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Central GABAergic Innervation of the Pituitary in Goldfish: A Radioautographic and Immunocytochemical Study at the Electron Microscope Level

OLIVIER KAH,* PIERRETTE DUBOURG,* MARIA-GRAZIA MARTINOLI,*
MOHAMMED RABHI,* FRANCIS GONNET,† MICHEL GEFFARD,‡ AND ANDRÉ CALAS*

*Laboratoire de Physiologie des Interactions Cellulaires, UA CNRS 339, Talence, France; †Laboratoire de Physiologie des Poissons, INRA, Campus de Beaulieu, Rennes, France; and ‡Laboratoire de Neuroimmunologie, IBCN CNRS, rue Camille Saint-Saëns, Bordeaux, France

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The GABAergic innervation of the goldfish pituitary was studied at the light and electron microscope levels by means of radioautography after *in vitro* incubation in tritiated γ -aminobutyric acid (GABA) and immunocytochemistry using antibodies against GABA. Following incubation of pituitary fragments in a medium containing tritiated GABA, a selective uptake of the tracer was observed within the digitations of the neurohypophysis. Silver grain clusters were also observed in the adenohypophyseal tissue. At the electron microscope level, this uptake was found to correspond to nerve endings containing small clear and dense-core vesicles. These labeled profiles were located mainly in neurohypophyseal digitations in close apposition with the basement membrane separating the neurohypophysis from the adenohypophysis. However, they were also encountered in direct contact with most adenohypophyseal cell types in the different lobes. These results were confirmed by immunocytochemical data demonstrating the presence of numerous GABA immunoreactive fibers in both anterior and neurointermediate lobes. They were found either in the digitations of the neurohypophysis or in the adenohypophysis in direct contact with the glandular cells with a distribution and an ultrastructural aspect similar to those observed by radioautography. These data demonstrate that the pituitary of teleosts receives a massive GABAergic innervation. Although physiological data providing a functional significance for such an innervation are lacking, the present study suggests that, as already documented in mammals, GABA may be involved in the neuroendocrine regulation of pituitary functions in teleosts. © 1987 Academic Press, Inc.

γ -Aminobutyric acid (GABA) is classically considered to be one of the major inhibitory neurotransmitters within the central nervous system of vertebrates. In addition, it is well documented in mammals that GABA is involved in the neuroendocrine regulations of pituitary functions either by a direct action at the level of the hypophysis or by modulating the activity of hypophysiotrophic factors at the hypothalamic level (for reviews see Racagni *et al.*, 1982; Tappaz, 1984; McCann *et al.*, 1984). In support of these data, significant levels of GABA and GABA transaminase activity have been detected in the anterior and neurointermediate (NIL) lobes of the pitu-

itary (Racagni *et al.*, 1979), while L-glutamate decarboxylase (GAD) activity could be measured only in the NIL (Racagni *et al.*, 1979; Oertel *et al.*, 1982). As a morphological support for the role of GABA in the neuroendocrine control of pituitary functions in mammals, GAD-immunoreactive (Ir) fibers were observed in close association with portal vessels of the median eminence and in the NIL of rats (Vincent *et al.*, 1982; Oertel *et al.*, 1982; Tappaz *et al.*, 1983; Berod *et al.*, 1984) and mice (Stoeckel *et al.*, 1985). The recent introduction of antibodies against GABA itself (Seguela *et al.*, 1984) confirms these results by the direct visualization of GABA itself

in the median eminence–pituitary complex of rats (C. Decavel, unpublished results) and mice (Rabhi *et al.* 1987).

To date, there is very little information concerning the eventual participation of GABA in the regulation of pituitary functions in lower vertebrates. In the carp, it was shown that GABA inhibits the stress-induced elevation of cortisol (Follénus, 1977) and that [³H]GABA is selectively taken up by nerve endings located in the rostral neurohypophysis (NH; Follénus, 1972). More recently, GABA was demonstrated to be a potent inhibitor of melanocyte-stimulating hormone (MSH) secretion from the pars intermedia (PI) of amphibians, where GAD-Ir fibers could be visualized (Verburg-Van Kemenade *et al.*, 1986).

Because of the direct innervation of its secretory cells, not only in the intermediate lobe (IL) but also in the pars distalis (PD), the pituitary of teleosts is unique among vertebrates (see Ball, 1981) and provides a model that allows the search for neurohormones and/or neurotransmitters, which, if present, are putative candidates involved in the regulation of hypophyseal functions. In the present work, we report our observations on the GABAergic innervation of the goldfish pituitary using radioautography following *in vitro* [³H]GABA administration or immunocytochemistry of GABA itself.

MATERIALS AND METHODS

Goldfish of the common variety were collected from a local pond and were acclimated in large tanks under running water and a natural photoperiod.

Radioautography. The fish were decapitated and the rapidly dissected pituitaries were cut into three pieces and transferred in an oxygenated Ringer solution adapted for fish (see Kah *et al.*, 1984) for a 5-min preincubation period. [³H]GABA (10^{-5} or 10^{-6} M; sp act, 30 Ci/mmol; Amersham) was then added for 15 min. After washing for 5 min, the pieces were processed for light and electron microscope radioautography as described by Kah *et al.* (1984). Exposure duration for light microscopy was 15–20 days and for electron microscopy was 40 days.

Immunocytochemistry. The fish were anesthetized with MS-222 (Sandoz) and either perfused with 6.25% glutaraldehyde in phosphate buffer (0.1 M, pH 7.2) or decapitated for rapid dissection of the pituitary and immersion in the same fixative. After fixation, the tissues were washed for 3–4 hr to remove free aldehyde and were cut at 40 μ m with a Vibratome. The sections were first incubated with antibodies against GABA coupled to bovine serum albumin with glutaraldehyde prepared by Dr. M. Geffard according to Seguela *et al.* (1984). The antiserum was routinely diluted 1:15,000 in phosphate buffer. After washing, the sections were exposed to sheep anti-rabbit peroxidase-labeled Fab fragments (Byosis) diluted 1:2000. Peroxidase activity was revealed using 0.025% diaminobenzidine (Sigma) containing hydrogen peroxide (0.003%). Sections were then either mounted on glass slides for light microscopy or processed for electron microscopy as previously reported (Kah *et al.*, 1986).

The specificity of the primary immune serum was checked by routine immunocytochemical controls and by competition experiments *in vitro* as previously described (Seguela *et al.*, 1984). Competitors included GABA, β -alanine, glycine, taurine, glutamate, and aspartate, all coupled to lysine by glutaraldehyde.

RESULTS

Radioautography. At the light microscope level an important uptake of the tracer was noted in all parts of the pituitary, mostly overlapping the digitations of the neurohypophysis (Fig. 1). However, silver grain clusters were also detected in the adeno-hypophyseal tissue. At the electron microscope level, this uptake was seen to correspond only to the neural tissue (Fig. 2) either in the digitations of the neurohypophysis or among the adeno-hypophyseal cells. No cellular uptake could be visualized either by pituicytes or by glandular cells. In all cases, the silver grains were located over part of the so-called type B fibers whose neurosecretory granules have a diameter of 60–80 nm. In the rostral NH, where there is a clear separation from the AH, the labeled profiles were often located facing the so-called basement membrane (Fig. 3). In the other parts of the gland, they were encountered more randomly either in small nervous tracts or among the different cell types.

Immunocytochemistry. Incubation of pi-

tuitary Vibratome sections with antibodies against GABA resulted in an important labeling of the NH and its digitations (Fig. 4). A large tract of Ir fibers entered the NH, crossed the PD, and reached the NIL. From the rostral NH, smaller tracts were seen to invade the rostral (RPD) and proximal (PPD) components of the pars distalis. Isolated individual positive profiles could be detected in all parts of the adeno-hypophysis (AH).

At the electron microscope level, Ir profiles were also seen to correspond to part of the type B fibers. As for radioautography, the labeling was located in the digitations of the neurohypophyseal tissue, but many positive profiles were encountered in direct contact with secretory cells.

In the rostral NH, positive profiles were observed in direct apposition with pituitocytes and on some occasions figures suggesting synaptic-like contacts could be observed (Fig. 5). The Ir profiles were frequently found facing the basement membrane separating the neurohypophysis from the adeno-hypophysis in a position similar to that described previously for radioautography. However, they were also observed in direct contact with the secretory cells, in particular the prolactin (PRL) cells (Figs. 6 and 7). Although no really clear pictures of synaptic contacts could be observed, slight thickenings of the fiber and PRL cell membranes were detected on some occasions (Fig. 7).

In the PPD, both gonadotrophs (GTH) and growth hormone (GH) cells were frequently found to be in direct contact with the Ir fibers (Figs. 8–11). Occasionally, pictures suggesting possible synaptic-like contacts were observed (Fig. 11).

As shown in Fig. 4, numerous GABA-positive fibers reached the NIL. Electron microscope examination showed that these fibers were located in the nervous tissue (Fig. 12), but also in close apposition with both cell types of the intermediate lobe, the MSH-secreting cells (Fig. 13), and the calcium-sensitive cells.

DISCUSSION

The present investigation demonstrates by two fundamentally different approaches that the goldfish pituitary receives a massive GABAergic innervation of central origin. Indeed, radioautography is based on the visualization of the cellular uptake of an exogenously applied tracer, while immunocytochemistry reveals the cellular content in an endogenous antigen.

The specificity of [³H]GABA uptake by GABA neurons is largely documented. *In vitro* studies (Iversen and Johnston, 1971; Martin and Smith, 1972) of the kinetics of GABA accumulation have shown that there are two transport systems of different affinities: one of high affinity (K_m around 10^{-5} – 10^{-6} M) and another with low affinity (K_m around 10^{-3} – 10^{-4} M). The high-affinity transport is due to an active uptake most likely involved in the inactivation of neurotransmission (Roskoski, 1978) and thus is specific to GABAergic neurons (Martin, 1976). However, it has been shown that glial cells can take up tritiated GABA with an affinity similar to that of neurons (Schon and Kelly, 1974), but this transport is much slower. Therefore, under the conditions of our study (low concentrations of [³H]GABA, short incubation times), we may assume that the uptake of the tracer is selective for GABA neurons. In addition, no cellular uptake, in particular by pituitocytes, has been observed. Concerning the radioautographic approach, our results are similar to those obtained by Follénus (1972) demonstrating that GABA terminals, also of type B, are present in the rostral neurohypophysis of the stickleback. In addition, we found evidence that this GABAergic innervation is not limited to the rostral NH but occurs in other parts of the pituitary as well.

The validity of using GABA antibodies to study the distribution of GABA within the central nervous system is now largely documented (Ottersen and Storm-Mathisen, 1984; Seguela *et al.*, 1984, 1985;

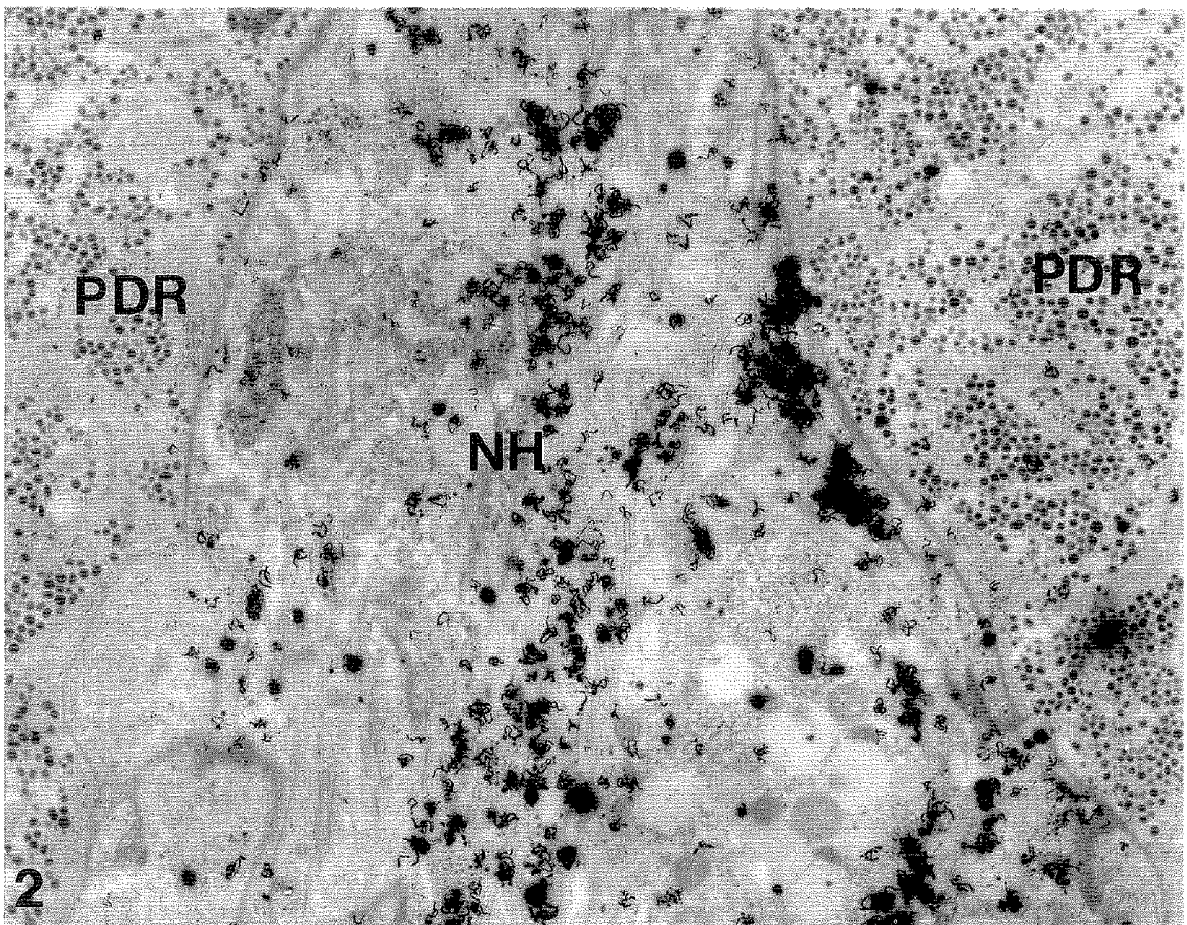
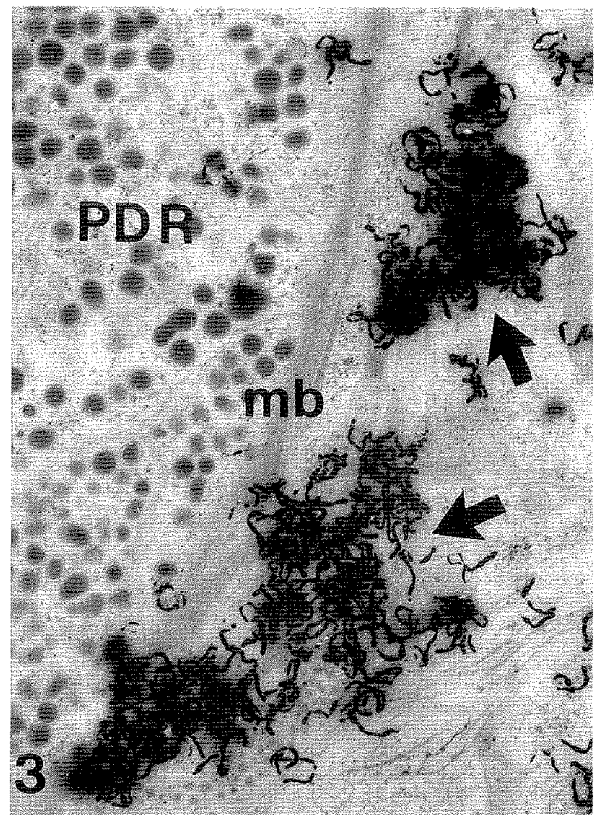
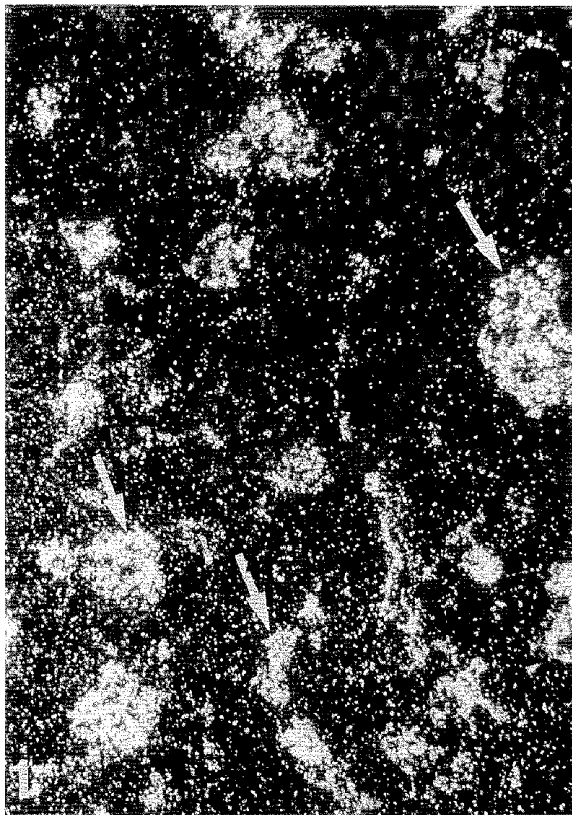


FIG. 1. Semithin section at the level of the pars distalis under dark-field illumination showing the important uptake of [^3H]GABA within the digitations of the neurohypophysis (arrows). $\times 435$.

FIG. 2. At the electron microscope level, the tracer appears accumulated over the neurohypophysial tissue (NH) only, here at the level of the rostral pars distalis (PDR). $\times 6700$.

FIG. 3. High-power view showing type B endings (arrows) upon the basement membrane (mb) facing the rostral pars distalis (PDR). $\times 14500$.

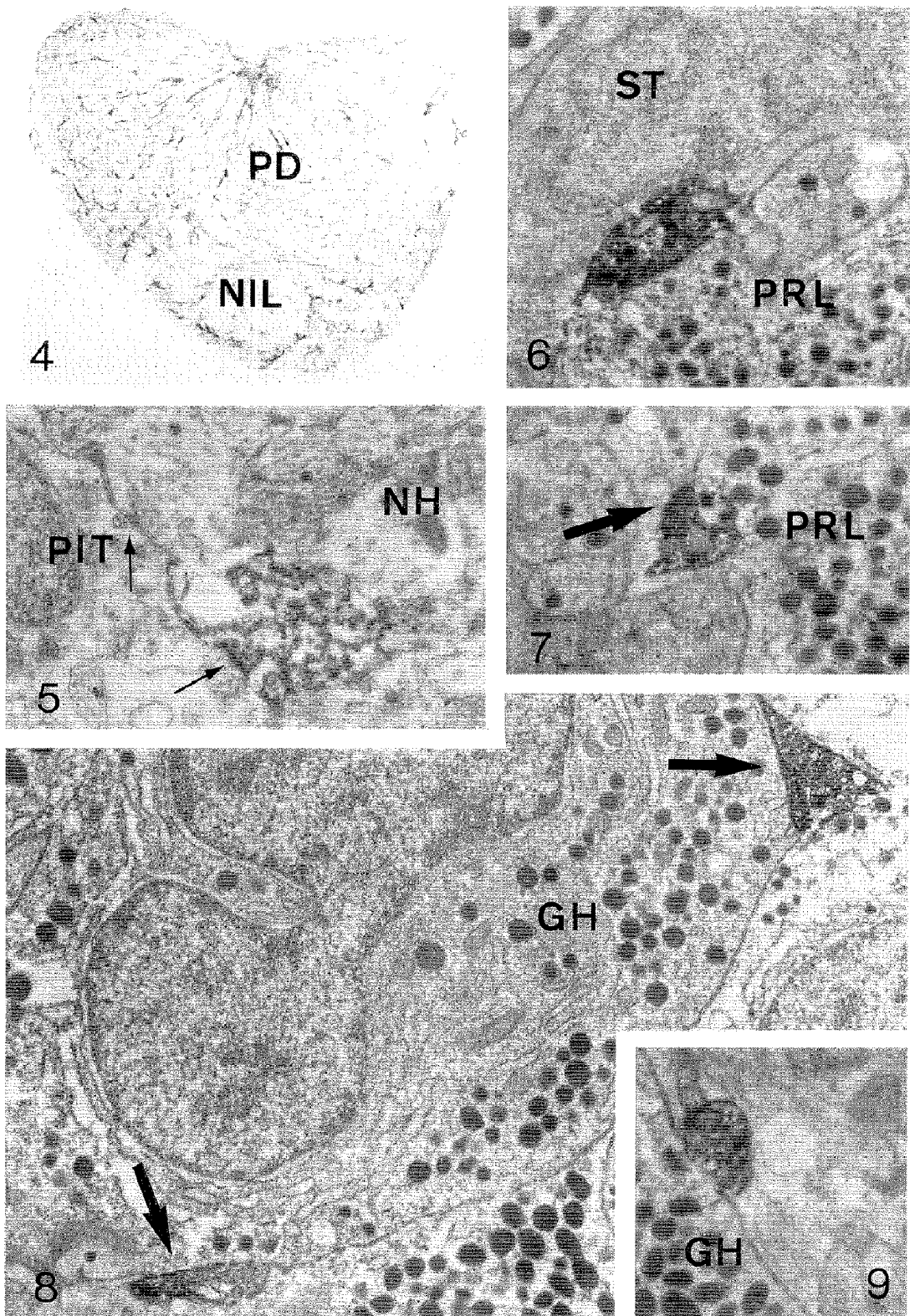
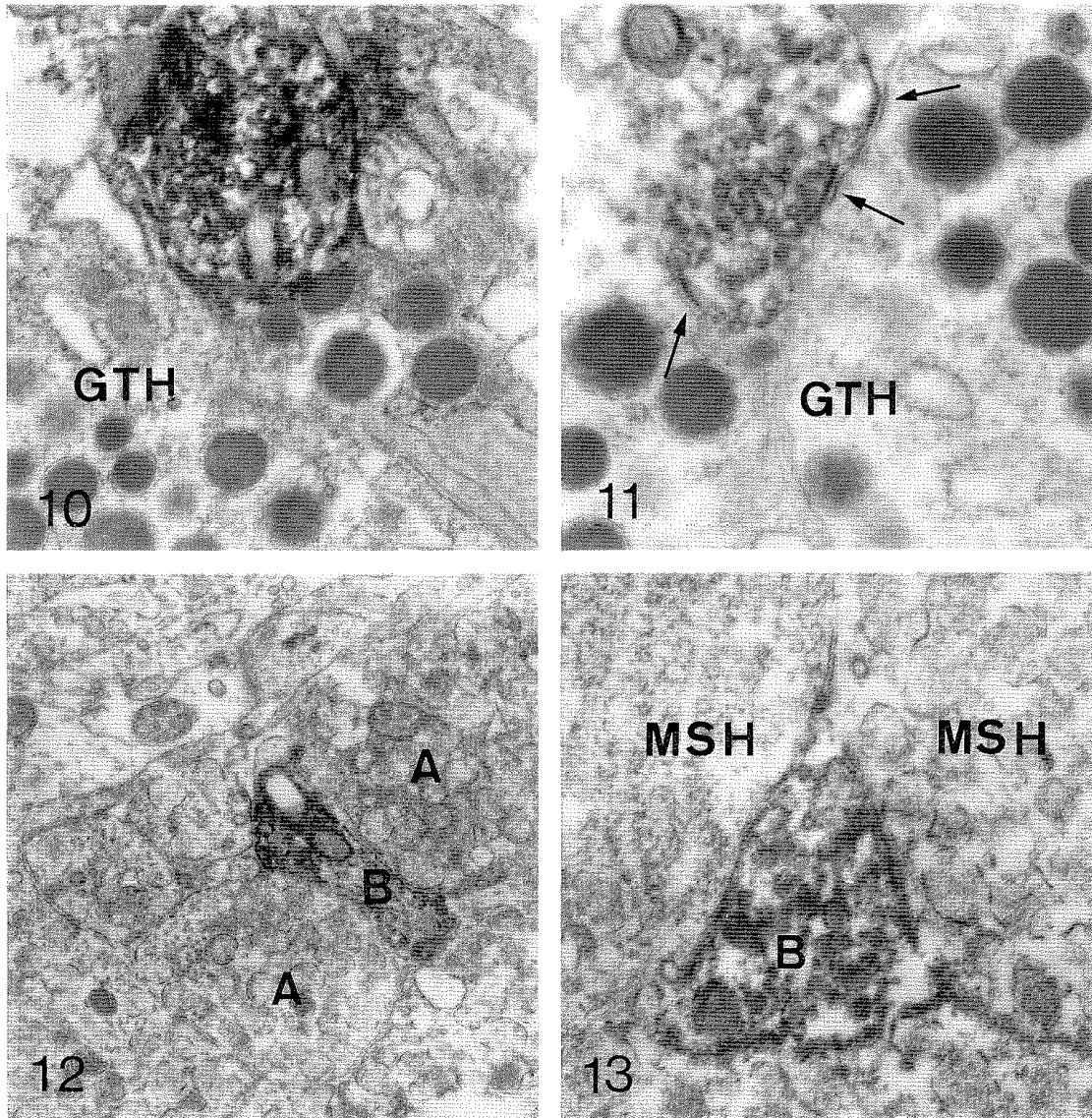


FIG. 4. Transverse Vibratome section (40 μ m). GABA-positive profiles invade all parts of the pituitary. PD, pars distalis; NIL, neurointermediate lobe. $\times 30$.

FIG. 5. In the neurohypophysis (NH), positive and negative profiles establishing synaptoid contacts (arrows) on a pituicyte (PIT). $\times 23600$.

FIGS. 6 AND 7. In the rostral pars distalis, type B positive fibers were frequently observed in direct contact with prolactin cells (PRL). On some occasions, slight membrane thickenings (arrow) could suggest synaptic-like contacts. ST, stellate cell. Fig. 6: $\times 17,000$; Fig. 7: $\times 19,000$.

FIGS. 8 AND 9. GABA-immunoreactive profiles were often observed in close apposition with the growth hormone cells (GH). Fig. 8: $\times 14300$; Fig. 9: $\times 18000$.



FIGS. 10 AND 11. GABA-positive fibers in direct contact with the gonadotrophs (GTH). In some cases, membrane thickenings (arrows) suggest synaptoid contacts. Figs. 10: $\times 23700$; Fig. 11: $\times 35500$.

FIGS. 12 AND 13. In the neurointermediate lobe, type B positive profiles were observed either in the nervous tissue (Fig. 12) or in direct contact with the secretory cells (Fig. 13). A, type A fiber; MSH, melanotrophs. Fig. 12: $\times 18700$; Fig. 13: $\times 17500$.

Gamrani *et al.*, 1986; Onteniente *et al.* 1987; Kah *et al.*, 1987; Rabhi *et al.*, 1987). The antiserum used in this study, prepared against GABA coupled to a carrier protein with glutaraldehyde, has been shown by *in vitro* competitions and by immunocytochemical controls to be highly specific for GABA (Seguela *et al.*, 1984, 1985; Gamrani *et al.*, 1986; Onteniente *et al.*, 1987; Rabhi

et al., 1987; Kah *et al.*, 1987). In addition, the good correlation between radioautography and immunocytochemistry strongly supports the conclusion that type B GABA fibers have a widespread distribution in the goldfish pituitary. Such an innervation is most likely present in other teleosts according to the data obtained in the rostral NH by Follénus (1972) or to our

own observations in the rainbow trout (F. Gonnet and O. Kah, unpublished data). From a quantitative point of view, despite underestimation of the number of labeled profiles due to the limitations of the two techniques (diffusion of the tracer, penetration of antibodies), this innervation appears to be important. With the two methods used in this study, positive endings were very easy to find at the electron microscope level if compared, for instance, with the number, in the same condition, of dopaminergic fibers (Kah *et al.*, 1984, 1986). Nevertheless, the number of Ir profiles was inferior to that of fibers labeled by radioautography, most likely because of the reduced penetration of immunoreagents into the glutaraldehyde-fixed tissue. This abundance of GABA-containing profiles in the goldfish pituitary led us to raise the question of a possible coexistence of GABA with other neurohormones within the same fiber. Experiments in this direction are in progress in our laboratory.

Another point of interest concerns the possible existence of synaptic contacts between GABA endings and the secretory cells. Using conventional electron microscopy, synaptoid contacts between type B fibers and adeno-hypophyseal cells are occasionally observed but they are weakly differentiated (Kaul and Vollrath, 1974). Nevertheless the observation in the present study of accumulations of small clear vesicles facing membrane thickenings could suggest that GABA type B fibers may establish synaptoid contacts with pituicytes or secretory cells, further allowing the assumption that GABA influences their activity.

From a comparative point of view, it is interesting to mention that this GABAergic innervation is very similar to that demonstrated in other vertebrates. Indeed, using GABA and GAD immunocytochemistry a direct GABA innervation was demonstrated in the intermediate and posterior lobes of *Xenopus* (Verburg-Van Kemenade

et al., 1986), rat (Oertel *et al.*, 1982; Vincent *et al.*, 1982; Tappaz *et al.*, 1983; Kah *et al.*, 1987) and mice (Stoeckel *et al.*, 1985; Rabhi *et al.*, 1987). In mammals, a rich innervation of the external layer of the median eminence has also been described following GABA or GAD immunocytochemistry (Vincent *et al.*, 1982; Tappaz *et al.*, 1983; Berod *et al.*, 1984; Stoeckel *et al.*, 1985; Rabhi *et al.*, 1987; Kah *et al.*, 1987) and radioautography (Tappaz *et al.*, 1980). According to these authors, such terminals, frequently observed to surround the capillaries of the primary plexus, are ideally placed for releasing GABA in the portal blood. Supporting this assumption, GABA has been measured in the portal circulation (Mulchahey and Neill, 1982; Guldelsky *et al.*, 1983) and in the anterior lobe of the pituitary (Racagni *et al.*, 1979). Furthermore, GABA transaminase is also present in the anterior pituitary (Racagni *et al.*, 1979). Most likely, the GABAergic innervation of the median eminence in mammals is the functional equivalent of that of the *pars distalis* in teleosts which lack the hypothalamo-hypophyseal portal system (Ball, 1981). From this homology, we may presume that this neuroanatomical feature is phylogenetically ancient and was already developed in early gnathostomes and that the functions, largely unknown, carried out by GABA fibers in the pituitary of teleosts are similar to those now well documented in mammals.

In the rat pituitary, high-affinity receptors for GABA have been reported (Grandison and Guidotti, 1979; Anderson and Mitchell, 1986a,b) and could mediate the diverse effects of GABA on the functions of the anterior and intermediate lobes. Indeed, a number of studies have shown that GABA acts on the secretion of prolactin (Pass and Ondo, 1977; Vijayan and McCann 1978; Locatelli *et al.*, 1979; Racagni *et al.*, 1979, 1984), growth hormone (Anderson and Mitchell, 1986a), thyrotrophin (Jordan *et al.*, 1983), lutein-

izing hormone (Ondo, 1974; Vijayan and McCann, 1978; Anderson and Mitchell, 1986a), and corticotrophin (Anderson and Mitchell, 1986a). In the intermediate lobe, a dual population of receptors has been described (Demeneix *et al.*, 1984) and it is known that GABA regulates MSH release by affecting the electrical properties of the cells (Taraskevitch and Douglas, 1982; Tomiko *et al.*, 1983). A similar effect of GABA on MSH secretion has been recently reported in *Xenopus* (Verburg-Van Kemenade *et al.*, 1986).

In teleosts, the role of GABA on pituitary functions is poorly documented. In the carp, GABA was shown to inhibit the cortisol increase associated with stress (Follénus, 1977) and to inhibit the release of prolactin (F. Gonnet and P. Prunet, personal communication). Preliminary *in vitro* experiments in our laboratory suggest that GABA also influences other pituitary functions in goldfish.

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