Visual Neuroscience (1990), 4, 399–412. Printed in the USA.

Neural elements in the pineal complex of the frog, *Rana esculenta*, II: GABA-immunoreactive neurons and FMRFamide-immunoreactive efferent axons

P. EKSTRÖM,[1] T. ÖSTHOLM,[1] H. MEISSL,[2] A. BRUUN,[3] J.G. RICHARDS[4], AND H. MÖHLER[4]

[1]Laboratory of Molecular Neuroanatomy, Department of Zoology, University of Lund, Lund, Sweden.
[2]Max-Planck-Institute for Physiological and Clinical Research, W.G. Kerckhoff Institute, Bad Nauheim, FRG.
[3]Department of Ophthalmology, University of Lund, Lund, Sweden.
[4]Pharmaceutical Research Department, F. Hoffmann-LaRoche & Co., Ltd., Basel, Switzerland.

(RECEIVED September 22, 1988; ACCEPTED February 19, 1990)

Abstract

The photosensory pineal complex of anurans comprises an extracranial part, the frontal organ, and an intracranial part, the pineal organ proper. Although the pineal organ functions mainly as a luminosity detector, the frontal organ may monitor the relative proportions of short and intermediate/long wavelengths in the ambient illumination. The major pathway of information processing in the pineal and frontal organs is the photoreceptor to ganglion cell synapse. It is not known whether interneurons form part of the neural circuitry. In the present study, we demonstrate GABA-immunoreactive (GABA-IR) neurons in the pineal and frontal organs of the frog, *Rana esculenta*. No GABA-IR axons were observed in the pineal nerve between the frontal and pineal organs, or in the pineal tract that connects the pineal complex with the brain. The GABA-IR neurons differed in morphology from centrally projecting neurons visualized by retrograde labeling with horseradish peroxidase. Thus, we suggest that the GABA-IR neurons in the pineal and frontal organs represent local interneurons.

Axons of central origin, immunoreactive with a sensitive antiserum against the tetrapeptide Phe-Met-Phe-Arg-NH_2 (FMRFamide), were observed in the intracranial portion of the photosensory pineal organ. The immunoreactive axons enter the caudal pole of the pineal organ via the posterior commissure. The largest density of axons was observed in the caudal part, while fewer axons were detected in the rostral portion. The uneven distribution of the FMRFamide-immunoreactive axons may be related to the distribution of different types of intrapineal neurons. FMRFamide-immunoreactive varicose axons were observed in the extracranial frontal organ. A central innervation of the pineal organ, previously known exclusively from amniotes, is probably not *per se* linked with the evolutionary transition of the pineal organ from a directly photosensory organ to a neuroendocrine organ. It could rather represent a centrifugal input to a sensory system which has been retained when the directly sensory functions have changed, during phylogeny, to neuroendocrine functions.

Keywords: Pineal complex, γ-aminobutyric acid, $GABA_A$/benzodiazepine receptor, FMRFamide, *Rana esculenta* (Amphibia)

Introduction

In evolution, the pineal organ has been transformed from a directly photosensory organ, analogous to the lateral eye retinae, to a neuroendocrine organ (Studnicka, 1905; Bargmann, 1943; Collin, 1971; Oksche, 1971; Korf & Oksche, 1986). This transformation has been accompanied by a change in innervation pattern: the afferent fibers (pinealofugal fibers) of the photosensory pineal organ, conveying neural signals related to ambient illumination (Dodt, 1973), have gradually been lost and efferent fibers (pinealopetal fibers) have become the dominant nervous connection with the brain. The sympathetic innervation of the mammalian pineal organ is the efferent system that has been the most extensively studied. Its main function appears to be the regulation of indoleamine synthesis, synchronizing it with the daily dark–light cycle (Wurtman et al., 1964; Klein et al., 1971). However, evidence has accumulated that the pineal organ of mammals also receives innervation from other sources, both peripheral (Shiotani et al., 1986) and central (Korf & Møller, 1984). The discovery of axons, immunoreactive with an antiserum against the neuropeptide FMRFamide, that innervate the photosensory pineal organ in teleost fish (Ekström et al., 1988) raised the possibility that the presence of a centrifugal in-

Reprint requests to: Peter Ekström, Laboratory of Molecular Neuroanatomy, Department of Zoology, University of Lund, Helgonavägen 3, S-223 62 Lund, Sweden.

nervation of the pineal organ may represent a pattern common to all vertebrate groups. To explore this possibility further, we investigated the possible presence of FMRFamide-immunoreactive axons in the pineal complex of the frog, *Rana esculenta*.

Another question that is largely unresolved is whether interneurons form an integral part of the neural circuitry in the photosensory pineal organ of anamniotes. The neural signals conveyed to the brain by projection neurons, that are analogous to retinal ganglion cells, carries simple information about the level of ambient illumination (Dodt, 1973; Meissl, 1986). These signals could conceivably be elaborated by a simple sign-conserving photoreceptor-to-neuron synapse (Meissl & Dodt, 1981). However, neuroanatomical data indicate the presence of intrapineal neurons that have no axonal connections with the brain (e.g. Paul et al., 1971; Wake, 1973; Wake et al., 1974; Korf, 1974; Ekström & Korf 1986*a,b*; Ekström & Meissl, 1988).

Here, we present evidence for putative GABAergic interneurons in the extracranial and intracranial portions of the pineal complex of the frog, *Rana esculenta*. We also report that although putative GABA receptor sites may be visualized in the retina and certain brain areas with monoclonal antibodies against $GABA_A$/benzodiazepine receptor subunits (Häring et al., 1985; Richards et al., 1986), no such sites were observed in the pineal complex.

Material and methods

Fixation

Eyes, brains, pineal organs, and frontal organs from frogs (*Rana esculenta*) were fixed either by immersion or by perfusion followed by immersion with one of the following fixatives: (1) 2% paraformaldehyde (w/v) + 0.1% glutaraldehyde (v/v) in phosphate-buffered saline (PBS), (2) 2% paraformaldehyde + 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB), (3) 4% paraformaldehyde + 0.1% glutaraldehyde in PB, (4) 4% paraformaldehyde + 0.25% glutaraldehyde + 0.25% picric acid (v. saturated sol./v) in PB (only for GABA immunocytochemistry), or (5) 4% paraformaldehyde and 0.25% picric acid in 0.1 M PB (only for FMRFamide immunocytochemistry). All fixatives were held at pH 7.4. Total fixation time was 24 h for all fixatives used. Fixations (1), (2), and (3) gave essentially identical results with the monoclonal antibodies.

Tissue processing and immunocytochemistry

Tissue fixed in solutions 1, 2, 3, or 4, that was intended for cryostat sectioning, was thoroughly rinsed in Tyrode buffer, and infiltrated with Tyrode buffer containing 25% sucrose and 0.1% (w/v) sodium azide (Sigma Chemicals, St. Louis, MO). The brains were placed in Tissue Tek embedding medium and frozen on a metal plate cooled with liquid nitrogen. They were then mounted on metal chucks for sectioning in a Reichert-Jung cryostat. Serial 20–25 μm sections were cut in the frontal and sagittal planes, thaw-mounted on chrome alum-gelatin-covered slides, and allowed to air dry.

Pineal organs and frontal organs fixed in solution 4 and frontal organs fixed in solution 5 were washed in Tyrode buffer, dehydrated in a graded alcohol series, and embedded in soft Araldite (Durcupan, Fluka Chemie AG, Buchs, Switzerland) with propylene oxide as intermediate medium. After polymerization, serial 3-μm sections were cut and mounted on chrome alum-gelatinized slides.

Before incubation with the primary antiserum, the resin was removed from the sections (Maxwell, 1978). Briefly, 40 g KOH was slowly dissolved in a mixture of 200 ml absolute methanol and 100 ml propylene oxide, and filtered. The resin was removed by immersing the slides in the filtered solution for 6 min. It is important that the mixture is cooled on ice throughout the procedure. After removal of the resin, the sections were rinsed in absolute methanol, and transferred to rinse buffer (PBS containing 0.25% Triton-X 100).

GABA immunocytochemistry

The slides were thoroughly rinsed in rinse buffer (see above). They were then processed for GABA immunocytochemistry, essentially as previously described (Ekström et al., 1987). Briefly, the sections were covered with rabbit anti-GABA, diluted 1:1000 in rinse buffer containing 1% bovine serum albumin. Incubations at room temperature for 18 h and at 8°C for 72 h gave identical results. Following 2 × 10 min in rinse buffer, swine anti-rabbit IgG (Dakopatts, Copenhagen) diluted 1:50 was applied for 30 min at room temperature. After 2 × 10 min in rinse buffer, rabbit PAP-complex (Dakopatts, Copenhagen) diluted 1:50 was applied for 30 min at room temperature. The slides were then rinsed for 10 min in rinse buffer.

For visualization of the immunoreaction product, the slides were transferred to Tris buffer (Tris-HCl, 0.05 M, pH 7.6) for 10 min. They were then incubated in a solution of 0.05% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemicals, St. Louis, MO; grade VI) and 0.025% (v/v) H_2O_2 in Tris buffer for 6 min at room temperature. After thorough rinsing in Tris buffer, the sections were dehydrated in a graded alcohol series, cleared in xylene, and coverslipped with Permount mounting medium.

$GABA_A$/benzodiazepine receptor immunocytochemistry

Frozen sections of tissue fixed in solutions 1, 2, or 3 were thoroughly rinsed in rinse buffer. They were then incubated overnight at room temperature with either of the hybridoma supernatants bd17 (against the α-subunit of the receptor) or bd24 (against the β-subunit) (Häring et al., 1985). Both supernatant bd17 and bd24 were used undiluted, or diluted 1+1 with phosphate-buffered saline (pH 7.2) containing 0.25% Triton X-100 and 1% bovine serum albumin. Following 2 × 10 min in rinse buffer, the sections were incubated in rabbit anti-mouse IgG (Dakopatts, Copenhagen) diluted 1:50 for 30 min, rinsed 2 × 10 min in rinse buffer, and incubated in mouse PAP-complex (Dakopatts, Copenhagen) diluted 1:50 for 30 min. After 10 min in rinse buffer, the sections were transferred to Tris buffer (see above) whereupon peroxidatic activity indicating immunoreactive sites was visualized by reacting with DAB (0.25 mg/ml), ammonium nickel sulphate (3 mg/ml), and H_2O_2 (final concentration 0.0075%) in Tris buffer for 6 min at room temperature.

FMRFamide immunocytochemistry

After thorough rinses in rinse buffer (see above), the slides were incubated overnight at room temperature with rabbit anti-FMRFamide antiserum (Incstar Corp., Stillwater, OK, USA) diluted 1:1500 in rinse buffer containing 1% bovine serum albumin. Following 2 × 10 min rinses in rinse buffer, swine anti-rabbit IgG (Dakopatts, Copenhagen) diluted 1:50 was applied for 30 min at room temperature. After 2 × 10 min in rinse buffer, rabbit PAP-complex (Dakopatts, Copenhagen) diluted 1:50 was applied for 30 min at room temperature. The slides were then rinsed for 10 min in rinse buffer.

https://doi.org/10.1017/S0952523800005162

For visualization of the immunoreaction product, the slides were transferred to Tris buffer and reacted with DAB as for GABA immunocytochemistry (see above). They were then dehydrated, cleared in xylene, and mounted in Permount.

Controls

To control the method specificity (van Leeuwen, 1986), the entire incubation protocol was performed after liquid-phase absorption of the antiserum: FMRFamide or GABA (Sigma Chemicals, St. Louis, MO) was added in excess to the FMRFamide or GABA antisera, respectively.

In the case of the monoclonal antibodies bd17 and bd24, parallel sections were incubated with culture medium in which monoclonal antibody-producing cells had not been grown. In addition, C3H pigmented mouse retina (generously supplied by Dr. S. Sanyal, Department of Biochemistry, University of Rotterdam), fixed in 4% paraformaldehyde in PB, was used as control tissue.

Neuronal counting and measurements of somatal sizes

Calculation of the numbers of GABA-immunoreactive neurons in the pineal and frontal organs, as well as measurements of the sizes of GABA-immunoreactive somata, were performed as described (Ekström & Meissl, 1990, pp. 389–397 in this issue).

Results

GABA-immunoreactive elements in the pineal organ and frontal organ

Incubation with the GABA antibody resulted in a distinct labeling of different types of neurons in four of the five pineal organs, and in all six frontal organs investigated. The numbers of GABA-immunoreactive (GABA-IR) neurons varied between individual organs (Table 1). It could be assessed from reconstructions of camera lucida drawings of serial thin sections that most GABA-IR neurons in the pineal organ are unipolar or multipolar, although bipolar neurons were also observed in smaller numbers. For all types, the primary dendrites are coarse and branch abruptly into numerous thin processes ending with rather large varicosities (Figs. 1 and 2). The neurons do not seem to possess any centrally projecting axons; no GABA-IR axons were traced to the brain. However, several dorsally situated, smaller neurons extend neurites towards the unlabeled pineal tract (Fig. 3a–c). Such neurites exhibit swellings that encircle unlabeled intrapineal neurons (Fig. 3c and 3e) or the unlabeled pineal tract (Fig. 3d and 3f). Sometimes, small swellings were observed within the pineal tract (Fig. 3f). It could not be ascertained whether these short neurites constitute dendrites or short branching axons.

In the rostral part of the pineal organ, a few neurons were observed extending thin neurites towards the habenular commissure. The habenular commissure contained numerous GABA-IR axons that formed a bundle consisting of crossing fibers. The GABA-IR neurites that emerged from the pineal organ did not appear to join the GABA-IR axons in the habenular commissure. Rather, they terminated with small swellings in the vicinity of the axon bundle.

Most GABA-IR neurons in the pineal organ were situated in the dorsal wall. They often occurred in small groups, where neurites from one neuron often could be seen to form swellings in close apposition to the soma of another (Figs. 1c, 1e, 1f and 2b). Also, adjacent neurons often possessed closely intertwined neurites with numerous swellings (Fig. 1d and 1f). Furthermore, neuronal somata were often in close apposition to each other (Fig. 1f).

GABA-IR neurons in the frontal organ were similar in morphology to those of the pineal organ. They encompassed two basic types: multipolar neurons with large dendritic trees (Figs. 4a–d, 5a and 5b) and unipolar neurons with extensively branching, although small, dendritic trees emerging from one primary dendritic trunk located in close apposition to the pineal nerve (Figs. 4e, 4f and 5c). No GABA-IR axons were observed in the pineal nerve.

As in the intracranial pineal organ, GABA-IR neurons in the frontal organ were situated close together, and neurites from adjacent neurons were often entwined, apparently sharing dendritic fields (Fig. 4f).

GABA-IR somata in the frontal organ were somewhat larger than those in the pineal organ (Table 2). The difference was, however, not statistically significant.

$GABA_A$/benzodiazepine receptor-immunoreactive elements

In all parts of the brain, except for the cerebellum (see below), immunolabeling with antibodies against the $GABA_A$/benzodiazepine receptor was very weak, and could only be discerned above background staining when using heavy metal amplifica-

Table 1. *Comparison of the numbers of HRP-backfilled projection neurons and photoreceptor-like bipolar cells (PRC) with the numbers of GABA-immunoreactive neurons in pineal and frontal organs of the frog* Rana esculenta[a]

	Pineal organs					Frontal organs					
	PO1	PO2	PO3	PO4	PO5	FO1	FO2	FO3	FO4	FO5	FO6
HRP											
$N_{i(neurons)}$	111	120	43	165	172	—	29	—	—	—	—
$N_{i(PRC)}$	6	21	9	48	34	—	1	—	—	—	—
GABA											
$N_{i(neurons)}$	45	0	13	29	36	10	14	9	8	7	3
$N_{i(PRC)}$	0	0	0	0	0	0	0	0	0	0	0

[a]Note that HRP-backfilling and GABA-immunocytochemistry was performed in different pineal organs. Numbers of neurons are given as numbers of counted profiles corrected according to Abercrombie's (1946) formula.

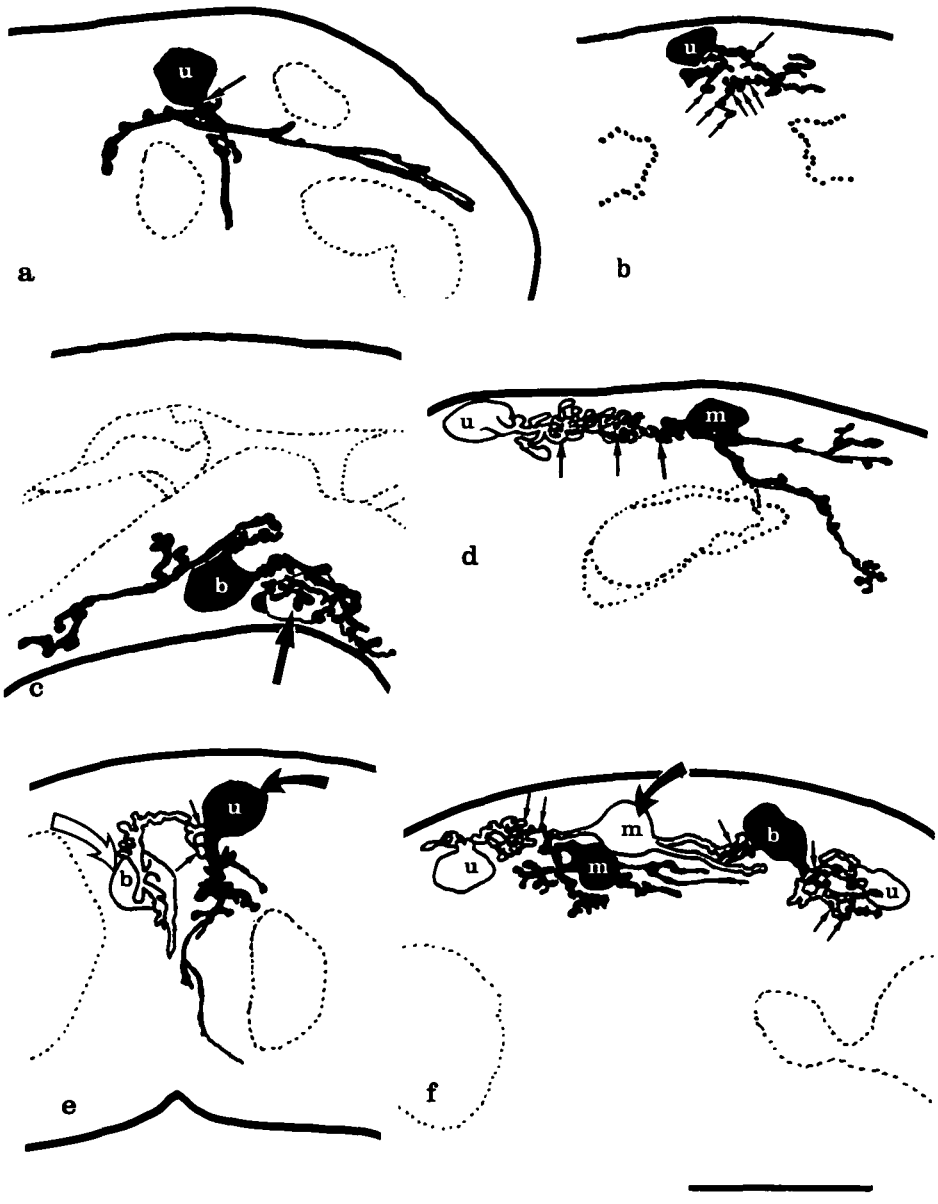


Fig. 1. Pineal organ. Reconstructions of GABA-immunoreactive neurons from camera lucida drawings of serial 3-μm-thick frontal sections. [a] Unipolar neuron. The primary dendrite (arrow) gives off three main branches. Cf. Fig. 3a. [b] Unipolar neuron exhibiting neurites with numerous varicosities (small arrows). [c] Bipolar neuron with varicose neurites, one of which exhibits terminal-like swellings on another GABA-IR cell body (arrow). [d] Two dorsally situated GABA-IR neurons with closely intertwined neurites (arrows). [e] The primary dendrite of a unipolar neuron (black curved arrow) is in close apposition to the terminal-like swellings (small arrows) of a bipolar neuron (open curved arrow). [f] Dorsally situated GABA-IR neurons have neurites in close apposition (small arrows). The dotted lines denote the border of the central lumen of the pineal organ. *b*: bipolar neuron; *m*: multipolar neuron; and *u*: unipolar neuron. Scale bar = 50 μm.

tion of the diaminobenzidine-peroxidase reaction. In the pineal organ, only a few cells with a weak labeling (only slightly more intense than the background; Fig. 6) were observed after incubation with bd17. The staining in the pineal organ lacked the somewhat granular appearance associated with the specific immunoreactivities observed in the retina and in the brain (Figs. 6–10) and was thus considered to be unspecific background staining. No labeling was observed in the frontal organ with bd17, and no labeling was observed in either the pineal or frontal organs with bd24.

No attempt was made to map the total distribution of bd17 immunoreactivity in the frog brain. Instead a few areas, known to receive input from the pineal organ and/or the retina, will be briefly described (nomenclature according to Neary & Northcutt, 1983).

A diffuse bd17 immunoreactivity was observed in the central neuropil of the dorsal habenular nucleus (Fig. 7a). Its distribution coincided with that of GABA-IR terminals (Fig. 7b). Caudally, the strongest labeling was confined to the lateral part of the medial subdivision (cf. Kemali & Lazar, 1985). In the

https://doi.org/10.1017/S0952523800005162

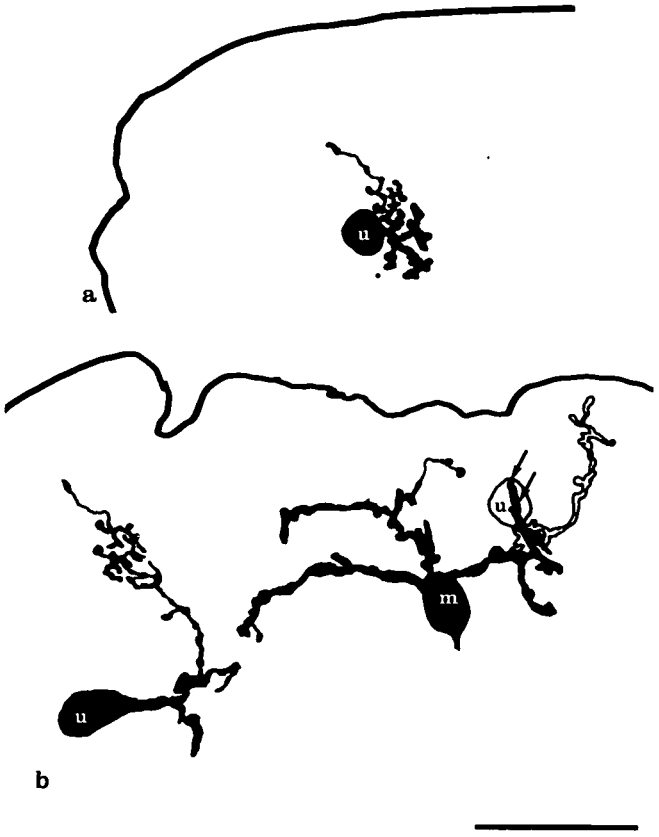


Fig. 2. Pineal organ. Reconstructions of GABA-immunoreactive neurons from camera lucida drawings of 3-μm-thick horizontal serial sections. [a] A unipolar neuron with short dendritic branches. [b] Some GABA-IR neurons extend their neurites in the horizontal plane. One neurite of a multipolar neuron is seen in close contact (arrows) with the cell body of a unipolar neuron. *m*: multipolar neuron; and *u*: unipolar neuron. Scale bar = 50 μm.

ventral habenular nucleus, a relatively distinct band of immunoreactivity was observed only caudally, in the lateral part (Fig. 6). The immunoreactivity was consistently strongest in the right habenular nuclei. In the anterior thalamic nucleus very weakly immunoreactive cell somata were observed in the anterior part. Posteriorly, diffuse non-perikaryal labeling extended into the neuropil of the central thalamic nucleus. The corpus geniculatum thalamicum contained diffuse immunoreactivity.

Strong immunoreactions with bd17 were observed in the granular layer of the cerebellum (Fig. 8a). Only weak immunoreaction was observed in the molecular layer, whereas the Purkinje cells were immunonegative. No immunoreaction was observed with bd24. The granular layer and some of the cell bodies in the molecular layer were GABA-IR (Fig. 8b).

Of the monoclonal antibodies against the two $GABA_A$/benzodiazepine receptor (BZR) subunits, only bd17 labeled neurons in the frog retina. Cell somata in the inner nuclear layer that were immunoreactive with bd17 were confined to the middle sublayer (Figs. 9 and 10). Their immunoreactive processes penetrated the inner portion to reach the inner plexiform layer. Here, immunoreaction was sometimes confined to three bands, of which the two innermost merged in most parts of the retina (Fig. 10). A diffuse immunoreactivity was associated with the outer plexiform layer (Fig. 10). No immunoreactive cell bodies were observed in the ganglion cell layer.

In the mouse retina, cell somata immunoreactive with bd17 were located in the inner nuclear layer. The largest numbers appeared to be situated approximately in the inner half of this layer. The somata were often very weakly labeled (Fig. 11a). Weakly labeled cell bodies were situated in the ganglion cell layer (Fig. 11b). Immunoreactivity was distributed throughout the inner plexiform layer, with a somewhat heavier labeling apparently associated with sublayers 2, 3, and 5 (Fig. 11).

FMRFamide-immunoreactive elements

FMRFamide-like immunoreactive (FMRF-IR) axons were observed in large numbers throughout the brain. FMRF-IR cell bodies were located in the periventricular hypothalamus: relatively large neurons (15–20 μm diameter) were located close to the ependyma of the third ventricle (Fig. 12). Incubation with liquid phase-absorbed antiserum greatly decreased the number of immunoreactive structures in the brain and pineal organ, but did not abolish all immunoreactivity. Since it is known that FMRFamide antisera may have affinities for pancreatic polypeptide-like substances, as well as other RFamides (see Discussion), we can only conclude that the structures recognized by the FMRFamide antibody contain one or more substances related to, but possibly different from, FMRFamide.

FMRFamide-IR axons could be followed from the habenular commissure into the ventral wall of the pineal organ (Fig. 13a). However, the largest number of axons appeared to enter the pineal organ at its caudal pole from the posterior commissure. Here, some axons branched and apparently terminated in the pineal parenchyma (Fig. 13b and 13c), while others only seemed to make a detour through the pineal parenchyma on their way to the contralateral side of the brain via the posterior commissure. Although a small number of FMRFamide-IR axons could be followed rostrally in the pineal organ for some distance, most axons were confined to the caudal (proximal) portion of the pineal organ.

FMRFamide-IR axons were mostly located close to the basal lamina of the pineal parenchyma. It was, however, not possible to ascertain whether they were associated with any special cell type. No FMRFamide-IR cell bodies were observed in the pineal organ.

Only very weak FMRF-immunoreactivity was observed in the frontal organ. Immunoreactive punctae, indicative of varicose fibers, were sparsely distributed throughout the parenchyma.

Discussion

Are the GABAergic neurons interneurons?

We have demonstrated, by means of immunocytochemistry, the presence of putatively GABAergic neurons in the photosensory pineal complex of the frog. The GABA-IR neurons were of similar appearance in the extracranial frontal organ and in the intracranial pineal organ proper. Comparison of numbers, morphology, and location of GABA-IR neurons with retrogradely HRP (horseradish peroxidase)-labeled neurons led to the conclusion that at least the large majority of GABA-IR neurons constitute interneurons. This interpretation was supported by the absence of GABA-IR axons in the pineal tract.

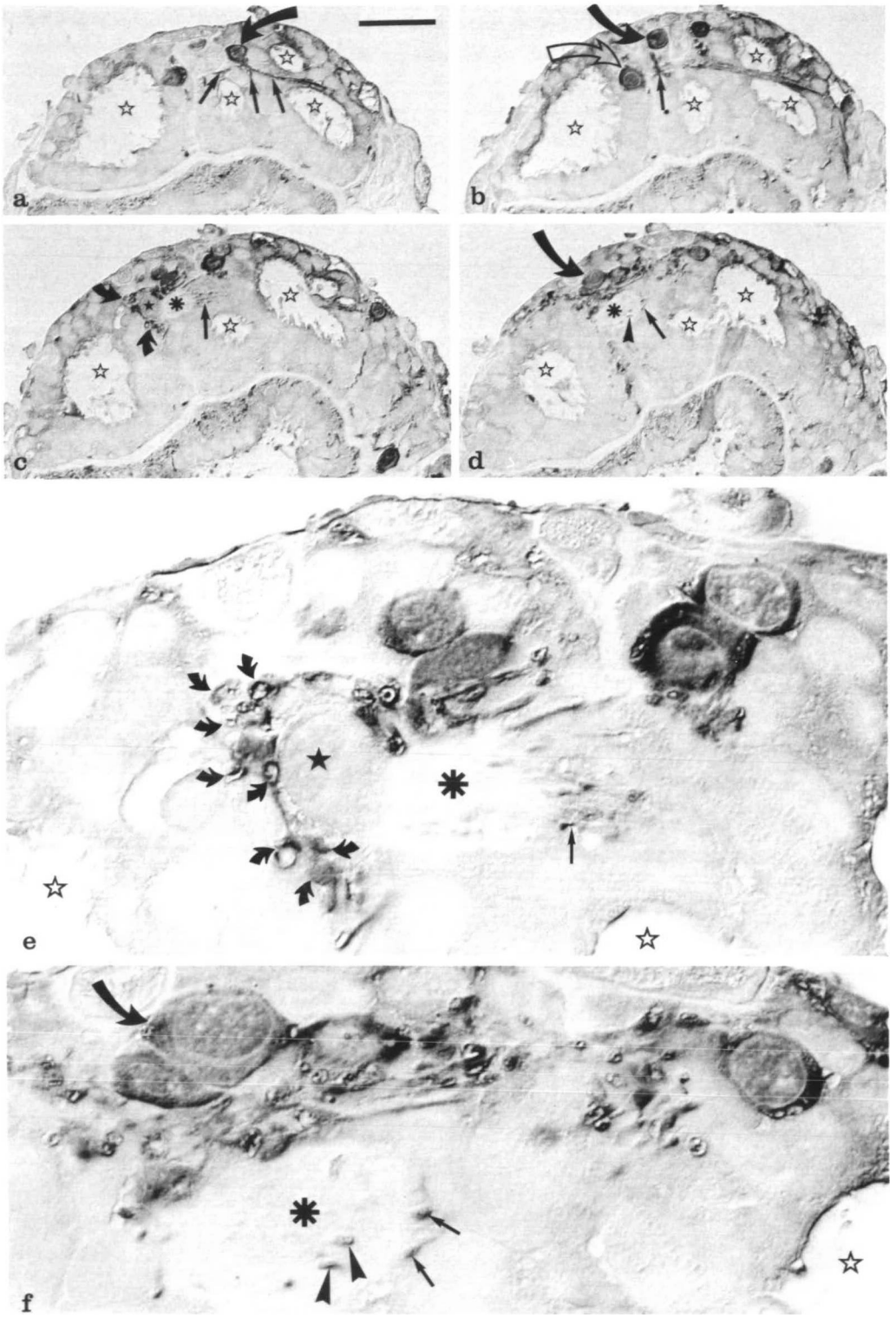


Fig. 3. Pineal organ. GABA-immunoreactive neurons. Frontal sections (3 μm). [a] The primary dendrite of a dorsally situated unipolar neuron (curved arrow) emits three branches (arrows) into the pineal parenchyma. This neuron is the one in Fig. 1a. [b] Dorsomedially situated neurons. One neuron (black curved arrow) extends its primary dendrite (arrow) ventrally. This neuron is the unipolar in Fig. 1e, whereas the one marked with the open curved arrow is the bipolar one. [c] Thin GABA-IR neurites (arrow) course towards the pineal tract (asterisk). GABA-IR neurites end with terminal-like swellings (small curved arrows) adjacent to an immunonegative cell body (black star; higher magnification in Fig. 3e). [d] The thin neurites from Fig. 3c end with terminal-like swellings around (arrows) and within (arrowhead) the pineal tract (asterisk; higher magnification in Fig. 3f). The neuron marked with a curved arrow is the one identically labeled in Fig. 1f. [e] Higher magnification of Fig. 3c. [f] Higher magnification of Fig. 3d. Central lumen of the pineal organ, *stars*; pineal tract, *asterisk*. Scale bar is 50 μm for Fig. a–d, and 10 μm for Fig. e–f.

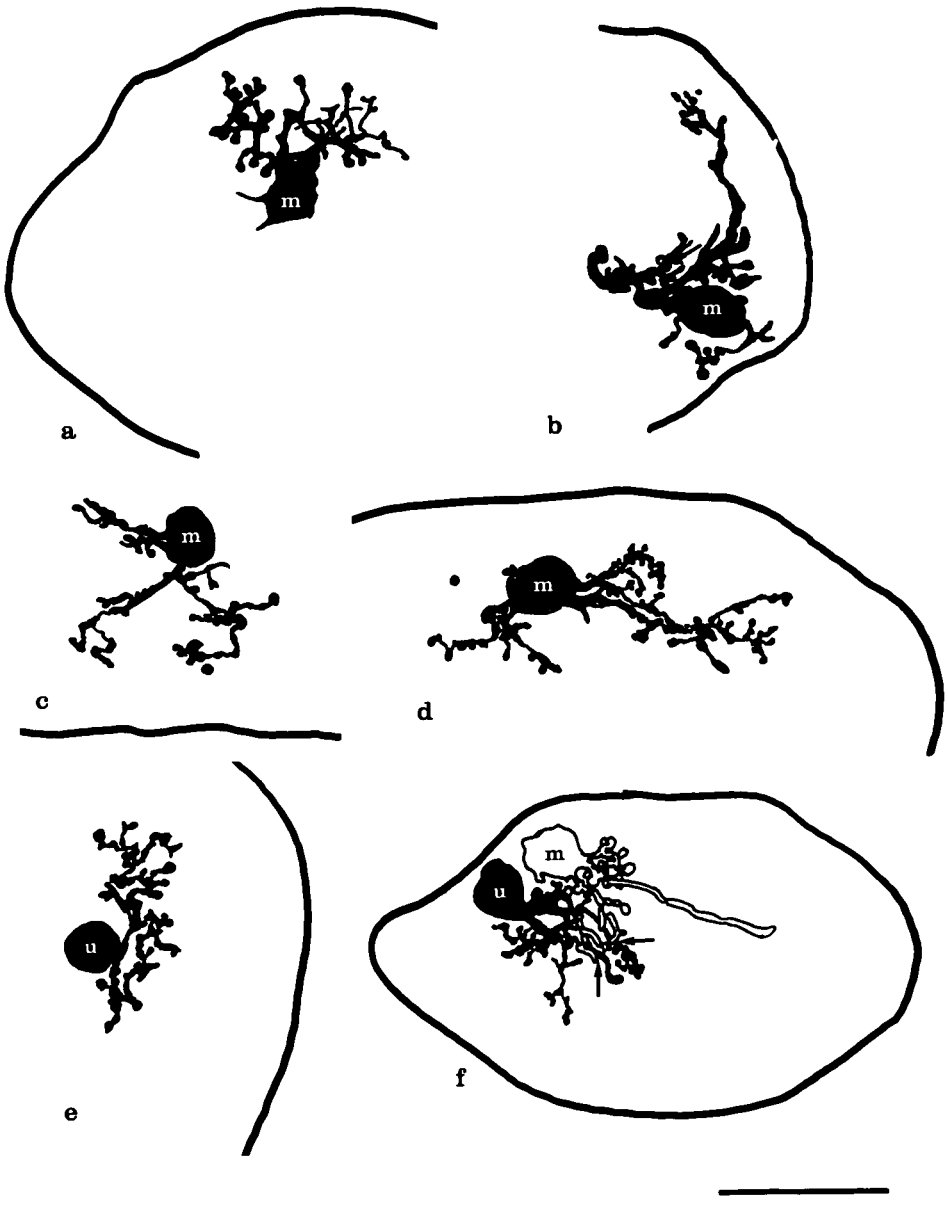


Fig. 4. Frontal organ. Reconstructions of GABA-immunoreactive neurons from camera lucida drawings of 3-μm-thick horizontal serial sections. [a–d] Different morphological varieties of multipolar neurons (m). All have irregularly branching varicose neurites. [e] Unipolar neuron (u). [f] A unipolar neuron (u) and a multipolar neuron (m) with closely intertwined neurites (arrows). Scale bar = 50 μm.

Pineal ganglion cells (projection neurons), as visualized when backfilled with HRP (Ekström & Meissl, 1990), tend to be located directly below the basal lamina of the pineal epithelium with dendrites forming a superficial plexus. GABA-IR neurons have also peripherally located cell bodies, but do not contribute neurites to a superficial plexus. Also, typical bipolar (photoreceptor-like) cells with a short dendritic protrusion towards the pineal lumen appear to constitute a class of pineal ganglion cells. No GABA-IR bipolar, photoreceptor-like, cells were observed in the pineal organ. It is worth noting that some of the so-called amacrine-like neurons visualized in the frog pineal organ, with supravital methylene blue-staining (Paul et al., 1971; Fig. 4g and 4h), have a morphology more similar with the GABA-IR neurons than neurons with axonal projections into the pineal tract.

The absence of GABA immunoreactivity in the pineal nerve and tract is not *per se* evidence that all GABA-IR neurons are interneurons. Nevertheless, it is a strong indication, since the antibody employed is a powerful marker of GABAergic neurites in the retina (Agardh et al., 1987; Östholm et al., 1988), teleost pineal organ (Ekström et al., 1987; Östholm et al., 1988), and brain (Ekström, in preparation).

In the pineal organ, the numbers of GABA-IR neurons are much smaller than the numbers of HRP-backfilled ganglion cells (Table 1). Taken together with the morphological differences, it appears that at least the majority of the GABA-IR neurons

https://doi.org/10.1017/S0952523800005162

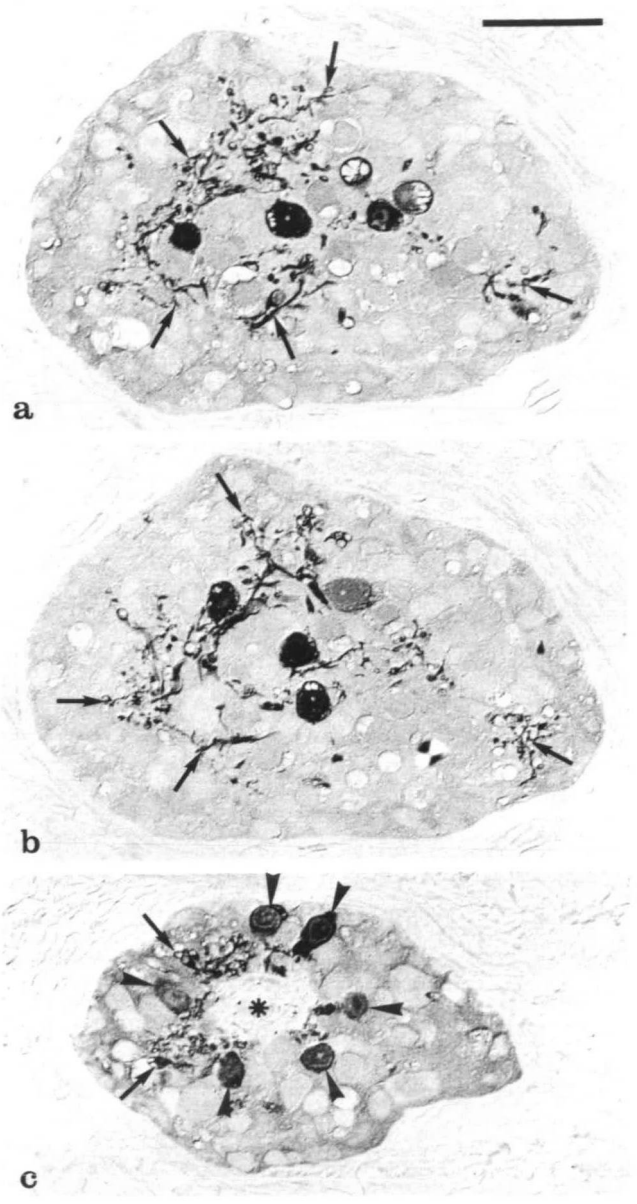


Fig. 5. Frontal organ. GABA-immunorcactive neurons. Horizontal sections (3 μm). [a,b] Multipolar neurons with large dendritic trees (arrows). [c] Neurons with cell bodies (arrowheads) and dendrites (arrows) in close apposition to the exit of the pineal nerve (asterisk). Scale bar = 50 μm.

constitute a population different from the ganglion cells. If any GABA-IR neurons in the pineal organ are projection neurons, the most likely candidate would be the dorsally situated neurons that emit neurites toward the pineal tract.

Also in the frontal organ, the numbers of GABA-IR neurons are smaller than the number of HRP-backfilled neurons (although more difficult to compare with only one frontal organ sufficiently well backfilled; Table 1). However, when comparing the morphology of centrally projecting neurons (Eldred & Nolte, 1981; see part Ekström & Meissl, part I, in this issue) with the GABA-IR neurons, it appears that most projection neurons are ventrally located with dorsally and laterally extending dendrites. Cell somata and dendrites would then probably not appear in the same (semithin) horizontal section. However, in semithin horizontal sections through the frontal organ, GABA-IR cell somata and dendrites can be observed in the same section. Also, GABA-IR neurons seem to be relatively evenly distributed along the dorsoventral axis. Taken together, these data suggest that also the GABA-IR neurons of the frontal organ are interneurons.

GABA-IR neurons in the frontal organ appeared to be of approximately the same size (Table 2), and to possess dendrites with a branching pattern closely similar to those of the pineal organ. Thus, morphological differences reflecting differences in the physiological responses to light stimulation should probably not be sought in neuronal shapes and/or dendritic arborizations but at the subcellular level, e.g. by electron-microscopic analysis of the synaptic organization of identified neural elements.

The close apposition of neurites from neighboring GABA-IR neurons indicate communication between these cells, perhaps reciprocal inhibitory synapses. However, this interpretation must await confirmation by immunocytochemical studies at the electron-microscopic level.

A direct way to prove that the GABA-IR neurons and the projection neurons constitute different neuronal populations would be to combine retrograde tracing with immunocytochemistry in order to visualize both types of neurons in the same section. So far, this has not been successful in the pineal organ of the frog (unpublished observations). The reason for this is probably that the conditions for optimal HRP backfilling is not compatible with optimal GABA immunocytochemistry in the pineal organ.

Comparison with earlier studies of GABA in the pineal complex

With the same GABA antiserum as in the present study, Ekström et al. (1987) demonstrated two populations of GABA-IR neurons in the pineal organ of the rainbow trout: large (20–30 μm diameter) multipolar interneurons and small (*ca.* 10 μm diameter) centrally projecting neurons. The numbers of neurons observed were small and varied greatly between specimens. In addition, a pronounced GABA immunoreaction was observed in glial elements (Ekström et al., 1987). In the present study, we observed no GABA-IR glial elements, although background labeling was somewhat higher in the pineal and frontal organs than in surrounding tissue (e.g. skin, paraphyseal complex), perhaps indicating diffusion of GABA during tissue processing. Further support for the presence of GABAergic neurons in pineal complexes may be gained from the study of the parietal eye of a lizard by Engbretson and Battelle (1985). Here it was shown that lizard parietal eyes may synthesize GABA from its precursor glutamic acid, and that [^{3}H]-GABA is taken up by neurons (and lens cells and glial elements) in the parietal eye.

Physiologic action of GABA in the pineal organ

The pineal ganglion cells—the centrally projecting neurons—show a spontaneous firing of action potentials that is highest in the dark and is suppressed by light (Dodt & Jacobson, 1963). Iontophoretic application of GABA also suppresses the spontaneous firing in the dark. However, the suppressive effects of GABA and light are not additive. Rather, a brief light stimu-

Table 2. *Comparison of the mean sizes of neuronal somata revealed in the pineal and frontal organs of the frog,* Rana esculenta, *by means of retrograde filling with HRP or GABA-immunocytochemistry. HRP-backfilled somata are divided in two classes, neurons and photoreceptor-like bipolar cells (PRC)*[a]

	Pineal organs						Frontal organs						
	PO1	PO2	PO3	PO4	PO5	ΣPO	FO1	FO2	FO3	FO4	FO5	FO6	ΣFO
HRP													
Neurons	$n = 21$	$n = 33$	$n = 7$	$n = 13$	$n = 15$	$n = 89$		$n = 19$					
$\bar{s}$	10.29	11.54	9.4	10.48	9.24	10.53		12.12					
S.D.	(2.4)	(2.23)	(2.35)	(2.06)	(1.65)	(2.30)		(2.11)					
$\bar{l}$	13.88	14.95	13.09	16.09	13.63	14.5		15.21					
S.D.	(3.86)	(3.1)	(4.04)	(3.82)	(3.10)	(3.51)		(2.52)					
PRC	$n = 1$	$n = 5$	$n = 5$	$n = 7$	$n = 6$	$n = 24$		$n = 1$					
$\bar{s}$	7.05	7.77	9.4	8.39	7.44	8.23		7.05					
S.D.	—	(1.29)	(3.71)	(1.84)	(1.77)	(1.7)		—					
$\bar{l}$	17.62	24.44	28.2	21.82	31.33	25.9		23.5					
S.D.	—	(2.10)	(4.7)	(1.78)	(6.07)	(5.55)		—					
GABA													
Neurons	$n = 30$	$n = 0$	$n = 13$	$n = 18$	$n = 28$	$n = 89$	$n = 22$	$n = 22$	$n = 17$	$n = 10$	$n = 8$	$n = 4$	$n = 83$
$\bar{s}$	10.88		11.75	11.35	11.16	11.2	11.54	14.1	16.73	13.87	14.98	15.28	14.07
S.D.	(2.43)		(1.66)	(1.20)	(1.98)	(1.97)	(1.61)	(1.45)	(3.51)	(1.33)	(1.22)	(1.36)	(2.71)
$\bar{l}$	15.98		16.08	13.84	14.93	15.24	18.37	17.84	22.67	17.16	17.62	18.21	18.88
S.D.	(3.11)		(2.85)	(2.12)	(2.32)	(2.75)	(3.53)	(2.48)	(5.51)	(2.49)	(1.26)	(1.18)	(3.93)

Measures are given as mean lengths in microns for the short ($\bar{s}$) and long ($\bar{l}$) axes of the cells (see also the text and Ekström & Meissl, 1990). n = the number of measured profiles; this may thus be larger than the actual (corrected) number of neurons (cf. Table 1). Standard deviations (S.D.) of the samples are given in parentheses.

lus interferes with the suppressive effect of GABA, eliciting an OFF response and generally shortening the duration of inhibition by GABA (Meissl & George, 1985).

The suppressive effect of light on the spontaneous firing of ganglion cells may be explained by taking the major pathway of information processing in the pineal organ as the photoreceptor to ganglion cell synapse. Similar to retinal photoreceptor cells, pineal photoreceptors appear to release excitatory amino acid(s) in the dark (Meissl & George, 1984) that stimulate the postsynaptic ganglion cells. Light hyperpolarizes the photoreceptor cell membrane, the release is reduced, and the excitatory input to the ganglion cells is reduced.

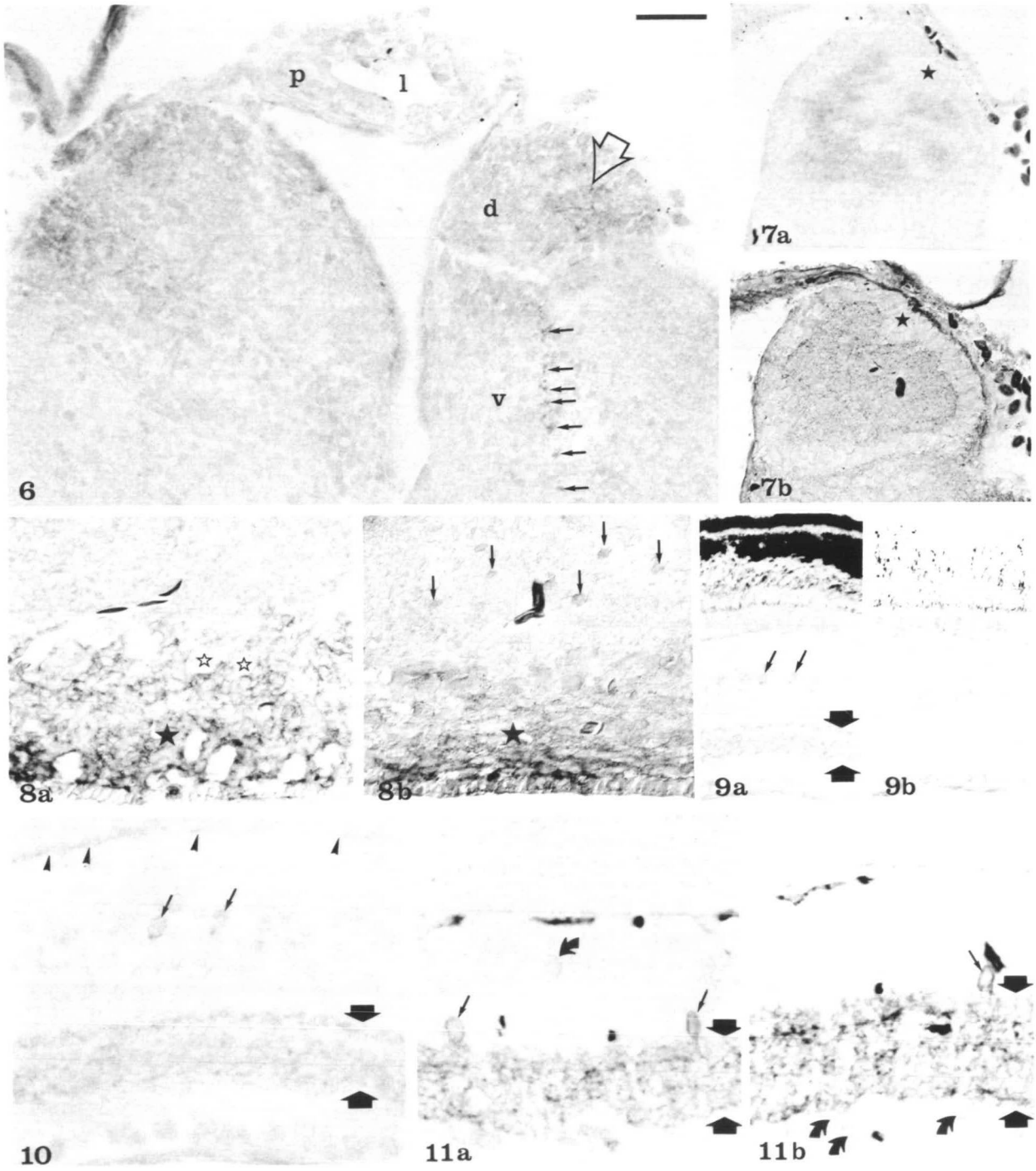
To explain the interactive effects of light and GABA on ganglion cell activity, we have to introduce a mini-circuit where the excitatory feedforward pathway from photoreceptor to ganglion cell is balanced by an inhibitory feedforward pathway via a GABAergic neuron that itself is postsynaptic to the photoreceptor cell. In order to achieve this balance, the GABAergic neuron can be assigned several possible identities: GABAergic interneurons that are postsynaptic to photoreceptor cells and make (1) feedforward inhibitory contact on ganglion cells or (2) feedback inhibitory contacts on photoreceptor cells, GABAergic interneurons that receive input from excitatory ganglion cell axon collaterals and make feedback inhibitory contacts with (3) ganglion cells or (4) photoreceptor cells, or GABAergic ganglion cells that are postsynaptic to photoreceptors and inhibit (5) ganglion cells or (6) photoreceptor cells by axon collaterals. Our results indicate that at least the large majority of GABA-IR neurons in the pineal organ are interneurons, and that at least some of the GABA-IR neurons are presynaptic to other neurons (Fig. 3e).

Assigning to the GABA-IR neurons the role of inhibitory interneurons that are presynaptic to ganglion cells and postsynaptic to photoreceptor cells [(1) above], the interactive effects of light and GABA may be explained as follows. Ganglion cells are activated in the dark by direct excitatory inputs from photoreceptor cells, but this activation is balanced by the inhibitory input from GABAergic interneurons that are activated by input from photoreceptor cells. A light flash would then reduce the direct excitatory input to ganglion cells from photoreceptor cells, but would also reduce the excitation of GABAergic interneurons, thus reducing their inhibition of ganglion cell activity. In this way, the mini-circuit possesses two pathways for information transfer from photoreceptor cells to ganglion cells, first, the direct feedforward excitatory pathway from photoreceptor to ganglion cells, and second, the indirect feedforward inhibitory pathway to ganglion cells via GABAergic interneurons.

If the GABAergic interneurons make inhibitory feedback on photoreceptor cells [(2) above] that are presynaptic to ganglion cells, the same net result would ensue: a light flash would decrease the activation of the ganglion cells, but also the tonic feedback inhibition of photoreceptors in the dark, thus attenuating the net decrease in release of excitatory transmitter. We cannot, on the basis of our immunocytochemical results, exclude the possibility that GABA-IR neurons form feedback inhibitory synapses on photoreceptors.

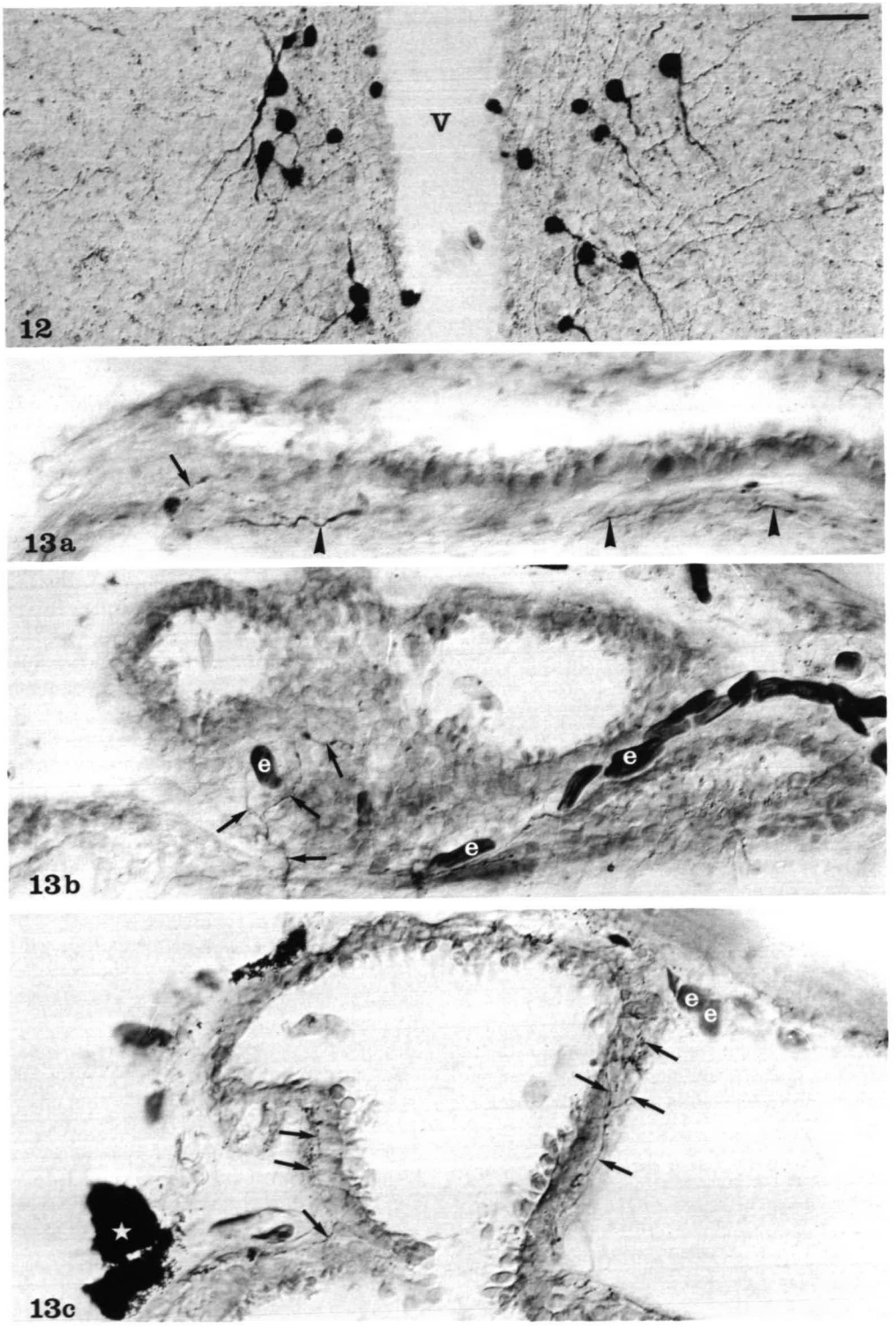
If the GABAergic neurons are interneurons that are activated by input from axon collaterals of excitatory ganglion cells, and make feedback inhibitory synapses on the ganglion cells [(3) above] or photoreceptor cells [(4) above], the light-induced decrease in direct activation of ganglion cells by photoreceptors would be balanced by a decreased inhibition by the GABAergic elements.

Also, if the GABAergic neurons are ganglion cells with recurrent axon collaterals that make inhibitory synapses on themselves or on other ganglion cells [(5) above] or on photo-



Figs. 6–11. bd17-immunoreactive (bd17-IR) and GABA-immunoreactive elements in the CNS and retina of frog and mouse. **[6]** The frog pineal organ (p) contains no labeling above background, whereas a weak bd17 immunoreactivity is observed in the dorsal habenular nucleus (open arrow) and the ventral habenular nucleus (small arrows). *d*: dorsal habenular nucleus; *l*: central lumen of the pineal organ; and *v*: ventral habenular nucleus. **[7]** Dorsal habenular nucleus, frog. Adjacent sections showing that the distribution of bd17-IR in the neuropil (a) corresponds well with that of GABA-IR (b). Note that the peripherally situated cell bodies (star) are unlabeled. **[8]** Cerebellum, frog. Adjacent sections showing that the granular layer (black star) is both bd17-IR (a) and GABA-IR (b). Note that the cell bodies of the Purkinje cells (open stars) are surrounded by bd17-IR processes, and that several cell bodies in the molecular layer are weakly GABA-IR (small arrows). **[9]** Frog retina. [a] bd17-IR cell bodies (small arrows) are located in the middle of the inner nuclear layer (INL), whereas the inner plexiform layer (between *arrows*) is interspersed with bd17-IR neurites, that in some locations can be seen to occur in higher densities in sublaminae 1, 3, and 5. [b] Control section: culture medium in which no monoclonal antibody-producing cells were grown was substituted for bd17. **[10]** Frog retina. Higher magnification of Fig. 9a. Note the diffuse immunoreactivity in the outer plexiform layer (arrowheads). **[11a,b]** Mouse retina. bd17-IR cell bodies occur primarily in the inner half of the INL, where strongly immunoreactive cells are located close to the IPL (small arrows) and weakly immunoreactive cells are farther away from it (curved arrow). Weakly bd17-IR cell bodies are located in the ganglion cell layer (curved arrows). Scale bar is 50 μm for Figs. 6, 8, and 9; 75 μm for Fig. 7; and 25 μm for Figs. 10 and 11.

https://doi.org/10.1017/S0952523800005162



Figs. 12–13. FMRFamide-immunoreactivity (FMRF-IR) in the frog brain and pineal organ. **[12]** FMRF-IR neurons located close to the ependyma lining the third ventricle. Note the large number of varicose processes. **[13]** Pineal organ. [a] At its rostral pole, close to the habenular commissure, a varicose FMRF-IR axon (arrow) enters the pineal parenchyma. Numerous FMRF-IR processes (arrowheads) course in the diencephalic roof. [b,c] Numerous FMRF-IR axons (arrows) enter the pineal organ from its caudal pole, close to the posterior commissure. Dark structures are erythrocytes (e) and pigment cells (star). Scale bar is 50 μm for Fig. 12, and 28 μm for Fig. 13.

receptors [(6) above], the net result of an interaction between light and GABA would be the same.

Here, a word of caution is appropriate. The interaction of GABA and light was observed when exogenous GABA was introduced to the pineal neural circuitry (Meissl & George, 1985). Thus, the effects of the exogenous GABA must be expected to have been superimposed on the effects of endogenous GABA release, and to have saturated the GABA response. Although picrotoxin and bicuculline were able to block the inhibition by GABA of ganglion cell activity in the dark, these substances did not appear to affect the ganglion cell activity by themselves. Unfortunately, their effect on the interactive effects of GABA and light was not investigated. To explain the attenuation by light of GABA-induced inhibition, we probably have to include non-GABAergic inhibitory neurons that are connected to photoreceptor cells and ganglion cells in the same way(s) as neurons (1)–(6) above.

Unfortunately, we have no information about the actions of GABA in the frontal organ. Although the intracranial pineal organ mainly functions as a luminance meter, the extracranial frontal organ could be a chromaticity detector, at least within a moderate range of light intensities (Dodt & Heerd, 1962; Dodt & Jacobson, 1963; Eldred & Nolte, 1978; Meissl & Dodt, 1981). The different actions of short and midspectral wavelengths on the projection neurons of the frontal organ might be mediated by inhibitory interneurons (Meissl & Dodt, 1981), although alternative mechanisms have been proposed. Hamasaki (1970) hypothesized that there may be two receptor populations with different photopigments that make synaptic contacts with a common ganglion cell. To date, we have no direct physiological recordings from pineal photoreceptors with different spectral sensitivities that may support this theory, although three types of photoreceptor cells may be distinguished on the basis of ultrastructure and opsin immunoreactivity (Vigh & Vigh-Teichmann, 1986; cf. Eldred & Nolte, 1981). Dodt (1963) and later Eldred and Nolte (1978) suggested that the chromaticity response may be due to photointerconversion of two states of a single visual pigment in a single type of photoreceptor. For this, again, we have no direct evidence.

$GABA_A$/benzodiazepine receptors

A direct way to investigate whether pineal projection neurons are postsynaptic to GABAergic interneurons would be to demonstrate GABA receptors in their plasma membranes. Histological analysis of the distribution of GABA receptors is usually performed by exposing unfixed tissue to radioactively labeled GABA or GABA ligands, whereupon the binding sites are visualized by autoradiography (Richards et al., 1986). This approach gives a poor resolution and would not allow identification of cell types in the minute pineal organ. Instead, we have taken advantage of the recent development of monoclonal antibodies against the α and β subunits of the $GABA_A$/benzodiazepine receptor complex ($GABA_A$/BZR; Häring et al., 1985; Richards et al., 1986). Immunocytochemical detection of this receptor complex yields a resolution at the subcellular level (Richards et al., 1986).

$GABA_A$/BZR-IR cell bodies and neurites were readily detectable with bd17 in control tissue, the mouse retina, with a distribution similar to that reported from the rat retina (Richards et al., 1986). In frog brain and retina, bd17 immunoreactivity was distinct but much weaker. The concentration of $GABA_A$/BZR-IR to the inner plexiform layer of the frog retina agrees with radiohistochemical studies of other vertebrates, using tritiated benzodiazepines (Yazulla, 1986). In light of this, the absence of immunoreactivity in the pineal organ is somewhat surprising, since Meissl and George (1985) showed that the $GABA_A$ receptor antagonists bicuculline and picrotoxin blocked the effect of GABA on pineal projection neurons. On the basis of the present results, we can only conclude that even if GABA receptors, with a structural similarity to the $GABA_A$/BZR of mammals, are present in the central nervous system (CNS) of the frog, their concentration in the frog pineal and frontal organs are too low to allow immunocytochemical detection by use of the monoclonal antibodies bd17 or bd24.

FMRFamide-like immunoreactive efferent axons

Although it is of great importance for the understanding of the functions of the photosensory pineal organ to identify the neuroactive substance of the FMRF-IR axons*, is it of less importance for the problem addressed in the present investigation: is there a central innervation of the pineal complex in the frog, the most extensively studied anamniote pineal organ? Earlier studies have not been unequivocal on this point (see below), and we have explored the possibility that axons innervating the pineal complex of the frog may be immunoreactive with a commercial FMRFamide antiserum, as in teleosts (Ekström et al., 1988).

One difficulty connected with the interpretation that the FMRF-IR axons are efferent to the pineal and frontal organs lies in the possibility that absence of immunoreactivity in pineal nerve cell bodies only reflects lower levels of antigen than that present in the axons. This difference relates to the process of packaging of a propeptide in secretory granules, in which the propeptide molecules undergo posttranslational modifications during transport down the axon. Thus, an antibody directed against the final signal peptide may label only axons and terminals, unless (1) secretory granules containing processed signal peptide are accumulated in the cell body, or (2) the signal peptide epitope recognized by the antibody is present and accessible also in the propeptide before packaging in secretory granules.

Accumulation of secretory granules in the cell bodies may be achieved by interruption of the axonal transport systems, e.g. by local application of colchicine. Unfortunately, the minute size of the frog pineal organ makes this approach impractical. Also, analysis of the synthesis pathways of peptides likely to cross-react with the FMRF antibodies gives no further clue to the origins of the intrapineal FMRF-IR axons: pancreatic polypeptides that cross-react with FMRFamide antisera (Triepel & Grimmelikhuijzen, 1984) are C-amidated in the secretory granules along their passage down the axon (Boel et al., 1984), and could give rise to a similar staining of axons but not cell bod-

*The question whether the FMRF-IR axons in the intracranial, directly photosensory, pineal organ of the frog (*Rana esculenta*) really contain FMRFamide cannot be answered by simple absorption tests; such tests only indicate the degree of recognition of similar epitopes on related molecules, i.e. the specificity of the antiserum. Even laborious solid-phase pre-absorptions with all substances that might conceivably be recognized by the polyclonal antiserum do not exclude the possibility that the purified antiserum still cross-reacts with unknown substances with epitope(s) very similar to the antigen. However, it is important to bear in mind that FMRFamide antisera may recognize pancreatic polypeptides, and some neuropeptides that have the carboxyterminus-RFamide (Triepel & Grimmelikhuijzen, 1984; Ebberink et al., 1987).

ies. Instead, we have to look for correlative evidence from morphological studies of frog pineal organs.

Neuroanatomical evidence for the presence of axons of central origin innervating the frontal organ has, until now, been somewhat contradictory. After applying HRP to the frontal organ, Eldred et al. (1980) found no retrogradely filled perikarya in the brain, and claimed that Zilles and Nickeleit (1979) had misinterpreted neuromelanin granules for HRP reaction product when describing widespread cell groups innervating the frontal organ. Later Kemali and De Santis (1983), using cobaltic lysine tracing, described neurons in the pineal organ, hypothalamus, and retina that would provide an innervation of the frontal organ. The location of these neurons did not coincide with those described by Zilles and Nickeleit (1979).

In a comparative study of different types of nerve fibers in the pineal organ, Vigh-Teichmann et al. (1973) described nerve terminals containing large granular vesicles apparently terminating on the basal parts of pinealocytes (photoreceptor cells) in the pineal organ of an urodele amphibian, *Pleurodeles waltlii*. These axons, which may well represent a peptidergic innervation of the pineal parenchyma, were similar to axons of hypothalamic neurons (Vigh-Teichmann et al., 1973).

Observations of axonal degeneration after lesions of the pineal nerve (i.e. the nerve connecting the intracranial pineal organ with the frontal organ) led Böttger and Böttger (1973) to the conclusion that axons, both afferent and efferent to the frontal organ, course in this nerve. This conclusion supported the physiological experiments of Morita (1971), who interpreted changes in the photic responses of the frontal organ to electrical stimulation of the pineal nerve as evidence for the presence of central axons innervating the projection neurons ("ganglion cells") of the frontal organ. The efferent impulses seemed to potentiate the light responses of the ganglion cells in the frontal organ (Morita, 1971). These results may be compared with the observations by Engbretson and Lent (1976) who were able to elicit efferent impulses in the parietal nerve of a lizard by exposing the intracranial pineal organ to norepinephrine and serotonin. Notably, these substances had differential effects on the light responses of the parietal eye, and the effects depended on whether the experiments were conducted during the animals' natural photophase or scotophase.

Because of the large numbers of FMRF-IR axons in the brain, it was not possible to follow the FMRF-IR axons from the pineal organ to their cell bodies of origin. Thus, our results do not allow the identification of the cell group(s) that supply the FMRF-IR innervation of the pineal complex in the frog. A recent study of the pineal organ in teleost fish demonstrated an innervation of FMRF-IR axons that seemed to originate, at least partly, in the telencephalic nucleus of the terminal nerve or nucleus olfactoretinalis (Ekström et al., 1988). No similar distribution of terminal nerve fibers is known from amphibians (Wirsig & Getchell, 1986). It seems probable that the FMRF-IR axons in the pineal complex of the frog originate elsewhere in the brain.

A central innervation is apparently not a feature directly linked with the evolution of the pineal organ from a photosensory to a photoneuroendocrine structure, but may represent a phylogenetically older trait. It is probably more related to the general pattern of centrifugal feedback innervations of sensory structures. However, there is so far no evidence for homologies either with the innervation of the pineal organ from the hypothalamus and/or habenular complex, as known from amniotes (Korf & Wagner, 1980, 1981; Korf et al., 1982), or the innervation by the nervus terminalis as known from teleosts (Ekström et al., 1988).

Conclusions

We have demonstrated the presence of GABA-immunoreactive neurons and FMRFamide-like immunoreactive axons in both the intracranial and extracranial portions of the pineal complex of the frog *Rana esculenta*. On the basis of neuronal morphology, it appears likely that at least the majority of the GABA-IR neurons represent a neuronal population different from the centrally projecting neurons. Thus, they appear to constitute a population of inhibitory interneurons. The FMRF-IR axons may represent a central innervation of this photosensory organ, although the location of their neuronal somata was not identified in the present study.

Acknowledgments

The technical assistance of Ms. C. Rasmussen and Ms. R. Wallén, and photographical assistance of Ms. I. Norling are gratefully acknowledged. The authors would like to thank the two anonymous reviewers for a number of very useful suggestions to improve the manuscript. This study was supported by a Research grant from the Swedish Natural Science Research Council (BI 8554-103) to P. Ekström.

References

Agardh, E., Bruun, A., Ehinger, B., Ekström, P., van Veen, Th. & Wu, J.-Y. (1987). Gamma-aminobutyric acid- and glutamic acid decarboxylase-immunoreactive neurons in the retina of different vertebrates. *Journal of Comparative Neurology* **258**, 622–630.

Bargmann, W. (1943). Die Epiphysis cerebri. In *Lehrbuch der Vergleichenden Mikroskopischen Anatomie des Menschen, Vol. VI/4*, ed. von Möllendorf, W., pp. 309–502. Berlin: Springer-Verlag.

Boel, E., Schwartz, T.W., Norris, K.E. & Fiil, N.P. (1984). A cDNA encoding a small common precursor for human pancreatic polypeptide and pancreatic icosapeptide. *EMBO Journal* **3**, 909–912.

Böttger, W.V. & Böttger, E.-M. (1973). Degenerationsstudien am Nervus pinealis von *Rana esculenta* L. nach stirnorgannaher und -ferner Durchtrennung. *Zeitschrift für Zellforschung* **136**, 365–391.

Collin, J.-P. (1971). Differentiation and regression of the cells of the sensory line in the epiphysis cerebri. In *The Pineal Gland*, ed. Wolstenholme, G.E.W. & Knight, J. pp. 79–120. Edinburgh, England: Churchill Livingstone.

Dodt, E. (1963). Photosensitivity of the pineal organ in the teleost, *Salmo irideus* (Gibbons). *Experientia* **19**, 642–643.

Dodt, E. (1973). The parietal eye (pineal and parietal organs) of lower vertebrates. In *Handbook of Sensory Physiology, Vol. VII/3B*, ed. Jung, R., pp. 113–140. New York, Berlin, Heidelberg: Springer-Verlag.

Dodt, E. & Heerd, E. (1962). Mode of action of pineal nerve fibers in frogs. *Journal of Neurophysiology* **25**, 405–429.

Dodt, E. & Jacobson, M. (1963). Photosensitivity of a localized region of the frog diencephalon. *Journal of Neurophysiology* **26**, 752–758.

Ebberink, R.H.M., Price, D.A., van Loenhout, H., Doble, K.E., Riehm, J.P., Geraerts, W.P.M. & Greenberg, M.J. (1987). The brain of Lymnaea contains a family of FMRFamide-like peptides. *Peptides* **8**, 515–522.

Ekström, P. & Korf, H.W. (1986*a*). Putative cholinergic elements in the photosensory pineal organ and retina of a teleost, *Phoxinus phoxinus* L. (*Cyprinidae*). Distribution of choline acetyltransferase immunoreactivity, acetylcholinesterase-positive elements, and pinealofugally projecting neurons. *Cell and Tissue Research* **246**, 321–329.

Ekström, P. & Korf, H.W. (1986*b*). Substance P-like-immunoreactive neurons in the photosensory pineal organ of the rainbow trout, *Salmo gairdneri* Richardson (*Teleostei*). *Cell and Tissue Research* **246**, 359–364.

Ekström, P. & Meissl, H. (1988). Intracellular staining of physiologically identified photoreceptor cells and hyperpolarizing interneurons in the teleost pineal organ. *Neuroscience* **25**, 1061–1070.

Ekstrom, P. & Meissl, H. (1990). Neural elements in the pineal complex of the frog, *Rana esculenta*, I: Centrally projecting neurons. *Visual Neuroscience* **4**, 389–397.

Ekström, P., van Veen, Th., Bruun, A. & Ehinger, B. (1987). GABA-immunoreactive neurons in the photosensory pineal organ of the rainbow trout form two distinct neuronal populations. *Cell and Tissue Research* **250**, 87–92.

Ekström, P., Honkanen, T. & Ebbesson, S.O.E. (1988). FMRFamide-like immunoreactive neurons of the nervus terminalis of teleosts innervate both retina and pineal organ. *Brain Research* **460**, 68–75.

Eldred, W.D. & Nolte, J. (1978). Pineal photoreceptors: evidence for a vertebrate visual pigment with two physiologically active states. *Vision Research* **18**, 29–32.

Eldred, W.D. & Nolte, J. (1981). Multiple classes of photoreceptors and neurons in the frontal organ of *Rana pipiens*. *Journal of Comparative Neurology* **203**, 269–295.

Eldred, W.D., Finger, T.E. & Nolte, J. (1980). Central projections of the frontal organ of *Rana pipiens*, as demonstrated by the anterograde transport of horseradish peroxidase. *Cell and Tissue Research* **211**, 215–222.

Engbretson, G.A. & Battelle, B.A. (1985). Identification of putative neurotransmitters in the lizard parietal eye. *Investigative Ophthalmology and Visual Science* **26**, 670–678.

Engbretson, G.A. & Lent, C.M. (1976). Parietal eye of the lizard: neuronal photoresponses and feedback from the pineal gland. *Proceedings of the National Academy of Sciences of the U.S.A.* **73**, 654–657.

Hamasaki, D.I. (1970). Interaction of excitation and inhibition in the Stirnorgan of the frog. *Vision Research* **10**, 307–316.

Häring, P., Stähli, C., Schoch, P., Takacs, B., Staehelin, T. & Möhler, H. (1985). Monoclonal antibodies reveal structural homogeneity of γ-aminobutyric acid/benzodiazepine receptors in different brain areas. *Proceedings of the National Academy of Sciences of the U.S.A.* **82**, 4837–4841.

Kemali, M. & De Santis, A. (1983). The extracranial portion of the pineal complex of the frog (frontal organ) is connected to the pineal, the hypothalamus, the brain stem, and the retina. *Experimental Brain Research* **53**, 193–196.

Kemali, M. & Lazar, G. (1985). Cobalt injected into the right and left fasciculi retroflexes clarifies the organization of this pathway. *Journal of Comparative Neurology* **233**, 1–11.

Klein, D., Weller, J. & Moore, R. (1971). Melatonin metabolism: neural regulation of pineal serotonin: acetyl coenzyme A N-acetyl transferase activity. *Proceedings of the National Academy of Sciences of the U.S.A.* **68**, 3107–3110.

Korf, H.W. (1974). Acetylcholinesterase-positive neurons in the pineal and parapineal organs of the rainbow trout, *Salmo gairdneri* (with special reference to the pineal tract). *Cell and Tissue Research* **155**, 475–489.

Korf, H.-W. & Møller, M. (1984). The innervation of the mammalian pineal gland with special reference to central pinealopetal projections. *Pineal Research Reviews* **2**, 41–86.

Korf, H.-W. & Oksche, A. (1986). In *Vertebrate Endocrinology: Fundamentals and Biomedical Implications*, ed. Pang, P.K.T. & Schreibman, M.P., pp. 105–145. Orlando, Florida: Academic Press.

Korf, H.-W. & Wagner, U. (1980). Evidence for a nervous connection between the brain and the pineal organ in the guinea pig. *Cell and Tissue Research* **209**, 505–510.

Korf, H.-W. & Wagner, U. (1981). Nervous connections of the parietal eye in adult *Lacerta s. sicula* Rafinesque as demonstrated by anterograde and retrograde transport of horseradish peroxidase. *Cell and Tissue Research* **219**, 567–583.

Korf, H.-W., Zimmerman, N.H. & Oksche, A. (1982). Intrinsic neurons and neural connections of the pineal organ of the house sparrow, *Passer domesticus*, as revealed by anterograde and retrograde transport of horseradish peroxidase. *Cell and Tissue Research* **222**, 243–260.

Maxwell, M.H. (1978). Two rapid and simple methods used for the removal of resins from 1.0-μm-thick epoxy sections. *Journal of Microscopy* **112**, 253–255.

Meissl, H. (1986). Photoneurophysiology of pinealocytes. In *Pineal and Retinal Relationships*, ed. Klein, D.C. & O'Brien, P.J., pp. 33–45. New York: Academic Press.

Meissl, H. & Dodt, E. (1981). Comparative physiology of pineal photoreceptor organs. In *The Pineal Organ: Photobiology–Biochronometry–Endocrinology*, ed. Oksche, A. & Pévet, P., pp. 61–80. Amsterdam: Elsevier/North-Holland Biomedical Press.

Meissl, H. & George, S. (1984). Electrophysiological studies on neuronal transmission in the frog's photosensory pineal organ. The effect of amino acids and biogenic amines. *Vision Research* **24**, 1727–1734.

Meissl, H. & George, S. (1985). Effect of GABA and its antagonists, bicuculline and picrotoxin, on nerve cell discharges of the photosensory pineal organ of the frog, *Rana esculenta*. *Brain Research* **332**, 39–46.

Morita, Y. (1971). Post-tetanic activity changes of the frog's neurosensory pineal end vesicle (Stirnorgan). *Pflügers Archiv* **328**, 135–144.

Neary, T.J. & Northcutt, R.G. (1983). Nuclear organization of the bullfrog diencephalon. *Journal of Comparative Neurology* **213**, 262–278.

Oksche, A. (1971). Sensory and grandular elements of the pineal organ. In *The Pineal Gland*, ed. Wolstenholme, G.E.W. & Knight, J. pp. 127–146. Edinburgh, England: Churchill Livingstone.

Östholm, T., Ekström, P., Bruun, A. & van Veen, Th. (1988). Temporal disparity in pineal and retinal ontogeny. *Developmental Brain Research* **42**, 1–13.

Paul, E., Hartwig, H.-G. & Oksche, A. (1971). Neurone und zentralnervöse Verbindungen des Pinealorgans der Anuren. *Zeitschrift für Zellforschung* **112**, 466–493.

Richards, G., Möhler, H. & Haefely, W. (1986). Mapping benzodiazepine receptors in the CNS by radiohistochemistry and immunohistochemistry. In *Neurohistochemistry: Modern Methods and Applications*, pp. 629–677, New York: Alan R. Liss, Inc.

Shiotani, Y., Yamano, M., Shiosaka, S., Emson, P.C., Hillyard, C.J., Girgis, S. & Macintyre, I. (1986). Distribution and origins of substance P (SP)-, calcitonin gene-related peptide (CGRP)-, vasoactive intestinal polypeptide (VIP)-, and neuropeptide Y (NPY)-containing nerve fibers in the pineal gland of gerbils. *Neuroscience Letters* **70**, 187–192.

Studnička, A.K. (1905). Die Parietalorgane. In *Lehrbuch der vergleichende Anatomie der Wirbeltiere, Vol. 5*, ed. Oppel, A., pp. 1–254. Jena: Springer-Verlag.

Triepel, J. & Grimmelikhuijzen, C.J.P. (1984). A critical examination of the occurrence of FMRFamide immunoreactivity in the brain of guinea pig and rat. *Histochemistry* **80**, 63–71.

van Leeuwen, F. (1986). Pitfalls in immunocytochemistry with special reference to the specificity problems in the localization of neuropeptides. *American Journal of Anatomy* **175**, 363–377.

Vigh, B. & Vigh-Teichmann, I. (1986). Three types of photoreceptors in the pineal and frontal organs of frogs: ultrastructure and opsin immunoreactivity. *Archivum Histologicum Japonica* **49**, 495–518.

Vigh-Teichmann, I., Vigh, B. & Aros, B. (1973). CSF contacting axons and synapses in the lumen of the pineal organ. *Zeitschrift für Zellforschung* **144**, 139–152.

Wake, K. (1973). Acetylcholinesterase-containing nerve cells and their distribution in the pineal organ of the goldfish, *Carassius auratus*. *Zeitschrift für Zellforschung* **145**, 287–298.

Wake, K., Ueck, M. & Oksche, A. (1974). Acetylcholinesterase-containing nerve cells in the pineal complex and subcommissural area of the frogs, *Rana ridibunda* and *Rana esculenta*. *Cell and Tissue Research* **154**, 423–442.

Wirsig, C.R. & Getchell, T.V. (1986). Amphibian terminal nerve: distribution revealed by LHRH and AChE markers. *Brain Research* **385**, 10–21.

Wurtman, R.J., Axelrod, J. & Fischer, J.E. (1964). Melatonin synthesis in the pineal gland: effect of light mediated by the sympathetic nervous system. *Science* **143**, 1328–1330.

Yazulla, S. (1986). GABAergic mechanisms in the retina. *Progress in Retinal Research* **5**, 1–52.

Zilles, K. & Nickeleit, V. (1979). Efferent connections from the brain to the frontal organ in *Rana temporaria* demonstrated by labeling with horseradish peroxidase. *Cell and Tissue Research* **196**, 189–192.