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Immunocytochemical localization of a galanin-like peptidergic system in the brain of two urodele and two anuran species (Amphibia)

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Summary. Galanin-like immunoreactivity was localized in the brain of Urodela (*Ambystoma, Pleurodeles*) and Anura (*Bufo, Xenopus*) by immunocytochemistry with anti-porcine galanin antiserum. In the four species, immunoreactive perikarya were observed in the telencephalon (striatum, amygdala), diencephalon preoptic area mainly along the anterodorsal wall of the preoptic recessus, suprachiasmatic nucleus, lateral hypothalamus, ventral and dorsal infundibular nuclei, paraventricular organ, and rhombencephalon (nucleus of the solitary tract). Galaninergic fibres extended in similar regions and in the medial septum, ventral telencephalon, ventral hypothalamus, median eminence, and various mesencephalic and rhombencephalic regions. Contacts with the cerebrospinal fluid cavity occurred along the preoptic recessus (*Ambystoma*) and the ventral infundibular wall (all species). Fibres were scarce in the neurohypophysis. The distal and intermediate lobes of the pituitary were virtually devoid of immunoreactivity. The galaninergic system appeared more developed in adult amphibia than in young animals, suggesting the stimulating influence of sex steroids on the expression of galanin as previously described in *Anguilla*. The extensive distribution of the galanin-like immunoreactive neurons in amphibian brains suggests that this peptide may act as a neuromodulatur and/or neurotransmitter.

Introduction

The amino acid sequences of porcine (Rökaeus and Brownstein 1986), bovine (Rökaeus and Carlquist 1988) and rat (Kaplan et al. 1988) galanins (GAL) show 90% homology amongst the three species. Human GAL appears unique in structure (with 30 amino acids; Bersani et al. 1991; Evans and Shine 1991; Schmidt et al. 1991). Chicken (Norberg et al. 1991) and blowfly (Lundquist et al. 1991) galanins also differ from mammalian galanins. Radioimmunoassays of GAL show high concentrations in various brain regions. The distribution of GAL-immunoreactive (ir) neurons has been determined in the mammalian brain and pituitary by immunocytochemistry (review in Merchenthaler 1991; Olivereau and Olivereau 1991a; Vrontakis et al. 1991).

In the amphibian *Necturus maculosus*, reverse phase high-pressure liquid chromatography showed that the major peak of GAL observed in heart, urinary bladder, small intestine and brain eluted just prior to porcine GAL; minor peaks probably represented extended or truncated forms of GAL. Radioimmunoassays and immunofluorescence techniques confirmed the presence of GAL-like peptide in the same tissues (McKeon et al. 1990).

The localization of GAL-containing neurons was briefly described in the brain of a few anuran (*Rana temporaria:* Wolfbauer and Skofitsch 1989; Horsten and Vandesande 1991; *Xenopus laevis:* Lazar et al. 1991) and urodele species (*Triturus carnifex:* Mulatero et al. 1991) and in more detail in *R. esculenta* (Lazar et al. 1991). In the toad *Bufo marinus*, GAL detected in sympathetic and parasympathetic neurons (Morris et al. 1989) induced some hypertension but slightly affected the heart rate (Courtice 1991). High concentrations of ir-GAL were reported in cardiac ganglia of *Necturus maculosus* (Parsons et al. 1989; McKeon and Parsons 1990). GAL application to parasympathetic post-ganglionic neurons induced a calcium-dependent membrane hyperpolarization and decreased excitability (Konopka and Parsons 1989; Konopka et al. 1989; Parsons and Konopka 1990, 1991). The GAL-like peptide seemed to act as an inhibitory transmitter (McKeon et al. 1991). In the present paper, the distribution of GAL-immunoreactive neurons has been investigated in the brain of two urodele (*Ambystoma* and *Pleurodeles*) and two anuran (*Bufo* and *Xenopus*) species.

Materials and methods

Animals

The following number and species of Amphibia were studied. Urodela: 9 *Ambystoma mexicanum* Shaw (axolotl; body wt. 17–20 g

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or 50 g); 24 *Pleurodeles waltlii* Michah [18 were in late metamorphosis (body wt. 1 g) and 6 were adults (body wt. 24–29 g)]. Anura: 5 *Bufo vulgaris* Laur (body wt. 25–30 g); 12 juvenile *Xenopus laevis* Daudin (body wt. 1.5–2 g) and 4 adults (body wt. 22–29 g). All the animals were kept in tap water.

Immunocytochemical procedure

All animals were decapitated in the morning without anaesthesia. The brain with the pituitary attached was rapidly dissected, fixed by immersion in sublimated Bouin-Hollande fluid and embedded in paraffin. Sagittal (Urodela, Anura) and frontal (Anura) sections (4- or 5-μm-thick) were successively incubated with primary immune goat serum to prevent non-specific immunostaining, rabbit antiserum to synthetic porcine galanin (batches 06165/3520 and 07233/3221; Cambridge Research Biochemicals, Euromedex, Strasbourg, France) at 1/1500 dilution for 18 h at room temperature, goat anti-rabbit serum and the peroxidase-antiperoxidase (PAP) complex. *Xenopus* sections were also incubated for 48 h with the anti-galanin serum at 1/1500 or 1/2000 dilution, or 24 h at a 1/1000 dilution. The immunostaining was revealed with the 3,3'-diaminobenzidine technique. The specificity of the antiserum was demonstrated by replacing the immune serum with normal rabbit serum and antiserum absorbed with porcine galanin (10^{-6} *M*), procedures which abolished the immunostaining.

Results

Ambystoma mexicanum

Immunocytochemical techniques demonstrated a dense network of GAL-like fibres and several groups of ir-perikarya in the brain. The galaninergic system will be described from the cephalic to the caudal part of the central nervous system, up to the level of the nucleus of the solitary tract. In the dorsal and ventral telencephalon, ir-fibres were not detected in the olfactory bulb and the medial, dorsal and lateral pallium. They were scarce in the ventral part of the medial septum and close to the accumbens nucleus. More posteriorly their number increased in the medial septum, ir-fibres extending through the lateral septum. Some perikarya were scattered in the striatum, mainly the pars ventralis. Most were weakly labelled, appeared unipolar and their axons were not visible (Fig. 1). They never seemed to contact the cavity of the telencephalic ventricle. Fibres were more abundant in the amygdala and ir-perikarya still showed a variable immunostaining intensity.

At the level of the rostral part of the recessus preopticus, the longitudinal ventral bundle was denser and wider (Fig. 2). Dorsally in the vicinity of the anterior commissure, ir-fibres arose from a group of more or less intensely labelled perikarya often located in or close to the ventricular wall (Figs. 3, 4). Some fine axons ran rostrally in parallel to the ventricular border under the ependymal cells (Fig. 4). In contrast with the predominant longitudinal direction of the fine GAL-ir fibres, much thicker fibres with large varicosities (Fig. 5) were running ventrodorsally, perpendicular to the basal surface of the brain (Figs. 2, 3). These thick fibres were followed up close to the anterior commissure, but did not seem to end close to the ventricle (Figs. 3, 4) and did not cross the commissure. The ventrodorsal system of large fibres arose from the numerous perikarya located around the recessus preopticus (Fig. 2), mainly the rostral (Fig. 6) and dorsal areas. Two kinds of ir-perikarya could be distinguished. The first type possessed a thin rim of highly labelled cytoplasm and fine axons visible over a short distance. Some perikarya sent fine axons running under the ependymal cells of the recess (Fig. 7) and might join the bundle in the ventral wall, homologous to the lamina terminalis of mammals. The second type of perikarya appeared larger and contained more intensely immunostained cytoplasm (Figs. 6, 7). They were observed in small numbers in the ventral wall and more frequently in the rostral and anterior half of the dorsal part of the preoptic area around the recess. These darkly stained neurons possessed thick and occasionally sinuous axons visible over quite a long distance (Fig. 6). The ventral axons turned rostrally around the recess and then dorsally up to the anterior commissure. Some cell bodies sent a thick or thin process between ependymal cells which reached the recess cavity (Fig. 7), suggesting that GAL might be released into the cerebrospinal fluid (CSF). The distribution of GAL-ir cell bodies and fibres in the forebrain is schematically presented in Fig. 8.

In medial sagittal sections, the posterior wall of the recess and the adjacent preoptic area as well as the optic chiasm and optic nerves did not contain ir-perikarya and fibres. Ir-fibres with fine varicosities were detected above the optic chiasm running in thin parallel bundles toward the basal hypothalamus. Moderately immunoreactive perikarya were observed in the suprachiasmatic nucleus (Fig. 9) and the amygdala (Fig. 10). GAL immunoreactivity was not homogeneously distributed in these perikarya, but concentrated in some cell portions and

Figs. 1–7. Galanin (GAL)-like immunoreactivity in the brain of *Ambystoma*. *Thick arrow*, dorsal direction

Fig. 1. Striatum (*St*) showing a ventral tract of fibres (*F*) and heavily or lightly labelled perikarya (*arrows*). *TV*, telencephalic ventricle. ×150

Fig. 2. Anterodorsal region along the recessus preopticus. Immunoreactive (ir) perikarya (*long arrows*) give rise to thick fibres (*arrows*) running ventrodorsally. Fine fibres (*F*) run longitudinally. ×150

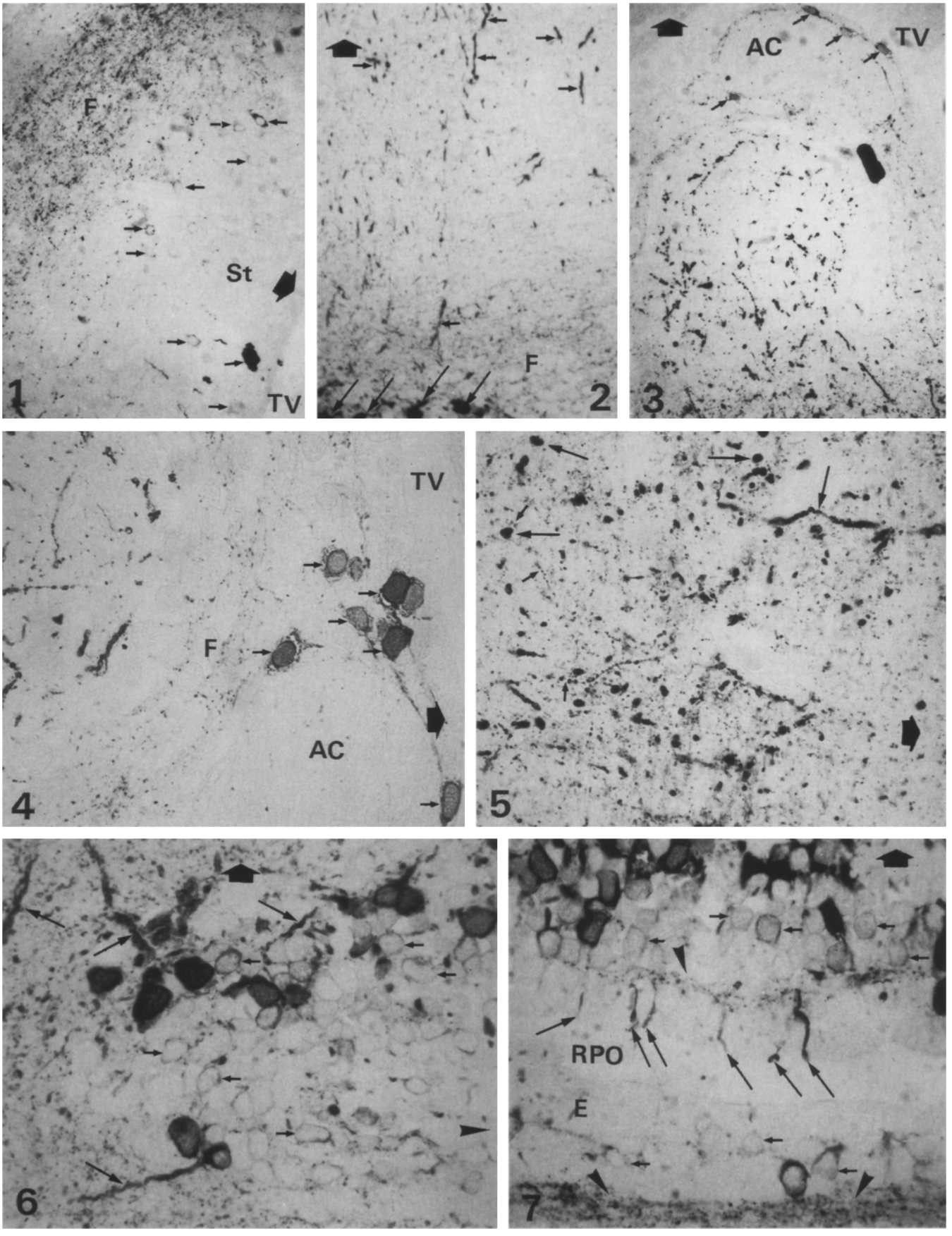
Fig. 3. Anterior preoptic area with thick fibres reaching the anterior commissure (*AC*) and a few perikarya (*arrows*) along the telencephalic ventricle. ×150

Fig. 4. Same region as in Fig. 3. Presence of thick varicous or undulating fibres (on the left), fine fibres (*F*) passing under the anterior commissure (*AC*) and ir-perikarya (*arrows*). ×375

Fig. 5. Same region as in Fig. 3. Large fibres and some thick axonal sections (*long arrows*) and fine fibres (*arrows*). ×375

Fig. 6. Tangential section of the rostral part of the recessus preopticus (*arrowhead*). Heavily labelled perikarya with thick and long axons (*long arrows*) and some pale perikarya (*arrows*). ×375

Fig. 7. Longitudinal section through the recessus preopticus (*RPO*) with pale (*arrows*) and darker perikarya. Long processes (*long arrows*) reach the *RPO* cavity. Fine fibres (*arrowheads* run under the ependymal layer (*E*) and in the ventral wall of the *RPO*. ×375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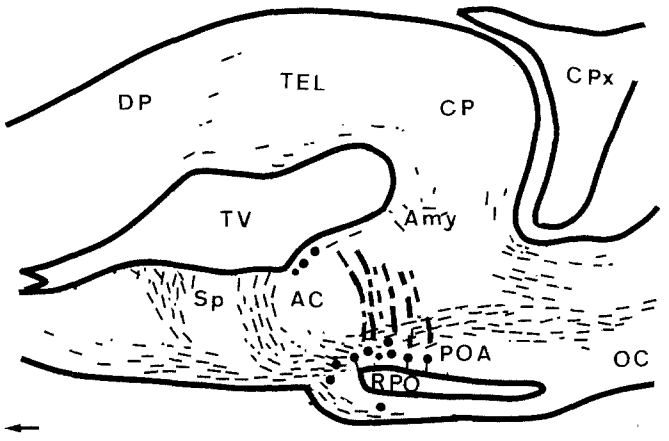


Fig. 8. Immunoreactive GAL-like system in the telencephalon and preoptic area of *Ambystoma*. Line drawing of a paramedian sagittal section. *Arrow*, Rostral direction; *dots*, GAL-like perikarya; *fine dashed lines*, fine GAL-like fibres; *thick dashed lines*, thick GAL-like fibres. Abbreviations as given in the list

Abbreviations used in the figures: AC, anterior commissure; *Acc*, nucleus accumbens; *Amy*, amygdala; *APOA*, anterior preoptic area; *AVH*, anteroventral hypothalamus; *BO*, bulbus olfactorius; *CE*, cerebellum; *CP*, caudal pallium; *CPx*, choroid plexus; *D*, dorsal; *DH*, dorsal habenula; *DM*, dorsomedian; *DP*, dorsal pallium; *DSt*, dorsal striatum; *E*, ependyma; *ELa*, external layer of the median eminence; *F*, fibers; *FH*, foramen of Munroe; *H*, habenula; *HC*, habenular commissure; *HT*, hypothalamus; *IL*, intermediate lobe of the pituitary; *ILa*, internal layer of the median eminence; *Ist*, isthmus; *LP*, lateral pallium; *LSp*, lateral septum; *M*, meningeal layers; *ME*, median eminence; *MES*, mesencephalon; *MP*, medial pallium; *MPT*, mesencephalic posterior tuberculum; *MSp*, medial septum; *NH*, neurohypophysis; *NID*, nucleus infundibularis dorsalis; *NIP*, nucleus interpeduncularis; *NIV*, nucleus infundibularis ventralis; *NST*, nucleus of the solitary tract; *OC*, optic chiasm; *ON*, optic nerve; *OV*, optic ventricle; *P*, paraphysis; *PC*, posterior commissure; *PD*, pars distalis of the pituitary; *POA*, preoptic area; *PVO*, paraventricular organ; *RH*, rhombencephalon; *RI*, recessus infundibularis; *RPO*, recessus preopticus; *SCN*, suprachiasmatic nucleus; *SL*, sulcus lateralis infundibularis; *Sp*, septum; *St*, striatum; *TC*, tuber cinereum; *TEL*, telencephalon; *THAL*, thalamus; *TO*, tectum opticum; *TRH*, thyrotropin-releasing hormone; *TS*, torus semicircularis; *TV*, telencephalic ventricle; *V*, ventral; *3V*, third ventricle; *4V*, fourth ventricle; *VH*, ventral habenula; *VM*, ventromedian; *VSt*, ventral striatum

barely detected in the thin rims along the nuclear membrane (Fig. 9). Axons were rarely visualized. Few perikarya contacted the CSF of the third ventricle facing the choroid plexus, which deeply penetrated inside the ventricular system of the axolotl. GAL was not detected in the magnocellular preoptic nucleus.

Posteriorly to the optic chiasm, fine ir-fibres formed a dense network in the ventrolateral hypothalamus. The longitudinal tracts running through the tuber cinereum (or ventral hypothalamus or infundibulum) seemed to end in the median eminence. Numerous ir-fibres surrounded unlabelled cell bodies. A large number of moderately or intensely labelled perikarya were observed in the paraventricular (or periventricular) organ and the ventral and dorsal infundibular nuclei (or ventral and dorsal hypothalamic nuclei), along the infundibular recess. Most peripheral cells were oriented perpendicularly to the recess wall. A few cells were very close to or directly in contact with the ventricular surface, and sent a short and thick dendrite up to the lumen. Some of these processes had a terminal nodule inside the ventricular lumen (Fig. 11). Densely stained perikarya were located in the posterodorsal portion of the tuber cinereum close to the median eminence. They frequently contacted the infundibular recessus, and sent thick and occasionally undulating axons into the ventral hypothalamus (Fig. 11). However, these axons could not be seen over a long distance and disappeared close to the optic chiasm, so that their endings were not identified.

Fibres were observed in the median eminence, mainly in the external layer, often surrounding capillaries of the portal plexus. Fibres were more abundant in the central than in the lateral parts of the median eminence. A small number of ir-fibres entered the neural lobe. No immunostaining was observed in the intermediate and distal lobes and endocrine cells were always unlabelled. Abundant fibres were coursing through the interpeduncular nucleus, the thalamus and the torus semicircularis. Some ir-fibres were observed in different layers of the optic tectum, but did not present a laminar distribution. The cerebellum appeared free of GAL, but numerous ir-fibres coursed dorsoventrally between the posterior end of the optic tectum and the cerebellum, in the isthmus, then continuing into the medulla oblongata. Ir-perikarya were not detected in the mesencephalon and the origin of the isthmic fibres remained undetermined.

Figs. 9–11. GAL-like immunoreactivity in the brain of *Ambystoma*. ×375

Fig. 9. Ir-perikarya (*arrows*) in the suprachiasmatic nucleus (*SCN*). Heterogeneous cytoplasmic labelling sometimes restricted to a few loci. *Long arrow*, long axon

Fig. 10. Ir-perikarya in the amygdala (*Amy*) and fine fibres

Fig. 11. Posterodorsal region of the tuber cinereum (*TC*) with heavily or lightly-ir perikarya, some processes reaching the recessus infundibularis (*RI*) cavity, and some thick and undulating fibres (*long arrows*)

Figs. 12–17. GAL-like immunoreactivity in the brain of late metamorphosing (Figs. 12–14) and adult (Figs. 15–17) *Pleurodeles*. Ventral direction on the left. ×375

Fig. 12. Posterior telencephalon. Ir-perikarya (*arrows*) along the anterior commissure (*AC*) and towards the telencephalic ventricle (*TV*). *St*, striatum

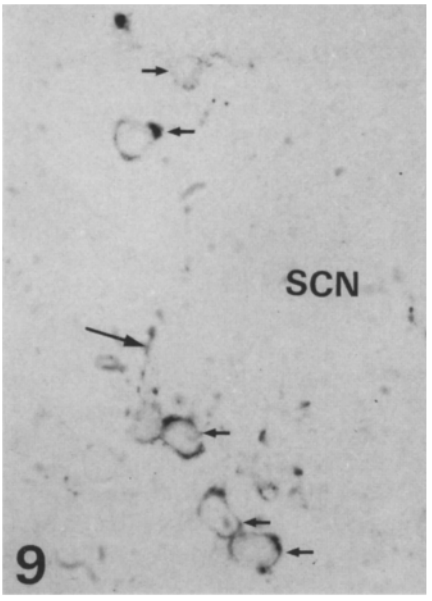
Fig. 13. Posterior tuber cinereum (*TC*). Ventral fibres (*long arrows*) seem to end in the internal layer (*ILa*) and external layer (*ELa*) of the median eminence. Pars distalis (*PD*) of the pituitary

Fig. 14. Small islet of ir-perikarya (*arrows*) in the nucleus of the solitary tract (*NST*). *Long arrow*, a long axon; *M*, meningeal layers; *Thick arrow*, dorsal direction

Fig. 15. High density or ir-fibres in the ventral telencephalon

Fig. 16. Posterodorsal portion of the tuber cinereum (*TC*) with a dense network of ir-fibres and pale or strongly immunostained perikarya, some sending processes (*arrows*) reaching the recess cavity (*RI*)

Fig. 17. Median eminence: ir-GAL is present in the internal layer (*ILa*) but mostly accumulates in the external layer (*ELa*). The pars distalis (*PD*) is free of galanin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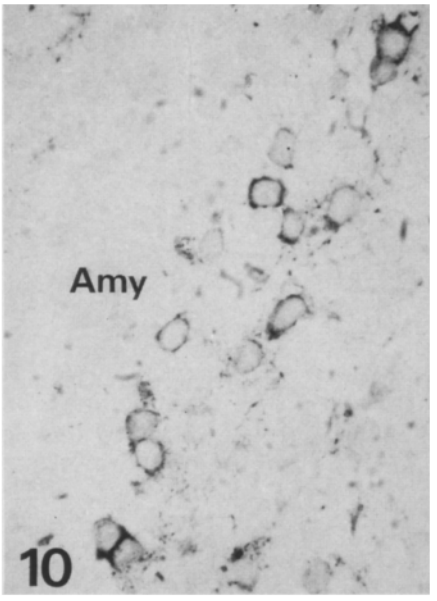
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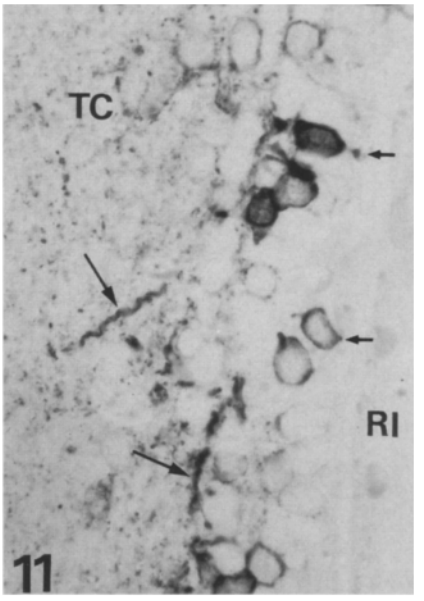
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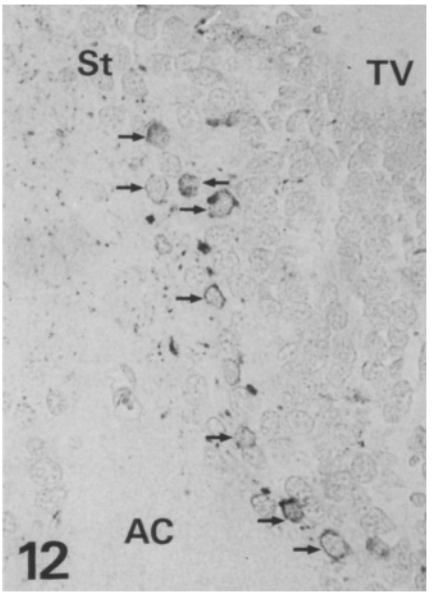
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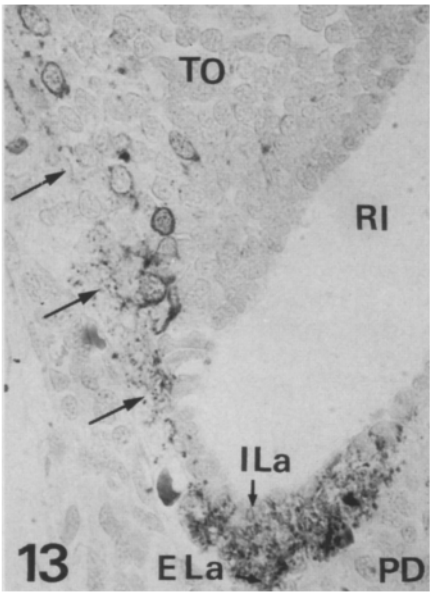
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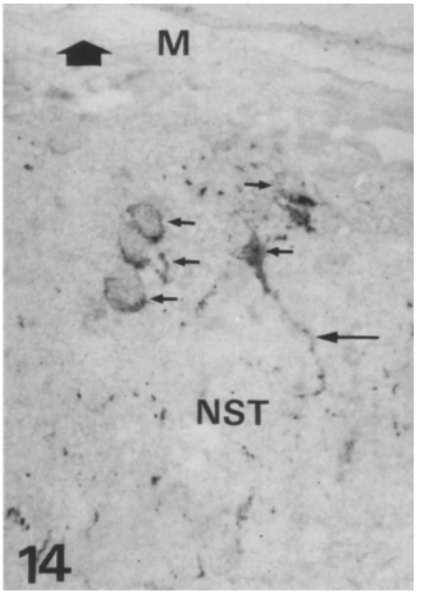
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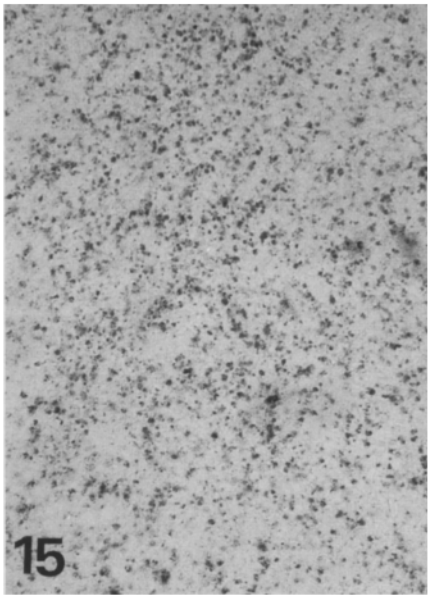
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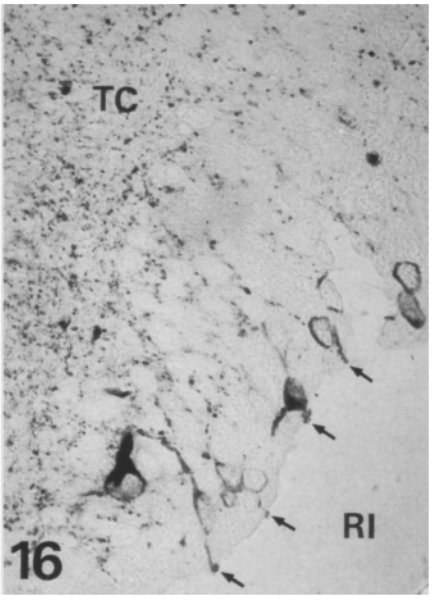
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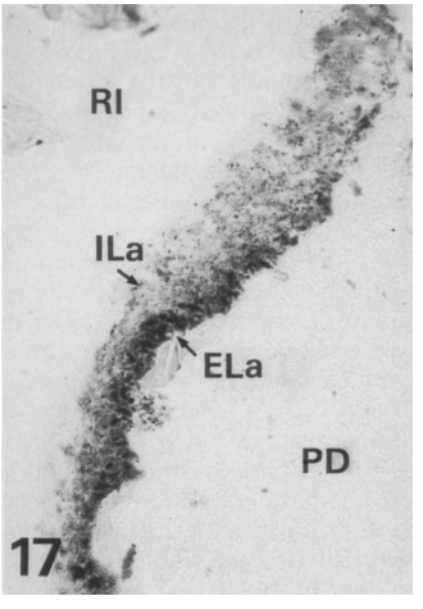
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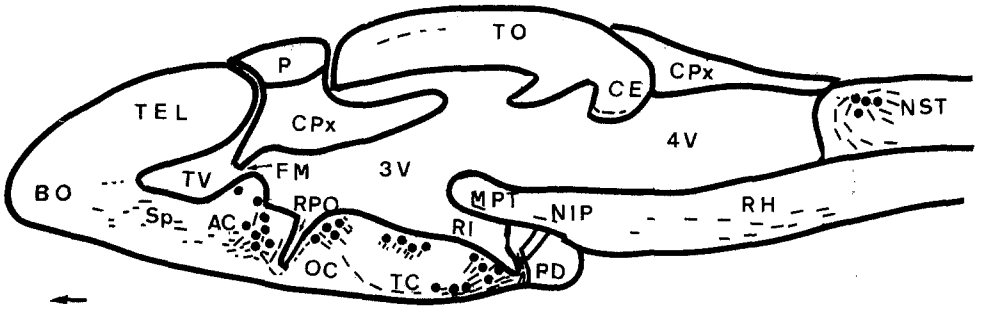


Fig. 18. Immunoreactive GAL-like system in the brain of a late metamorphosing *Pleurodeles*. Line drawing of a paramedian sagittal section. *Dots*, GAL-like perikarya; *dashed lines*, tracts of GAL-like fibres. Abbreviations as given in the list

More posteriorly, at the level of the caudal end of the large choroid plexus of the 4th ventricle, several ir-perikarya were observed in the nucleus of the solitary tract among fibres that ran caudally in the spinal cord, at first dorsally, then ventrally.

Pleurodeles waltlii

Late metamorphosing stage. Ir-fibres were observed in the ventral telencephalon, mainly in the septal and amygdalian areas and in the vicinity of the recessus preopticus from which they extended dorsally among unlabelled perikarya of the preoptic nucleus and in the suprachiasmatic nucleus. Ir-fibres formed small parallel bundles above the optic chiasm and spread into the tuber cinereum, giving rise to two symmetric ventrolateral bundles and a dense network in the caudal portion. A large number or ir-fibres ended in the median eminence, mainly in the external layer. A small number entered the neurohypophysis while pituitary cells were unlabelled. Sparse fibres seemed to contact the CSF of the infundibular cavity. From the infundibular region, fibres passed around the third ventricle and through the mesencephalic posterior tuberculum, reaching the rhombencephalon and the ventral spinal cord. Ir-fibres were also noted in the thalamus and under the habenula although they did not enter it. Some fibres were observed in the optic tectum, often coursing in parallel to the dorsal surface of the mesencephalon. A few fibres were observed in the torus semicircularis, under the cerebellum, in the isthmus and the tegmentum, continuing in the ventral and dorsal spinal cord. A large group of fibres arose from the nucleus of the solitary tract, ran ventrally and then caudally, either above or under the central canal. All these fibres had fine varicosities. Some fibres with large varicosities were occasionally observed in the vicinity of the preoptic and infundibular recesses.

Ir-perikarya were observed in the amygdala close to the anterior commissure (Fig. 12), in the vicinity of the recessus preopticus although in a much lower number than in the axolotl, in the preoptic area where they were scarce and slightly labelled, in the posterior portion of the suprachiasmatic nucleus along the chiasmatic ridge. In the ventral hypothalamus, ir-perikarya occasionally occurred in the lateral hypothalamic nucleus, and mainly in the dorsal and ventral hypothalamic nuclei and the paraventricular organ. Fibres ended in the median eminence, some in the internal layer and most in the external layer (Fig. 13). Perikarya located close to the posteroventral surface of the tuber were in a subependymal situation and occasionally sent a thin process reaching the ventricular cavity. Caudally, in the nucleus of the solitary tract, moderately ir-perikarya were observed with ventrally oriented axons (Fig. 14). A paramedian sagittal section of the brain of a larval *Pleurodeles* is illustrated in Fig. 18.

Adult Pleurodeles. The general distribution of the galaninergic system was similar to that described in larval *Pleurodeles*. However the density of ir-fibres was often much higher as in the ventral telencephalon, anteriorly to the recessus preopticus (Fig. 15), the ventral hypothalamus (Fig. 16), the median eminence (Fig. 17) and the optic tectum (Fig. 19). Despite the larger size of ir-perikarya, they were not observed in the striatum and their number in the vicinity of the preoptic recess and anterior commissure was still lower than in *Ambystoma*. Fibres

Fig. 19. GAL-immunoreactivity in the optic tectum (*TO*) of an adult *Pleurodeles*, often more abundant in the superficial layers (on the right side) without typical laminar structure. ×375

Figs. 20–25. GAL-like immunoreactivity in the brain of *Bufo*. Ventral direction on the left. ×375

Fig. 20. Ir-fibres (*arrows*) run close to the ependymal layer (*E*) in the dorsal (*D*) and ventral (*V*) walls of the recessus preopticus (*RPO*) but do not contact the cavity

Fig. 21. Dense network of ir-fibres in the amygdala (*Amy*) pars lateralis

Fig. 22. Anterior preoptic area (*APOA*) above the recessus preopticus with ir-perikarya (*arrows*) and fibres

Fig. 23. Anterior portion of the ventral hypothalamus (*AVH*) behind the optic chiasm, containing ir-perikarya (*arrows*)

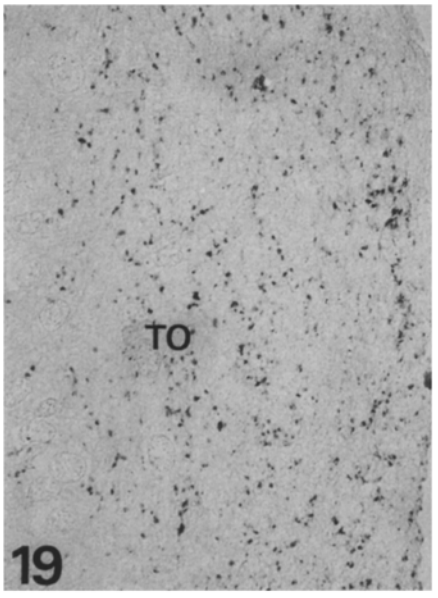
Fig. 24. Anterodorsal portion of the tuber cinereum (*TC*) along the recessus infundibularis (*RI*) with longitudinal fibres, ir-perikarya (*arrows*) and two contacts (*long arrows*) with the cavity

Fig. 25. Posterodorsal portion of the tuber cinereum: ir-perikarya (*arrows*) and long processes (*long arrows*) towards the recessus (*RI*)

Figs. 26, 27. GAL-like immunoreactivity in the brain of young *Xenopus*. Frontal sections. ×375

Fig. 26. Telencephalon: ir-perikarya (*arrows*) in the ventral striatum (*VSt*) and fibres (*long arrows*) running along the telencephalic ventricle (*TV*) in the lateral septum (*LSp*). *Dst*, Dorsal striatum

Fig. 27. Ir-fibres in the medial septum (*MSp*). *Arrowheads*, septal wall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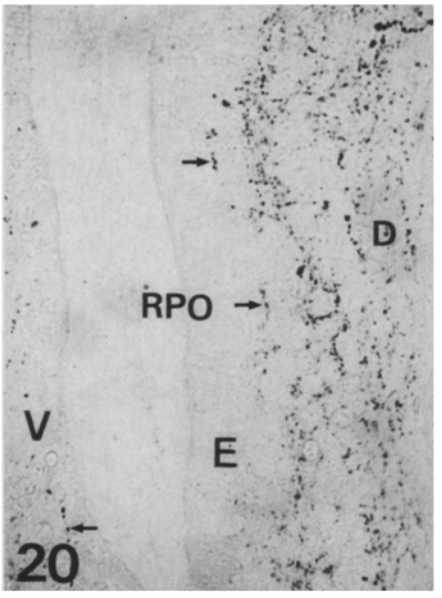
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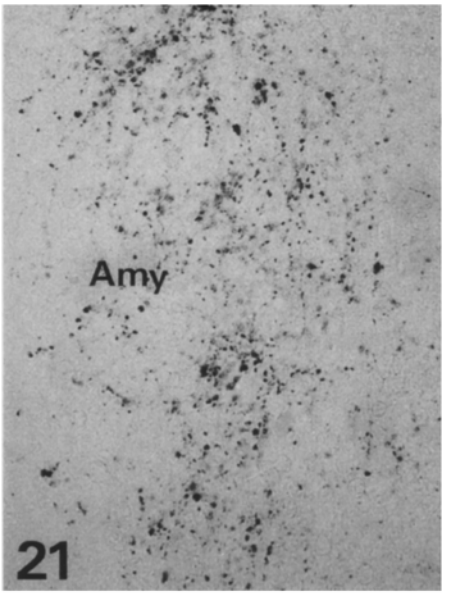
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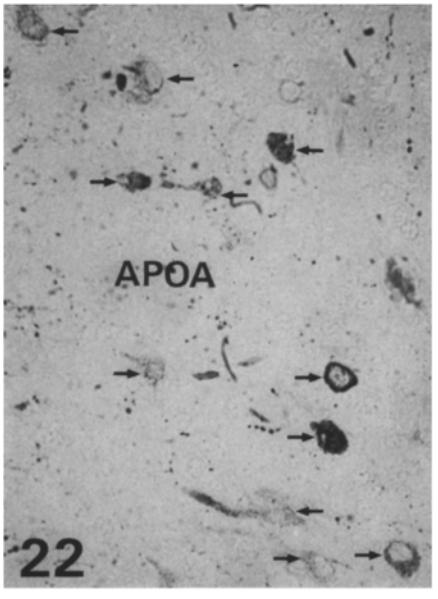
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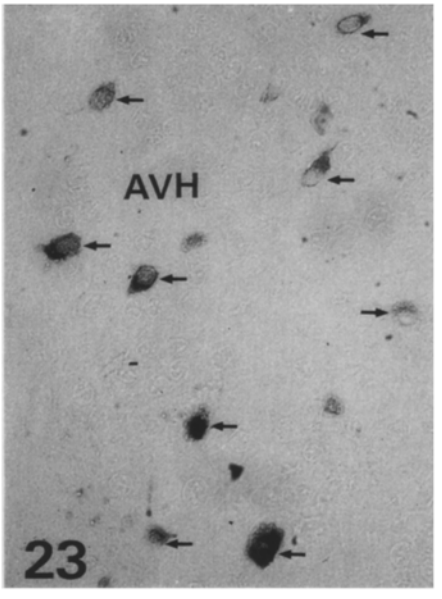
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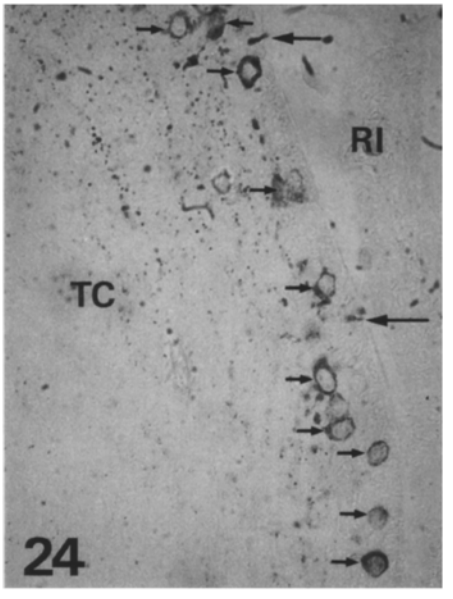
RI
TC
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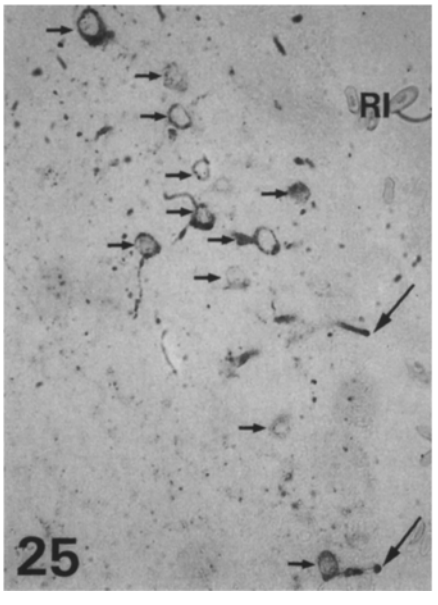
RI
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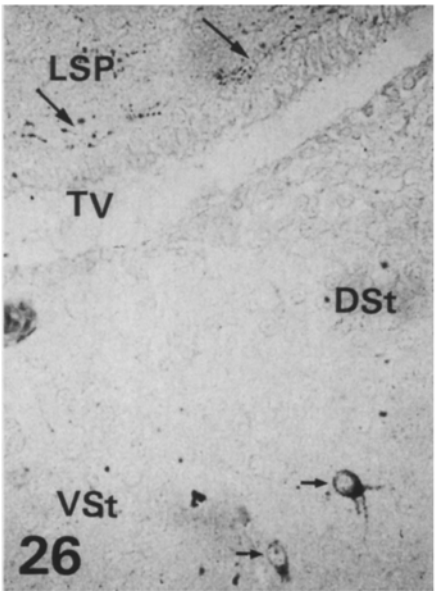
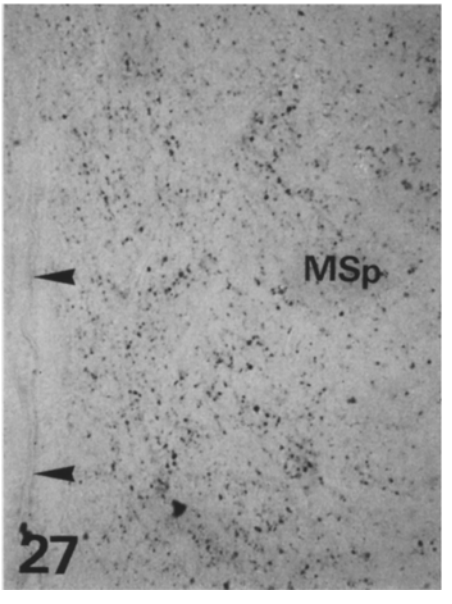

LSP
TV
DSt
VSt
26


MSp
27

with thick varicosities were not visible above the preoptic recess. Evident contacts with the CSF were still limited to cell bodies in the ventral hypothalamus (Fig. 16) but appeared more frequent than in young *Pleurodeles*. The predominant localization of GAL in the external layer of the median eminence was often more characteristic (Fig. 17) and the number of ir-fibres was also clearly increased in the neurohypophysis of adults. In the optic tectum, abundant fibres were observed in several layers, mainly in the superficial ones although they occurred more deeply (Fig. 19) but did not exhibit a typical laminar distribution. Fibres with larger varicosities were observed in the interpeduncular nucleus and the isthmus, and fine fibres in the torus semicircularis, tegmentum and rhombencephalon.

Bufo

Ir-fibres were observed in the ventral telencephalon up to the olfactory bulb, but the majority was located in the caudal portion, mainly in the lateral and medial septum. They were not detected in the various subdivisions of the pallium. They were more abundant close to the preoptic recess, but rarely entered the ependymal layer and then ran in parallel to the recess border, apparently without contact with the cavity (Fig. 20). Although ir-perikarya were rarely detected in the telencephalon, abundant ir-fibres were present in the amygdala (Fig. 21). Some unipolar cell bodies with strong or moderate labelling, occurred in the preoptic area along the rostral and anterodorsal wall of the preoptic recess (Fig. 22). In the preoptic area, fibres were coursing either ventrodorsally or longitudinally passing close to the optic chiasm in small parallel bundles. Perikarya were noted in the caudal region of the suprachiasmatic nucleus and fibres coursed along the third ventricle wall but did not contact the ventricular cavity. Other perikarya were observed in the lateral hypothalamus (Fig. 23), behind the optic chiasm, and the ventral tuber cinereum. Perikarya located along the infundibular recess occasionally contacted the CSF through a more or less elongated process (Figs. 24, 25). Most ir-fibres issued from the paraventricular organ ran rostrally, those in the ventral hypothalamus ran either rostrally, or turned ventrally and joined the ventral tract ending in the median eminence. Apparently axons also joined the ventral tract coursing posteriorly into the spinal cord. Ir-fibres did not appear to enter the neurohypophysis and adenohypophysial cells were not labelled. Ir-fibres were also present in the thalamus, under the habenula, in the interpeduncular nucleus, torus semicircularis, isthmus and spinal cord, either ventrally or dorsally, where they showed some larger varicosities. They were not detected in the habenula, cerebellum and were scarce in the optic tectum.

Xenopus

Juvenile Xenopus. In serial frontal sections, an extensive network of ir-fibres was detected in the following structures: lateral and medial septum (Figs. 26, 27), striatum, amygdala, preoptic area close to the preoptic recess, suprachiasmatic area, hypothalamus, forming two dense ventrolateral tracts, torus semicircularis, isthmus, ventral and dorsal rhombencephalon. Fibres were more abundant around the foramen of Munroe and amygdala, along the anterodorsal wall of the preoptic recess and in the posteroventral hypothalamus. Abundant ir-fibres passed along unlabelled cell bodies in the ventral hypothalamus. Occasional ir-fibres were observed in the medial and dorsal pallium and in some portions of the mesencephalon. In the optic tectum, ir-fibres were more abundant in the internal layers than in the dorsal ones. In the pituitary, ir-fibres were only observed in the pars distalis of a single animal. A few fibres were present in the neurohypophysis of all specimens. Adenohypophysial cells were unlabelled.

A small number of weakly ir-perikarya was observed in the lateral septum, with axons oriented towards the medial septum. Ir-perikarya with a variable immunostaining intensity occurred from the dorsal to the ventral striatum occasionally reaching the accumbens nucleus. They sent axons toward the periphery of the telencephalon. Fusiform cell bodies were more numerous in the amygdala pars medialis (Fig. 28) and extended close to the anterior and dorsal walls of the recessus preopticus. In these loci, contacts of fibres and perikarya with the CSF were not visible (Fig. 29).

Ir-fibres coursed either laterally or in parallel to the recess cavity and did not penetrate among ependymal

Figs. 28–33. GAL-like immunoreactivity in the brain of young *Xenopus*. Frontal sections. × 375

Fig. 28. Ir-perikarya in the amygdala pars medialis (*Amy*). *Arrowhead*, direction of the third ventricle

Fig. 29. Ir-perikarya (*arrows*) along the recessus (*RPO*) and fibres running in parallel to the recess wall, under the ependymal layer, but not contacting the cavity

Fig. 30. Lower part (on the left) of the recessus (*RPO*) surrounded by fibres (*F*) not contacting the recess cavity

Fig. 31. Posterior region of the tuber cinereum (*TC*); *arrowhead*, lateral sulcus. Presence of ir-perikarya (*arrows*) and ir-fibres surrounding unlabelled cell bodies, without contact with the recess cavity

Fig. 32. Posterior region of the tuber with ir-perikarya (*long arrows*), fibres and ir-processes (*arrows*) in direct contact with the recess cavity (*RI*)

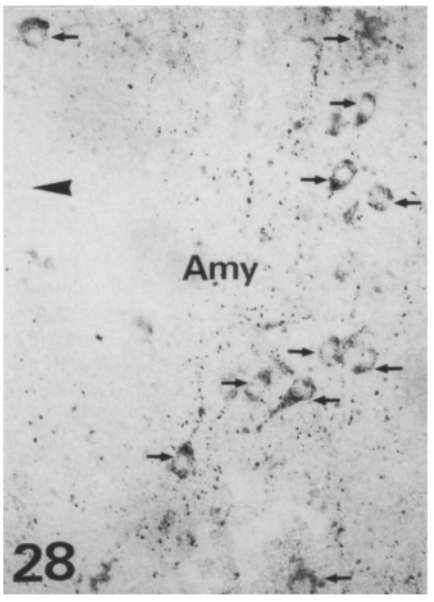
Fig. 33. Median eminence (*ME*) with an ir-perikarya (*long arrow*) and accumulation of ir-fibres in the internal layer (*ILa*) and mostly in the external layer (*ELa*). *RI*, Recessus infundibularis

Fig. 34. Posterodorsal portion of the tuber cinereum with abundant ir-fibres, ir-perikarya and some processes (*arrows*) in contact with the infundibular cavity (*RI*)

Figs. 35–36. GAL-like immunoreactivity in the brain of adult *Xenopus*. Sagittal sections. × 375

Fig. 35. Ir-fibres in the torus semicircularis (*TS*). Optic ventricle on the right

Fig. 36. Ir-fibres in the tectum opticum (*TO*) mainly in the superficial layers (on the right)


Amy
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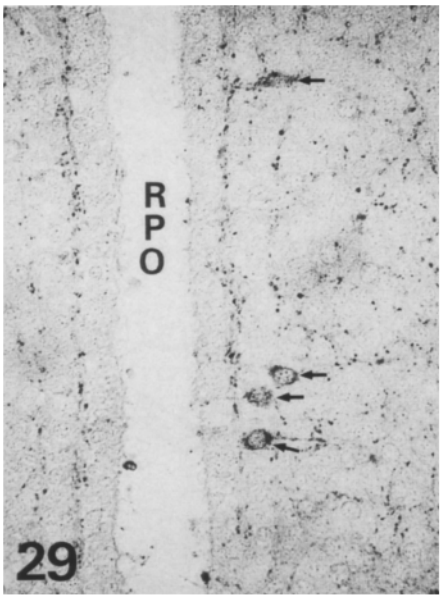
RPO
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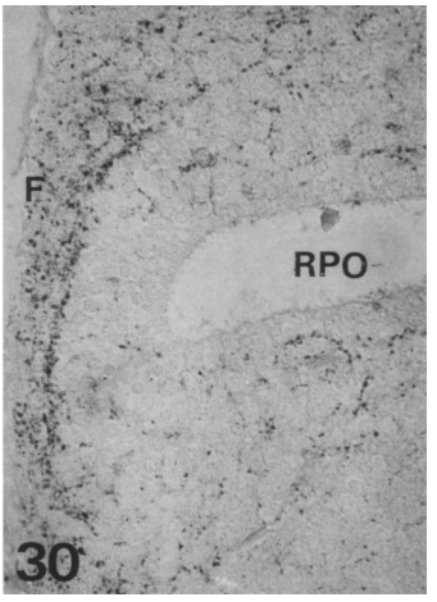
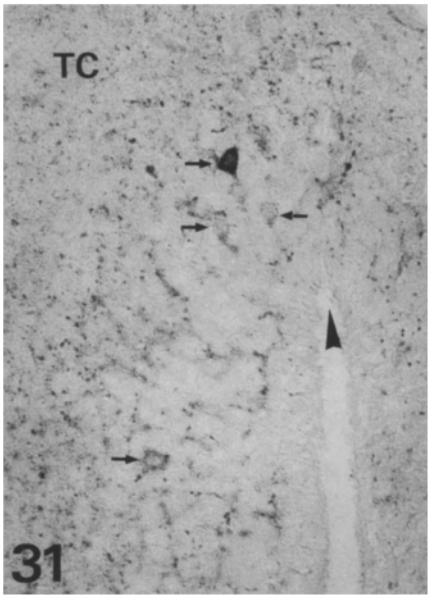
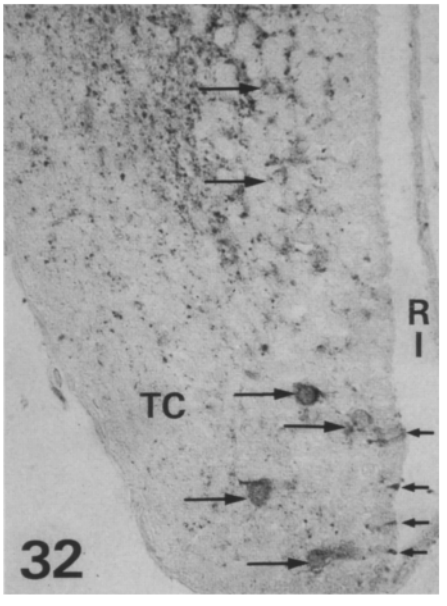
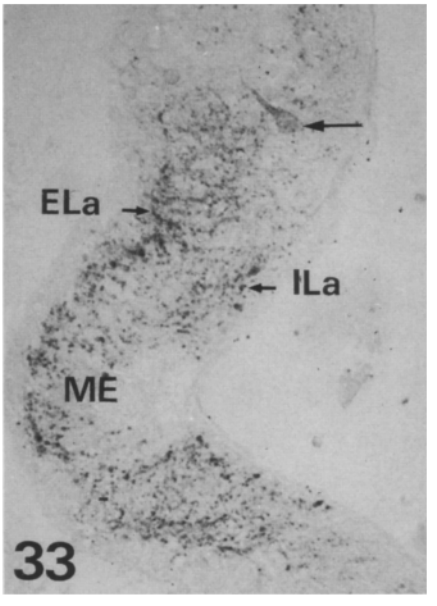
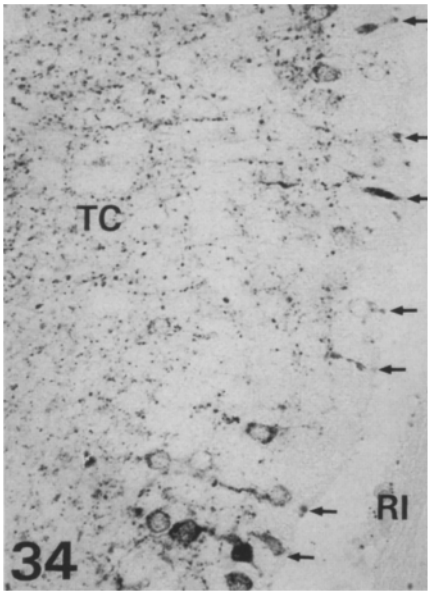
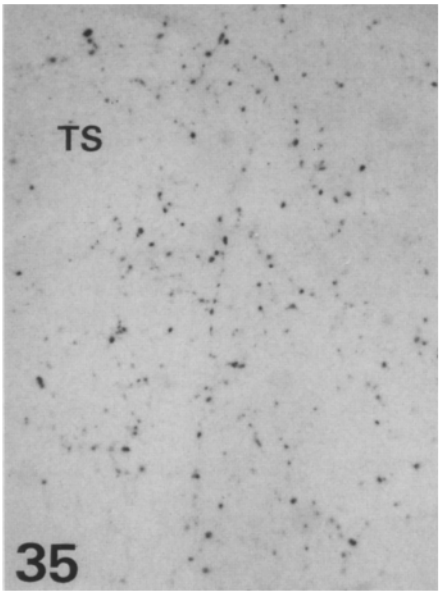

F
RPO
30

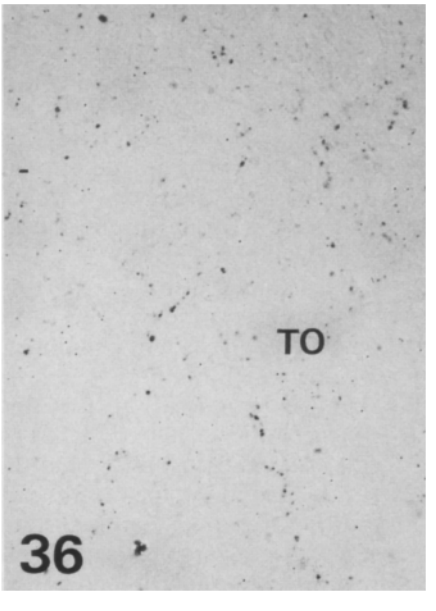

TC
31


RI
TC
32


ELa
ILa
ME
33


TC
RI
34


TS
35


TO
36

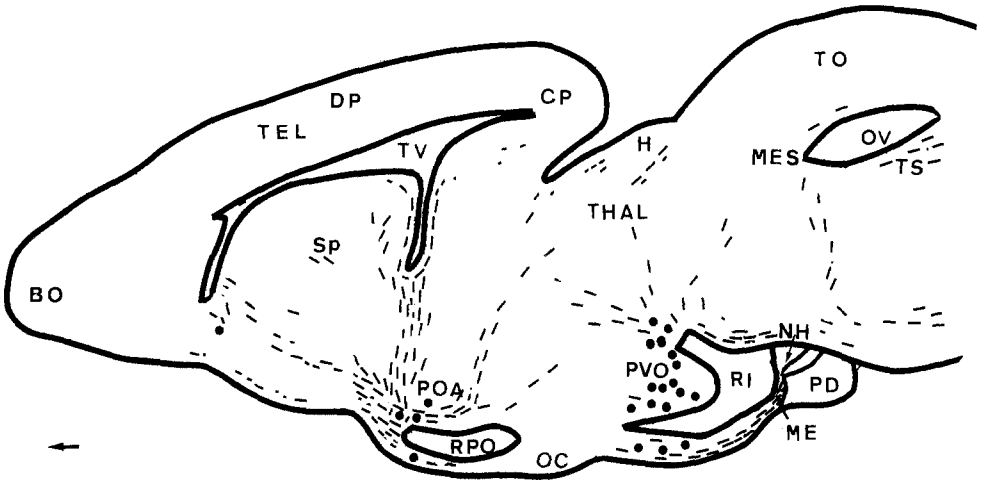


Fig. 37. Immunoreactive GAL-like system in the telencephalon, diencephalon and mesencephalon of young *Xenopus*. Line drawing of a paramedian sagittal section. *Dots*, GAL-like perikarya; *dashed lines*, tracts of GAL-like fibres; *arrow*, rostral direction. Abbreviations as given in the list

cells, even in the ventral part of the recessus preopticus (Fig. 30). Ir-perikarya also occurred in the tuber cinereum and in the paraventricular organ and around the lateral sulcus (Fig. 31). These neurons sent long axons towards the lateral tuber. In the ventral hypothalamic nucleus, perikarya showed immunostaining of variable intensity. Some perikarya contacted the CSF through a short process (Fig. 32). A few perikarya were observed close to the median eminence. Numerous ir-fibres ended in the external and internal zones of the median eminence (Fig. 33). Caudally, various tracts of ir-fibres coursed through the rhombencephalon and some perikarya were observed in the nucleus of the solitary tract. The distribution of GAL-like immunoreactivity is illustrated with schematic drawings of longitudinal section (Fig. 37) or frontal sections through the telencephalon (Figs. 38 A–D), the preoptic area (Figs. 38 E–G), the optic chiasm (Fig. 38 H), the tuber cinereum, the mesencephalon (Figs. 38 I–L) and the rhombencephalon (Figs. 38 M, N).

Adult Xenopus. The general distribution of the galaninergic systems in the brain was similar to that described in juvenile *Xenopus*, although fibres appeared more abundantly and the immunostaining of perikarya was often more intense. Ir-fibres were observed in the ventral telencephalon, close to the anterior commissure and the amygdala, in the preoptic and suprachiasmatic nuclei, ran in fine parallel tracts above the optic chiasm and formed a dense network around the infundibular recess in the ventral tuber (Fig. 34). Fibres of the two lateral tracts seemed to end at least in part in the median eminence. In some sections only, rare ir-fibres were detected in the neurohypophysis and glandular cells of the pituitary were unstained. Ir-fibres were noted under the ventral habenular ganglion, in the torus semicircularis (Fig. 35), the optic tectum (Fig. 36) mainly in internal and medial layers, the isthmus, and the rhombencephalon, ventrally and along the central canal.

In addition to weakly immunostained cell bodies in the striatum, ir-perikarya were mainly located in the posterior region of the anterior commissure, the preoptic area close to the recess and along the third ventricle, the suprachiasmatic nucleus, the lateral and ventral hypothalamus and the paraventricular organ. Neurons close to the infundibular recess were elongated and showed axons or short processes oriented towards the cavity. Contacts of GAL-perikarya with the CSF (Fig. 34) appeared more frequenly than in juvenile *Xenopus*, but were still limited to cell bodies in the posteroventral tuber. A paramedian sagittal section of the brain of adult *Xenopus* (Fig. 39) summarizes the distribution of the galaninergic-like peptide. Data obtained on the localization of the GAL-like systems in the four species are compared in the Table 1.

Discussion

The immunocytochemical study of the brain of four species of amphibia reveals a similar distribution of GAL-like fibres and perikarya: (i) perikarya around the rostrodorsal wall of the recessus preopticus, extending rostrally around the anterior commissure and in the amygdala and suprachiasmatic nucleus; (ii) abundant perikarya in the ventral hypothalamus; and (iii) some perikarya in the nucleus of the solitary tract. GAL fibres belonged to several tracts (preoptico-amygdalian, preoptico-neurohypophysial, preoptico-habenular, ventro-hypothalamo-neurohypophysial) although these need to be confirmed by retrograde tracing.

Comparison of the description of the four species with that of *R. esculenta* and *X. laevis* (Lazar et al. 1991) shows some differences, which may be related to the various technical procedures used. In Lazar's study, some animals were given an intraventricular injection of colchicine 48 h before they were deeply anaesthetized, perfused and fixed in paraformaldehyde. Most brains were cut either with a vibratome (30- to 40-μm-thick sections) or after embedding in Polywachs 1000 (20-μm-thick sections). Antiserum dilution varied from 1/2000 to 1/4000. In most cases, immunostaining was followed by a silver or nickel intensification procedure. However specific effects of colchicine, Polywachs versus non em-

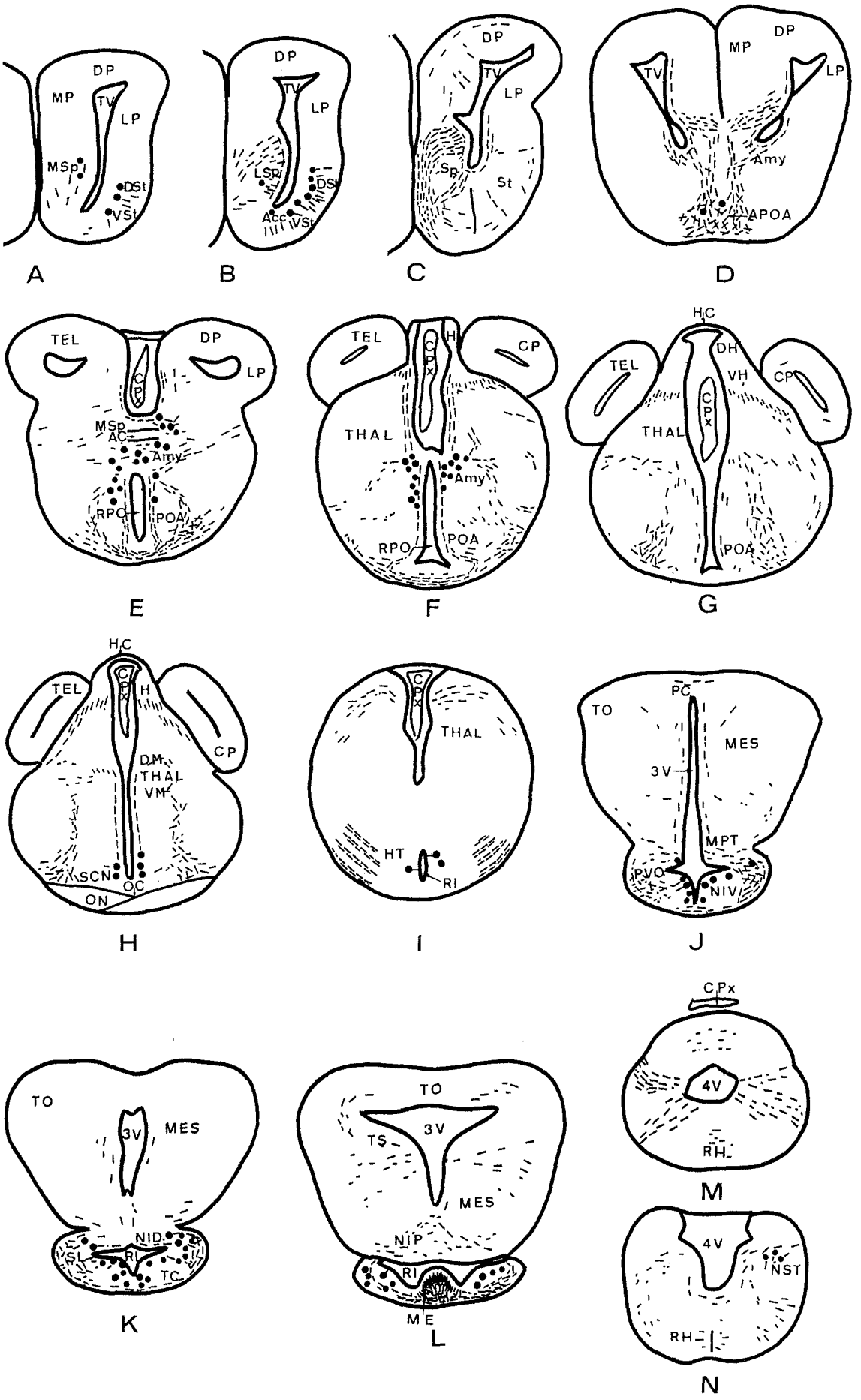


Fig. 38 A–N. Immunoreactive GAL-like system in the brain of young *Xenopus*. Line drawings of frontal sections through **A–D** telencephalon, **D–G** preoptic area, **H–L** hypothalamus and **M**, **N** rhombencephalon. *Dots*, GAL-like perikarya, *dashed lines*, tracts of GAL-like fibres

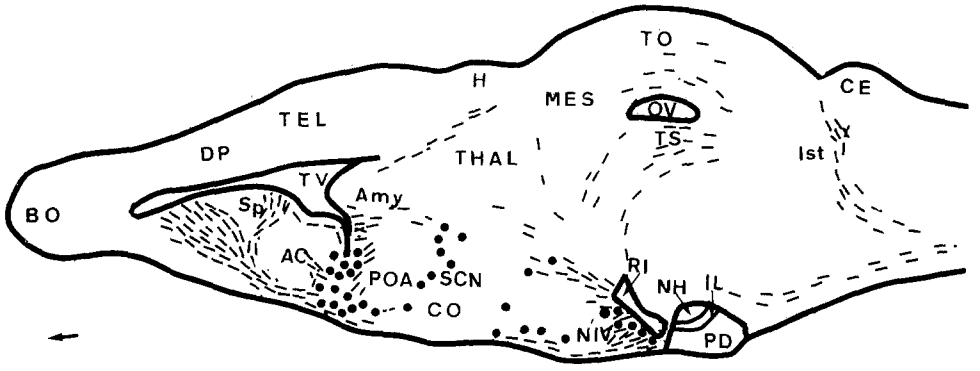


Fig. 39. Immunoreactive GAL-like system in the brain of adult *Xenopus*. Line drawing of a lateral sagittal section. *Dots*, GAL-like perikarya; *dashed lines*, tracts of GAL-like fibres; *arrow*, rostral direction. Abbreviations as given in the list

Table 1. Distribution of galanin-like immunoreactivity[a] in the brain and pituitary of four adult amphibian species

	Urodela				Anura			
	Ambystoma		*Pleurodeles*		*Bufo*		*Xenopus*	
	Perikarya	Fibres	Perikarya	Fibres	Perikarya	Fibres	Perikarya	Fibres
Telencephalon:								
Pallium	−	(+)	−	+	−	−	−	+
Septum	−	++	−	+	−	+	+	++
Striatum	+	++	−	+	−	+	+	++
Amygdala	+++	+++	++	++	+	+	+++	+++
Diencephalon:								
Preoptic area	+++	+++	++	+++	++	++	+++	+++
CSF-contacting type	+		−		−		−	
Thick fibres		++		−		−		−
Fine fibres		++		++		++		++
Suprachiasmatic nucleus	+	+	+	++	+	+	+	+
Habenula	−	−	−	−	−	−	−	−
Thalamus	−	+	−	+	−	+	(+)	+
Infundibulum	+++	+++	+++	+++	++	+++	+++	+++
CSF-contacting type	+		+		+		+	
Median eminence	−	+++	−	+++	−	+++	−	+++
Pituitary cells	−	−	−	−	−	−	−	−
Neurohypophysis	−	+	−	++	−	−	−	+
Mesencephalon:								
Optic tectum	−	+	−	++	−	(+)	−	++
Torus semicircularis	−	+	−	+	−	+	−	+
Isthmus	−	++	−	++	−	+	−	++
Tegmentum	−	+	−	++	−	+	−	+
Nucleus interpeduncularis	−	+	−	+	−	+	−	+
Metencephalon:								
Cerebellum	−	−	−	−	−	−	−	−
Rhombencephalon:								
Medulla oblongata	−	++	−	++	−	++	−	++
Nucleus of the solitary tract	+	++	+	+	(+)	+	+	++

[a] −, No; (+), very scarce; +, few; ++, medium; +++, many immunoreactive perikarya or fibres

bedded material, and the intensification procedure are not commented upon. In *Xenopus*, ir-perikarya were not seen in sections stained with the conventional PAP technique. A few very pale cells were occasionally seen following silver intensification (Lazar et al. 1991). In contrast, ir-perikarya of the present study were observed in young and adult *Xenopus* without colchicine pretreatment and although brains were fixed by immersion only, embedded in paraffin and stained without an intensification procedure on much thinner sections.

In 5-μm-thick paraffin sections, scattered pale perikarya were present in the striatum of *Ambystoma* and *Xenopus* as well as in *Triturus carnifex* (Mulatero et al. 1991) but were not detected in *Rana* and *Xenopus* (Lazar

et al. 1991), *Bufo* and *Pleurodeles*. In agreement with Wolfbauer and Skofitsch (1989) ir-fibres were present in the dorsal and ventral striatum, the nucleus accumbens, in the species presently studied. In the medial and lateral septal areas, ir-fibres were abundant in the four species and in *R. esculenta* (Lazar et al. 1991), *R. temporaria* (Wolfbauer and Skofitsch 1989) and the newt, but occasional cell bodies were detected in the newt (Mulatero et al. 1991) and *Xenopus* (present study). Perikarya were observed in the amygdala, suprachiasmatic nucleus and preoptic area in *R. esculenta* (Lazar et al. 1991), *R. temporaria* (Wolfbauer and Skofitsch 1989; Horsten and Vandesande 1991), *Triturus* (Mulatero et al. 1991) and four other species (present data). Similar concordant observations were reported in these same species on the localization of ir-fibres and perikarya in the ventral and lateral hypothalamus, infundibular nuclei and nerve terminals in the median eminence by the same authors.

The recessus preopticus and the anterior preoptic area containing ir-perikarya belonged to the ventral diencephalon (e.g. Neary and Northcutt 1983) although they were considered as part of the telencephalon in *Rana* (Lazar et al. 1991). Variations were more apparent in the distribution of ir-fibres in the mesencephalon and optic tectum: a laminar distribution in several layers (mainly internal) of the optic tectum in *R. esculenta*, scattered fibres without stratification in *Xenopus* (Lazar et al. 1991), and presence in *R. temporaria* (Horsten and Vandesande 1991). In *Ambystoma* ir-fibres occurring in the optic tectum were not restricted to specific layers and did not show a characteristic laminar distribution. Ir-fibres were more numerous in the optic tectum of *Pleurodeles* and *Xenopus* and occasionally visualized in *Bufo*. They were predominantly located in the internal layers in *Xenopus*. Very pale perikarya were described in the optic tectum, tegmentum and torus semicircularis of *R. esculenta* (Lazar et al. 1991), but were not observed in the four species studied. Perikarya were not detected in the thalamus of *R. esculenta* (Lazar et al. 1991) and *R. temporaria* (Horsten and Vandesande 1991). However they were described in this latter species (Wolfbauer and Skofitsch 1989), in the newt (Mulatero et al. 1991), and in adult *Xenopus* (present work) a few perikarya were located close to and in the ventral thalamus.

Ir-fibres were scattered in the neurohypophysis of *R. esculenta* (Lazar et al. 1991) and *Ambystoma*. They appeared more abundant in adult *Pleurodeles*, scarce in young *Pleurodeles* and young or adult *Xenopus* and seemed to be absent in *Bufo*. As these four species were submitted to identical procedures, these minor differences seem to reflect a species specificity rather than an artefact. The abundance of ir-fibres may also vary according to the physiological condition of the animals. In a single young *Xenopus*, isolated fibres were observed in the pars distalis, along the intermediate lobe, in an area rich in growth hormone-secreting cells. The absence of immunoreactivity in the adenohypophysial cells and the cerebellum is common to *R. esculenta* and the four species studied. As regards the dorsal and ventral habenular ganglia, ir-fibres were described in *R. temporaria* (Wolfbauer and Skotfitsch 1989) but were not observed in the present four species and *R. esculenta* (Lazar et al. 1991). Ir-perikarya and ir-fibres in the nucleus of the solitary tract were seen in four species (present study) and in *Rana* by Lazar et al. (1991) who observed ir-fibres only in *Xenopus*.

Some variability was also noted in the frequency of cells contacting the CSF. Around the recessus preopticus, ir-perikarya often contacted the CSF, either directly or through a short or elongated process as in *Rana* (Lazar et al. 1991) and *Ambystoma*. The contacts were not evident at this level in *Pleurodeles*, *Bufo* and *Xenopus*. Peripheral perikarya along the posterior wall of the infundibular recess clearly contacted the CSF in all species studied so far. However, in late metamorphosing *Pleurodeles* and juvenile *Xenopus*, cells of the CSF-contacting type were less frequent than in adults. These contacts suggest that GAL may be released in the CSF in amphibia as in human subjects (Berrettini et al. 1988).

Comparison with the galaninergic system of fish

In both Anura and Urodela, a high density of ir-perikarya occurs in the tuber and along the infundibular recess. In teleost fish, only scattered and moderately immunostained perikarya were observed in the posteroventral hypothalamus. Close to the recessus preopticus, intensely immunostained nuclei, which strongly react to the administration of sex steroids (Olivereau and Olivereau 1991 b), are mixed with smaller and less strongly labelled perikarya. A similar heterogeneity of immunoreactivity is also obvious in amphibian preoptic perikarya.

Ir-perikarya have been described in the magnocellular cells in the preoptic area of the dogfish (Vallarino et al. 1991) as in the rat (Arai et al. 1990), but were not observed in teleosts (Batten et al. 1990; Olivereau and Olivereau 1991 a; Holmqvist and Ekström 1991), *Rana* (Lazar et al. 1991) and in the present work. In a selachian *Scyliorhinus canicula*, ir-perikarya around the preoptic and the posterior recesses can contact the CSF (Vallarino et al. 1991) as in amphibia. In contrast, ir-cells of the preoptic and posterior hypothalamus never made contact with the ventricular cavities in teleosts (Olivereau and Olivereau 1991 a). Ir-fibres also showed some different localizations, for example presence in the teleost habenula, and absence in amphibia and some variability in the optic tectum: presence in amphibia, *Anguilla* (Olivereau and Olivereau 1991 a) and absence in salmon (Holmqvist and Ekström 1991). Sex steroid hormones stimulate the preoptic galaninergic system of *Anguilla* (Olivereau and Olivereau 1991 b). The abundance of GAL in adult *Pleurodeles* and *Xenopus* compared to juveniles may be related in part to the development of the gonadal activity.

Teleost fish do not posses a classical median eminence similar to that of tetrapodes. The neural digitations penetrating the adenohypophysial subdivisions are homologous to an internalized median eminence. These neural ramifications contain some galaninergic fibres in the neurohypophysis. Adenohypophysial cells (intermediate lobe and pars distalis) were never labelled, even after

treatment with sex steroids (Olivereau and Olivereau 1991 b). Amphibia resemble teleosts, showing scarce neurohypophysial ir-fibres and immunonegative cells in the pituitary.

Comparison with the galaninergic system in mammals

The amphibian pituitary clearly differs from that of mammals, which possesses a rich galaninergic innervation of the neural lobe and a positive staining in several cell types (review in Olivereau and Olivereau 1991 b). In rats, a combined retrograde tracing and immunohistochemical study showed that 56–60% of the fibres projecting to the neurohypophysis arose from the supraoptic nucleus, 18–23% from the retrochiasmatic nucleus, and 8–10% from the lateral magnocellular portion of the paraventricular nucleus (Arai et al. 1990). Although these data cannot be extended to *Ambystoma*, they suggest that some of the fine fibres reaching the median eminence arise from the suprachiasmatic and preoptic areas. The proportion of fibres issued from the ventral tuber cannot be evaluated. In *Ambystoma* perikarya around the preoptic recessus gave rise to thick fibres running ventrodorsally which were quite easy to follow. A high percentage appeared to end close to the amygdalian region, but did not seem to reach the median eminence. Such a distinction of two different pathways has not been possible in *Pleurodeles*, *Bufo* and *Xenopus* in which most fibres possess varicosities of a similar diameter.

Role of the galanin-like peptide and possible interactions

Various actions of GAL are demonstrated in mammals (review in O'Halloran et al. 1990; Vrontakis et al. 1991) and some effects reported in *Bufo* and *Necturus* (see the Introduction). The extensive network of galaninergic fibres often pass along unlabelled perikarya, which may be influenced by axosomatic or axo-axonal contacts through synaptic or non-synaptic structures. The anterior preoptic area, mainly around the recessus preopticus, contains a large number of perikarya labelled with antisera to neurotransmitters such as monoamines (Terlou and Ploemacher 1973; Prasada Rao and Hartwig 1974) and their enzymes (tyrosine hydroxylase: Franzoni et al. 1986; Gonzalez and Smeets 1991). Neurons also react with antisera to neuropeptides: alpha- and beta-endorphins (Doerr-Schott et al. 1981), alpha-melanocyte-stimulating hormone (Benyamina et al. 1986), melanin-concentrating hormone (Andersen et al. 1986), calcitonin gene-related peptide (Wolfbauer and Skofitsch 1989; Mulatero and Fasolo 1991), somatostatin (Vandesande and Dierickx 1980), corticotropin-releasing factor (Fasolo et al. 1984; Tonon et al. 1985; Olivereau et al. 1987), gonadotropin-releasing hormone (Andersen et al. 1988; Chieffi et al. 1991), thyrotropin-releasing hormone (TRH; Seki et al. 1983; Mimnagh et al. 1987; Lamacz et al. 1989) and TRH mRNA (Zoeller and Conway 1989) in addition to GAL. Furthermore, this particular region contains cells concentrating ^{3}H-labelled testosterone and/or ^{3}H-labelled estradiol in *Xenopus* (Kelley et al. 1975; Morrell et al. 1975), *R. pipiens* (Kelley et al. 1978) and *R. esculenta* (Di Meglio et al. 1987).

The posteroventral hypothalamus is another site where numerous perikarya contain monoamines (Terlou and Ploemacher 1973; Prasada Rao and Hartwig 1974), tyrosine hydroxylase (Gonzalez and Smeets 1991), neuropeptide Y (Danger et al. 1985; Perroteau et al. 1988), atrial natriuretic factor (Netchitailo et al. 1987), proenkephalin (Merchenthaler et al. 1989), substance P (Gaudino and Fasolo 1980), corticotropin-releasing factor (Fasolo et al. 1984; Olivereau et al. 1987), TRH (Lamacz et al. 1989) and GAL. These perikarya are often located in the paraventricular organ. In addition, in the posterodorsal region of the ventral hypothalamus, perikarya which may contact the CSF contain somatostatin (Vandesande and Dierickx 1980; Blähser et al. 1982; Olivereau et al. 1987), a gastrin-like peptide (Doerr-Schott et al. 1979), galanin (Lazar et al. 1991; present work) and sex steroid hormone-concentrating cells (Morell and Pfaff 1978; Di Meglio et al. 1987).

Although double immunostaining with antisera to neurotransmitters or neuropeptides and anti-galanin have not been applied to the amphibian brain, various co-localizations appear conceivable. Moreover, the high density of ir-fibres suggest an interaction between galaninergic axons and these neurotransmitter- and neuropeptide-containing perikarya. A large number of ir-fibres and axonal endings were observed in the median eminence where numerous ir-peptidergic fibres were also demonstrated as in *R. catesbeiana* (Yui 1983) and mix with fibres arising from the preoptic nucleus containing vasotocin and mesotocin. Some cases of co-localization have been reported such as TRH and mesotocin in *R. catesbeiana* (Shioda et al. 1989) and *R. ridibunda* (Lamacz et al. 1989) in nerve endings of the neurointermediate lobe. It seems evident that complex interferences among these neurotransmitters and neuropeptides occur during their release into portal capillaries.

Ir-GAL is co-localized with a large number of neuropeptides in various nuclei of the mammalian brain as reviewed elsewhere (Olivereau and Olivereau 1991 a). Coexistence of CRF and GAL in some cells of the anterior preoptic area has been described in cyprinodont fish only (Batten et al. 1990) among teleost species studied so far. In *R. esculenta*, there is an extensive overlap between the distribution of GAL and several other peptides in some layers of the optic tectum, but the identification of GAL-ir perikarya in this region requires further confirmation (Lazar et al. 1991). A paracrine or autocrine action of GAL has been proposed in rat and human pituitaries as GAL coexists with several hypophysal hormones (O'Halloran et al. 1990; Vrontakis et al. 1991). According to the available anatomical data, such a paracrine or autocrine influence is not apparent either in teleosts (Olivereau and Olivereau 1991 a) or in amphibia. GAL released in the median eminence and to a much reduced extent in the neurohypophysis can reach pituitary cells only through a vascular route.

Conclusion

Results of the present study demonstrate the existence of a galaninergic-like system in the brain of two Urodela and two Anura, showing some similarities with that previously described in several teleost species. The extensive distribution of galanin in the brain, mainly the hypothalamus, suggests that GAL may play a role of neuromodulator and/or neurotransmitter in amphibia. As GAL is not detected in adenohypophysial cells, it does not seem to act on the pituitary through a paracrine or autocrine route.

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