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The distribution of corticotropin-releasing factor immunoreactive neurons and nerve fibres in the brain of *Gambusia affinis* and *Salmo trutta*

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Summary. The study was carried out on the distribution of neurons and fibres which contain the Corticotropin releasing factor-like (CRF) immunoreactivity in the encephalon of two species of teleosts, *Gambusia affinis* and *Salmo trutta*. The peroxidase-antiperoxidase (PAP) immunocytochemical technique was employed. The present study has shown differences between both species. In *Gambusia affinis*, positive neurons were observed in the area ventralis telencephali pars lateralis (VLT), in the nucleus praeopticus (NPO) and in the nucleus lateralis tuberis (NLT). The immunoreactive fibres were in the area ventralis telencephali, in the preoptic hypophyseal tract and in the hypophysis.

In *Salmo trutta* the immunoreactive cells were seen in the pars magnocellularis and pars parvocellularis of the NPO and in cerebrospinal fluid (CSF) contacting neurons of the NLT. The pattern of distribution of immunoreactive fibres in *Salmo trutta* was different from that in *Gambusia affinis*. In addition to the distribution of perikarya in *Gambusia affinis* (in the VLT and in the preoptic hypophyseal tract), fibres were also observed in the tubero-hypophyseal tract and in the posterior hypothalamus. The hypophysis of *Salmo trutta* also presents an extensive labelling.

The interspecific differences shown in the present study should be due to the different degree of evolution in the two species studied and to other causes, such as environmental ones.

Key words: Corticotropin Releasing Factor (CRF), Immunocytochemistry, Diencephalon, *Gambusia affinis*, *Salmo trutta*

Introduction

The corticotropin-releasing factor (CRF) is a polypeptide of 41-amino acid whose functions comprise stimulation of the liberation of adrenocorticotropin (ACTH) and ß-endorphins in the adenohypophysis (Vale et al., 1981). Some immunocytochemical studies have demonstrated the distribution of CRF-positive neurons and fibres in the central nervous system of mammals (Merchenthaler et al., 1982; Kawata et al., 1983; Swanson et al., 1983). The majority of these neurons are situated in a portion of the supraoptic nucleus (SON) and paraventricular nucleus (PVN), projecting their axons toward the hypophysis. However, hypothalamic and extrahypothalamic zones express CRF receptors, e.g. the lateral nucleus of the amygdala and cingulate cortex in the telencephalon of the rat (De Souza et al., 1984) or the locus coeruleus and parabranchial nucleus in the encephalon of rabbit (Chai et al., 1990). Due to their extensive distribution in the central nervous system of mammals it is believed that the functions of CRF, besides those mentioned above, could be those of neuromodulator and central neurotransmitter (De Souza et al., 1984; Olivereau et al., 1984; Mancera et al., 1991).

In contrast, studies on the neuroanatomic distribution of CRF-immunoreactive(-ir) cells in lower vertebrates are very few: teleosts, Olivereau et al. (1984), Olivereau and Olivereau (1988), Yulis et al. (1987); amphibians, Olivereau et al. (1987), González and Lederis (1988); and reptiles, Mancera et al. (1991). The majority of these studies have been carried out on specific zones of the encephalon.

An immunocytochemical study of the distribution of CRF-ir cells and fibres in the whole brain of a primitive teleost, *Salmo trutta*, (salmonidae), and in the advanced teleost *Gambusia affinis*, (poecilidae) is presented in this study. We also discuss the interspecific differences between them and propose possible causes of these variations.

Materials and methods

Four adult *Gambusia affinis* and eight *Salmo trutta* were used for this study. They were kept in an aquarium at room temperature and natural photoperiod. The

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specimens were anaesthetized with tricaine methanesulphonate (MS-222, Sigma) at a dilution of 1:10,000 and they were sacrificed at the same time of day (14:00 h), in order to eliminate the possible influence of the photoperiod. *Salmo trutta* were fixed by transcardiac perfusion, while *Gambusia affinis*, due to their small size, were fixed by immersion.

The fixatives used were: Bouin and 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Brains were dissected and postfixed for 12 hours at room temperature in the same fixative and then transferred to 0.1M phosphate buffer, pH 7.4, overnight. After dehydration in graded alcohols they were embedded in paraffin and cut in serial 10 µm sections in frontal plane. In addition, four *Salmo trutta* brains were directly cut with a vibratome in 50 µm sections. These were mounted on gelatin-coated slides and processed for immunocytochemistry according to the peroxidase-antiperoxidase (PAP) technique (Sternberger, 1986).

After 3 x 10 minute rinses in phosphate buffer, endogenous peroxide within the tissue was blocked by a solution of 0.3% hydrogen peroxidase in PBS at room temperature for 30 min. This was followed by 3 washes in PBS and incubation for 30 min at room temperature in a solution of normal pig serum 1:30 in PBS. Sections were treated with rabbit polyclonal antisera directed against rat CRF (kindly supplied by Dr. E.M. Rodriguez, Valdivia, Chile) at a dilution of 1:500 in PBS and incubated for 48 hours at 4 °C in a humid atmosphere. Sections were then rinsed in PBS and incubated with pig antirabbit immunoglobulin diluted 1:100 in PBS for 1 hour and then with rabbit PAP complex (Sigma) diluted 1:100 in PBS for 1 hour. The sites of peroxidase attachment were demonstrated by incubation in 0.005% 3,3'diaminobenzidine-tetrahydrochloride (DAB, Sigma) solution in tris-HCl buffer (0.05 M, pH 7.6) containing 0.025% hydrogen peroxide. Finally, the sections were rinsed in water, dehydrated and coverslipped.

To ensure method specificity, controls were performed by incubating parallel sections with normal non-immune serum replacing CRF antibody or by omitting one of the steps of the immunohistochemical procedure.

Immunostained sections were observed and photographed using a Leitz Orthoplan photomicroscope, and Agfa Copex film.

Results

In the present study, we have found differences in the distribution of CRF in the encephalon of two teleosts; advanced teleost, *Gambusia affinis* (Fig. 1) and a primitive teleost, *Salmo trutta* (Fig. 2).

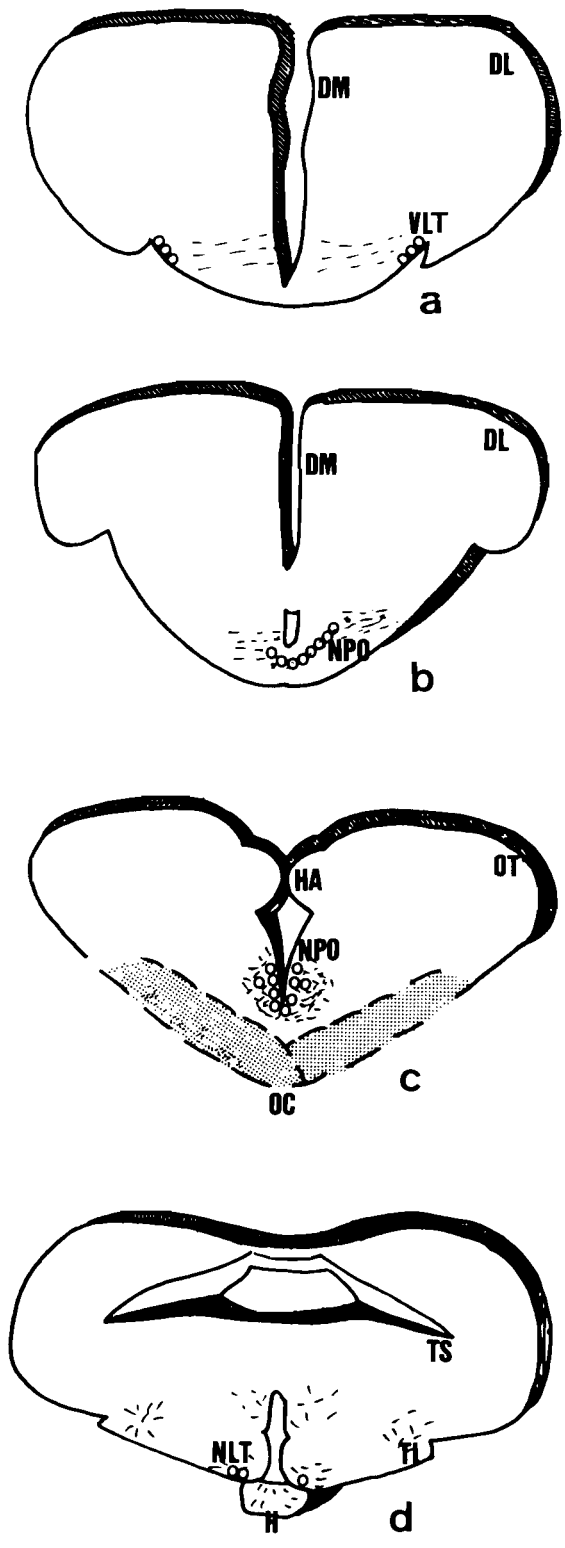


Fig. 1. Schematic drawings of successive rostrocaudal transverse sections trough the brain of *Gambusia affinis* showing the localization of CRF-ir cell bodies (circles) and fibres (dashes). DL: area dorsolateralis telencephali; DM: area dorsomedialis telencephali; H: hypophysis; HA: habenula; NLT: nucleus lateralis tuberis; NPO: nucleus praeopticus; OC: optic chiasma; OT: optic tectum; TL: torus lateralis; TS: torus semicircularis; VLT: area ventralis telencephali, pars lateralis.

Gambusia affinis

CRF-ir cell bodies were observed in the area ventralis telencephali, pars lateralis (VLT), dorso-medial to the sulcus entopeduncularis (Figs. 1a, 3). These CRF-ir neurons did not constitute a defined nucleus. They were

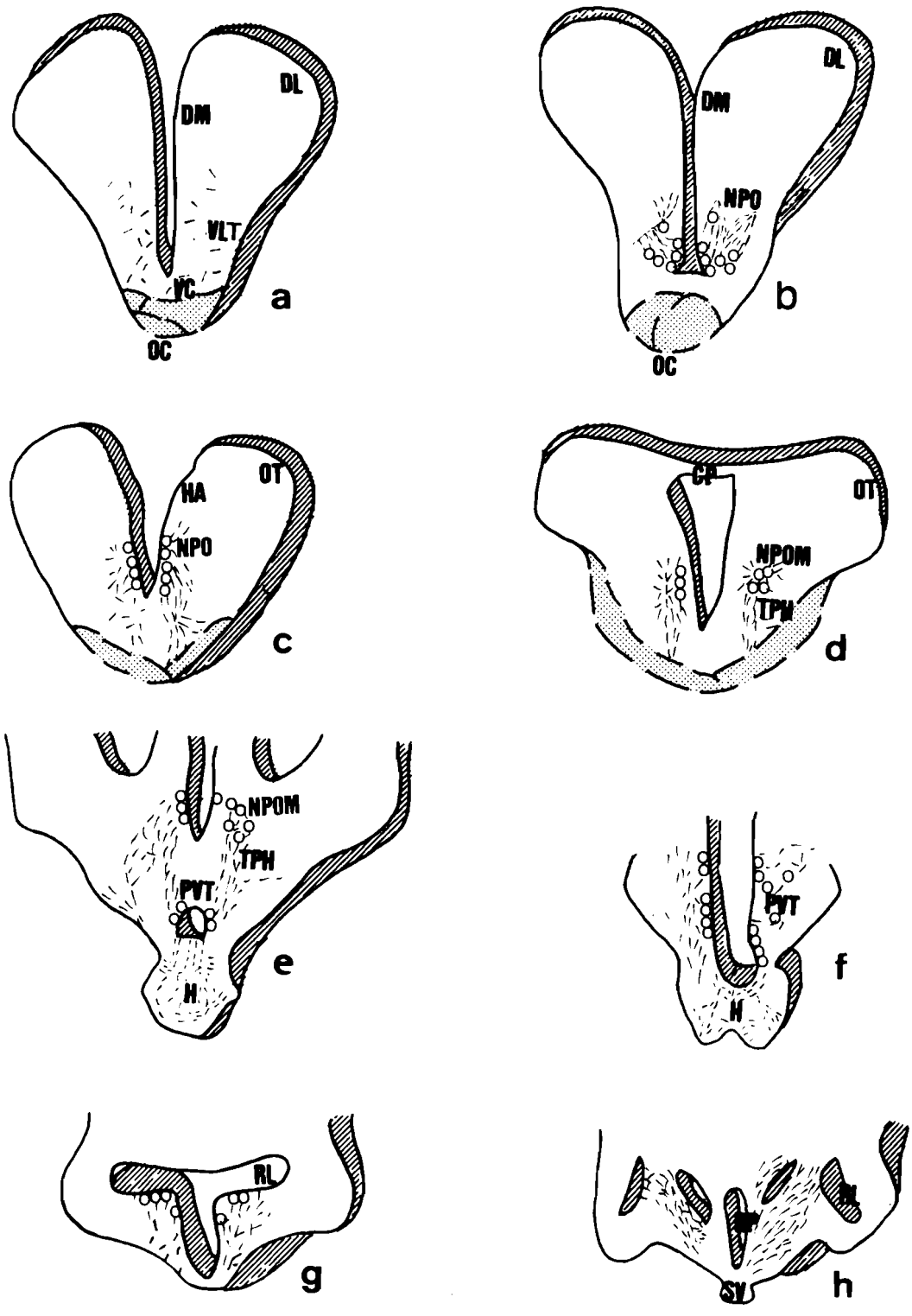


Fig. 2. Schematic rostrocaudal sections through the *Salmo trutta* brain. Note the localization of CRF-ir neurons (circles) and fibres (dashes). CP: commissura posterioris; DL: area dorsolateralis telencephali; DM: area dorsomedialis telencephali; H: hypophysis; HA: habenula; NPO: nucleus praeopticus; NPOM: pars magnocellularis of the nucleus praeopticus; OC: optic chiasm; OT: optic tectum; PVT: periventricular zone of the tuber; RL: recessus lateralis; RP: recessus posterioris; SV: saccus vasculosus; TPH: tractus praeoptico-hipophisheos; VC: pars caudalis of the area ventralis telencephali; VLT: pars lateralis of the area ventralis telencephali

small (with an average diameter of 4 μm) monopolar and with a pyriform morphology, (Fig. 3a,b) with a process leading toward the medial zone of the telencephalon.

A few stained perikarya were located in the nucleus praeopticus (NPO) parvocellularis, pars anterioris. These CRF-ir cells, with a pyriform shape, were found together with fibre running caudally (Fig. 1b). In the posterior sections, CRF-ir neurons were found throughout the whole NPO. These neurons, with a pyriform shape, were near the ventricular surface, but no projections were seen going towards the ventricular space (Figs. 1c, 4). A small number of immunopositive fibres were detected running toward the hypophysis, forming part of the preopticohypophyseal tract (TPH).

A small number of pyriform CRF-ir neurons with a diameter of 10 μm were located in the nucleus lateralis tuberis (NLT) (Figs. 1d, 5).

In the hypophysis of *Gambusia affinis* were only found some CRF-ir interdigitations extending toward the proadenohypophysis and rostral pars distalis (Fig. 6). However, the dorsal and central neurohypophysis, near the entry of the stalk, was never stained.

Salmo trutta

On carrying out a rostro-caudal study in *Salmo trutta* in order to compare the results with those described in *Gambusia affinis* CRF-ir fibres were seen in the pars lateralis and pars caudalis of the area ventralis telencephali (VLT and VC respectively) (Figs. 2a, 7). CFR-ir cell bodies appeared most rostrally in the pars parvocellularis of the NPO (Figs. 2b, 8). Two distinct populations of labelled cells were observed in this area: the first type were small parvocellular neurons situated in a periventricular position, they had a pyriform morphology with a process leading toward the ventricle. The immunoreactivity was found occupying the entire somata, except the nucleus (Fig. 9); the second type of CRF-ir neuron was located in a more lateral position. These were of similar size to the previous ones (diameter of 12 μm), with the same labelling distribution, but of ovoid shape (Fig. 10). More caudally, these immunoreactive cells were seen near the ventricle and in a dorsal position of the hypothalamus. The intracellular distribution of the immunoreactivity was similar to that previously described. A large number of parallel immunoreactive fibres was found in this area. These fibres were found running laterally and caudally (Figs. 2d, 8).

The immmunoreactive neurons of the pars magnocellularis of the nucleus praeopticus (NPOM) were located closer to the ependyma than in *Gambusia affinis*. These magnocellular immunoreactive cell bodies had a diameter of approximately 30 μm and a spheroid or pyriform shape. Some of these neurons presented one or two processes clearly labelled by the antibody (Figs. 2d, 11). The majority of these CRF-ir cells were situated near the ventricle, but without processes towards this cavity, and other CRF-ir cells were seen more laterally. Their immunoreactive fibres formed the preoptico-hypophyseal tract. It was directed to the hypophysis and the labelling was seen throughout all the neuro-hypophysis with large positive digitations toward the proadenohypophysis, rostral pars distalis and the neurointermedial lobule (Figs. 2e, 12).

In the tuberal hypothalamic region small-sized CRF-ir neurons (approximate diameter 10 μm) were observed surrounding the ventricular limit of the tuberal recess. These cells were round, bipolar, and liquor contacting (Figs. 2e, 13). In a transverse section, CRF-ir spheroidal monopolar neurons were seen within the periventricular zone of the tuber (PVT), dorsal and medial to the NLT (Figs. 2e, 14). CRF-ir fibres in this area were found between the periventricular neurons and the hypophysis, forming a tubero-hypophyseal tract.

This immunopositivity extended from the anterior tuberal zone to the posterior one with the same density of positive neurons distributed parallel to the ependyma.

More caudally, CRF-ir periventricular neurons were observed at the beginning of the lateral recess. The labelled cells were round and bipolar and situated in a subependymal position. They were oriented perpendicularly to the ventricular surface and contacted the cerebrospinal fluid via a process that penetrated the ependymal layer and terminated in a single knob-like swelling (Figs. 2g, 15). The other processes of these CRF-ir neurons was seen to run laterally to a zone of fibres. These immunoreactive fibres were oriented parallel to the ventricular border and increased in a number in successive caudal sections. They were found forming a dense plexus located on the edge of the lateral recess on both sides of the posterior recess

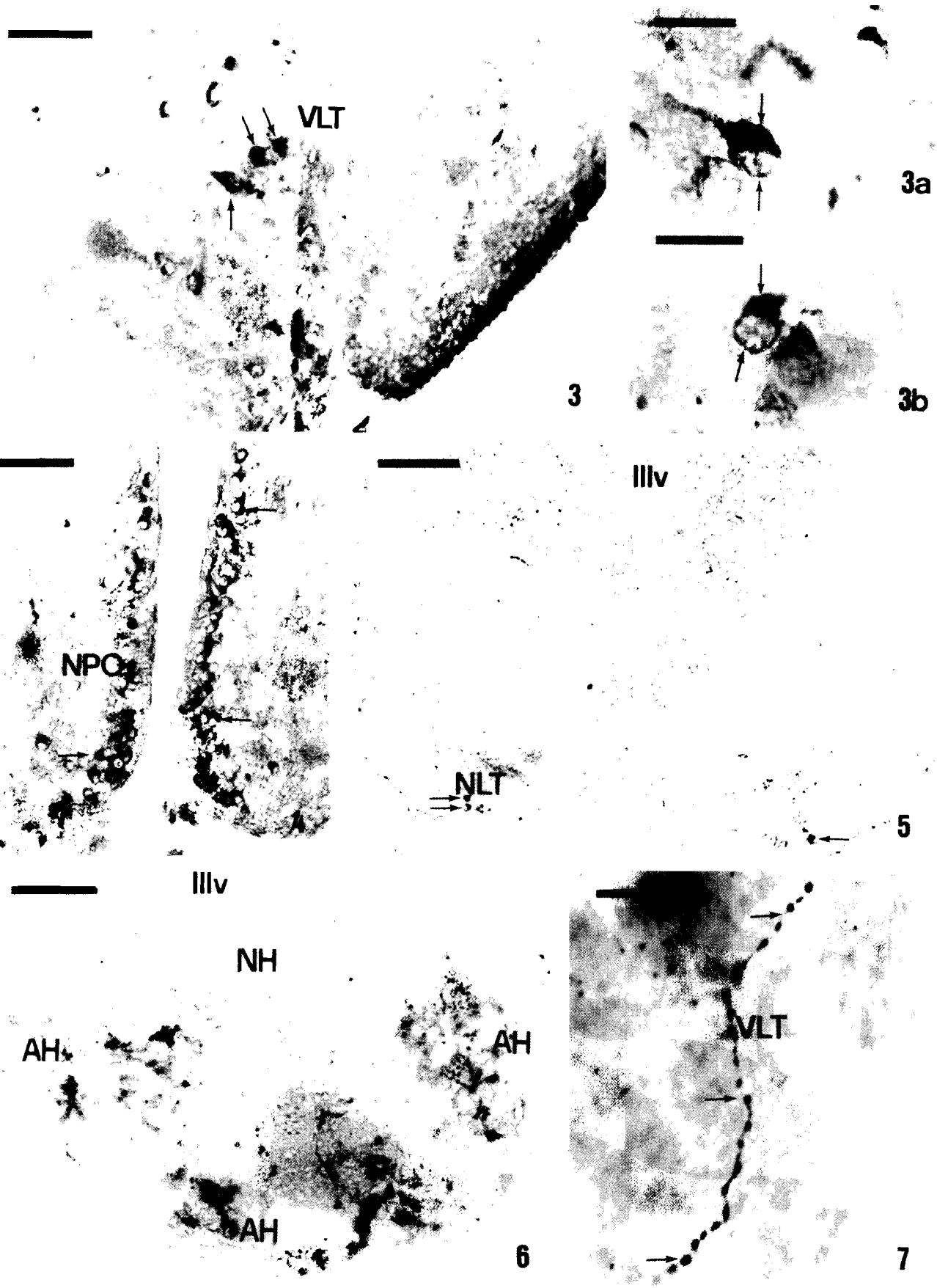
Fig. 3. Transversal section of *Gambusia affinis* telencephalon showing CRF-ir neurons (arrows) in the lateral part of area ventralis telencephali (VLT). Bar = 18 μm. x 840. **a,b.** Detail of CRF-ir neurons (arrows) in the area ventralis telencephali. Bar = 9 μm. x 1,680

Fig. 4. Positive neurons with a pyriform shape (arrows) in the nucleus praeopticus (NPO) near the ventricular surface. Bar = 125 μm. x 120

Fig. 5. Paraffin section showing a small number of CRF-ir neurons (arrows) in the nucleus lateralis tuberis (NLT). These neurons also have a pyriform shape. Third ventricle (IIIv). Bar = 63 μm. x 240

Fig. 6. General aspect of *Gambusia affinis* hypophysis (H) with CRF-ir interdigitations in the adenohypophysis (AH) while the neurohypophysis (NH) is not stained. Third ventricle (IIIv). Bar = 25 μm. x 640

Fig. 7. Vibratome section showing positive fibres in the pars lateralis of the area ventralis telencephali (VLT). Bar = 33 μm. x 450


VLT
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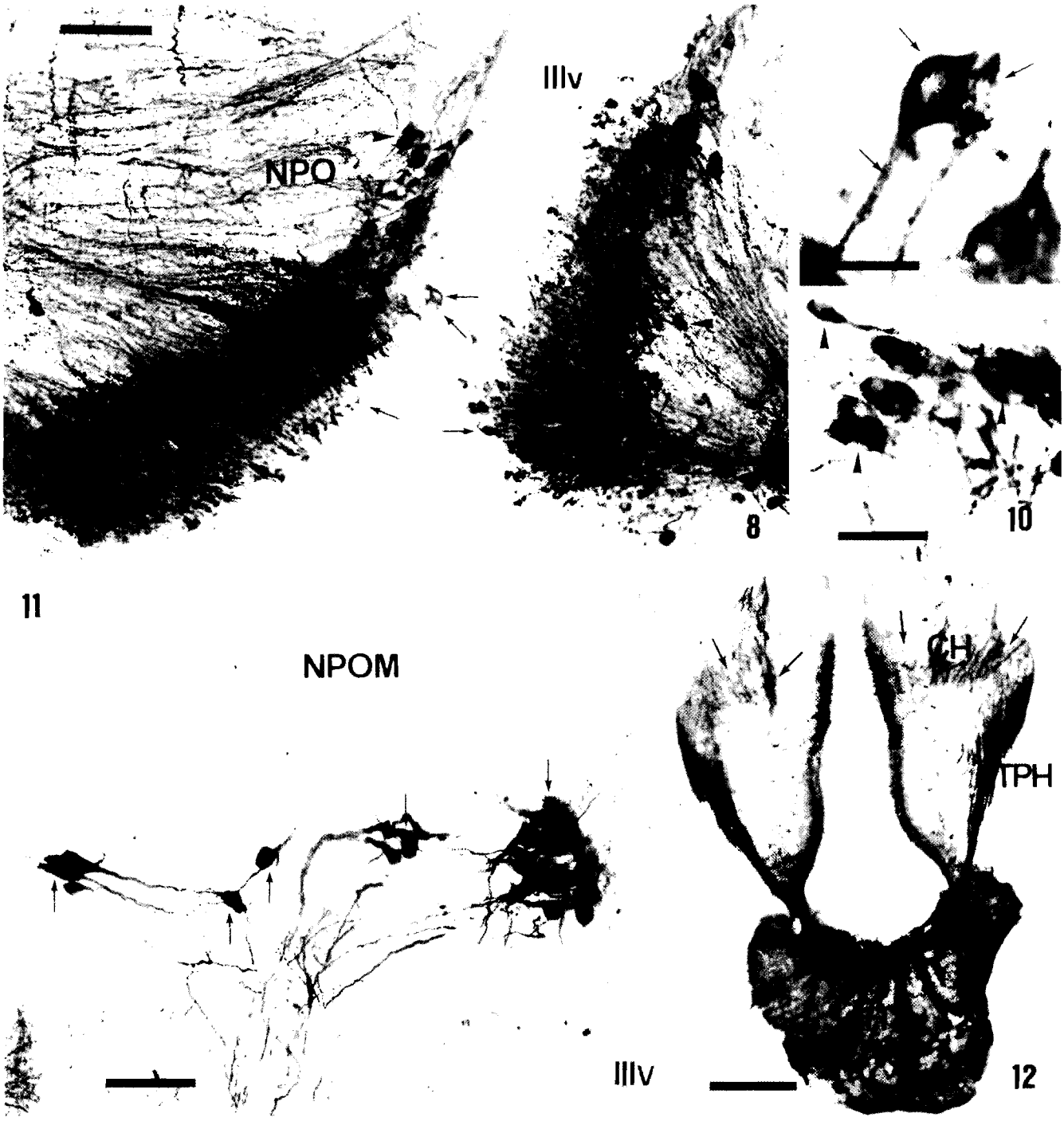
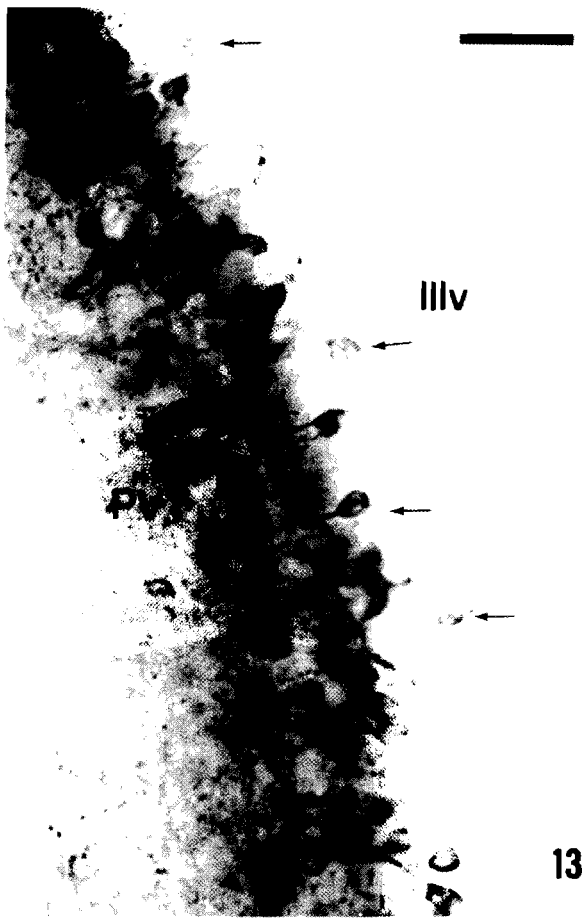


Fig. 8. Two distinct populations of CRF-ir neurons. One type is situated in a periventricular position (arrows) and another type is situated in a more lateral position (arrowheads). A large number of immunoreactive fibres is found running laterally and caudally in the pars parvocellularis of the nucleus praeopticus (NPO). Third ventricle (IIIv). Bar = 123 μm. x 123

Fig. 9. Detail of the CRF-ir neurons (arrows) with a process leading toward the ventricle in the pars parvocellularis of the NPO. Bar = 24 μm. x 623

Fig. 10. Detail of the CRF-ir neurons (arrowheads) without a process leading toward the ventricle in the pars parvocellularis of the NPO. Bar = 44 μm. x 342

Fig. 11. CRF-ir neurons (arrows) in the pars magnocellularis of the nucleus praeopticus (NPOM). Some of these neurons present one or two processes labelled by the antibody. Third ventricle (IIIv). Bar = 27 μm. x 560



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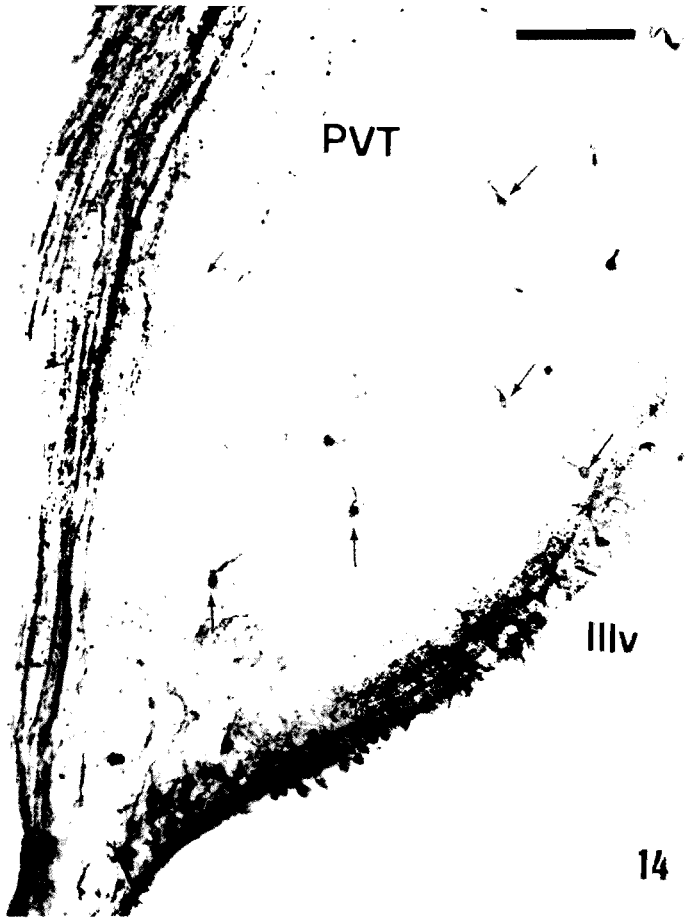

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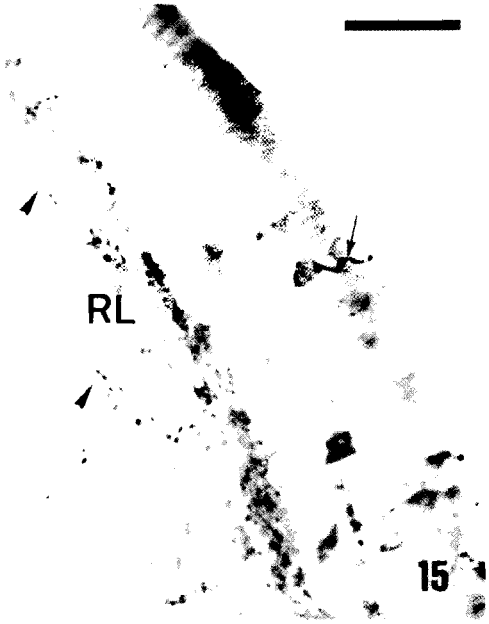

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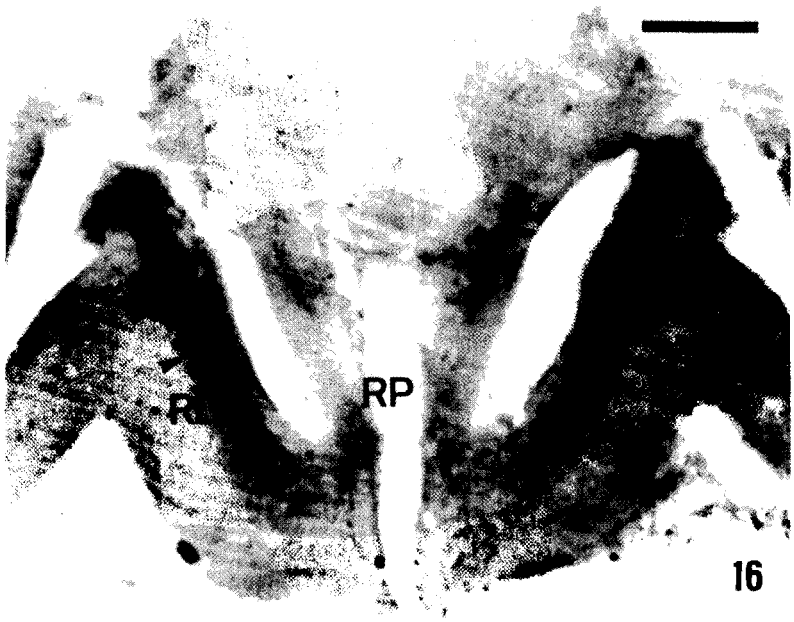

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(Figs. 2h, 16).

Discussion

The immunocytochemical study of paraffin and vibratome sections using antiserum against CRF has demonstrated differences between *Gambusia affinis* and *Salmo trutta* brains. The disparity of the results between both specimens was first seen in the telencephalon, a place where *Gambusia affinis* shows CRF-positive neurons in VLT; in the rest of the fish studied positive neurons in the telencephalon have not been found (Yulis and Lederis, 1987; Olivereau et al., 1988; Vallarino et al., 1990); only in *Poecilia latipinna* some positive neurons in the lateral part of the ventral telencephalon have been found (Batten et al., 1990), which is homologous to the VLT. However, Mancera et al. (1991), in the reptile *Natrix maura*, found positive neurons in the dorsal cortex, accumbens nucleus and the amygdala. Even if the localisation of positivity in *Gambusia affinis* is not the same as that described in *Natrix maura*, it is necessary to bear in mind, following the nomenclature of Northcutt and Davis (1983), that the amygdala of reptiles is homologous to the ventro-lateral telencephalic region of ray-finned fish and to some types of teleosts.

De Souza et al. (1984), using immunohistochemical and autoradiographic techniques, found receptors of high affinity for CRF in the amygdala. Perhaps equivalent receptors exist in our species and it is toward where the fibres observed in *Salmo trutta* and the axons of the neurons found in *Gambusia affinis* lead. This fact supports the hypothesis that CRF, besides the functions related to the hypophysis, acts on the central nervous system of vertebrates as a modulator or neurotransmitter, a theory already suggested by various authors (Olivereau et al., 1984; Chai et al., 1990; Mancera et al., 1991).

The NPO in its parvocellular and magnocellular parts shows a greater number of CRF-positive neurons in the case of *Salmo trutta* than in *Gambusia affinis*. Further studies should be done in order to find out if this variation can be due to physiological conditions, such as shown by Olivereau and Olivereau (1988), who demonstrated than in the eel the coexistence of Arginine-Vasotocine (AVT) and CRF in the same cells of the NPO varied according to the degree of osmotic stress. In similar situations of stress, the production of CRF by vasopressinergic cells has been observed (Batten et al., 1990). If we compare the disposition and the number of cells that in our study appear CRF positive in the NPO of *Salmo trutta* with the work of Olivereau et al. (1984) differences can be seen, since some of our positive neurons of the NPO are CSF-contacting. The causes of these differences should be a result of the stress that is spontaneously produced in our specimens, since they showed a hyperkinetic behaviour and became violent when removed for their natural habitat and moved to the environment imposed in the laboratory. A stressful behaviour was seen in all the trout used. Captivity and immobilization are causes of stress which have already been studied by Fagerlund (1967) in adult sockeye salmon and Redgate (1984) in *Cyprinus carpio*, who describe in all causes an activation of the hypophyseal-adrenal axis in teleosts.

Stress could also be the cause of the high density of positive neurons observed in the PVT. If we refer to the studies carried out by Fryer and Peter (1977a,b) in which they tried to locate the CRF productive zone in stressful situations by the use of stereotaxic lesions, we can see that the zone they were looking for could be situated very near to the NLT, as it was here that they tried to make the lesions. Fryer and Peter (1977c) also carried out experiments using cortisol implants and demonstrated that corticosteroids exercise negative feedback effects in the brain on suppressing the release of ACTH. Implants which contain cortisol in the hypothalamic ventricle suppress the stress induced by the increase of circulating adrenocorticoids. These experiments could also explain the fact that all the periventricular neurons of the PVT are CSF-contacting.

The hypophysis of *Salmo trutta* and *Gambusia affinis* do not present the same labelling since the neurohypophisis is CRF-positive in *Salmo trutta* but not in *Gambusia affinis*. We do not know the reason for the absence of immunoreactivity which is present in other fish inside the same family (Batten, 1986).

This leads us to suppose that the tubero-hypophyseal tract is towards this zone of the pituitary. Based on Batten (1986) we can complete this information with the hypothesis which this author proposes, i.e., that these

Fig. 12. General aspect of the hypothalamus with immunoreactive fibres forming the preoptic hypophyseal tract (TPH) directed to the hypophysis (H) that also shows CRF-ir interdigitations. Horizontal Comissure (CH). Bar = 455 μm. x 33

Fig. 13. CRF-ir liquor contactant neurons (arrows) in the periventricular zone of the tuber (PVT). These neurons are round, bipolar and liquor contactant. Third ventricle (IIIv). Bar = 23 μm. x 640

Fig. 14. Transverse section with CRF-ir monopolar neurons (arrows) within the periventricular zone of the tuber (PVT) dorsal and medial to the nucleus lateralis tuberis. Third ventricle (IIIv). Bar = 98 μm. x 153

Fig. 15. Detail of the zone with a CSF-contacting CRF-ir neuron (arrow) and CRF-ir fibres (arrowheads) oriented parallel to the ventricular border at the beginning of the ventral side of the lateral recess (RL). Bar = 23 μm. x 640

Fig. 16. A dense plexus of CRF-ir fibres (arrowheads) in the ventral side of the lateral recess (RL) on both sides of the posterior recess (RP). Bar = 98 μm. x 153

fibres are going to synapse directly with the target cells. However, not all fibres of the PVT lead toward the hypophysis, since we have to bear in mind those which appear positive on both sides of the posterior recess. The work of Olivereau et al. (1984), in which the presence of ACTH-positive cells in the lateral recess (RL) are indicated, suggest to us that the fibres which appear labelled in this posterior zone innervate these adreno-corticotropic cells in the same way as occurs in the hypophysis. In the same work Olivereau et al. add that it is probable that the ACTH-positive cells of the RL release into the ventricle. If we relate this data to the hypothesis proposed by Ruhmann-Wennhold and Nelson (1981), in which it is stated that ACTH carries out a feedback on the secretion of CRF controlled by the level of ACTH in the blood at the level of the hypothalamus we could suppose that in non-experimental conditions the cortisol or ACTH directly is distributed through the cerebro-spinal fluid and its fluctuating concentration is detected by the cells of the PVT area and even the liquor-contactants in the NPO, informing in this way, the necessity to release CRF or not.

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