mical staining, instead of primary antiserum. These conjugates were added to the diluted antiserum (1/500) at a molar ratio of 2:1. In sections, absorption with homocarnosine-BSA as well as carnosine-BSA abolished the immunostaining (fig., e), but addition of other conjugates (GABA-BSA, L-histidine-BSA, β-alanine-BSA) did not affect the staining. For further analysis of cross reactivity, an enzyme-linked immunosorbent assay (ELISA) was performed. This ELISA method was based on our previous report[13]. First, 50 µl of poly-L-lysine solution (1 mg/ml) was dropped into each well of a microtest plate (FALCON 3072) left for 12 h, rinsed with PBS-Tween, and activated by adding 100 µl of 10% GA for 1 h. After rinsing with PBS-Tween, 10 µl of each amino acid solution (1 mM) was placed in the well and left for 12 h. Carnosine (Carn), homocarnosine (h-Carn), GABA, L-histidine (His), β-alanine (β-Ala), glutamate (Glu), aspartate (Asp), glycine (Gly), taurine (Tau), glutamine (Gln), proline (Pro), and cystein (Cys) were prepared as antigens. After blocking free aldehyde with L-lysine (1 M), and preincubation with 200 µl of 2% gelatin, the PAP immunohistochemical method was applied to detect the presence of amino acid-GA-polylysine complexes (coloring with o-phenylenediamine). Cross-reactivities towards these amino acids were obtained by reading the absorbance (OD 490 nm) of each well in the auto-analyzer (Immunoreader, InterMed). The cross-reactivity of this antiserum towards h-Carn was 84%. No cross-reaction was detectable with other amino acids (GABA, His, β-Ala, Glu, Asp, Gly, Tau, Gln, Pro and Cys).

Results and discussion. Immunohistochemical investigation using the antiserum to carnosine (IgG, 3.0 mg/ml, 1/1000–1/2000) revealed that the primary olfactory neuron was specifically labeled, but no labeled neuronal element was found in the remaining regions of the brain. This immunoreactivity was not observed after incubation with antiserum preabsorbed with antigen (carnosine-BSA) (fig., e). In the olfactory epithelium, many olfactory cells of bipolar shape and fiber bundles also showed a positive reaction (fig., b, d). Strongly labeled apical dendrites and terminal swelling of dendrites were noticed in the lumen of the nasal cavity, and basal thin axons could be followed into the axonal bundles. In the olfactory bulb, immunoreactivity was restricted exclusively to areas within both the olfactory nerve and glomerular layer, but no neuronal perikaryon was stained. Dot-like axon terminals demarcated the profiles of the glomerulus, where numerous labeled nerve fibers terminated (fig., a, c). Concentration of carnosine in the olfactory nerve or olfactory bulb was reported to be 50–100-fold as much as that of homocarnosine by a biochemical assay[4]. This suggest that immunoreaction detected in the present investigation is mostly due to carnosine, though our antiserum does not differentiate carnosine from homocarnosine. A selective decrease (5–15%) in the carnosine level in peripherally deafferentiated olfactory bulb[4] also supports our result. It should also be noted that dendrites toward the olfactory epithelium and their terminal swellings have such an amount of carnosine (fig., d). Carnosine may have some function in this region.

The present immunohistochemical study shows that carnosine-like immunoreactivity is specifically localized within the primary olfactory nerve. This evidence strongly supports the hypothesis that carnosine is the neurotransmitter in the primary olfactory neuron.

1 This study was supported by grants from the Ministry of Education, Science and Culture, Japan (No. 61218016). Reprint requests to I. N., Department of Anatomy, School of Medicine, Fujita-Gakuen Health University, Toyoake, Aichi 470-11, Japan.

2 Margolis, F. L., Science *184* (1974) 909.
3 Neidle, A., and Kandera, J., Brain Res. *80* (1974) 359.
4 Ferriero, D., and Margolis, F. L., Brain Res. *94* (1975) 75.
5 Margolis, F. L., and Grillo, M., Neurochem. Res. *2* (1977) 507.
6 Burd, G. D., Davis, B. J., Macrides, F., Grillo, M., and Margolis, F. L., J. Neurosci. *2* (1982) 244.
7 Macrides, F., and Davis, B. J., in: Chemical Neuroanatomy, p. 391. Ed. P. C. Emson. Raven Press, New York 1983.
8 Rochel, S., and Margolis, F. L., J. Neurochem. *38* (1982) 1505.
9 Gonzales-Estrada, M. T., and Freeman, W. J., Brain Res. *202* (1980) 373.
10 Storm-Mathisen, J., Leknes, A. K., Bore, A. T., Vaaland, J. L., Edminson, P., Haug, F. M. S., and Ottersen, O. P., Nature *301* (1983) 517.
11 Geffard, M., Henrich-Rock, A.-M., Dulluc, J., and Seguela, P., Neurochem. Int. *7* (1985) 403.
12 Nagatsu, I., Karasawa, N., Yoshida, M., Kondo, Y., Sato, T., Niimi, H., and Nagatsu, T., Biomed. Res. *3* (1982) 623.
13 Yoshida, M., Karasawa, N., Ito, M., Sakai, M., and Nagatsu, I., Neurosci. Res. *3* (1986) 356.

0014-4754/87/0298-03$1.50 + 0.20/0

Morphological evidence for a direct neuroendocrine GABAergic control of the anterior pituitary in teleosts

O. Kah, P. Dubourg, M.-G. Martinoli, M. Geffard* and A. Calas

*Laboratoire de Physiologie des Interactions Cellulaires, UA 339 CNRS, Avenue des Facultés, F-33405 Talence (France), and *Laboratoire de Neuroimmunologie, IBCN CNRS, rue Camille Saint-Saëns, F-33077 Bordeaux (France), 6 May 1986*

Summary. The anterior pituitary of teleosts is unique among vertebrates in receiving a direct innervation which represents the morphological support of the neuroendocrine control of pars distalis functions. The participation of GABAergic fibers in this innervation was studied by means of immunocytochemistry at the light and electron microscopic levels, using antibodies against GABA. Immunoreactive fibers, characterized by the presence of small clear and dense cored vesicles, were detected in all parts of the gland. Immunopositive terminals were found in close, sometimes synaptic-like, contact with most glandular cell types in the anterior lobe. The data strongly suggest that in teleosts, as in mammals, GABA is involved in the neuroendocrine control of anterior pituitary functions.

Key words. GABA; immunocytochemistry; anterior pituitary; neuroendocrine control; teleost.

GABA is classically considered to represent one of the major inhibitory neurotransmitters in the central nervous system. Furthermore, a growing body of evidence obtained from mammals suggests that GABA may also be involved in the neuroendocrine regulation of adenohypophyseal functions[1–4]. The suggestion that GABA plays a role in the release of MSH[5,6] is supported by the fact that GABAergic fibers have been detected in the intermediate lobe[7–9]. Because of the absence of nerve fibers in the

anterior lobe, the only morphological evidence favoring the concept of a GABAergic control of anterior pituitary (AP) functions was furnished by the observation of glutamate decarboxylase (GAD) immunoreactive (ir) fibers[8,9] and the selective uptake of ^{3}H-GABA[10] in the external layer of the median eminence (ME). In this context, the pituitary of teleosts provides an interesting model, since the direct innervation of the pars distalis represents the morphological support of the neuroendocrine regulation of anterior pituitary functions[11]. The participation of GABAergic fibers in this innervation was studied at the light and electron microscope levels by immunocytochemistry, using antibodies against GABA.

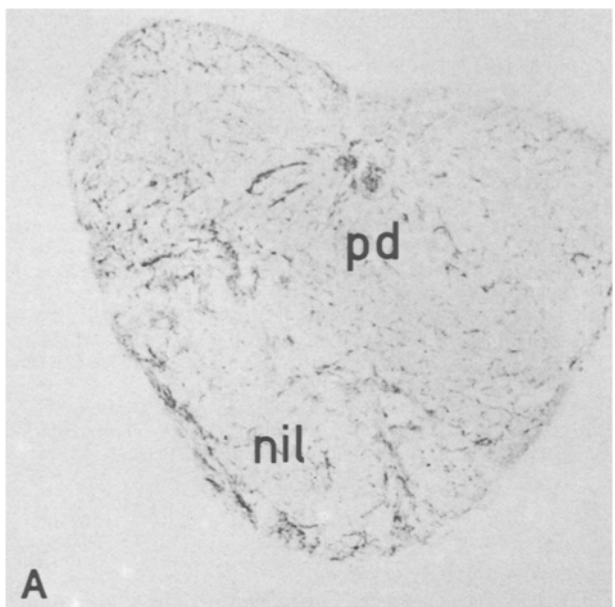
Material and methods. Antisera against GABA were prepared by immunizing rabbits with a mixture of Freund's complete adjuvant and GABA coupled with glutaraldehyde (G) to alternatively bovine serumalbumin (BSA), hemoglobin and poly-(L-lysine), as described [12]. The specificity of the antiserum was carefully monitored with an enzyme-linked immunoabsorbant assay as previously reported[13]. Competition experiments were performed between the conjugate GABA-G-BSA coated on well plates and each of the following competitors: GABA-G-lysine, beta-alanine-G-lysine, glycine-G-lysine, taurine-G-lysine, glutamate-G-lysine, aspartate-G-lysine preincubated with a GABA antiserum dilution. The final antibody dilution used in our Elisa tests was 1:20,000. As expected, the most immunoreactive compounds were GABA-G-lysine and then, at factor 750, beta-alanine-G-lysine. The other conjugates were not recognized (at factors higher than 50,000).

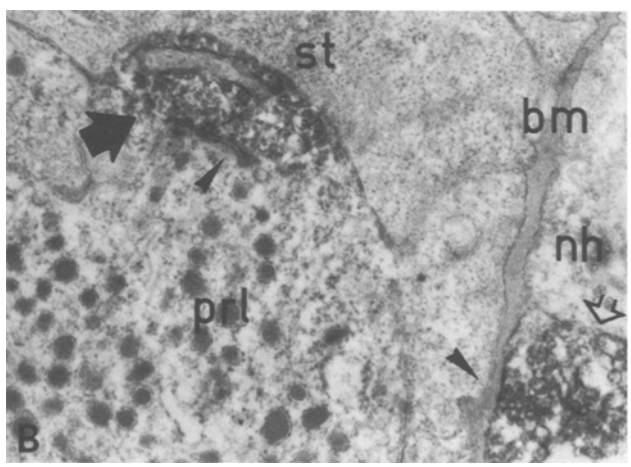
For light microscopy, twelve adult goldfishes were perfused via the aortic bulb with 5% glutaraldehyde in cacodylate buffer (0.1 M; pH 7.2). The pituitaries were postfixed in the same fixative for 2 h and cut with a Vibratome. 50-µm sections were washed thoroughly in several changes of buffer to remove free aldehydes and were incubated overnight with the GABA antiserum diluted 1:5000 to 1:10,000. After washing, they were exposed for 2 h to peroxidase-labeled sheep anti-rabbit Fab fragments (Biosis; 1:2000). Peroxidase activity was demonstrated with 3-3′-diaminobenzidine (0.05%; Sigma) and hydrogen peroxide. For electron microscopy, the pituitaries were rapidly dissected out, divided into 3 pieces, immersed in 5% glutaraldehyde in Soerensen buffer (0.06 M; pH 7.4), and processed as above. The sections were postfixed in 1% osmium tetroxide after the peroxidase reaction had been carried out, and embedded in Epon. Immunocytochemical routine controls, including pre-absorption of the GABA antiserum with its immunogen, led to the extinction of the immunoreaction.

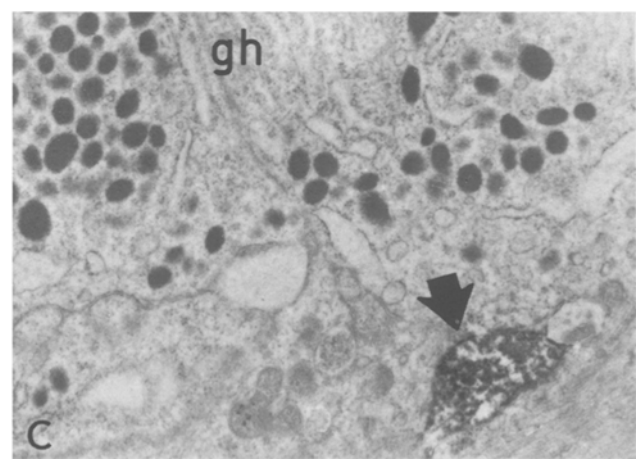
Results. Digitations of the teleost neurohypophysis (NH) penetrate deeply into the adenohypophysis (AH) from which it is separated by a basement membrane[14–16]. In many species, e.g. the goldfish, this membrane is interrupted, so that the fibers enter the AH and form direct contacts with the secretory cells. The neurosecretory fibers are usually divided into type A and type B, according to the diameter of their secretory vesicles (larger and smaller than 100 nm)[17].

At the light microscopic level, ir fibers were observed to enter the pituitary and to ramify in the rostral (RPD) and proximal pars distalis (PPD; fig., A). A prominent immunoreactive tract crossed the anterior pituitary and reached the neurointermediate lobe (NIL).

At the electron microscopic level, ir fibers contained numerous small clear (40–50 nm) and occasionally dense-cored vesicles (80–100 nm). Therefore, they belong to the type B. The electron dense reaction product was spread over the entire cytoplasm, on the vesicles and the membranes of cytoplasmic organelles, particularly mitochondria. Such ir terminals were detected either in the digitations of the NH or in close apposition to the secretory







A Transverse 50-µm vibratome section of a goldfish pituitary showing the overall distribution of GABAergic fibers not only in the neurointermediate lobe (nil), but also in the pars distalis (pd); ×32. *B* GABA immunoreactive type B fiber ending on the basement membrane (bm; open arrow) separating the rostral neurohypophysis (nh) from the adenohypophysis. GABAergic terminals are also found in direct contact with the glandular cells, in this case a prolactin cell (prl; large arrow). In both cases, a slight thickening of the fiber membrane (arrow head) could suggest a synaptic-like contact. st, stellate cell; ×25,200. *C* GABA immunoreactive terminal (arrow) in direct contact with a growth hormone cell (gh) of the proximal pars distalis. ×15,500.

cells. In the rostral part of the AP, which contains corticotroph (ACTH), thyrotroph (TSH) and prolactin (PRL) cells[14,15], ir fibers were encountered in digitations of the NH and were characterized by an accumulation of small clear vesicles facing the basement membrane (fig., B). In addition, ir profiles were also observed in close apposition to the AH cells, in particular PRL cells (fig., B). In some cases, an accumulation of small clear vesicles, facing a slight thickening of the fiber membrane, suggested a synaptic-like contact (fig., B). However, no really clear picture of membrane differentiation could be observed. The proximal part of the AP contains gonadotrophs (GTH) and growth hormone (GH) cells[18,19] which are easily differentiated at the electron microscopic level on the basis of their ultrastructural aspect. Both cell types were found in close apposition to type B ir fibers (fig., C) and synaptic-like contacts were sometimes encountered. In the NIL, ir fibers were present in the NH and in apposition to the two cell types of the pars intermedia, MSH-secreting cells and calcium-sensitive cells[20].

Discussion. The immunochemical and immunocytochemical controls described suggest that ir structures contain GABA. The results provide direct evidence that GABAergic terminals of CNS origin are present in the AP, establish direct synaptic-like contacts with the secretory cells, and thus, most likely, influence their activity. To date, except for a role in ACTH secretion[21], little is known about the effects of GABA on AP hormone secretion in teleosts. In mammals, it has been shown that GABA acts, either directly or via the hypothalamus, on the secretion of all AP hormones[1-4]. In support of a hypophysiotrophic role of GABA in the AP of mammals, the mediator itself[1], high-affinity receptors for GABA[22,23] and GABA-transaminase activity[1] have been found at the level of the AP, while GAD activity could be detected only in the NIL[1]. GAD-ir fibers in the external layer of the median eminence[8,10], and the presence of measurable amounts of GABA in the portal blood[24,25] were also reported. Nevertheless, except for a direct inhibitory effect at the level of the AP on PRL release[1,4], the site of action of GABA on other AP functions, either hypothalamic or hypophyseal, remains unclear. On the other hand, a function of GABA has been demonstrated in the IL of mammals where GAD-ir fibers were found in direct contact with the MSH cells[7,9]. At this level, GABA has been shown to modulate hormone output[6] by affecting the electrophysiological properties of the cells[5]. Although its functional significance remains to be established, the direct GABAergic innervation of all cell types in the pituitary of teleosts suggest that GABA is involved in the regulation of some, if not all, anterior pituitary functions.

Acknowledgment. This work was supported by the CNRS and the Foundation for Medical Research (A.C.).

1 Racagni, G., Apud, J.A., Locatelli, V., Cocchi, D., Nistico, G., di Giorgio, R.M., and Müller, E.E., Nature *281* (1979) 575.
2 Vijayan, E., and McCann, S.M., Endocrinology *103* (1978) 1888.
3 Pass, K.A., and Ondo, J.G., Endocrinology *100* (1977) 1437.
4 McCann, S.M., Vijayan, E., Negro-Vilar, A., Mizunuma, H., and Mangat, H., Psychoneuroendocrinology *9* (1984) 97.
5 Taraskevich, P.S., and Douglas, W.W., Nature *299* (1982) 733.
6 Tomiko, S.A., Taraskevich, P.S., and Douglas, W.W., Nature *301* (1983) 706.
7 Oertel, W.H., Mugnaini, E., Tappaz, M.L., Weise, V.K., Dahl, A.L., Schmechel, D.E., and Kopin, I.J., Proc. natn. Acad. Sci. USA *79* (1982) 675.
8 Vincent, S.R., Hökfelt, T., and Wu, J.Y., Neuroendocrinology *34* (1982) 117.
9 Tappaz, M.L., Wassef, M., Oertel, W.H., Paut, L., and Pujol, J.F., Neuroscience *9* (1983) 271.
10 Tappaz, M.L., Aguera, M., Belin, M.F., and Pujol, J.F., Brain Res. *186* (1980) 379.
11 Ball, J.N., Gen. comp. Endocr. *44* (1981) 135.
12 Seguela, P., Geffard, M., Buijs, R.M., and Le Moal, M., Proc. natn. Acad. Sci. USA *81* (1984) 3888.
13 Geffard, M., Dulluc, J., and Rock, A.M., J. Neurochem. *44* (1985) 1221.
14 Leatherland, J.F., Can. J. Zool. *50* (1972) 835.
15 Kaul, S., and Vollrath, L., Cell Tissue Res. *154* (1974) 211.
16 Kaul, S., and Vollrath, L., Cell Tissue Res. *154* (1974) 231.
17 Knowles, F., and Vollrath, L., Phil. Trans. R. Soc. Ser. B *250* (1966) 329.
18 Dubourg, P., Burzawa-Gérard, E., Chambolle, P., and Kah, O., Gen. comp. Endocr. *59* (1985) 472.
19 Cook, H., Cook, A.F., Peter, R.E., and Wilson, W., Gen. comp. Endocr. *50* (1983) 348.
20 Olivereau, M., Aimar, C., and Oliverau, J.M., Cell Tissue Res. *212* (1980) 29.
21 Follénius, E., C.r. Acad. Sci. Sér. D, Paris *285* (1977) 993.
22 Grandison, L., and Guidotti, A., Endocrinology *105* (1979) 754.
23 Apud, J.A., Cocchi, D., Masotto, C., Penalva, A., Müller, E.E., and Racagni, R., Brain Res. *361* (1985) 146.
24 Mulchahey, J.J., and Neill, J.D., Life Sci. *31* (1982) 453.
25 Gudelsky, G.A., Apud, J.A., Masotto, C., Cocchi, D., Racagni, G., and Müller, E.E., Neuroendocrinology *37* (1983) 397.

0014-4754/87/0300-03$1.50 + 0.20/0

Hormonal facilitation in the release of sperm from the spermatheca of the red-spotted newt

M.P. Hardy[1] and J.N. Dent

Department of Biology, Gilmer Hall, University of Virginia, Charlottesville (Virginia 22901, USA), 6 May 1986

Summary. Several neurotransmitters and hormones with potential to trigger a simultaneous contraction of the oviducts and the spermathecal myoepithelium were examined. Saline (0.05 ml), or 0.05 ml saline plus acetylcholine (9 mg), norepinephrine (50 µg), arginine-vasotocin (25 units), prostaglandin $F_{2\alpha}$(3 µg) were injected into the spermathecal region of female newts (n = 24 per group). The numbers of sperm present in the cloacae of prostaglandin-injected animals (107 ± 30 SEM) were significantly greater than the numbers detected in saline (27 ± 5 SEM) and in uninjected (14 ± 3 SEM) controls. Smaller and less consistent increases in the numbers of sperm were detected in the vasotocin- and norepinephrine-injected groups. Study of sections from ovulating female newts failed to produce evidence that pressure from the passage of ova through the posterior portion of the oviduct forced sperm from the spermatheca. Observations indicate an active role for the spermathecal myoepithelium in the discharge of stored sperm and of a role for prostaglandin $F_{2\alpha}$ in triggering that discharge.

Key words. Spermatheca; newt spermatheca; sperm storage; sperm discharge from storage; hormonal action in sperm discharge; prostaglandin and sperm discharge.