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Distribution of Neuropeptide Y-Like Immunoreactivity in the Brain of the Lizard *Gallotia galloti*

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ABSTRACT

The distribution of neuropeptide Y (NPY)-like immunoreactivity was studied in the brain of the lizard *Gallotia galloti,* in order to gain insight into the comparative topography of this peptide. Antisera against both NPY and its C-terminal flanking peptide (C-PON) were used, demonstrating a general coexistence of both peptides, as described in other vertebrates.

Most NPY-like immunoreactive (NPY-LI) cell bodies were observed in the telencephalon, specifically in various olfactory structures, all cortices, septum, basal ganglia (except for the globus pallidus), the nucleus of the diagonal band of Broca, the amygdaloid complex, and the bed nucleus of the anterior commissure. NPY-LI cells were also seen in the preoptic and hypothalamic regions and the dorsal thalamus (mainly in the perirotundal belt), as well as in the mesencephalic tegmentum (in the ventral tegmental area, the substantia nigra, and the retrorubral area). NPY-LI fibers and terminals were widely distributed in the brain. All visual and auditory neuropiles were densely innervated. Specially dense plexuses were seen in the nucleus accumbens, the ventral pallidum, the suprachiasmatic and ventromedial hypothalamic nuclei, the nucleus medialis thalami, the left habenula, and the central nucleus of the torus semicircularis.

Our analysis shows that the distribution of NPY-like immunoreactivity in the forebrain of *Gallotia* largely resembles that of other vertebrates, whereas differences are mainly observed in the brainstem. The widespread distribution of NPY in the lizard brain suggests several modulatory functional roles, either in local-circuit systems of the forebrain, or in various limbic, neuroendocrine, and sensory pathways. © 1992 Wiley-Liss, Inc.

Key words: C-PON, cortex, basal ganglia, visual centers, asymmetry, reptiles

Neuropeptide Y (NPY) is a member of the pancreatic polypeptide family that was first isolated and sequenced by Tatemoto et al. (Tatemoto, '82; Tatemoto et al., '82). The extensive distribution of NPY, together with data obtained by pharmacological studies, suggests that this peptide may play a role as a neurotransmitter or neuromodulator in the brain, and may regulate feeding behavior, luteinizing hormone releasing hormone release, sexual behavior, and vascular control (Gray and Morley, '86). Moreover, it may affect the electrophysiological properties of central catecholaminergic neurons (Illes and Regenold, '90).

The distribution of this peptide has been described in the mammalian brain (Adrian et al., '83; Allen et al., '83; Chronwall et al., '85; De Quidt and Emson, '86; Bons et al., '90; Walter et al., '90), and in the brain of various nonmammalian vertebrates (fishes: Vallarino et al., '88; Noe et al., '89; Pontet et al., '89; amphibians: Danger et al., '85; Cailliez et al., '87; birds: Anderson and Reiner, '90; Lorenz and Skofitsch, '90; Aste et al., '91). However, knowledge of the presence of NPY immunoreactivity in the reptilian central nervous system is limited (Reiner and Oliver, '87; Martí et al., '90; Dávila et al., '91).

The main goal of the present study is to provide a complete mapping of the distribution of NPY in the brain of a lizard, *Gallotia galloti.* We have analyzed in detail similarities and differences in the distribution of NPY immunore-

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active structures in lizards and other vertebrates, aiming to gain insight into the phylogeny of this peptidergic system.

MATERIALS AND METHODS

Tissues and fixation

Twenty adult specimens of *Gallotia gallotia* (both male and female) were deeply anesthetized with ether and transcardially perfused with 0.1 M, pH 7.4, phosphate-buffered saline (PBS), followed by 150–200 ml of 4% paraformaldehyde solution in 0.1 M, pH 7.4, phosphate buffer (PB). Brains were removed and postfixed in the same solution for 2 hours at 4°C. Tissues were then rinsed and cryoprotected in 0.1 M PB with increasing concentrations of sucrose (10%, 15%, and 30%) during 24 hours at 4°C, and finally frozen in isopentane cooled in liquid N_2. Transverse,

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Abbreviations

Acc	nucleus accumbens
Alh	area lateralis hypothalami
Amb	nucleus ambiguus
Amc	nucleus centralis amygdalae
Ame	nucleus externus amygdalae
Aml	nucleus lateralis amygdalae
Apol	area preoptica lateralis
AT	area triangularis
Bca	bed nucleus of the commissura anterior
Bmfb	bed nucleus of the medial forebrain bundle
Bst	bed nucleus of the stria terminalis
ca	commissura anterior
Cer	cerebellum
Cerl	nucleus cerebellaris lateralis
cl	cellular layer
Cmam	corpus mamillare
CP	cell plate of Unger
cp	cellular plate
cp*	commissura posterior
cpa	commissura pallii anterior
cpp	commissura pallii posterior
Cxd	cortex dorsalis
Cxla	cortex lateralis anterior
Cxlad	cortex lateralis anterior, pars dorsalis
Cxlav	cortex lateralis anterior, pars ventralis
Cxlp	cortex lateralis posterior
Cxml	cortex medialis, large-celled part
Cxms	cortex medialis, small-celled part
Dlh	nucleus dorsolateralis hypothalami
Dlm	nucleus dorsolateralis thalami, pars magnocellularis
Dlp	nucleus dorsolateralis thalami, pars parvocellularis
Dm	nucleus dorsomedialis thalami
DVR	dorsal ventricular ridge
EW	nucleus of Edinger-Westphal
flm	fasciculus longitudinalis medialis
fr	fasciculus retroflexus
GC	griseum centrale
Glv	nucleus geniculatus lateralis, pars ventralis
Gld	nucleus geniculatus lateralis, pars dorsalis
GP	globus pallidus
GT	griseum tectalis
Habl	nucleus lateralis habenulae
Habm	nucleus medialis habenulae
Ico	nucleus intercollicularis
IIId	nucleus oculomotorius dorsalis
IIIv	nucleus oculomotorius ventralis
Ipd	nucleus interpeduncularis dorsalis
ipl	inner plexiform layer
Ipv	nucleus interpeduncularis ventralis
Ism	nucleus isthmi, pars magnocellularis
Isp	nucleus isthmi, pars parvocellularis
IV	nucleus trochlearis
Jm	nucleus juxtacommissuralis medialis
Lc	locus coeruleus
Le	nucleus lentiformis thalami, pars extensa
lfb	lateral forebrain bundle
lfbd	lateral forebrain bundle, dorsal peduncle
lfbv	lateral forebrain bundle, ventral peduncle
Ll	nucleus lemnisci lateralis
Lm	nucleus lentiformis mesencephali
ln	lateral neuropile
Lp	nucleus lentiformis thalami, pars plicata
mfb	medial forebrain bundle
mn	medial neuropile
Mp	nucleus medialis posterior
Mt	nucleus medialis thalami
Ncpp	nucleus of the commissura pallii posterior
Ncsva	nucleus anterior of the commissura supraoptica ventralis
Ncsvp	nucleus posterior of the commissura supraoptica ventralis
NdB	nucleus of the diagonal band of Broca
Nflm	nucleus of the fasciculus longitudinalis medialis
nIII	nervus oculomotorius
nIV	nervus trochlearis
Noa	nucleus olfactorius anterior
Nsa	nucleus septalis anterior
Nsd	nucleus septalis dorsalis
Nsi	nucleus septalis impar
Nsl	nucleus septalis lateralis
Nsm	nucleus septalis medialis
Nsph	nucleus sphericus
nVIII	nervus vestibulocochlearis (octavus)
O	nucleus ovalis
opl	outer plexiform layer
oph	organon periventriculare hypothalami
Opt	nucleus opticus basalis
osc	organon subcommissurale
OT	optic tract
Pb	parabrachial region
pd	fasciculus predorsalis
Pd	nucleus posterodorsalis
Ph	nucleus periventricularis hypothalami
Phg	nucleus prepositus hypoglossi
Pl	Purkinje cell layer
Ppo	periventricular preoptic area
Prd	nucleus pretectalis dorsalis
Prmc	nucleus profundus mesencephali, pars caudalis
Prmr	nucleus profundus mesencephali, pars rostralis
pRot	perirotundal belt
Prv	nucleus pretectalis ventralis
Pth	pallial thickening
RA	retrorubral area
Ra	nucleus raphe
Ras	nucleus raphe superior
Riv	nucleus reticularis inferior, pars ventralis
Rm	nucleus reticularis medius
Rot	nucleus rotundus
Rub	nucleus ruber
Sch	nucleus suprachiasmaticus
sfgs	stratum fibrosum et griseum superficiale
sgc	stratum griseum centrale
sgp	stratum griseum periventriculare
SNc	substantia nigra, pars compacta
SNr	substantia nigra, pars reticulata
So	nucleus supraopticus
Sol	nucleus tractus solitarii
Sp	nucleus suprapeduncularis
Str	striatum
tect	tectum mesencephali
tolf	tractus olfactorius
Torc	torus semicircularis, nucleus centralis
Torl	torus semicircularis, nucleus laminaris
tsh	tractus septohypothalamicus
tub olf	tuberculum olfactorium
tVds	tractus descendens nervi trigemini
Vds	nucleus descendens nervi trigemini
Vedl	nucleus vestibularis dorsolateralis
Veds	nucleus vestibularis descendens
Vespl	tractus vestibulospinalis lateralis
Vevl	nucleus vestibularis ventrolateralis
Vmv	nucleus motorius nervi trigemini, pars ventralis
Vl	nucleus ventrolateralis thalami
Vh	nucleus ventromedialis hypothalami
Vm	nucleus ventromedialis thalami
VTA	ventral tegmental area

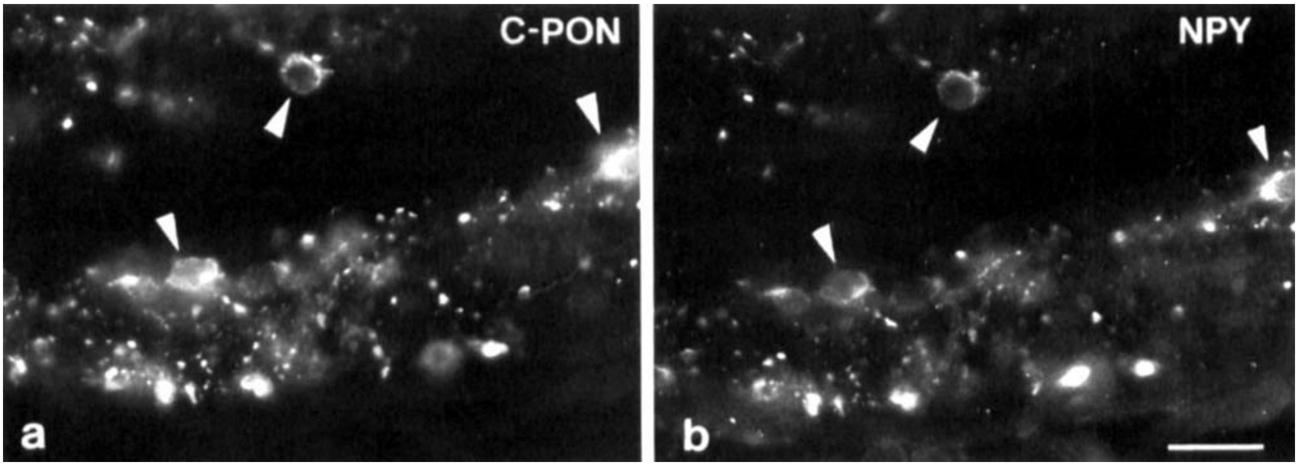


Fig. 1. Photomicrographs of immunoreactive fluorescent cell bodies and fibers in the median eminence of the lizard *Gallotia galloti,* reacted with anti-C-terminal flanking peptide (C-PON) serum (**a**) or anti-neuropeptide Y (NPY) serum (**b**). Note that all the C-PON immunoreactive elements (arrowheads) also contain NPY immunoreactivity. Scale bar = 0.1 mm.

sagittal, and horizontal sections, 20 or 40 μm thick, were made on a cryostat. Twenty micron thick sections were serially mounted on gelatin-coated slides, and 40 μm thick sections were processed free-floating.

Immunohistochemical procedures

Immunohistochemistry was performed with four primary antisera: two rabbit polyclonal anti-NPY sera (Martí et al., '87; Perroteau et al., '88); one sheep polyclonal anti-NPY serum (Furness et al., '85); and one rabbit polyclonal anti-C-flanking peptide (C-PON) serum (Martí et al., '87).

The peroxidase–anti-peroxidase (PAP) technique was used for single immunostaining, both on free-floating and on mounted sections. Polyclonal antisera were diluted 1:800 in 0.1 M, pH 7.4, PBS containing 0.01% Triton-X (PBS-TX). Briefly, the following sequence was used: sections were first blocked with normal goat serum diluted 1:100 in 0.1 M PBS, and subsequently incubated with the primary antiserum overnight at room temperature (with constant agitation for free-floating sections). After several rinses in 0.1 M, pH 7.4, PBS, followed by an incubation with goat anti-rabbit IgG (ICN Biomedicals Ltd., UK, 1:100 in PBS-TX) for 1 hour at room temperature, sections were rinsed again and incubated with PAP complex (ICN Biomedicals Ltd., 1:100 in PBS-TX). After additional rinses first in PBS and then in 0.05 M, pH 7.6, Tris-HCl buffer, the immunoreactivity was visualized by incubation in 0.03% 3,3′-diaminobenzidine tetrahydrocloride solution in 0.05 M, pH 7.6, Tris-HCl buffer, containing 0.025% H_2O_2 and 0.1% ammonium nickel sulphate.

Double immunostaining was performed by the indirect immunofluorescence method on sections mounted on slides. The sheep polyclonal anti-NPY (diluted 1:50 in 0.1 M PBS-TX) and the rabbit polyclonal anti-C-PON (diluted 1:400) were applied simultaneously and the sections were incubated overnight at room temperature. After rinsing, sections were incubated again in a mixture of secondary antibodies labeled with fluorescein or rhodamine isothiocyanate (FITC-donkey anti-goat immunoglobulin, Nordic; or TRICT-swine anti-rabbit immunoglobulin, Dakopatts) to a final dilution of 1:20 in 0.1 M, pH 7.4, PBS-TX for 1 hour at room temperature.

The specificity of the reactions was checked by omitting the primary antibodies or adsorbing them with the homologous synthetic peptides (1×10^{-5} to 1×10^{-6} M NPY, prepared by Dr. S. Saint-Pierre, Montreal, Canada; and 1×10^{-5} to 1×10^{-6} M C-PON; see Martí et al., '87). In double immunostaining, the reaction was also checked by incubating the sections with a mixture in which one of the two primary antibodies had been previously adsorbed with the homologous antigen or substituted by preimmune serum.

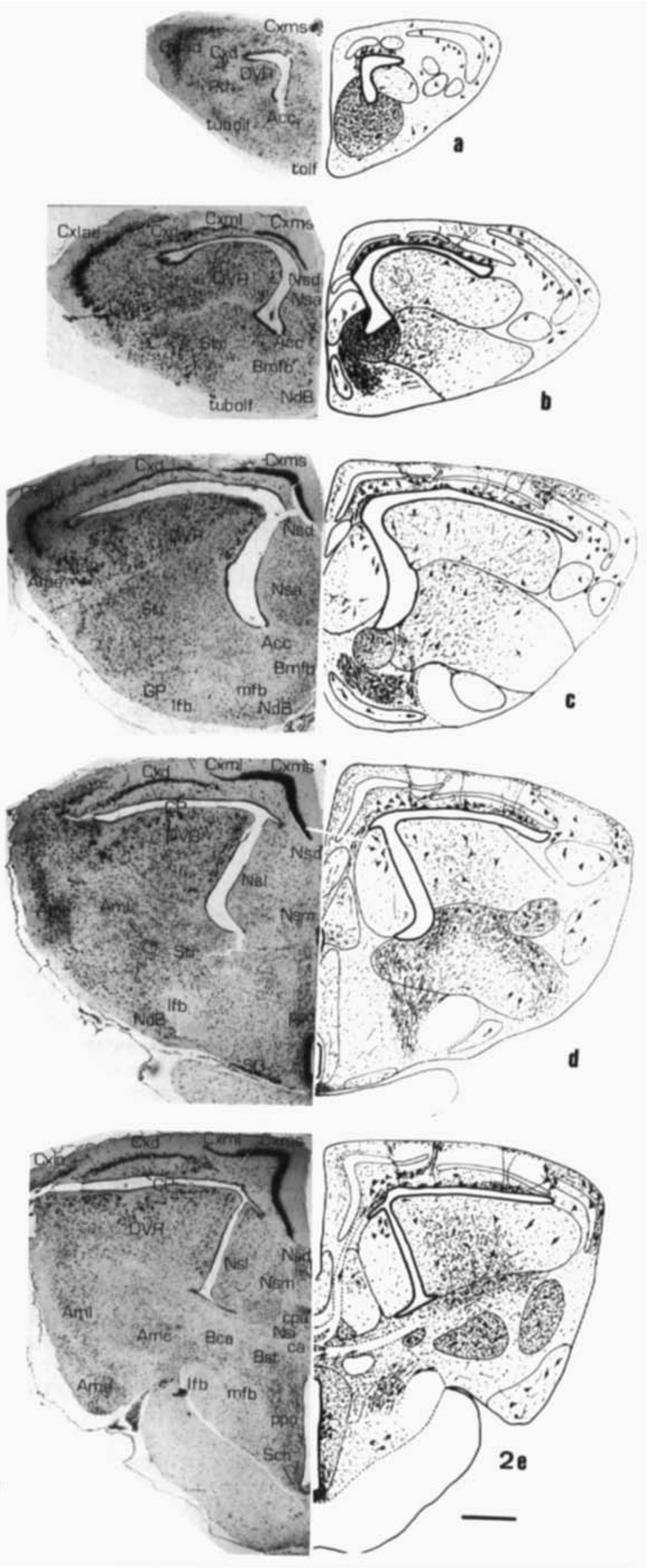
RESULTS

When sections were incubated with normal serum or with serum preadsorbed with the homologous antigens no labeling was observed. The labeling observed with all polyclonal antisera (anti-NPY and anti-C-PON) on consecutive sections appeared to be highly corresponding. Further, the double immunofluorescence labeling demonstrated that in all labeled structures, anti-NPY and anti-C-PON immunoreactivities are colocalized (Fig. 1). Thus, we shall use the expression *NPY-like immunoreactivity* to refer to elements containing both NPY and C-PON.

Figures 2–6 show the distribution of NPY-like immunoreactivity in representative brain sections of the lizard *Gallotia galloti.* The left-hand side of the figures shows cresyl violet staining, while the right-hand side shows camera lucida drawings of the NPY-like immunoreactive (NPY-LI) neurons and fibers observed in the adjacent sections. For clarity, some nuclei and fiber tracts are also indicated on the right-hand side of the figures. The nomenclature used follows that of Smeets et al. ('86a) for the forebrain, and that of ten Donkelaar et al. ('87) for the brainstem.

NPY-like immunoreactive (NPY-LI) cell bodies

Telecephalon. The most rostral NPY-LI cell bodies are observed in the olfactory peduncle and the nucleus olfactorius anterior (Fig. 7). These cell groups are caudally contin-



Figs. 2–6. Photomicrographs (left) and drawings (right) of representative transverse levels of the brain of *Gallotia*. Left side of the figures shows a cresyl violet-stained section, and selected nuclei and fiber tracts are indicated on it. Right side of the figures shows the distribution of NPY-LI neurons and fibers observed in adjacent sections. Scale bar (shown only in Fig. 2) = 0.2 mm.

Figure 2

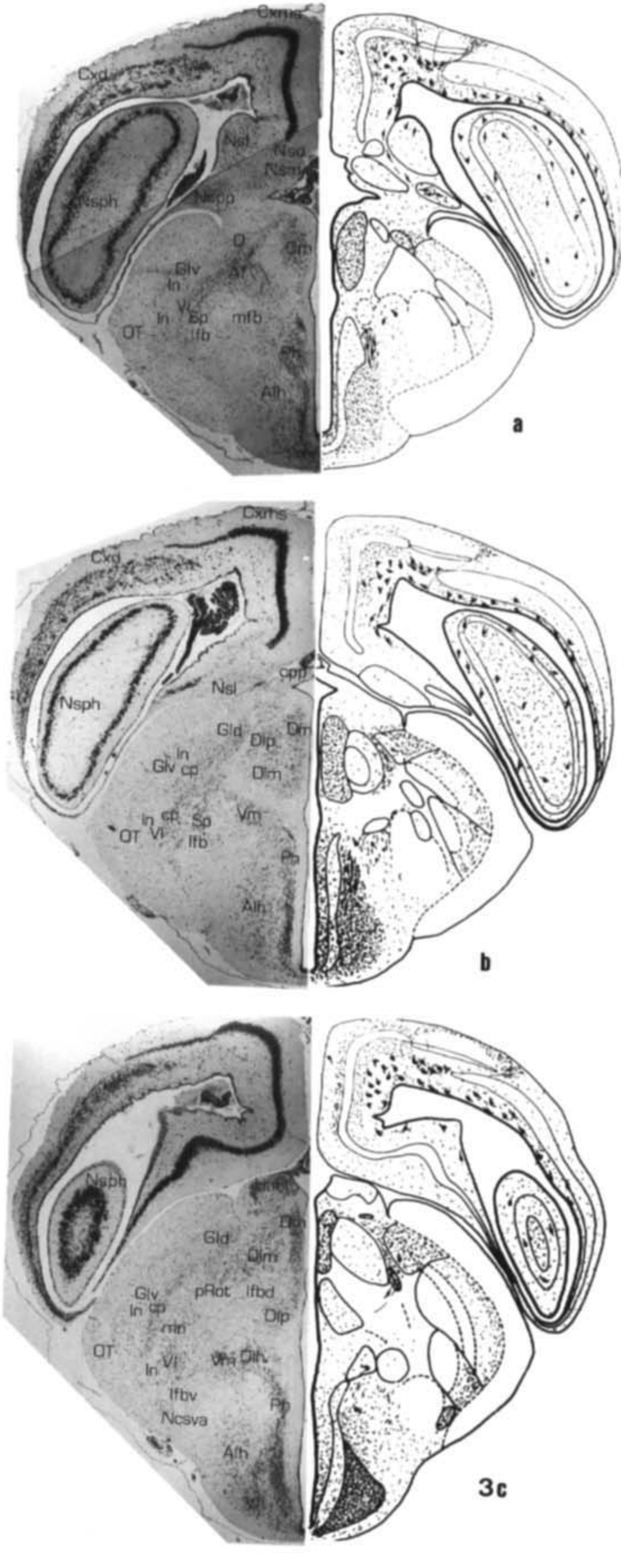

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Figure 3

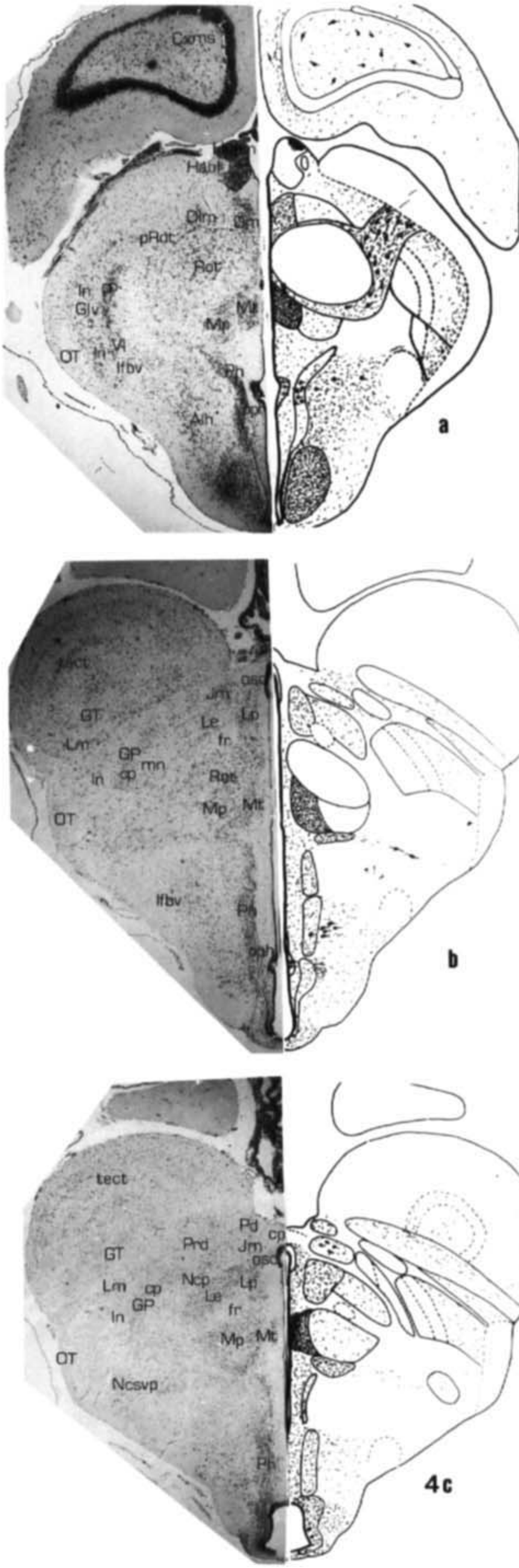
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Figure 4

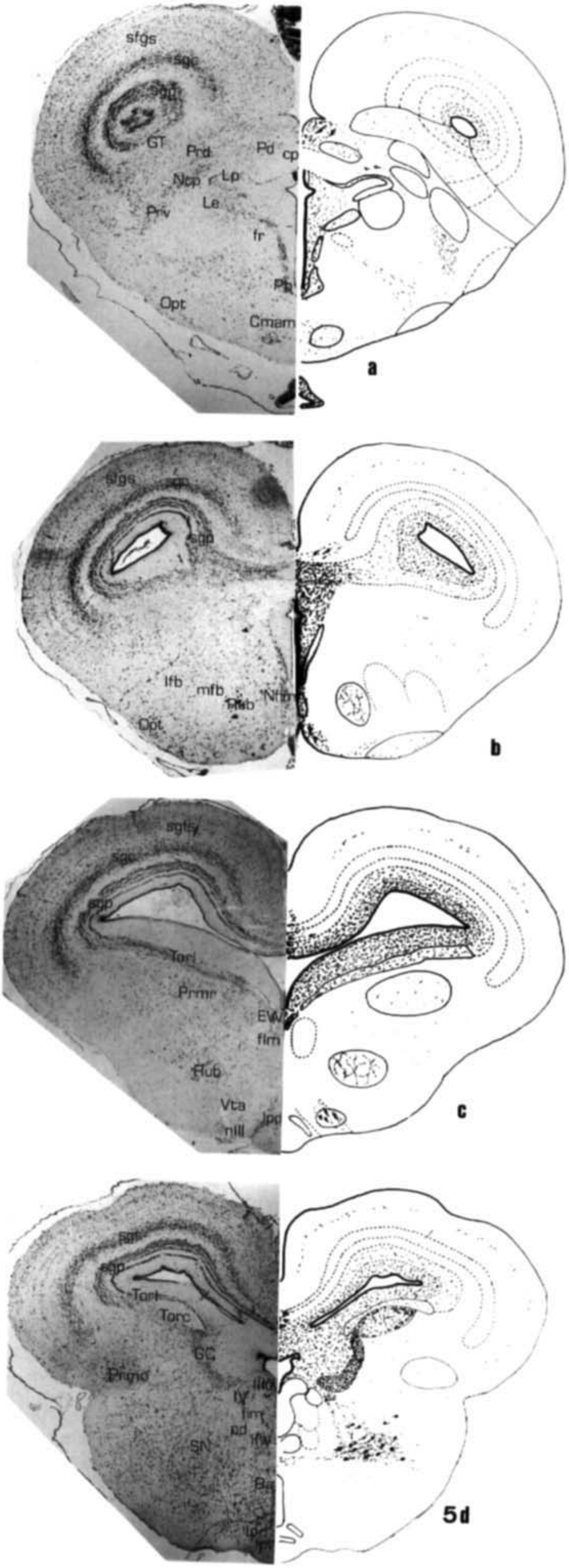
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Figure 5

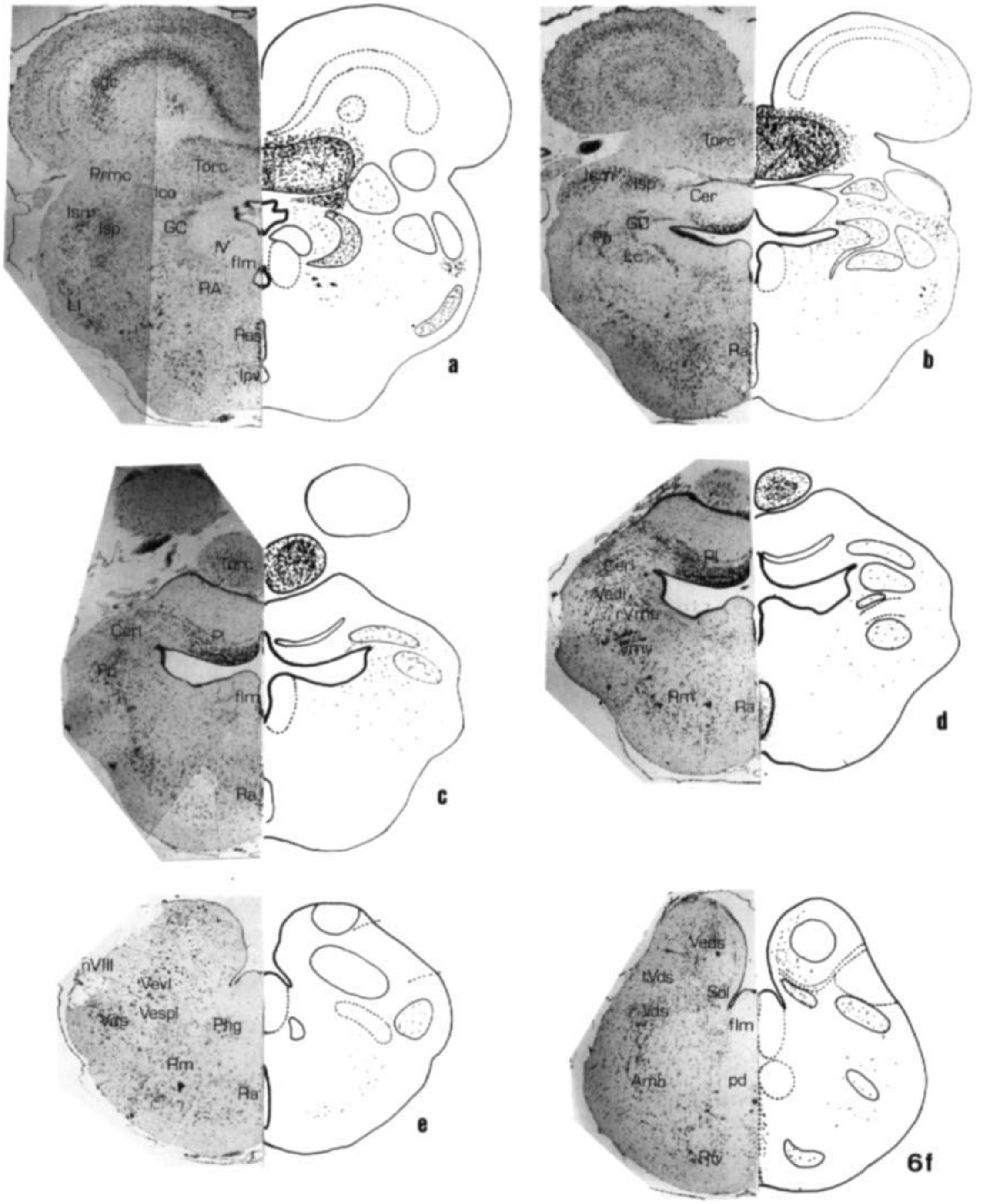

Figure 6

uous with the NPY-LI cell bodies of the olfactory tubercule and the lateral cortex (Fig. 2a).

More caudally in the telencephalon, NPY-LI cell bodies are widely distributed. Numerous immunoreactive neurons are observed in the cortical areas (Figs. 2, 3, 8). Most of them are multipolar (Figs. 8a–e), and lie in the inner plexiform layer of all cortices. Using the terminology proposed by Berbel ('88) for the lamination of the small-celled medial cortex, most NPY-LI cells in this cortical region are polymorphic neurons, located within the stratum V of the inner plexiform layer (Figs. 8a,b). Immunoreactive, fusiform neurons are observed adjacent and in parallel to the ventricle in the stratum VI or alveus of the small-celled medial cortex. In the large-celled medial cortex, sparse NPY-LI cell bodies are located in the alveus, whereas other cells are immediately subjacent to the inner dendritic field of the neurons of the cortical layer, forming a cortical lamina (Fig. 8a). These cells have apical processes ascend-

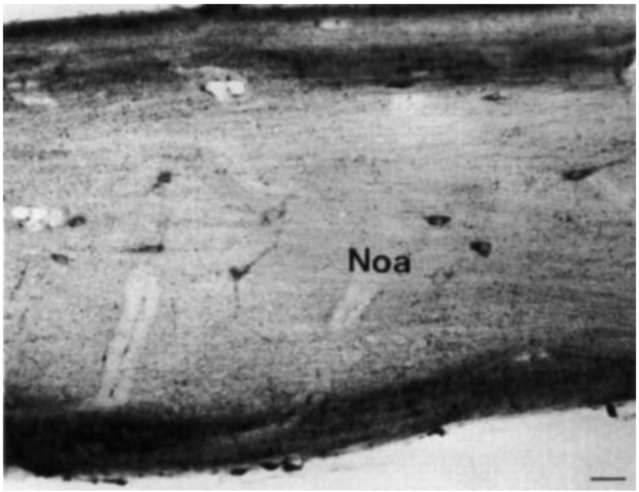

Fig. 7. Photomicrograph of NPY-LI cell bodies and fibers in the nucleus olfactorius anterior of *G. gallotti*. Horizontal section. Scale bar = 0.1 mm.

ing towards the superficialmost stratum of the outer plexiform layer (Figs. 2e, 3, 8a).

In the dorsal cortex, a few NPY-LI neurons are dispersed in the inner plexiform layer, and a larger number of them appears within the cell plate of Unger, particularly in its lateral portion (Figs. 8a,c,d). Some of these deeper cells have long apical processes that reach the cortical surface (Figs. 2b–e, 8c).

Cell bodies containing NPY-like immunoreactivity are also present in the outer plexiform layer, primarily in the lateral cortex (Figs. 2a–e, 8a). A few are found at caudal levels of the medial cortex (Figs. 3c, 4a). Moreover, a few immunoreactive neurons are seen in the cortical layer of the lateral cortex and in the pallial thickening (Figs. 2a–e).

NPY-LI cell bodies are abundant in the subcortical telencephalic areas. Dispersed immunoreactive neurons are present in the dorsal ventricular ridge (DVR), throughout its rostrocaudal extent (Figs. 2a–e, 9d). The striatum also contains numerous NPY-LI neurons, most of them located medially and dorsally, adjacent either to the ventricle or to the border with the DVR (Fig. 9c). This is obvious at caudal telencephalic levels (Fig. 2d,e). Small immunoreactive neurons are present in the nucleus accumbens, the bed nucleus of the medial forebrain bundle (or ventral pallidum), and the nucleus of the diagonal band of Broca (Figs. 2a–d, 9a,b).

In the caudal telencephalon, NPY-LI neurons are located in the amygdaloid complex, i.e., in the central, external, and lateral nuclei of the amygdala, as well as in the nucleus sphericus (Figs. 2c–e, 3, 9d,f). NPY-LI cell bodies are also seen in the bed nucleus of the anterior commissure (Figs. 2e, 10a).

The septal area contains small, lightly stained NPY-LI cell bodies that are located in the dorsal part of the nucleus septalis lateralis (Figs. 2c–e, 3a). Another group of NPY-LI cell bodies is observed in the bed nucleus of the posterior pallial commissure (Fig. 3a); these cells show a larger somatic size and are strongly labeled. This group of NPY-LI cells is continuous with the fusiform cells observed in the alveus traversing the septum and in the medial cortex.

Hypothalamus. Several NPY-LI cell groups are observed in the hypothalamus. Two distinct cell groups are seen in the preoptic periventricular nucleus (Figs. 2e, 10b); one lies ventrally, near the optic chiasm (Fig. 10d), whereas the other cell group is located more dorsally, with some cells lying adjacent to the ventricle. The latter group contains liquor-contacting cells (Fig. 10c). NPY-LI cell bodies are also seen in the area preoptica lateralis. A few fusiform immunoreactive neurons lie immediately adjacent to the medial forebrain bundle at the level of the preoptic region (Fig. 2e).

In caudal hypothalamic areas, several groups of NPY-LI cell bodies are distinguishable. A group of vertically disposed bipolar neurons appears in the area lateralis hypothalami (Figs. 3a,b, 10e,f). These cells lie within the area that is occupied by the cortico-hypothalamic bundle. Some immunoreactive cell bodies are present in the nucleus dorsolateralis hypothalami and within the medial forebrain bundle (Fig. 3). At the level of the periventricular organ, another group of immunoreactive, radially disposed neurons is found (Figs. 4a,b, 11a,d). These cells are located either in the periventricular hypothalamic nucleus or in the lateral hypothalamic area. Finally, the periventricular stratum of the median eminence contains NPY-LI cell bodies as well (Figs. 1, 4c).

Thalamus and pretectum. A very impressive group of NPY-LI cell bodies is present in the dorsal thalamus, in the perirotundal belt (Figs. 3c, 4a, 11a,c). Caudally, many NPY-LI neurons of the perirotundal belt separate from the nucleus rotundus and can be followed superficially to the optic tract, between the pretectal geniculate and the ventral lateral geniculate nuclei (Fig. 4a). Immunoreactive cells observed in the perirotundal belt are mainly bipolar, with their dendrites disposed parallel to the interthalamic limit. A few immunoreactive cell bodies are also present in the dorsal lateral geniculate nucleus (Fig. 3b,c). Immunoreactive cell bodies are absent in the pretectum of *Gallotia galloti*.

Mesencephalon. A few NPY-LI neurons appear in a rostral area adjacent to the tectal commissure (Fig. 5a,b). In the tegmentum, NPY-LI neurons appear within and around the fibers of the oculomotor nerve, in the ventral tegmental area (Fig. 5b,c). More caudally, immunoreactive neurons are observed in the lateral tegmentum, forming an oblique mediolateral band of cells located in the substantia nigra pars compacta (Figs. 5d, 12a). A few immunoreactive cell bodies, with smaller somatic sizes, are observed dorsally to these cells. Further caudally, NPY-LI cell bodies are present centrally in the tegmentum, in the retrorubral area, at the level of the trochlear motor nucleus (Fig. 6a).

Only a few immunoreactive cell bodies are present in the rhombencephalon, lying medial to the nucleus of the lemniscus lateralis (Fig. 6b).

Distribution of NPY-LI fibers

Telencephalon. The olfactory peduncle and the nucleus olfactorius anterior contain many immunoreactive fibers and terminals (Fig. 7). NPY-LI fibers are also observed in the tuberculum olfactorium or coursing in the tractus olfactorius lateralis (Fig. 2a,b).

NPY-LI fibers and terminals are abundant throughout the telencephalon. A laminar organization can be recognized in the cortical areas (Figs. 2, 3, 4a, 8a). The NPY-like immunoreactivity is mainly located in both the inner and outer plexiform layers of the medial, dorsal, and lateral cortices. Thick NPY-LI fibers cross the cortical areas radially and ramify in the outer plexiform layer (Fig. 8c).

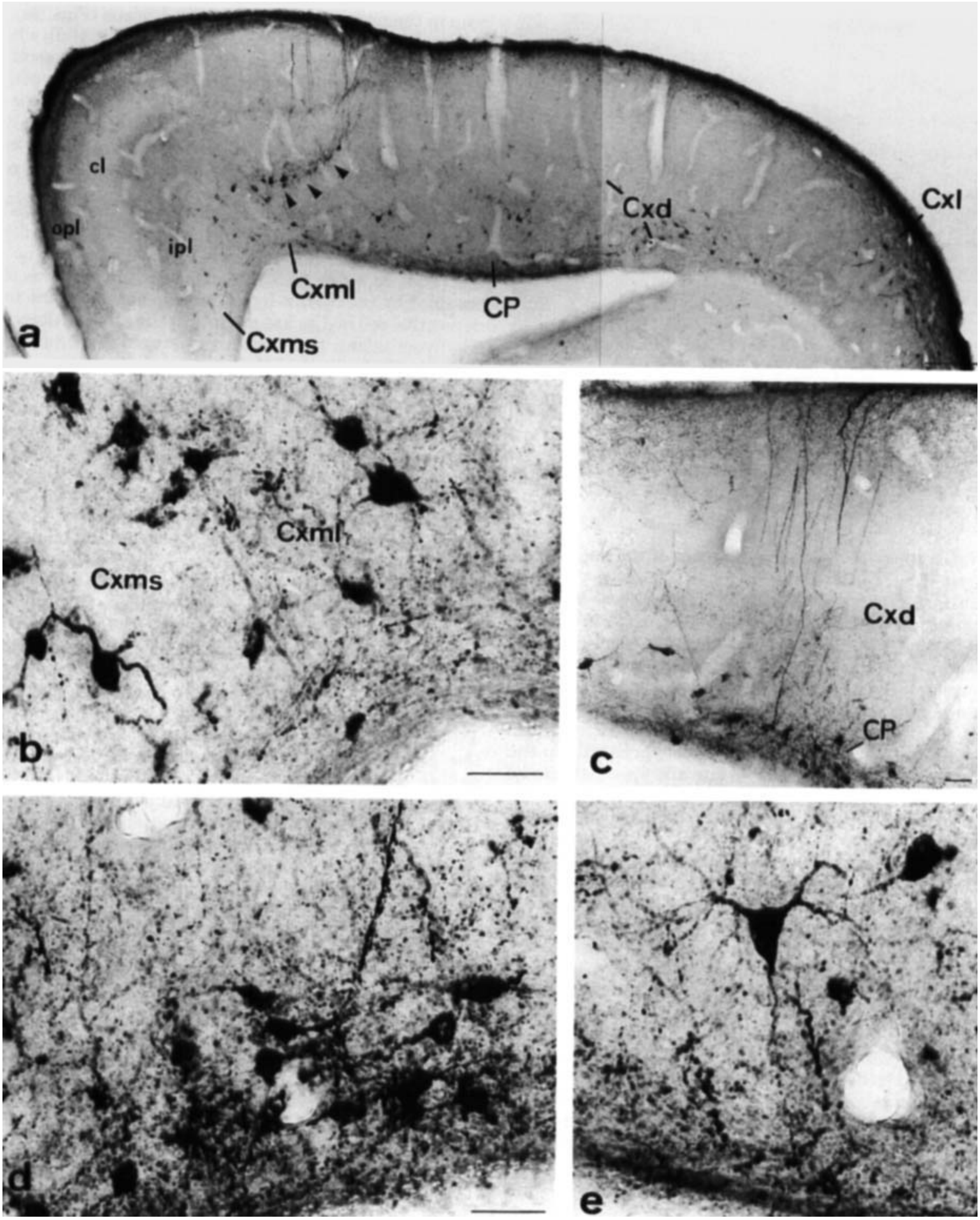

Fig. 8. **a:** Photomicrograph of the cortical areas of *G. galloti* shows NPY-LI fibers and cell bodies. Note the lamination at the level of the medial cortex, and the corticoid sheet of immunoreactive neurons (arrowheads) in the large-celled part of it. **b:** NPY-LI cell bodies and fibers in the inner plexiform layer of the medial cortex. Note that NPY-LI cell bodies are mostly segregated from the fibers in the small-celled medial cortex, but not in the large-celled medial cortex. **c:** NPY-LI processes crossing the dorsal cortex radially. These fibers arise in the NPY-LI cell bodies of the cell plate of Unger. **d,e:** Photomicrographs of NPY-LI neurons and fibers in the cell plate of Unger (**d**) and inner plexiform layer of the dorsal cortex (**e**). Scale bars: a = 0.5 mm; b–e = 0.1 mm.

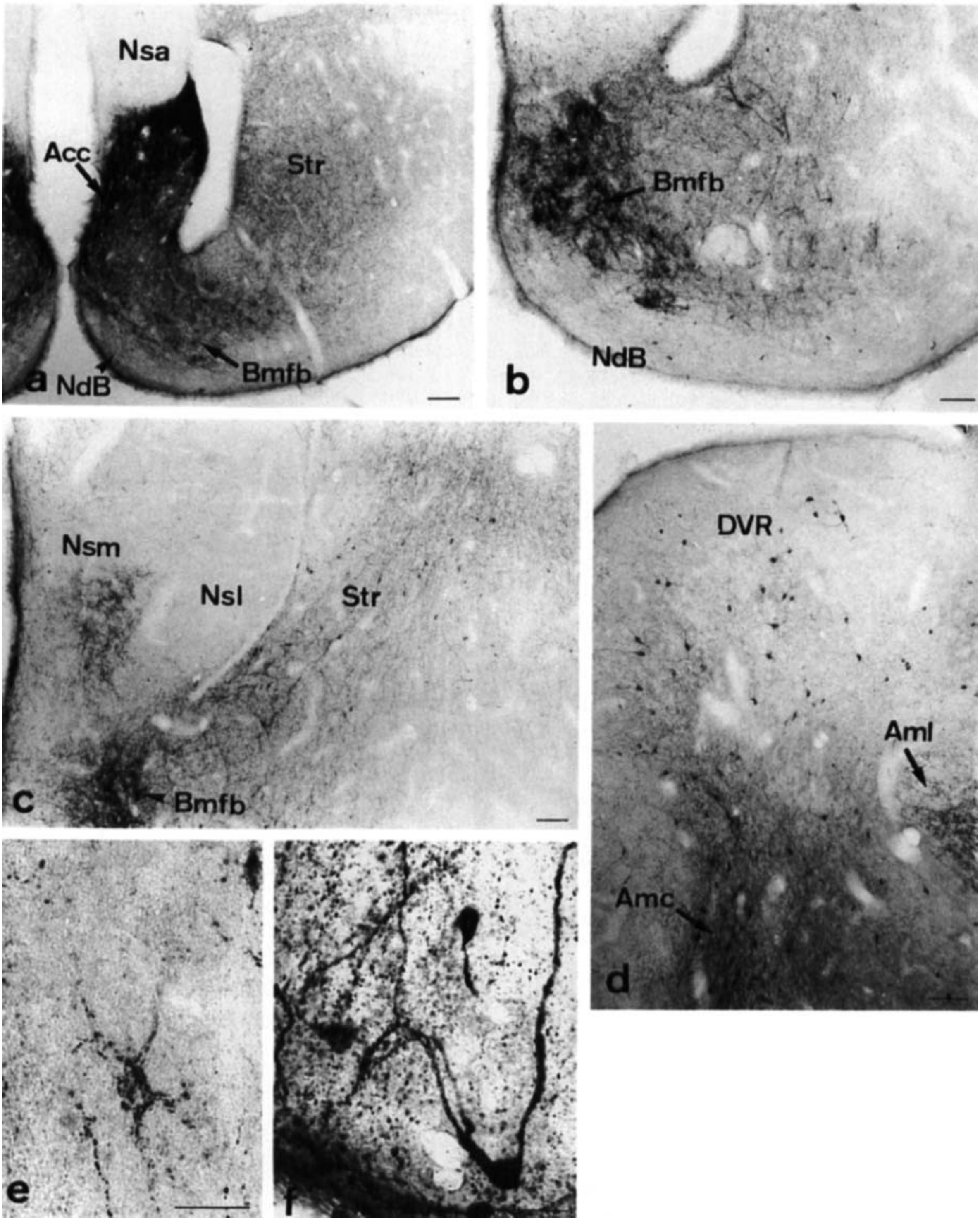

Fig. 9. Photomicrographs of NPY-like immunoreactivity in the subcortical areas of the lizard *G. galloti*. Note the dense plexuses in the nucleus accumbens (**a**) and the bed nucleus of the medial forebrain bundle (**b**). NPY-like immunoreactivity is also shown in the medial septum (**c**), striatum (**a–c**), dorsal ventricular ridge (**d**), and amygdala (**d,f**). A detail of NPY-LI fibers forming a nest around an unstained cell body of the septum is shown in **e.** Scale bars = 0.1 mm.

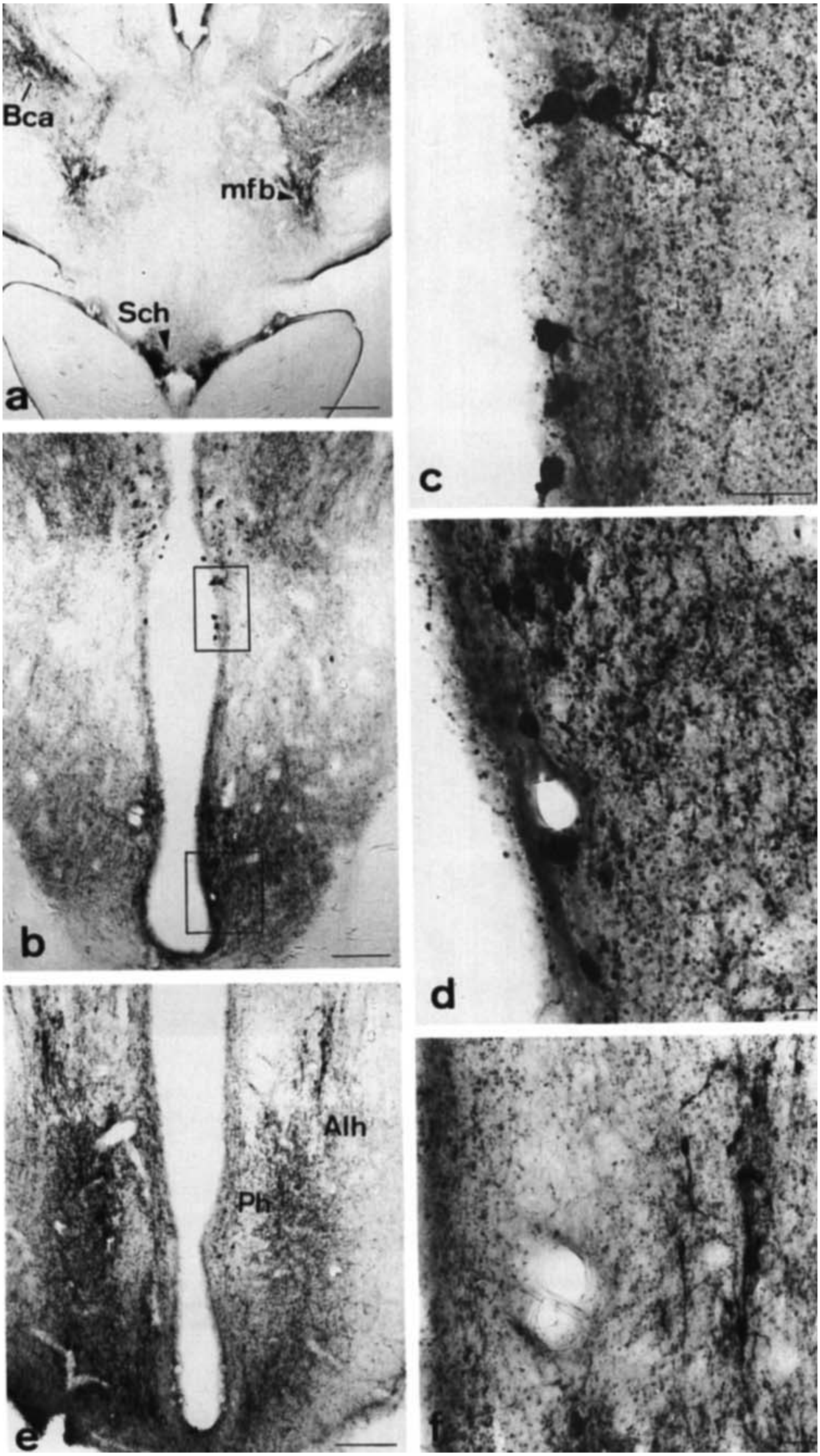

Figure 10

However, some differences are observed between the cortices. In the dorsal half of the small-celled medial cortex, several innervated laminae are distinguishable within the inner and outer plexiform layers (Figs. 2c–e, 3). According to Berbel's lamination ('88), these laminae coincide with the strata I and II in the outer plexiform layer, and the strata IV and VI (or alveus) in the inner plexiform layer. Stratum V shows scarce NPY-LI innervation and contains most of the immunoreactive cell bodies (Fig. 8b). In the ventral half of the small-celled medial cortex, the NPY-LI innervation is lighter than in the dorsal one, and the lamination referred to above is less clear. A segregation between immunoreactive cell bodies and fibers is not observed in the other cortices.

The large-celled medial cortex also shows a distinctive lamination of immunoreactivity. A very dense plexus is observed in the most superficial part of this cortex, in which cell processes of deeper immunoreactive neurons ramify (Figs. 2d,e, 3, 8a). On the contrary, the rest of the molecular layer, the pyramidal layer, and the external part of the inner plexiform layer are completely devoid of immunoreactive varicose fibers. These three layers coincide with the areas where the somata and dendritic fields of the so-called bipyramidal neurons are located. Numerous immunoreactive fibers are observed within the deep part of the inner plexiform layer and the alveus, surrounding the NPY-LI cells (Fig. 8b).

In the dorsal cortex, a specially dense NPY-LI plexus is observed in the medial part of the outer plexiform layer (Figs. 2c–e, 3). Apart from this superficial plexus, immunoreactive varicose fibers are dispersed throughout the inner plexiform layer (Figs. 2, 3); these become denser at the level of the cell plate of Unger (Fig. 8c,d). The lateral cortex mainly displays NPY-LI fibers and varicosities in the outer plexiform layer, and surrounding its immunoreactive neurons (Figs. 2, 8a).

The subcortical telencephalic areas are either moderately or densely innervated by NPY-LI fibers. The densest plexuses are found in the nucleus accumbens, the medial forebrain bundle and its nucleus, and some parts of the striatum (Figs. 2, 9a–c). Other subcortical structures, such as the DVR, the nucleus of the diagonal band of Broca, the amygdaloid complex (including the nucleus sphericus), and the septal region, are also moderately to densely innervated by NPY-LI fibers (Figs. 2, 3, 9c–f). In the nucleus septalis anterior and the nucleus septalis medialis, NPY-LI fibers form dense nests around the somata and proximal processes of unstained neurons (Figs. 2, 9c,e). Numerous immunoreactive fibers course in the medial forebrain bundle and the stria terminalis, while the lateral forebrain bundle and the globus pallidus are devoid of immunoreactivity (Figs. 2c–e, 10a).

Hypothalamus. The preoptic region contains many NPY-LI fibers and terminals (Fig. 10a–d). An extremely dense plexus is observed in the nucleus suprachiasmaticus (Figs. 2d,e, 10a). Further caudally, the hypothalamus contains numerous NPY-LI fibers and terminals. A dense plexus is observed in the lateral hypothalamic area and the ventromedial hypothalamic nucleus (Figs. 3, 4a, 10e, 11a). Numerous immunoreactive fibers and terminals are also observed in the periventricular neuropile located medially to the periventricular hypothalamic nucleus (Fig. 10e,f). The remaining hypothalamic areas show a moderate innervation. In the periventricular organ, immunoreactive fibers surround the somata and proximal processes of unstained cells (Fig. 4a,b).

Thalamus, epithalamus, and pretectum. In the ventral thalamus, the lateral neuropiles of the ventral lateral geniculate and the ventrolateral nuclei, as well as the nucleus ovalis, show a moderate number of NPY-LI fibers (Figs. 3, 4a).

In the dorsal thalamus, a larger amount of NPY-LI fibers is found. A very dense plexus is present in the nucleus medialis thalami (Figs. 4, 11a). Other nuclei of the dorsal thalamus, such as the nucleus dorsomedialis, the dorsal lateral geniculate nucleus, and the perirotundal belt, are also moderately innervated by NPY-LI fibers (Figs. 3, 4a, 11a,c). Immunoreactive fibers are also observed in the nucleus dorsolateralis thalami and the nucleus medialis posterior.

NPY-LI fibers are observed to innervate asymmetrically the epithalamus, in particular the nucleus medialis habenulae. A dorsolateral part of this nucleus contains a very dense immunoreactive plexus, which is present only on the left side of the brain (Figs. 4a, 11a,b).

A moderate number of NPY-LI fibers is observed in the pretectum. These fibers are dispersed in the lateral neuropile of the pretectal geniculate nucleus, the nucleus lentiformis thalami, pars plicata and pars extensa, the nucleus lentiformis mesencephali, the nucleus posterodorsalis, and in an area ventral to the nucleus pretectalis ventralis (Figs. 4b,c, 5a). A patch of denser terminals coincides with the nucleus juxtacommissuralis medialis, a medially lying accessory cell mass of the nucleus of the posterior commissure (Fig. 4c). NPY-LI fibers are also present in the periventricular stratum of the pretectal region, continuing into the mesencephalon.

Mesencephalon. The mesencephalon contains some NPY-LI fibers in the retino-recipient superficial and inner neuropiles of the griseum tectale (Figs. 4b,c, 5, 6a,b). The tectum also shows immunoreactive fibers, which are primarily located in the periventricular laminae. Tectal layer 2 shows a dense immunoreactive plexus, whereas a moderate number of fibers is observed in layers 3–5. A few NPY-LI fibers are also present in the superficial stratum, coinciding with tectal layer 12. The tectal periventricular stratum containing NPY-LI fibers is continuous with the tegmental periventricular zone (Fig. 5b–d). NPY-LI fibers are also seen to form nests around the large neurons of the mesencephalic trigeminal nucleus (Fig. 5c,d). Some terminals also surround the NPY-LI neurons in the rostral area adjacent to the tectal commissure (Fig. 5a,b).

The nucleus ruber contains some coarse NPY-LI fibers (Fig. 5b,c). Moderate plexuses are observed in the tegmental areas containing NPY-LI cell bodies, specially at the level of the substantia nigra, pars compacta (Figs. 5c,d, 12a). From the latter area, some immunoreactive fibers

Fig. 10. Photomicrographs of NPY-LI cell bodies and fibers in the preoptic and rostral hypothalamic regions. Note the dense plexus in the suprachiasmatic nucleus and fibers in the medial forebrain bundle (**a**). Two groups of periventricular NPY-LI cell bodies are observed in the preoptic region (**b**). One of them is located closer to the optic chiasm, and is shown at higher magnification in **d**. Another group is located more dorsally (higher magnification in **c**); some of its cells contact the cerebrospinal fluid (CSF-contacting cells). NPY-LI elongated cell bodies are also seen in the area lateralis hypothalami, related to a vertical fiber tract (**e,f**). NPY-LI fibers and terminals are also numerous in the area lateralis hypothalami and the neuropile located medially to the periventricular hypothalamic nucleus (**e,f**). Scale bars: a, b, e = 0.5 mm; c, d, f = 0.1 mm.

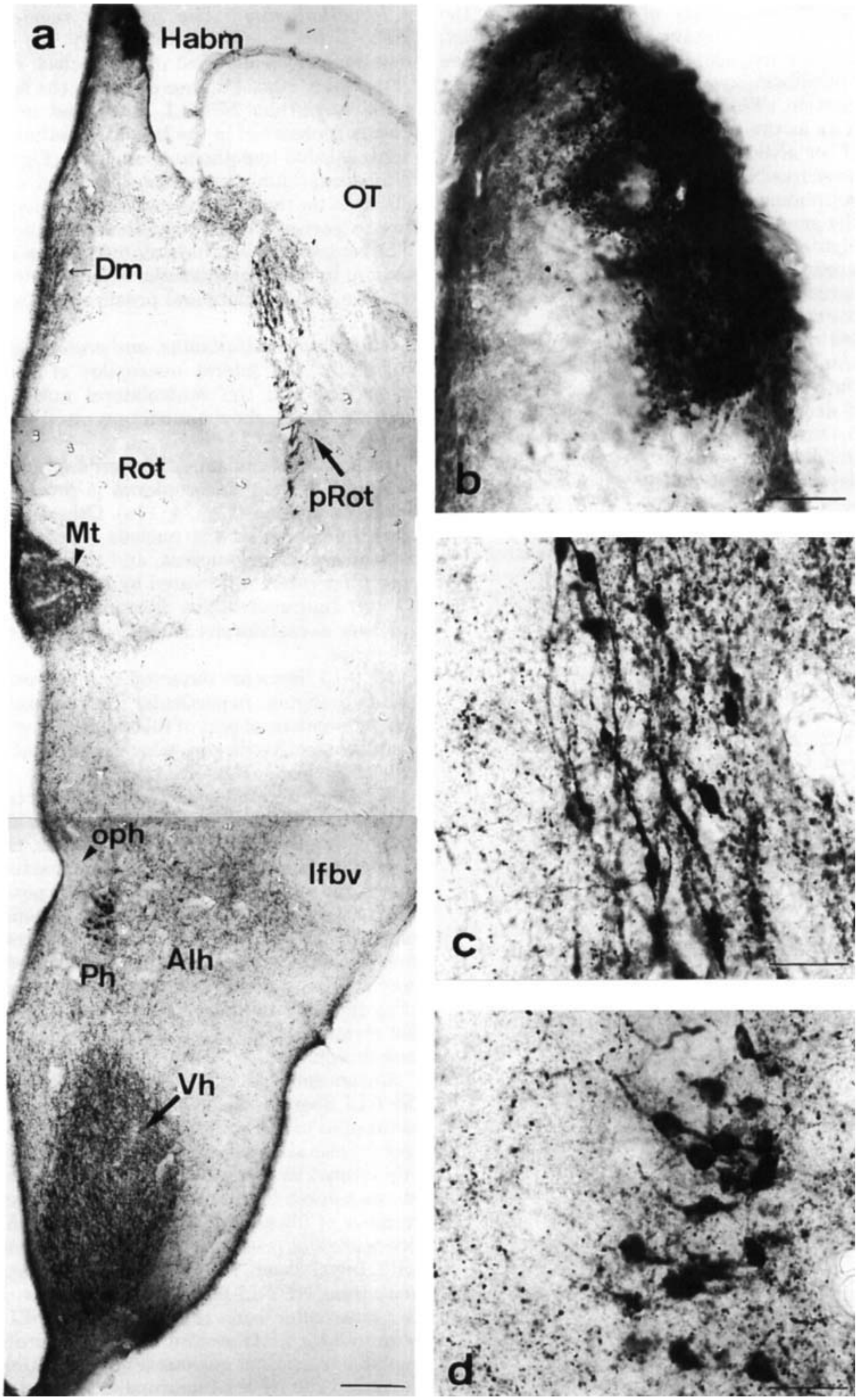

Fig. 11. Photomicrographs of NPY-LI fibers and cell bodies in the epithalamus, thalamus, and hypothalamus of *G. gallotia*. Note the dense plexuses in the habenula (**a**; higher magnification in **b**), nucleus medialis thalami (**a**), and nucleus ventromedialis hypothalami (**a**). NPY-LI cell bodies are observed in the perirotundic belt (**a,c**) and lateral to the hypothalamic periventricular organ (**a,d**). Scale bars: a = 0.5 mm; b–d = 0.1 mm.

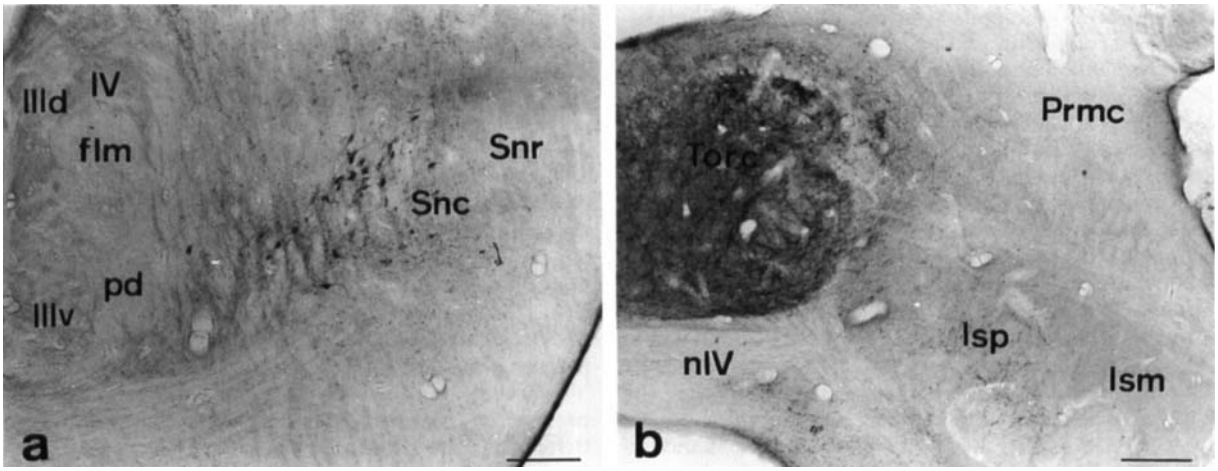

Fig. 12. Photomicrographs of NPY-LI cell bodies and fibers in the mesencephalon of *G. galloti*. The midline lies at the left. NPY-LI neurons are shown in the substantia nigra, pars compacta (**a**), while a dense innervation by NPY-LI fibers fills the torus semicircularis (**b**). Scale bars = 0.5 mm.

seem to course dorsally into the griseum centrale. A patch of terminals is observed lateral to the nucleus of Edinger-Westphal.

The nucleus centralis of the torus semicircularis contains a very high number of NPY-LI fibers and terminals throughout all its rostrocaudal extent (Figs. 5d, 6a–d, 12b). Many of these fibers form baskets of terminals around the somata and proximal processes of unstained cells. Surrounding this nucleus there is a capsular area showing a moderate innervation (Figs. 6a–d, 12b). The nucleus laminaris of the torus semicircularis contains a moderate number of immunoreactive fibers (Fig. 5c).

Rhombencephalon. In the isthmic region, the griseum centrale, the locus coeruleus, and a reticular region located just ventrocaudally to the nucleus isthmi pars magnocellularis contain a small to moderate number of NPY-LI fibers (Fig. 6a,b). The nucleus isthmi pars magnocellularis itself does not contain any immunoreactivity. A moderate number of immunoreactive fibers is observed in the nucleus isthmi pars parvocellularis, the nucleus intercollicularis, and the parabrachial region (Fig. 6a–c).

Further caudally, a moderate to light innervation by NPY-LI fibers is observed in the nucleus motorius nervi trigemini, pars ventralis, nucleus princeps nervi trigemini, nucleus descendens nervi trigemini, nucleus vestibularis dorsolateralis, nucleus salivatorius, and in the tractus solitarius and its accompanying nucleus (Fig. 6d–f).

DISCUSSION

Specificity of the methods

All polyclonal antisera used in this study (described in Materials and Methods) have been previously characterized and used specifically to identify NPY immunoreactive structures in the central nervous system of mammals (Martí et al., '87), lizards (Martí et al., '90) and amphibians (Perroteau et al., '88). In the present study, the immunohistochemical labeling obtained using these different antisera reliably resulted in a corresponding pattern, either by means of PAP immunolabeling or by immunofluorescence.

Comparative distribution of NPY and C-PON in the brain of *Gallotia*

The present study reveals that both peptides, NPY and C-PON, are widely distributed in the brain of the lizard *Gallotia galloti*. Moreover, using double immunolabeling we have shown that NPY and C-PON are colocalized in all regions of the brain of this lizard. A coexistence of NPY and C-PON has also been described in the frog (Cailliez et al., '87) and in mammals (Gulbenkian et al., '85), suggesting that both peptides are split from the same precursor molecule, which shows a high structural similarity within different tetrapod vertebrates. Supporting this hypothesis, the sequence of the NPY gene has recently been compared in mammals, birds, and fishes, showing a strong evolutionary conservation (Larhammar et al., '90).

Comparison with the NPY-LI distribution in other vertebrates

Similarly to turtles, in the lizard *Gallotia galloti* NPY-LI cell bodies and fibers are present in several secondary olfactory structures, suggesting that this peptide plays a role in the processing of olfactory information in reptiles. The presence of NPY-immunoreactive cell bodies in olfactory structures seems to be a constant character in higher tetrapods (birds and mammals), whereas it shows a high variation in anamniotes. Numerous cell bodies showing either NPY or C-PON immunoreactivities have been demonstrated in the olfactory bulbs and related structures of brain of teleost fishes, birds, and mammals (goldfish: Pontet et al., '89; pigeon: Anderson and Reiner, '90; rat: Chronwall et al., '85; Nakagawa et al., '85; De Quidt and Emson, '86; Villalba et al., '89; monkey: Bons et al., '90; human: Ohm et al., '88; Walter et al., '90). In contrast, olfactory structures of elasmobranch fishes and amphibians did not contain NPY-immunoreactive cell bodies (*Scyliorhinus:* Vallarino et al., '88; frog: Danger et al., '85; newt: Perroteau et al., '88).

Like in the lizard *Gallotia*, numerous NPY-LI neurons are present in the cortical areas of other reptiles (Reiner and Oliver, '87; Dávila et al., '91), birds (Anderson and

Reiner, '90), and mammals (Chronwall et al., '85; Nakagawa et al., '85; De Quidt and Emson, '86; Bons et al., '90; Walter et al., '90). The pallium of amphibians also contains a few NPY-immunoreactive neurons (Danger et al., '85; Cailliez et al., '87; Perroteau et al., '88). In contrast, pallial areas of fishes do not show any NPY-immunoreactive cell bodies (Vallarino et al., '88; Pontet et al., '89).

While in reptiles the NPY-LI cortical neurons are mainly located in the inner plexiform layer, in birds and mammals these neurons do not show a distinctive laminar segregation. During development of the rat, the earliest NPY-immunoreactive neurons appearing in the cortex are located in the inner part of the cortical plate (Woodhams et al., '85), and they become distributed homogeneously later on. This observation agrees with the suggestion that the early developing mammalian cortex is reminiscent of the reptilian cortex (Marin Padilla, '78). The equivalence of mammalian and reptilian NPY cortical cells is also supported by the finding that most cortical NPY neurons coexpress somastostatin and GABA and are thought to be local-circuit neurons in both mammals and reptiles (Vincent et al., '82a,b; Chronwall et al., '84; Hendry et al., '84; Reiner and Oliver, '87; Dávila et al., '91). The vast majority of NPY neurons present in subdivisions of the avian telencephalon that are presumed to be analogous to mammalian cortex also contain somatostatin (Anderson and Reiner, '90). This finding suggests a common origin of these NPY neurons in stem reptiles or perhaps even amphibians ancestral to mammals, birds, and modern reptiles.

The dorsal ventricular ridge of both reptiles (Reiner and Oliver, '87; present results in *Gallotia*) and birds (Anderson and Reiner, '90) contains some NPY-LI neurons. This observation cannot resolve whether the DVR is analogous to the mammalian neocortex (Reiner et al., '84a) or claustrum (Díaz et al., '90, for revision), since NPY-immunoreactive cell bodies have been found in both structures (Chronwall et al., '85).

The labeling pattern for NPY in the basal ganglia of *Gallotia* resembles those of turtles (Reiner and Oliver, '87), birds (Anderson and Reiner, '90), and mammals (Chronwall et al., '85; Nakagawa et al., '85; Smith et al., '85; De Quidt and Emson, '86; Smith and Parent, '86; Bons et al., '90; Reuss et al., '90; Schwartzberg et al., '90; Walter et al., '90) in the following points: 1) the striatum contains many NPY-LI cells and fibers, the immunoreactivity being more abundant medially, close to the ventricle; 2) other basal formations, such as the nucleus accumbens and the ventral pallidum, also have NPY-LI neurons and fibers; and 3) the globus pallidus is almost devoid of NPY immunoreactivity.

The fact that the reptilian globus pallidus and the substantia nigra pars reticulata—the major recipients of the striatal outflow (Russchen and Jonker, '88)—are almost devoid of NPY immunoreactivity indicates that NPY-LI striatal cells may be local circuit neurons. This conclusion is consistent with the absence of NPY-LI fibers in the lateral forebrain bundle of *Gallotia.* An intrinsic character of the striatal NPY-immunoreactive neurons has been proposed in mammals and birds (Smith and Parent, '86; Anderson and Reiner, '90), using double labeling after injections of a retrograde tracer in the globus pallidus or the substantia nigra. The NPY-LI neurons of the DVR may also be local circuit neurons.

In *Gallotia,* the caudal striatal region that borders the amygdaloid complex shows a dense plexus of NPY-LI fibers and numerous cell bodies. A dense dopaminergic innervation has been observed at this level in the lizards *Gekko gecko* (Smeets et al., '86b) and *Gallotia galloti* (personal observations), suggesting a separate architectonic entity for this caudal part. On the basis of connectivity data, this region may represent a transition area, called striato-amygdaloid complex in *Gekko* (Russchen and Jonker, '88).

The presence of NPY-LI neurons in the amygdaloid complex seems to be a very conservative feature in the brain of vertebrates. NPY-LI cell bodies have been described in the amygdala of mammals (Chronwall et al., '85; Nakagawa et al., '85; De Quidt and Emson, '86; Bons et al., '90; Reuss et al., '90; Schwartzberg et al., '90; Walter et al., '90), birds (Anderson and Reiner, '90), reptiles (Reiner and Oliver, '87; present results in *Gallotia*), and amphibians (Danger et al., '85; Perroteau et al., '88). In mammals, some of these cells provide an NPY projection to other telencephalic structures, like the striatum or the bed nucleus of the stria terminalis, as well as to the hypothalamus (Allen et al., '84; Tatehata et al., '84). The few data available on the connectivity of the reptilian amygdala suggest that a similar pattern may be present in reptiles (Stoll and Hoogland, '84; Gonzalez et al., '90). Moreover, many NPY-LI fibers coursing in the medial forebrain bundle and the stria terminalis of *Gallotia* seem to arise from the amygdaloid complex.

NPY-immunoreactive cell bodies have also been reported as a constant feature in the bed nucleus of the stria terminalis in mammals (see references above) and birds (Anderson and Reiner, '90). In mammals, this nucleus is a complex mass of gray matter with several subdivisions (Ju and Swanson, '89), and only a part of it contains NPY-LI neurons. Applying to the lizard brain the criteria used in the rat by Ju and Swanson ('89), it appears that the bed nuclei of the anterior commissure (Bca) and stria terminalis (Bst)—as identified by Smeets et al. ('86a) and here—may be parts of a larger complex analogous to the bed nucleus of the stria terminalis of mammals. In support of this notion, we have observed that the NPY-LI neurons in the central amygdaloid nucleus of *Gallotia* are medially continuous with those of the Bca. This situation resembles that described in mammals (Walter et al., '90). According to cyto- and chemoarchitectonic criteria, the centromedial amygdala of mammals is continuous with the grisea of the Bst and some parts of the substantia innominata. All these grisea are referred to as "extended amygdala" or "Bst-amygdala continuum" (see revisions by Alheid and Heimer, '88; De Olmos, '90; Martin et al., '91). The present study provides evidence that an extended amygdala may also be present in reptiles. This idea agrees with the observations on cells and fibers containing several peptides (like enkephalin, somatostatin, substance P, and colecystokinin-8) in this Bst-amygdala continuum of reptiles (Naik et al., '81; Bear and Ebner, '83; Reiner et al., '84b; Reiner and Beinfeld, '85; Reiner, '87) and mammals (see for review Martin et al., '91).

NPY-LI neurons and fibers have been described in preoptic and hypothalamic regions of the brain of fishes, amphibians, turtles, birds, and mammals (cf. Vallarino et al., '88; Pontet et al., '89; Danger et al., '85; Perroteau et al., '88; Reiner and Oliver, '87; Anderson and Reiner, '90; De Quidt and Emson, '86). On comparing our results in *Gallotia* with those of other authors, several points become apparent:

1. As in the lizard *Gallotia,* cerebrospinal fluid (CSF)-contacting NPY-immunoreactive neurons have been described in the hypothalamus of an elasmobranch fish (Vallarino et al., '88) and a newt (Perroteau et al., '88).

The presence of CFS-contacting neurons in the hypothalamus seems to be a normal condition in the brain of nonmammalian vertebrates. Such neurons have been described to contain monoamines (Parent et al., '84; Smeets and Gonzalez, '90) and peptides like vasotocine (Smeets et al., '90) or substance P (Reiner et al., '84b). The existence of CSF-contacting catecholaminergic neurons apparently was a primitive condition in the brain of vertebrates, and a reduction of these cells is observed during evolution to higher tetrapods. This evolutionary trend possibly holds for all liquor-contacting hypothalamic neurons, and may be true for neurons containing NPY.

2. In the lizard *Gallotia* and in other nonmammalian vertebrates, NPY-LI neurons are located in the hypothalamus in the neighborhood of the periventricular organ (Danger et al., '85; Perroteau et al., '88; Pontet et al., '89). Dopaminergic neurons have been described at the same site (Smeets et al., '86b; Ekström et al., '90; Gonzalez and Smeets, '91). These NPY-LI neurons may be analogous to those found in the hypothalamic nucleus dorsomedialis of mammals, which is topographically related to the A14 dopaminergic cell group (Hökfelt et al., '84; Chronwall et al., '85). In mammals, dopamine and NPY are not colocalized in the same neurons (Everitt et al., '84), a question that still has to be resolved in nonmammalian vertebrates.
3. As in the lizard *Gallotia,* NPY-LI neurons and fibers have been described in the median eminence of fishes, amphibians, birds, and mammals. The NPY innervation observed in the mammalian median eminence arises from hypothalamic neurons, indicating that this peptide plays a role in the neuroendocrine system (Walter et al., '90).
4. A particularly high density of fibers is present in the suprachiasmatic nuclei of mammals (De Quidt and Emson, '86), birds (Aste et al., '91), and the lizard *Gallotia.* In mammals, this innervation originates in the intergeniculate leaflet and is of major importance in the regulation of circadian rhythms (Harrington et al., '87). As we will discuss below, the perirotundal belt of reptiles seems to be homologous to the intergeniculate leaflet of mammals. Moreover, our preliminary observations in *Gallotia* show that the perirotundal belt projects to the suprachiasmatic nucleus (Medina et al., '91).

In the thalamus of the lizard *Gallotia,* the NPY-LI neurons located around the nucleus rotundus and externally to it form a single group of neurons called the perirotundal belt. Its location, immunohistochemical characteristics (present results), and direct retinal inputs (Medina, '91; Medina et al., '91) reveal a close correspondence with the perirotundal region of birds (Hamassaki and Britto, '90; Aste et al., '91; Martínez and Puelles, '91; Martínez et al., '91; Puelles et al., '91). This finding supports its possible homology with the mammalian intergeniculate leaflet. NPY-LI neurons have been described in a rostral region of the dorsal thalamus of fishes (Pontet et al., '89) and amphibians (Perroteau et al., '88), which, therefore, may correspond to those of the perirotundal region of lizards and birds, and the intergeniculate leaflet of mammals.

Neuropeptide Y distribution in the diencephalon and mesencephalon generally shows a remarkable association with visual structures. In addition to the suprachiasmatic nucleus and the perirotundal belt, other primary visual centers of the brain of *Gallotia* show a moderate NPY-LI innervation, like the ventrolateral nucleus and the lateral geniculate nuclei in the thalamus, the pretectal geniculate nucleus and the posterodorsal nucleus in the pretectum, and the griseum tectale and several tectal laminae in the mesencephalon. A high NPY innervation of the optic neuropiles has also been noted in the chicken (Martínez and Puelles, '91) and the quail (Aste et al., '91), but not in other vertebrates.

One remarkable observation of the present study is the existence of a dense asymmetric plexus of NPY-like immunoreactivity in the left habenula of *Gallotia.* Descriptions of structural and connective asymmetry in the lacertidian habenula are found in the literature (see Engbretson et al., '81, for review). Asymmetry is also well known in the habenula of anamniotes (Braitenberg and Kemali, '70; Kemali et al., '80; Smeets et al., '83). The meaning of a difference between both sides of the brain in NPY innervation remains unknown, although it is possible that it is related to the asymmetric connection of the parietal eye with the left habenula (Engbretson et al., '81), mirroring NPY innervation of other visual neuropiles.

The auditory system of *Gallotia* is also characterized by an abundant NPY-LI innervation at its mesencephalic (torus semicircularis) and diencephalic (nucleus medialis thalami) stations. NPY-LI cells and fibers have been observed in the avian torus semicircularis, but not in the nucleus ovoidalis (analogous to the nucleus medialis thalami of lizards) of the diencephalon (Martínez and Puelles, '91).

In contrast to reports of NPY neurons in the pretectum of birds (Aste et al., '91; Martínez and Puelles, '91), the pretectum of the lizard *Gallotia* does not contain NPY-LI cell bodies. However, one of us has observed NPY-LI neurons in the nucleus pretectalis dorsalis of the lizard *Gekko gecko* (Medina, unpublished observations). Although we are well aware that a pretreatment with colchicine could perhaps elicit NPY-LI cell bodies in the pretectum of *Gallotia,* this discrepancy may also represent an interspecies difference. The pretectum of *Gallotia* and *Gekko* otherwise also shows differing patterns of enkephalin immunoreactivity with and without the use of colchicine (Medina and Smeets, '91).

With respect to the mesencephalic tegmentum, we noted NPY-LI neurons in the ventral tegmental area (VTA) and substantia nigra, pars compacta (SNc) in the brain of *Gallotia.* Comparison of adjacent sections immunostained either for NPY or for catecholamine markers [tyrosine hydroxylase (TH); dopamine (DA); Medina, unpublished observations] assures us that an overlapping of both kinds of cells occurs in these areas. The mammalian SNc does not contain NPY-LI neurons, and thus NPY and DA are not colocalized at this site (Everitt et al., '84). However, NPY-LI neurons have been found in the adult and embryonic VTA of the rat (Chronwall et al., '85; Woodhams et al., '85). Moreover, NPY-LI neurons have been described in the mesencephalic tegmentum of the frog and the newt (Danger et al., '85; Perroteau et al., '88), in a location similar to that of dopaminergic neurons (Gonzalez and Smeets, '91). Comparison of NPY and DA series of the brain of *Gallotia* (Medina, unpublished observations), shows that the number of neurons containing NPY in the VTA/SNc is smaller than that of dopaminergic neurons. This observation, together with the differential location of NPY and DA in the mammalian brain, suggests that in reptiles VTA/SNc neurons containing NPY do not contain DA.

Finally, the dense NPY immunoreactivity observed in the brainstem of mammals contrasts with its scarce distribution in the rhombencephalon of the lizard *Gallotia* and other nonmammalian vertebrates. Most rhombencephalic NPY-immunoreactive cell groups described in mammals (Chronwall et al., '85; De Quidt and Emson, '86; Coveñas et al., '90) have not been found in nonmammalian brains.

CONCLUSIONS

The widespread distribution of NPY in the lizard brain, as well as in the other tetrapods, suggests that this peptide is related to several functional systems. Thus, for example, the role of NPY-LI neurons and plexuses in the cortex may be an intrinsic, regulatory function (coexistence with somatostatin and GABA), and similar local-circuit roles may operate in the DVR and striatum. Sensory systems appear to be extensively modulated by NPY-containing intrinsic cells or afferents (olfactory, visual, and auditory centers), as well as the limbic, amygdalo-hypothalamic, and neuroendocrine pathways. Conserved evolutionary distribution of many features of the NPY-LI structures in the brain of vertebrates probably indicates an ancestral origin and an essential functional role for this peptide.

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