, which is present in the central nervous system of vertebrates. The primary structure of this peptide is highly conserved among vertebrates (almost 90%), indicating the importance of the molecule (Wang and Conlon, 1994).

This peptide has multiple biological effects and many studies have demonstrated its involvement in several hypothalamic and hypophyseal functions. Galanin exerts strong neuroendocrine effects by modulating the release of

gonadotrophins, prolactin, growth hormone and somatostatin (Vrontakis, 2002). These regulatory actions of Gal are further supported by many reports that show galaninergic innervation of hypophyseal secretory cells in several vertebrate groups including mammals (Moons et al., 1989; Maiter et al., 1990; Olivereau and Olivereau, 1991, 1992; Józsa and Mess, 1993; Jiménez et al., 1994; Liu and Gao, 1998; Liu, 2002). Moreover, the expression of galanin is elevated following estrogen administration, neuronal activation, denervation and/or nerve injury, as well as during development (Vrontakis, 2002).

The use of immunohistochemical methods has revealed a wide distribution of galanin in the brain of several vertebrate groups (mammals: Pérez et al., 2001; Jacobowitz et al., 2004; birds: Józsa and Mess, 1993; Azumaya and Tsutsui, 1996; reptiles: Jiménez et al., 1994; amphibians: Lázár et al., 1991; Olivereau and Olivereau, 1992; González-Nicolini et al., 1995; fish: Batten et al., 1990; Olivereau and

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Olivereau, 1991; Jadhao and Meyer, 2000; Rodriguez et al., 2003; Adrio et al., 2005). These investigations have revealed a conserved pattern of distribution of galaninergic structures in preoptic–hypothalamic regions.

Furthermore, the developmental origin of Gal-immunoreactive (Gal-ir) neurons from the olfactory epithelium has been described in mice, where the Gal-ir neurons were demonstrated to migrate to the CNS along the olfactory/vomer nasal nerve (Key and Wray, 2000).

To our knowledge nothing is known about the development of the galaninergic system during metamorphic process in amphibians. Therefore here, we study in detail the development of the galanin system throughout the entire brain and the pituitary of the larva of the anuran *Rhinella arenarum* by means of immunohistochemistry.

2. Materials and methods

2.1. Animals

Rhinella arenarum tadpoles were obtained by *in vitro* fertilization according to Paz et al. (1995). Amphibian systematics is in a state of flux due to a series of large scale taxonomic changes recently proposed by several papers. Because of this reason many species formerly included in the genus *Bufo* have been recently accommodated in other genera. The new taxonomy is still changing as new data are gathered, and so many taxonomic changes are still expected in the near future (Frost, 2007). Be aware that the species that now is called *Rhinella arenarum* has recently been called *Chaunus arenarum*, and previously *Bufo arenarum*, the name that has been used in nearly most publications.

The tadpoles were held in tanks containing dechlorinated tap water, exposed to 12–12 h light–darkness cycles and constant temperature ($22 \pm 1^\circ\text{C}$). At least three individuals, in each stage of development after hatching and metamorphosis were used. The developmental stages were classified according to Gosner (1960).

All the procedures were in accordance with the principles of laboratory animal care of the Institutional Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, UBA Res CD: 140/00, and the principles of the NIH (publication 8523, revised 1985). The tadpoles were anesthetized by immersion in 0.1% MS222 (tricaine methanesulfonate, Sigma St. Louis, MI) and fixed in Bouin's solution for 24 h at 4°C . Then, they were dehydrated and embedded in Histo-plast (Biopack, Buenos Aires, Argentina). Serial transversal and sagittal sections were cut at $7\ \mu\text{m}$ and mounted on gelatin-coated glass slides.

2.1.1. Immunohistochemical procedures

Tissue sections were deparaffined, rehydrated and washed in phosphate-buffered saline. Sections were treated with 5% hydrogen peroxide (H_2O_2) solution to quench endogenous peroxidase activity. Non-specific binding sites were blocked by treating tissues with TNB blocking reagent (Cat. FP1020, NEN Life Science Products, Boston, MA) and subsequently incubated 24 h at 4°C with the primary antiserum. The antiserum used was rabbit anti-Galanin (human), (dilution 1:1000) (Peninsula, Belmont, CA). Then, sections were treated with the biotinylated Anti-Rabbit antibody (dilution 1:1000) (Vector Laboratories, Burlingame, CA) followed by avidin–horseradish peroxidase–biotin complex (Vectastain ABC kit, Vector Laboratories). The color reaction was visualized by exposure to 3,3'-diaminobenzidine

Table 1

Time table of appearance of Galanin immunoreactive cells groups (black lines) and fibers (gray lines) in the central nervous system of *Rhinella arenarum*

	PREMETAMORPHOSIS	PROMETAMORPHOSIS					CLIMAX
	26 - 30	32	34	36	38	41	42 - 46
OE							
ON							
OB							
DP							
MS							
Am							
PoA							
NPO							
Sc							
DH							
VH							
Avl							
Avm							
ME							
PD							
R							

OE: Olfactory epithelium; ON: olfactory nerve; OB: olfactory bulb; DP: dorsal pallium; MS: medial septum; Am: medial amygdala; PoA: anterior preoptic area; NPO: preoptic nucleus; Sc: suprachiasmatic nucleus; DH: dorsal hypothalamic nucleus; VH: ventral hypothalamus; Avl: ventrolateral thalamic area; Avm: ventromedial thalamic area; ME: median eminence; PD: pars distalis; R, rostral rhombencephalon.

tetrahydrochloride staining kit (Dako Cytomation, Carpinteria, CA). The slides were mounted in Permount (Fisher Scientific, Pittsburgh, PA). Specificity of the immunostaining was determined by omission of the primary antiserum and by preadsorption of the primary antiserum with $1 \mu\text{g}/\mu\text{l}$ of the synthetic antigen (Gal; Sigma). The control sections produced negligible background staining. The images were captured by a Reichert Polyvar microscope.

2.2. Double immunofluorescence

Sections were incubated with mouse anti- β -tubulin (E7, 1/1000) (Developmental Studies Hybridoma Bank, Univer-

sity of Iowa), an antibody that has been probed to be specific for *R. arenarum* olfactory neurons (Pozzi et al., 2006), followed by anti-mouse FITC, (dilution 1:40) (Vector Labs.) and subsequently with rabbit anti-Galanin (dilution 1:1000), followed by a goat anti-rabbit-rodamine, (dilution 1:40) (Chemicon Int., Temecula, CA). Sections were coverslipped with Vectashield (Vector Labs.).

Images of the sections were captured by a confocal laser microscope (Olympus FV-30 attached to a microscope Olympus Bx-61).

The terminology used for the different brain areas was adopted according to Scalia (1976), Lannoo (1999) and López et al. (2002).

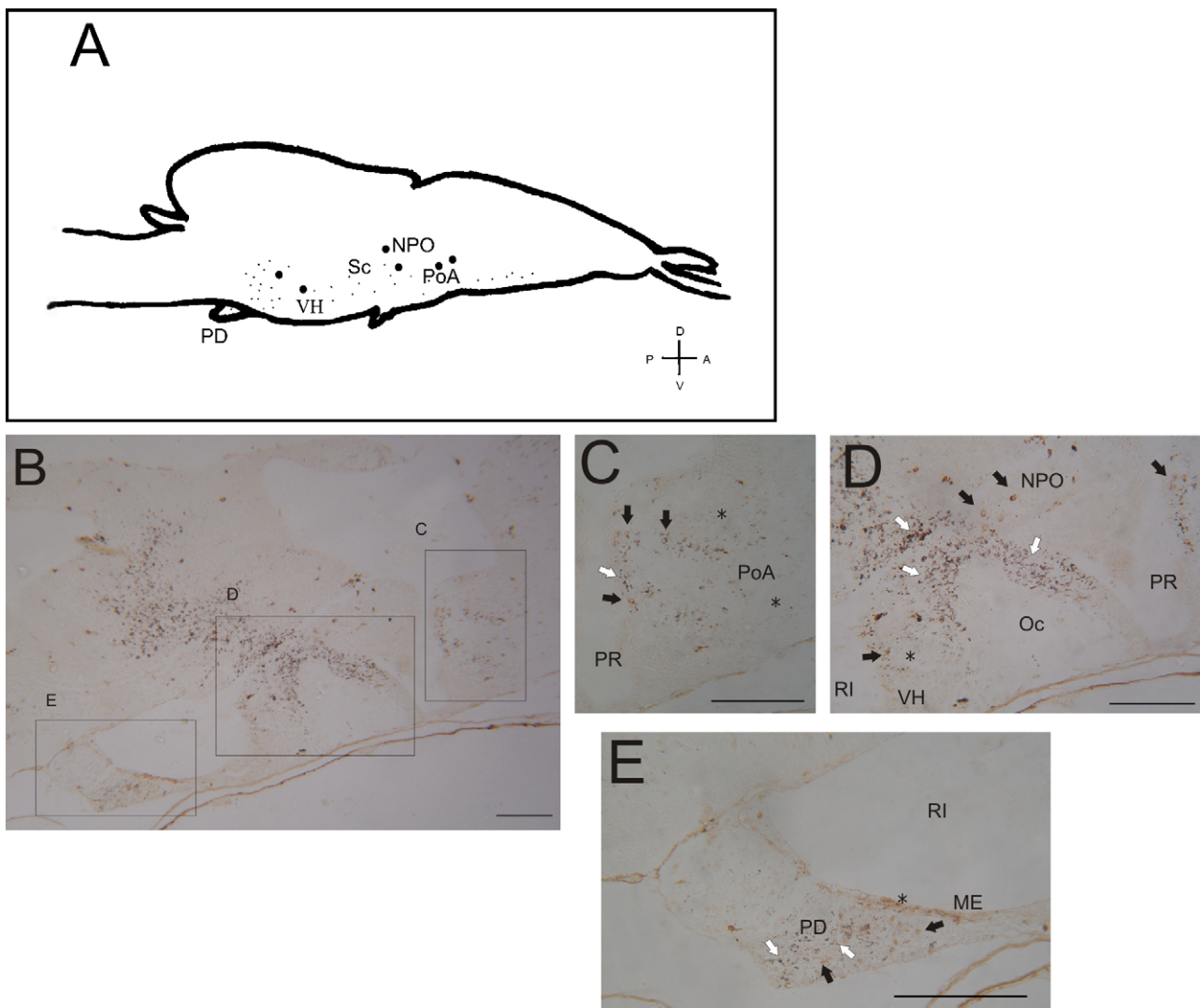


Fig. 1. Galanin distribution in the brain of *Rhinella arenarum* premetamorphic (stages 26–30) tadpole. (A) Schematic drawing of a representative sagittal section of the brain showing the distribution of Gal-ir neurons (black circles) and fibers (dotted areas). The intensity of dotting and the number of circles are roughly proportional in density among brain areas. (B) Panoramic view of a sagittal section of the tadpole brain showing Gal-ir cells and fibers. Note the abundant pigment granules present in this early stage (white arrows). (C) Detail of the Gal-ir cells (black arrows) in the PoA. These cells were scarce and weakly stained. (D) Detail of scattered Gal-ir cells and fibers (asterisk) in the NPO and VH. (E) Weakly stained Gal-ir cells in the pars distalis of the pituitary and fibers in the median eminence. Abbreviations: A: anterior; P: posterior; D: dorsal; V: ventral. For abbreviations see Table 1. Scale bar = $50 \mu\text{m}$.

3. Results

The larval period of anurans, which starts with independent feeding, is generally subdivided into three sets of stages: (1) premetamorphic stages (26–30); (2) prometamorphic stages (30–41); and (3) metamorphic climax stages (42–46). To help the description, the progress in appearance of Gal-ir cell groups and fibers in the developing *Rhinella arenarum* brain has been summarized in Table 1.

Premetamorphosis represents the start of the long period with active feeding. In this period, the hindlimbs buds develop on the side of the body but toes development is not initiated.

The distribution of Gal-ir perikarya and fibers in the premetamorphic brain of the tadpole is shown in Fig. 1A. During this period the first detectable Gal-ir elements were observed in several brain areas. In the olfactory epithelium, few Gal-ir cells were detected (data not shown).

From the telencephalon to the diencephalon, Gal-ir fibers were observed in a ventral position (Fig. 1A). The first Gal-ir neurons were observed in the anterior preoptic area. These cells were few in number, round in shape and weakly stained (Fig. 1B and C). A small group of Gal-ir neurons with the same characteristics was observed in the preoptic nucleus (Fig. 1A–D). The most numerous accumulations of Gal-ir cells and fibers were observed more caudally, in the ventral

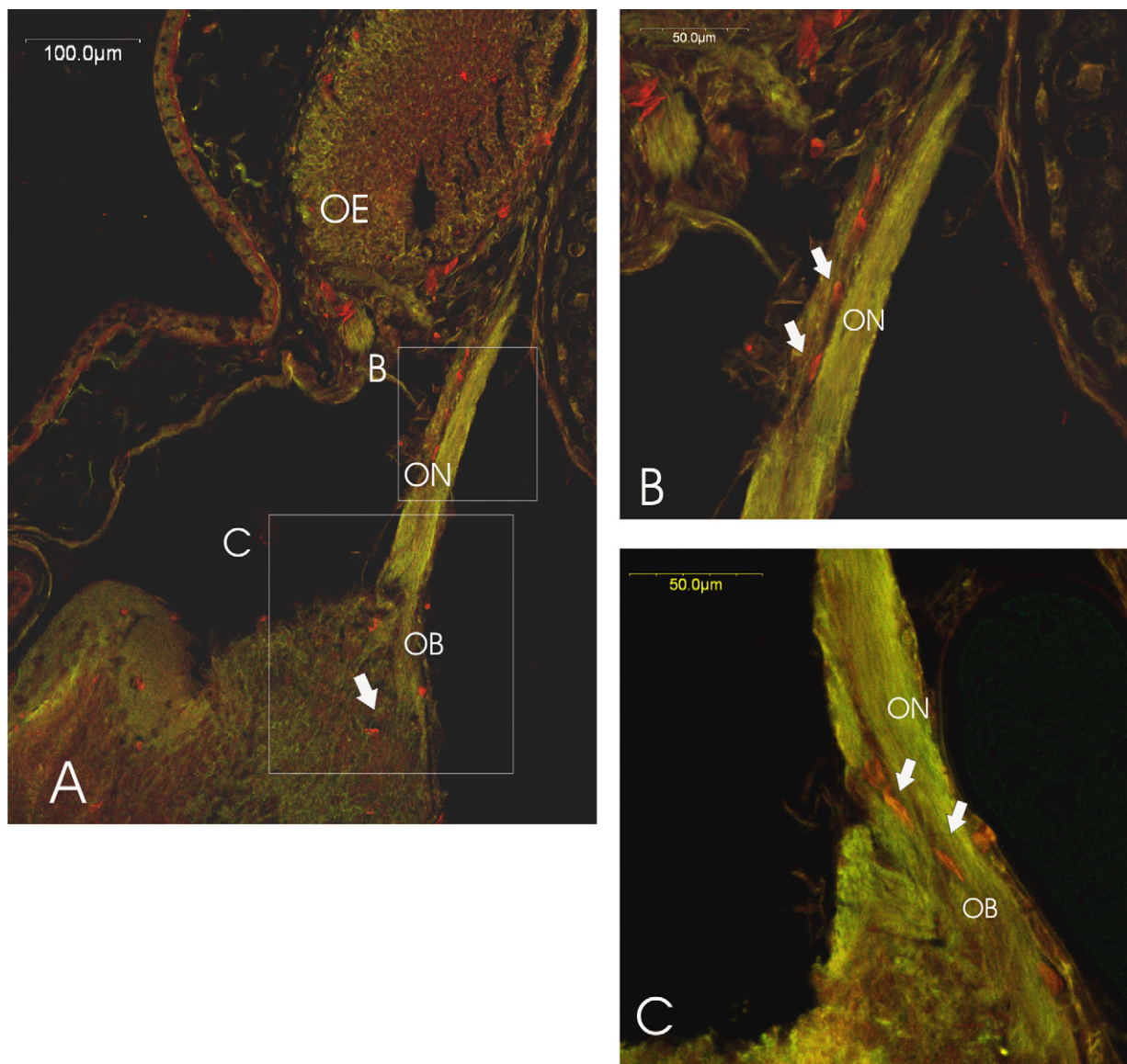


Fig. 2. Confocal laser scanning immunofluorescence microscopy of stage 32 tadpole olfactory system. Sagittal sections are double-labeled with anti-Gal (red) and anti- β -tubulin (green). A: olfactory epithelium (OE), olfactory nerve (ON) and olfactory bulb (OB). Note the presence of Gal-ir (white arrows) and β -tubulin in the same regions. B: detail of the ON. Note the typical migratory pattern of Gal-ir cells. C: detail of the nervous layer of the OB. For abbreviations see Table 1 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

hypothalamus around the infundibular region (Fig. 1A, B and D).

The median eminence was richly supplied with Gal-ir nerve terminals and in the pars distalis of the pituitary a few Gal-ir cells were observed (Fig. 1E).

The prometamorphic period is marked by the development of the hindlimbs and ends just before the drastic metamorphic changes that transform the tadpole into a froglet. At the beginning of this period, double-label immunofluorescence revealed scattered Gal-ir neurons in the olfactory epithelium (Fig. 2A). Moreover, single Gal-ir neurons were observed throughout the olfactory nerve (Fig. 2B) and some of them reached the nervous layer or the olfactory bulb (Fig. 2C).

The prometamorphic stages were characterized by the progressive maturation of the galaninergic system in the tadpole brain. Moreover, the pattern of fiber labeling became more conspicuous than before (Fig. 3A). In the olfactory epithelium Gal-ir cells were round in shape and scattered, as in

previous stages (Fig. 3B). In the telencephalon two well-defined groups of Gal-ir cells were observed. One group was formed by scattered Gal-ir cells in the medial pallium (Fig. 3C and D) while the second group was composed of few cells with strongly stained perikarya located mainly in the medial septum (Fig. 3C and E). In addition, Gal-ir fibers were densely distributed in the medial septum and became more sparsely distributed towards a caudal direction, the medial amygdala also exhibited a dense network of fibers (Fig. 3A). The ventromedial and lateral thalamus became richly supplied with fibers that extended caudally (Fig. 3F). Gal-ir neurons and fibers in the anterior preoptic area were more in number and became more intensely stained than in previous stages. These cells were found in the rostral and caudal area of the preoptic recess organ, in the prechiasmatic area (Fig. 4A and B). At the level of the optic chiasm, a new group of Gal-ir cells was observed in the suprachiasmatic nucleus. These cells were round in shape and presented very strong stained perikarya. Furthermore, Gal-ir fibers were found in

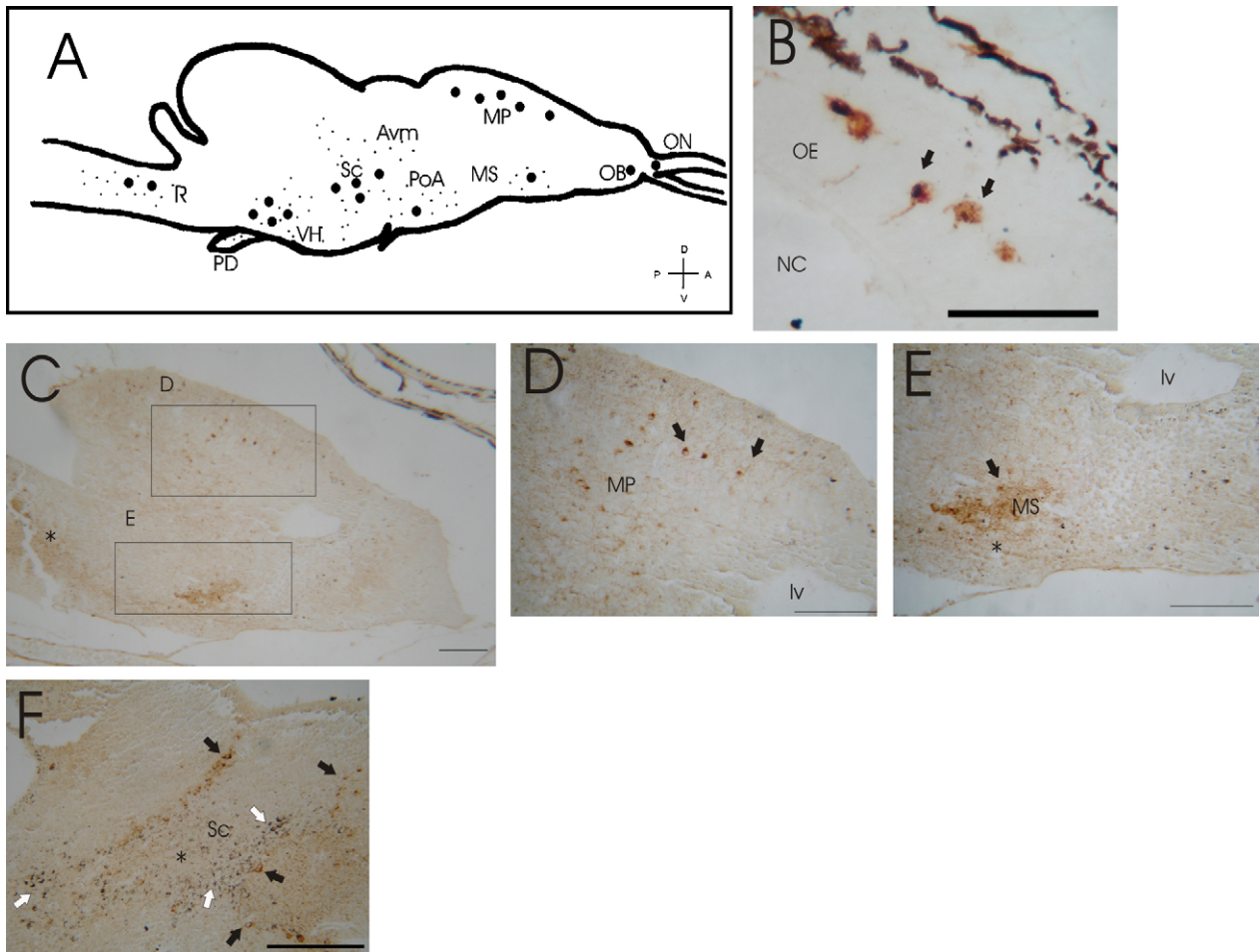


Fig. 3. Galanin distribution in the brain of *Rhinella arenarum* prometamorphic (stages 30–41) tadpole. (A) Schematic drawing of a representative sagittal section of the brain showing the distribution of Gal-ir neurons (black circles) and fibers (dotted areas). The intensity of dotting and the number of circles are roughly proportional in density among brain areas. (B) Round shaped Gal-ir cells in the olfactory epithelium. (C) Panoramic view of a sagittal section of the telencephalon showing Gal-ir cells and fibers. (D) Detail of the medial pallium showing many scattered Gal-ir cells (black arrows). (E) Detail of the medial septal area showing Gal-ir cells and fibers (asterisks). (F) Gal-ir cells and fibers in the medial thalamus and suprachiasmatic nucleus. Note the abundant pigment granules present in this area (white arrows). NC: nasal cavity. For abbreviations see Table 1. Scale bars = 50 μ m.

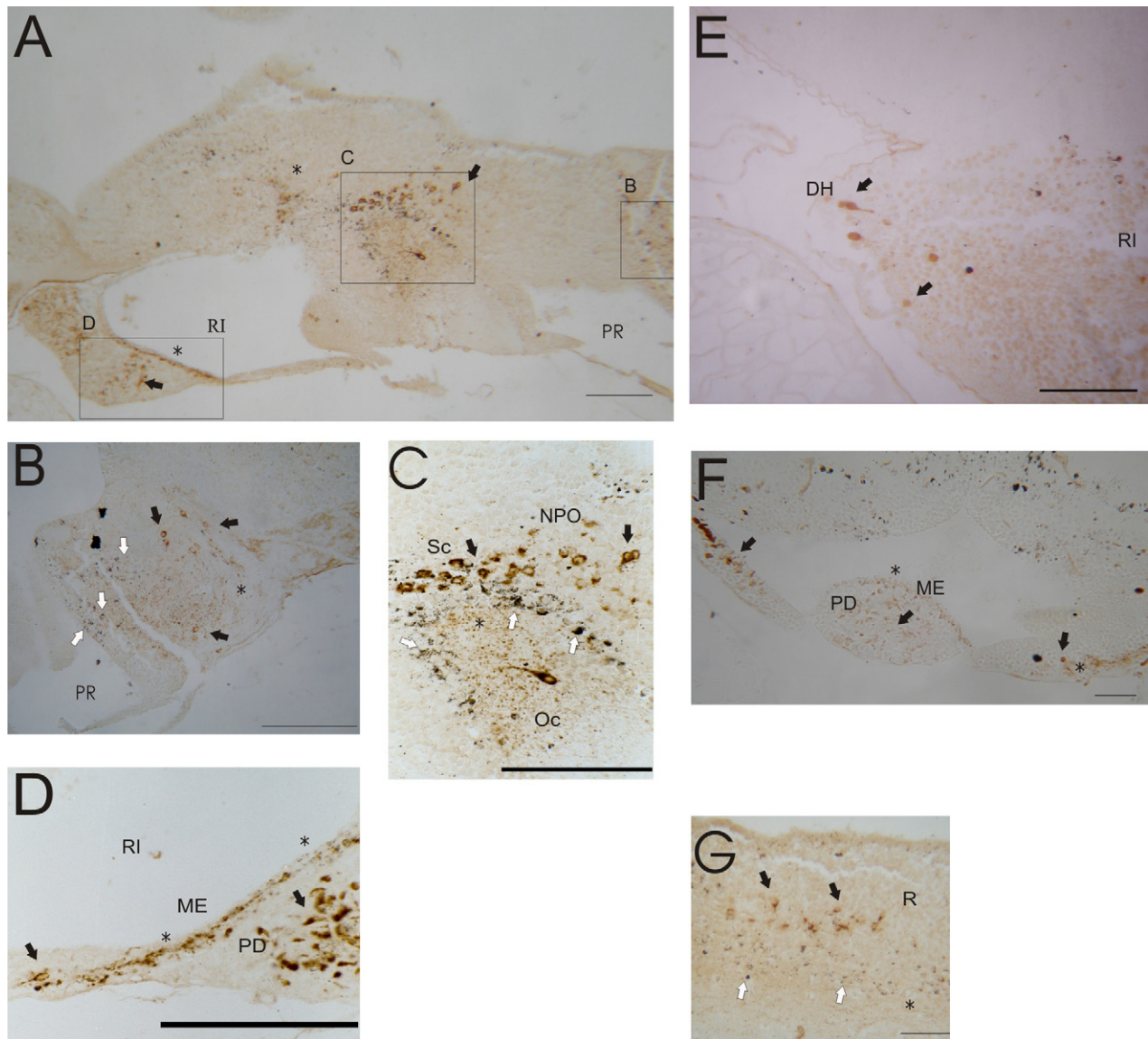


Fig. 4. (A) Presence of Gal-ir neurons and fibers in a panoramic sagittal section of the preoptic–hypothalamic area of *Rhinella arenarum* prometamorphic tadpole (stages 30–41). (B) Detail of the anterior preoptic area showing scattered Gal-ir cells (black arrows) and fibers (asterisk). (C) Detail of the suprachiasmatic area showing many strongly immunoreactive cells and fibers. (D) Dense innervation of Gal-ir in the median eminence and many cells in the pars distalis of the pituitary. (E) Transversal section showing few Gal-ir cells in the dorsal hypothalamus. (F) Gal-ir cerebrospinal fluid-contacting cells (CSF) and the typical Gal-ir cells in a transversal section of the ventral hypothalamus. (G) Detail of Gal-ir cells in the rhombencephalon. Also note the innervation in the median eminence and many Gal-ir cells in the pars distalis. For abbreviations see Table 1. Scale bars = 50 μ m.

the optic chiasm itself (Fig. 4A and C). In the hypothalamus, few Gal-ir neurons were observed in the dorsal hypothalamic nucleus (Fig. 4E), while many neurons were observed in the ventral hypothalamus. Many of these Gal-ir cells were of the cerebrospinal fluid-contacting (CSF-C) type, with their distal perikarya sending their dendrites to the ventricular space (Fig. 4E and F). More caudally, a very dense Gal-ir neuron/fiber tract courses caudally from the ventral hypothalamus through the pituitary stalk to the median eminence and pars distalis of the pituitary (Fig. 4D and F). In the pars nervosa of the pituitary only few Gal-ir fibers were observed (data not shown). More caudally, a new group of Gal-ir fibers

and neurons was observed in the rostral rhombencephalon (Figs. 3A and 4G).

Metamorphic climax. During this crucial period the tadpole loses its larval characteristics and takes on adult structures, the tail begins to atrophy, larval feeding structures are replaced by adults jaws and tongue, and forelimbs and hindlimbs become functional.

During the metamorphic climax and soon after, the frequency and distribution of Gal-ir cells slightly changed (Fig. 5A–G). In the telencephalon (Fig. 5A), preoptic–hypothalamic area (Fig. 5B–D) and ventromedial and lateral thalamus (Fig. 5D) the pattern of fiber labeling

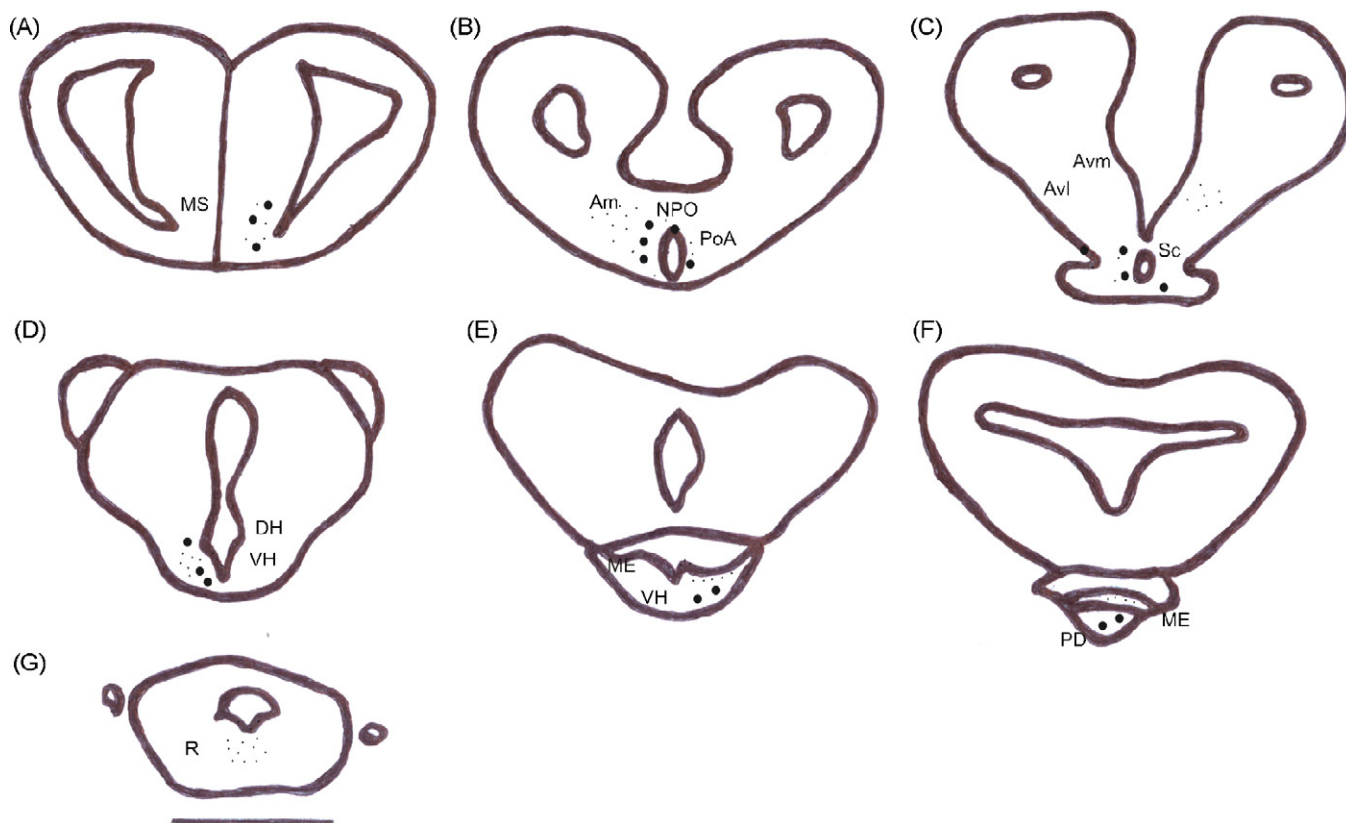


Fig. 5. (A–G) Schematic drawings of transversal sections of the brain of *Rhinella arenarum* tadpole in metamorphic climax stages (stages 42–46) showing the distribution of Gal-ir neurons (black circles) and fibers (dotted areas). The intensity of dotting and the number of circles are roughly proportional in density among brain areas. For abbreviations see Table 1. Scale bar: 100 μ m.

was less intense than before. Moreover, whereas few Gal-ir cells were observed in the olfactory epithelium, none were observed in the pallium (Fig. 5A) (see also Table 1).

In the anterior preoptic area, the relative number of Gal-ir neurons and fibers remained constant but the staining intensity was weaker (Fig. 6A and B). The relative abundance of Gal-ir neurons and fibers in the dorsal suprachiasmatic nucleus increased and many neurons were observed in a more lateral position (Fig. 6C). On the other hand, in the medial part of the diencephalon, the ventral hypothalamus, specially the infundibulum, presented the highest number of Gal-ir neurons, and within this group, CSF-C type cells became more abundant than before (Fig. 6C and D).

Gal-ir fibers in the median eminence and in the pituitary slightly decreased (Figs. 5F and 6E). More caudally, in the rostral rhombencephalon only Gal-ir fibers were observed (Fig. 5G).

4. Discussion

The present study shows the development of the Gal-ir neuronal system and fibers in the brain and pituitary of *Rhinella arenarum* larvae and metamorphs. Previous data on the development of the galaninergic system in amphibians

during development are lacking, however, there are studies of the Gal-system in the adult amphibian brain, which will be discussed in relation with the present findings.

In the olfactory epithelium we observed Gal-ir cells along the entire metamorphic process. Furthermore, in early prometamorphic stages (stages 31–33) Gal-ir neurons were also found along the olfactory nerve and bulb. These findings could be indicating an olfactory origin of a galanin cell population. This is true in mice where Gal-ir neurons were observed to migrating to the central nervous system along the olfactory/vomeroneasal nerve (Key and Wray, 2000). Nevertheless, a more detailed investigation with other techniques such as *in situ* hybridization is required to confirm this observation.

During the development of *R. arenarum* larvae, the highest densities of Gal-ir cells occurred in the preoptic–hypothalamic area: in the preoptic nucleus, suprachiasmatic nucleus and in the ventral hypothalamus, which is in accordance with previous data reported in the adult amphibian *Bufo (Rhinella) arenarum* (González-Nicolini et al., 1995) as well as in adults of other species such as *Rana esculenta* (Lázár et al., 1991) and *Xenopus laevis* (Lázár et al., 1991; Olivereau and Olivereau, 1992). The preoptic nucleus is a neurosecretory nucleus which contains several regulatory peptides in amphibia

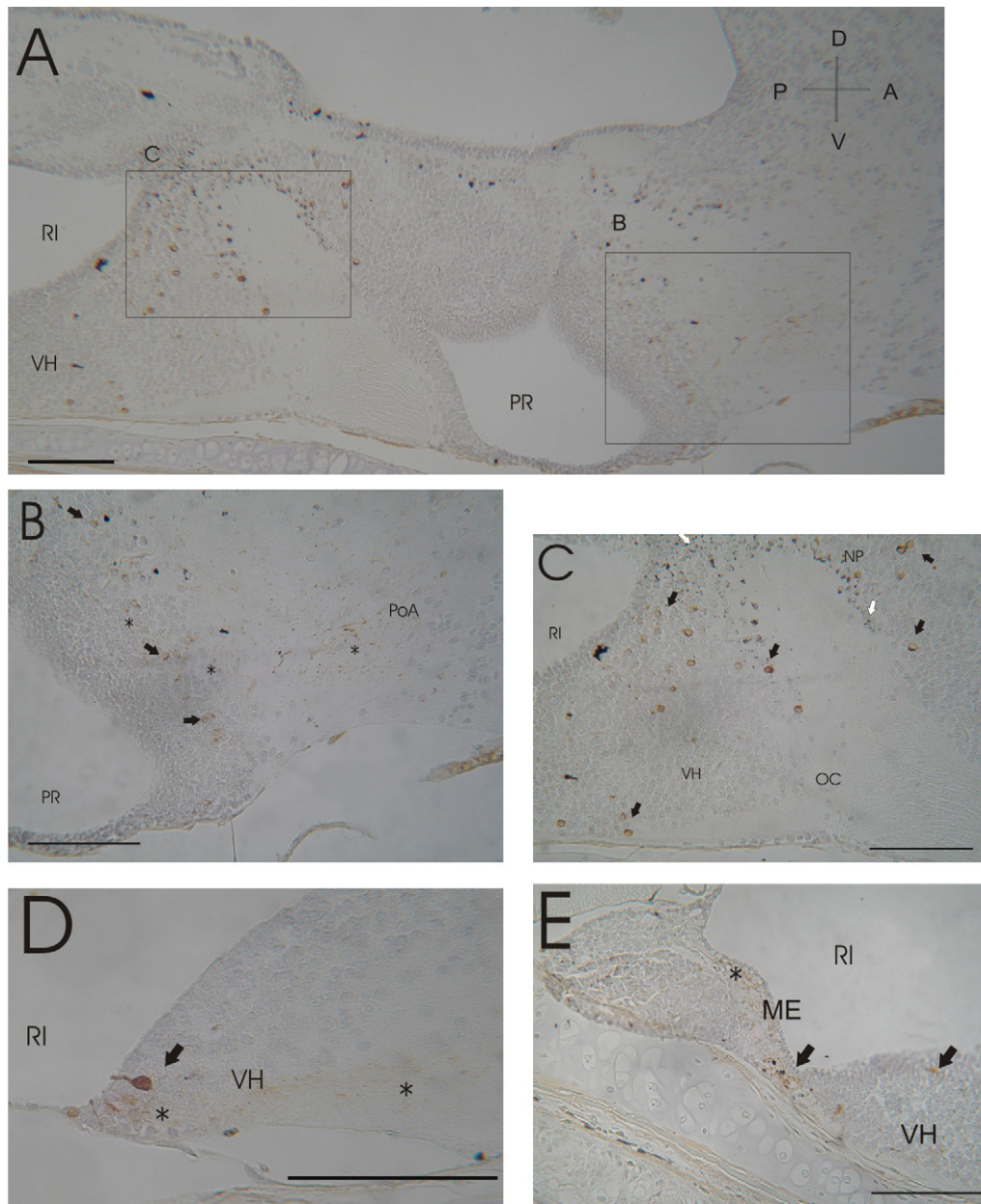


Fig. 6. Galanin distribution in the brain of *Rhinella arenarum* tadpole in metamorphic climax stages (stages 42–46). (A) Panoramic view of a sagittal section of *Rhinella arenarum* metamorphs (stages 42–46) showing the presence of Gal-ir neurons and fibers. Note the less abundant pigment granules present in late stages (white arrows). (B) Detail of the anterior preoptic area showing scattered Gal-ir cells (black arrows) and fibers (asterisk). (C) Gal-ir cells in the hypothalamic area and preoptic nucleus. (D) Detail of Gal-ir cerebrospinal fluid-contacting cells in the ventral hypothalamic area. (E) Detail of the pituitary and ventral hypothalamic area showing Gal-ir cells and fibers. For abbreviations see Table 1. Scale bars: 100 μm . Abbreviations: A: anterior; P: posterior; D: dorsal; V: ventral.

(Andersen et al., 1993). Previous work in the adult *Bufo* (*Rhinella*) *arenarum* suggests that the presence of galanin in the preoptic area may be related with a regulatory function on hypophyseal hormones, in particular growth hormone (González-Nicolini et al., 1997) and a possible role of Gal and nitric oxide synthase (NOS) in the regulation of hibernation in these animals (González-Nicolini et al., 1998).

The early expression of Gal in the hypothalamus seems to be a shared feature of the few vertebrates studied to date, indi-

cating that, in this region, this peptide plays a basic important role in the development, maybe related to growth and food intake (Murakami et al., 1987; Kyrkouli et al., 1990). In the present work, most of the Gal-ir cells in the ventral infundibular area were of the cerebrospinal fluid-contacting (CSF) type, exhibiting an apical dendrite that ended in a ventricular bulb. A similar observation was reported in the adult toad *Bufo* (*Rhinella*) *arenarum* (González-Nicolini et al., 1995) as well as in other vertebrate species including amphibians (Lázár et al., 1991; Jiménez et al., 1994; Adrio et al., 2005). These

CSF-contacting cells have been considered a primitive type of secretory neuron (Vigh-Teichmann and Vigh, 1989) with no clear function, but it probably connected with sensorial and/or secretory roles (Vigh et al., 2004). These neurons may also be involved in regulatory processes of the hypothalamus, control changes in the chemical composition of the CSF, or secretion of Gal into the CSF.

The presence of Gal in the mammalian pituitary has been described in rat (Palkovits et al., 1987) and human (Hsu et al., 1991) with different expression patterns (Cimini, 2000). We have not performed co-localization studies in order to analyze which cell populations are expressing Gal, but its distribution is coincident with the expression areas of different pituitary hormones in *Bufo (Rhinella) arenarum* (Miranda et al., 1995).

Our study shows Gal-ir cells and fibers in the pituitary gland, in both anterior (pars distalis) and posterior lobe (median eminence and pars nervosa). In their work, González-Nicolini et al. (1995) could only detect seldom single fibers in the anterior lobe of the pituitary of the adult toad *Bufo (Rhinella) arenarum*. This difference is in agreement with the hypothesis that the direct innervation on the pituitary is present in amphibian larval stages as a remnant character of the fish-like amphibian ancestor (Aronsson and Enemar, 1981).

In the brainstem, we only found Gal-ir cells in the rhombencephalon, while in the adult toad *Bufo (Rhinella) arenarum* González-Nicolini et al. (1995) observed a wider distribution.

The present study of the development of the galaninergic system in the brain and pituitary of *Rhinella arenarum* has demonstrated that this system develops gradually during larval stages. The distribution of Gal in the brain as well as the pituitary innervation by Gal-ir fibers suggest that this peptide play an important role in neuroendocrine regulation of the brain and pituitary functions during development. The differences observed in the galaninergic system between the tadpole and the adult could be due to a low expression of this neuropeptide during larval stages or to the fact that the galaninergic system maturation continues until sexual maturity.

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