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Localization and Identification of α-Melanocyte-Stimulating Hormone (α-MSH) in the Frog Brain

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The distribution of α-melanocyte-stimulating hormone (α-MSH) in the central nervous system of the frog *Rana ridibunda* was determined by immunofluorescence using a highly specific antiserum. α-MSH-like containing perikarya were localized in the infundibular region, mainly in the ventral hypothalamic nucleus. A rich plexus of immunoreactive fibers directed towards the ventral telencephalic region was detected. Reverse-phase high-performance liquid chromatography and radioimmunoassay were used to characterize α-MSH-like peptides in the frog brain. Chromatographic separation revealed that immunoreactive α-MSH coeluted with synthetic des-Nα-acetyl α-MSH, authentic α-MSH and their sulfoxide derivatives. The heterogeneity of α-MSH-like material in the frog brain was in marked contrast with the figure observed in the intermediate lobe of the pituitary gland where only des-Nα-acetyl α-MSH is present. These findings support the existence of discrete α-MSH immunoreactive neurons in the frog brain containing both desacetyl and authentic α-MSH.

INTRODUCTION

Alpha-melanocyte stimulating hormone (α-MSH) is an acetyltridecapeptide amide secreted by the intermediate lobe of the pituitary gland[9]. The structure of this peptide has been highly preserved in all vertebrate species examined so far[18,31,35]. However, in mammals, the physiological function for this hormonal peptide remains unknown. Conversely, in lower vertebrates, α-MSH is clearly involved in the dispersion of melanin in melanocytes and therefore plays an important role in skin color adaptation.

During the last few years it has become apparent that α-MSH is not confined to pars intermedia cells. Immunocytochemical techniques[3,4,11,20,23] as well as bioassays and radioimmunoassays[22,33] have revealed that this peptide is also present in discrete areas of the central nervous system of mammals. Neuronal cell bodies containing immunoreactive α-MSH are located in the arcuate nucleus[1,4,11,20,23,27] and in the dorsolateral region of the hypothalamus[6,7,39,40]. Concurrently, we have shown that the brain of amphibia contains a substance chemically related to α-MSH[32,34] and we have provided evidence that this peptide originated from the brain itself[12]. Therefore, we have decided to investigate further the chemical nature of the α-MSH-like peptide contained in the frog brain and to determine the distribution of α-MSH-producing neurons in this species.

MATERIALS AND METHODS

Tissue preparation

Adult male frogs (*Rana ridibunda*) of 30–40 g body weight were obtained from a commercial source (Couetard, Saint-Hilaire de Riez, France).

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The animals were maintained at constant temperature (8 ± 0.5 °C) for one week, with an established photoperiod of 12 h light per day (light from 06.00–18.00 h).

For immunocytochemical studies, the animals were stunned and transcardially perfused with 30 ml of 0.1 M phosphate buffer (pH 7.3) containing 0.025% xylocaine. Then the perfusion was carried on with 50 ml of McLeans's fixative as previously described[17]. The brains with the attached pituitary were dissected and postfixed in the same fixative solution for 3 h. The tissues were rinsed overnight in a 0.1 M phosphate buffer–15% sucrose solution and then transferred into a 0.1 M phosphate buffer–30% sucrose solution for 24 h. The brains were frozen in liquid nitrogen and sliced at 8 μm in the frontal or sagittal plane on a cryostat (Frigocut 2700, Reichert Jung).

For biochemical studies, the animals were decapitated and 3 regions of the brain were dissected: infundibulum, median eminence and telencephalon. The tissue samples from 3 animals were pooled together, immersed in 1 ml boiling 2 N acetic acid and maintained in a boiling water bath for 10 min. Then the tissues were sonicated for 2 min, the homogenates were centrifuged (10,000 *g*) at 4 °C for 15 min and the supernatants were collected and freeze-dried.

Immunohistochemistry of α-MSH

The tissue sections were incubated overnight at 4 °C in a humid atmosphere with α-MSH antiserum (code number 81-0103) at 1:200 dilution. The sections were rinsed for 1 h in phosphate buffer (PBS) and incubated at 20 °C for another hour with fluorescein-isothiocyanate-conjugated antirabbit γ-globulins (GAR/FITC; Nordic Immunology) at the working dilution of 1:600. Finally, the sections were rinsed in phosphate buffer and examined using a Leitz Orthoplan microscope equipped with a vario-orthomat photographic system. Several sections were used as controls, which were incubated with antiserum preabsorbed with synthetic α-MSH at a concentration of 10^{-6} M. The specificity of the antiserum has been also studied by radioimmunoassay[12,33]. The epitope read by the antibodies has been determined to be the dipeptide Gly^{10}-Lys^{11}, a determinant which is contained within the biologically active region of the α-MSH molecule.

High-performance liquid chromatography

α-MSH-related peptides were separated by reverse-phase HPLC on a Dupont Liquid Chromatograph (model 870) equipped with a Gradient Controller (model 8800) and a 0.46 × 25 cm column of Zorbax/C8. The mobile phase consisted of a linear gradient established with 0.1% trifluoracetic acid in water (pH 2.4) and acetonitrile. The gradient used is presented in Fig. 4. The flow rate was 1 ml/min and 1-min fractions were collected. Each fraction was evaporated in a Speed Vac Concentrator (Savant, Hicksvile, NY) at 45 °C and kept dry until radioimmunoassay. HPLC standards consisted of 1 μg each of synthetic des-Nα-acetyl α-MSH and α-MSH. Oxidation of the reduced standards was performed by adding 20 μl of chloramine T solution (1 mg in 1 ml 0.5 M, pH 7.4, phosphate buffer) to 1 μg of synthetic peptides diluted in 10 μl 0.004 N HCl. In these conditions, the oxidation was completed within 30 s. The reaction was stopped by adding 20 μl of a sodium metabisulfite solution (3 mg in 1 ml 0.5 M, pH 7.4, phosphate buffer). Synthetic standards were detected in the column effluent by ultraviolet absorption at 212 nm using an isco UV-detector (model 1 840) and by radioimmunoassay.

Radioimmunoassay

Standards and samples were reconstituted in 0.02 M veronal buffer (pH 8.6) and assayed in duplicate by means of a sensitive double-antibody radioimmunoassay method[33]. The specificity of the antiserum used for both radioimmunoassay and immunohistochemistry (code number 81-0103) has been studied in detail previously[12] using 37 synthetic α-MSH analogues and a large series of neuropeptides. The antibodies are C-terminally directed and thus, recognize on an equimolar basis des-Nα-acetyl α-MSH and α-MSH and their sulfoxide derivatives. The antiserum had very low cross-reactivity with other POMC-derived peptides and did not cross-react with any other neuropeptide. After purification of the iodinated tracer by reverse-phase HPLC, the detection limit of the assay was 2 pg.

RESULTS

Immunofluorescence studies

The distribution of α-MSH immunoreactive cell bodies and fibers in a parasagittal diagram of the frog

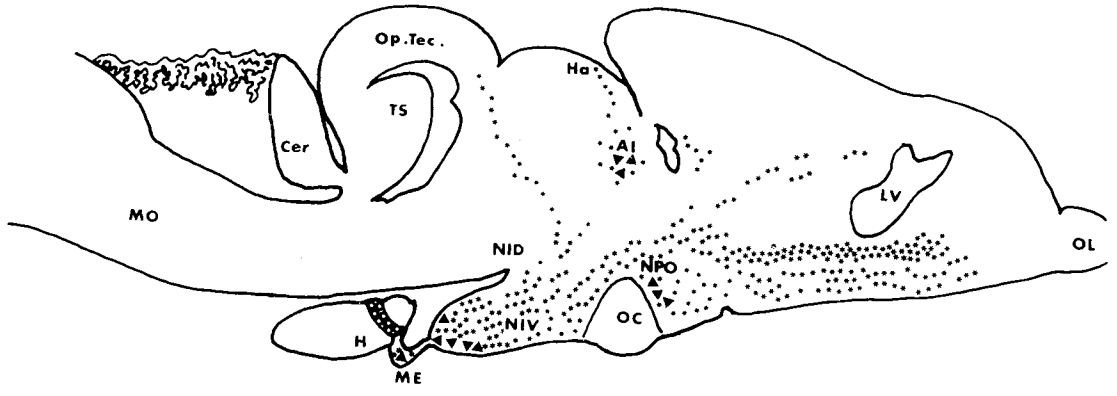

Fig. 1. Schematic parasagittal section through the brain of *Rana ridibunda* depicting the distribution of α-MSH-containing perikarya (triangles) and neuronal processes (asterisks). Al, amygdala, pars lateralis; Cer, cerebellum; H, hypophysis; Ha, habenula; LV, lateral ventricle; ME, median eminence; MO, medulla oblongata; NID, nucleus infundibularis ventralis; NPO, nucleus preopticus; OC, optic chiasma; OL, olfactive lobe; Op. Tec., optic tectum; TS, torus semicircularis.

brain is presented in Fig. 1. The perikarya are shown as triangles and the fibers as asterisks. The relative densities (sparse, moderate or dense) of α-MSH processes are suggested by the separation of asterisks.

Immunoreactivity was mainly concentrated in the hypothalamus. Many brightly fluorescent cell bodies were observed in the ventral infundibular nucleus (Fig. 3a, b). An abundant network of thick and brightly fluorescent fibers was present in the infundibulum (Fig. 3c), with a dense ventral tract coursing forwards to reach the preoptic area and the ventral telencephalon. Some fibers issuing from the ventral infundibular nucleus projected in the median eminence. Two groups of weakly fluorescent perikarya were also visualized in the preoptic nucleus and in the lateral amygdala. Fibers arising from lateral amygdala projected upwards to the habenular region. The major tract of fibers was observed in the ventral part of the telencephalon. Scarce fibers were found in the cerebral cortex (hippocampus). Some immunoreactive fibers originating probably from the infundibular region were observed in the mesencephalon where they projected in the optic tectum. No immunoreactivity was contained in the posterior part of the frog brain, i.e. tegmentum, cerebellum and medulla oblongata. All the cells of the intermediate lobe of the pituitary were labelled by application of the α-MSH antiserum (Fig. 3d). Conversely no immunoreactivity could be detected in distal and neural lobes of the pituitary.

In Fig. 2 (a–e) are presented 5 schemes of frontal sections indicating the distribution of α-MSH immunoreactive perikarya and nervous processes in the frog brain. Immunoreactive perikarya located in the medioventral part of the ventral nucleus of the infundibulum were concentrated under the floor of the third ventricle. Few cell bodies were found in the lateral part of the dorsal area of the ventral infundibular nucleus (Fig. 3a). The dense network of infundibular fibers projected laterally under the third ventricle towards the lateral part of the dorsal infundibular nucleus and forwards to the preoptic area. As shown in the parasagittal section, no fibers were found in the dorsal part of the mesencephalon. Only few fibers were visualized in the posterolateral nucleus of the thalamus (Fig. 2e).

Preincubation of the primary antiserum with synthetic α-MSH (10^{-6} M) resulted in complete loss of immunoreaction. When the α-MSH antiserum was substituted with either pre-immune rabbit serum or PBS, no immunofluorescence was observed. Unstained slices exhibited no autofluorescence.

Reverse-phase HPLC

The characterization of α-MSH immunoreactivity in the infundibulum, median eminence and telencephalon was carried out by a combination of reverse-phase HPLC and radioimmunoassay. As shown in Fig. 4, 3 peaks exhibiting α-MSH-like im-

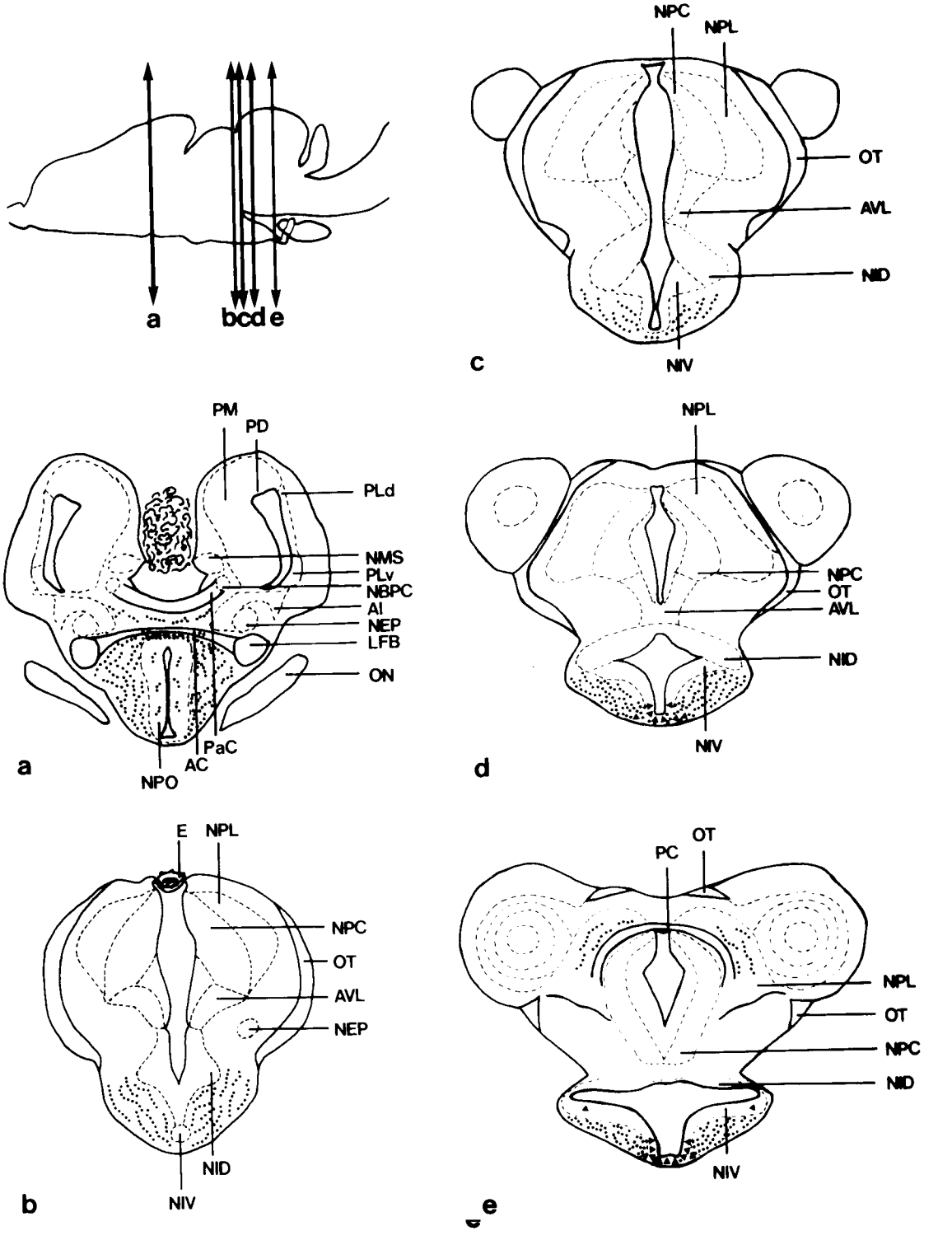

Fig. 2. Schematic frontal sections (a–e) through the brain of *Rana ridibunda* showing the distribution of cell bodies (triangles) and fibers (asterisks) labelled with α-MSH antiserum. Diagrams and abbreviations were used according to Wada et al.[37]. Al, amygdala, pars lateralis; AC, anterior commissure; AVL, area ventrolateralis thalami; E, epiphysis; LFB, lateral forebrain bundle; NBPC, bed nucleus of the pallial commissure; NEP, nucleus entopeduncularis; NID, nucleus infundibularis dorsalis; NMS, nucleus medialis septi; NPC, nucleus posterocentralis thalami; NPL, nucleus posterolateralis thalami; NPO, nucleus preopticus; ON, optic nerve; OT, optic tract; PaC, pallial commissure; PC, posterior commissure; PLd, pallium laterale, pars dorsalis; PLv, pallium laterale, pars ventralis; PM, pallium mediale.

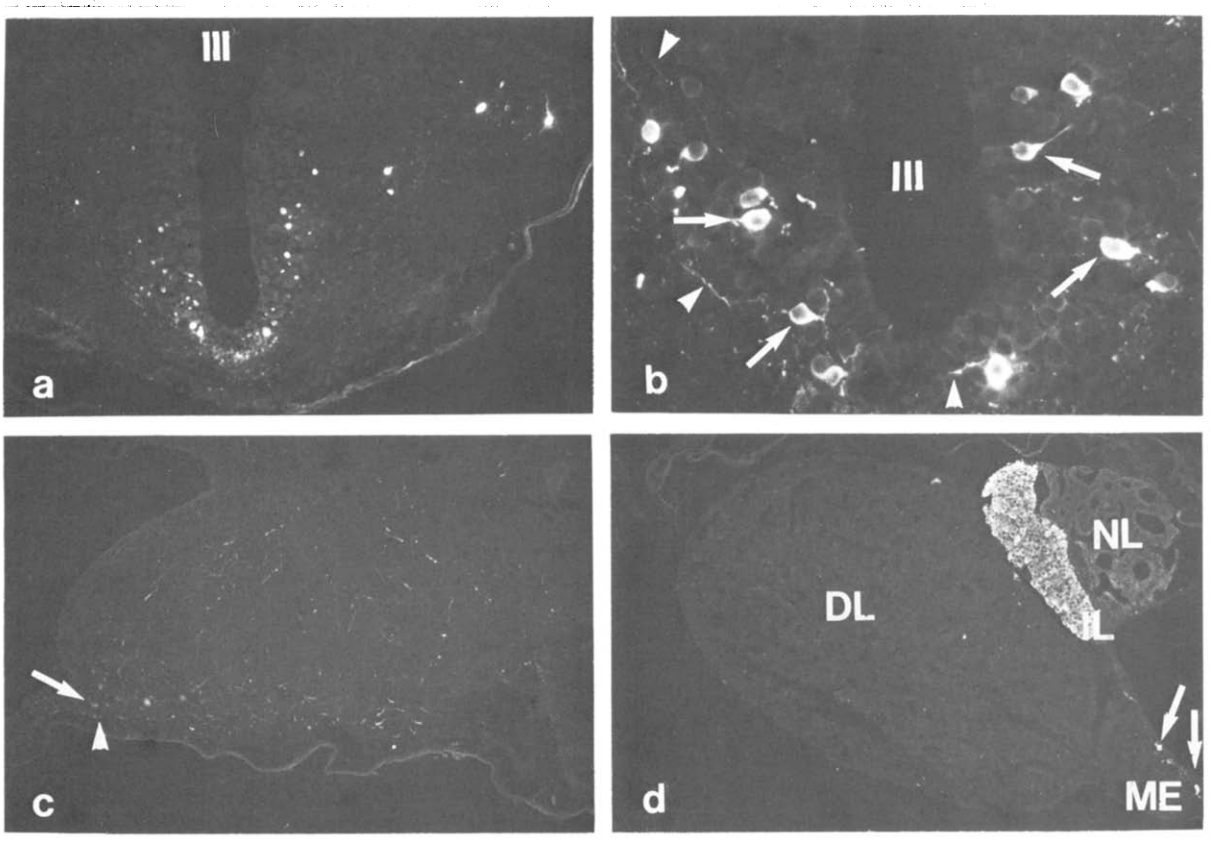

Fig. 3. a: photomicrograph of a frontal section, across frog infundibulum, labelled with anti-α-MSH antiserum showing the ventromedian distribution of α-MSH-like immunoreactivity. Some perikarya can be seen more dorsolaterally. b: higher magnification of a frontal section across the ventral nucleus of the infundibulum. Most representative positive cell bodies are indicated by arrows and positive fibers by arrow heads. c: photomicrograph of a parasagittal section of the infundibulum showing the widespread distribution of thick α-MSH-positive fibers and the caudal localization of cell bodies (arrows). d: sagittal section of frog hypothalamo-hypophysial tract labelled with anti-α-MSH antiserum. Two cell bodies, indicated by arrows, are present in the median eminence. All the cells of the intermediate lobe of the hypophysis are stained by the α-MSH antiserum. Abbreviations: III, third ventricle; ME, median eminence; NL, neural lobe; IL, intermediate lobe; DL, distal lobe.

munoreactivity were resolved by HPLC. In all 3 extracts, peptides coeluting exactly with synthetic des-Nα-acetyl α-MSH and authentic α-MSH were observed.

In infundibulum and median eminence extracts, the major peak corresponded to desacetyl α-MSH. A compound exhibiting α-MSH-like immunoreactivity and coeluting precisely with the sulfoxide forms of desacetyl α-MSH and α-MSH was detected in all 3 tissue extracts. In the telencephalon extract, the sulfoxide forms were predominant. It should be noted that the gradient of acetonitrile used for HPLC analysis did not allow to separate the sulfoxide forms of desacetyl α-MSH and α-MSH.

DISCUSSION

The results of the present study in the frog indicate that α-MSH-containing neurons are located in the infundibular region, mainly in the ventral hypothalamic nucleus. This is in agreement with previous biochemical studies from our laboratory, which demonstrated that the highest concentrations of α-MSH-like material were measured in the diencephalon[12,34]. Since the distribution of α-MSH-containing neurons has not been investigated previously in amphibia, our present results can be only compared to those reported for the mammalian and reptilian nervous system. In mammals, two neuronal systems containing

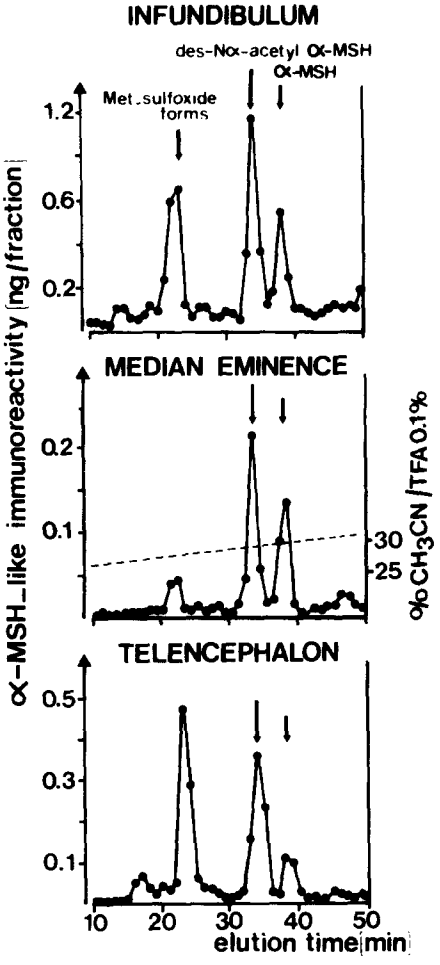

Fig. 4. Reverse-phase high-performance liquid chromatography analysis and RIA quantification of α-MSH-like peptides in the frog brain. Infundibulum, median eminence and telencephalon were extracted in 2 N acetic acid, lyophilized and submitted to HPLC separation on a Zorbax/C8 column at a flow-rate of 1 ml/min. The mobile phase consisted of 0.1% trifluoroacetic acid/water and a gradient of acetonitrile (25%–31%) over 48 min. Synthetic des-Nα-acetyl α-MSH, authentic α-MSH and their sulfoxide forms (1 μg each) were analyzed in the same conditions (arrows). All fractions collected were assayed for α-MSH immunoreactivity using a C-terminal-directed antiserum.

α-MSH-like material have been detected by immunonocytochemistry. The first group of neurons, which was identified in the arcuate nucleus, stained positively not only for α-MSH, but also for various pro-opiomelanocortin (POMC)-derived peptides including the 16-K fragment[6,26], γ-MSH[2], ACTH[25–27,29,39], β-LPH[1,3,19,27,42] and β-endorphin[3,29,38,41]. The second group of neurons was located in the dorsolateral region of the rat hypothalamus and contained specifically α-MSH[6,7,13,38,40]. Labelling of serial sections of the frog hypothalamus with antiserum to α-MSH or to other POMC-derived peptides should establish whether all α-MSH neurons observed in the frog hypothalamus are also immunoreactive for ACTH and β-endorphin. Recently, Vallarino[31] has investigated the distribution of α-MSH-containing neurons in the hypothalamus of a lizard (*Lacerta muralis*), using an antiserum produced in our laboratory. The study has shown the existence of two groups of immunoreactive cell bodies located in the ventrolateral part of the preoptic area and in the posterior region of the supraoptic nucleus. In another lizard species (*Anolis carolinensis*), Khachaturian et al.[16] have observed the existence of a mesencephalic cell population exhibiting immunoreactivity to β-endorphin, ACTH and α-MSH. Such POMC-containing perikarya are not found in the mammalian and in the amphibian mesencephalon. Conversely, in the present study, we have detected a second group of perikarya exhibiting a weak α-MSH-like immunoreactivity in the frog brain. This neuronal population, located in the lateral amygdala, seemed to contribute to the innervation of the habenular region. Such a telencephalic–diencephalic α-MSH-containing population has never been observed in reptiles[16,31] or mammals[11]. Thus, our results, while supporting the view that most peptidergic neurons in the frog brain originate in the anterior hypothalamus[30], indicate the existence of neuropeptide cell populations in more rostral regions.

In mammals, α-MSH-positive fibers are widely distributed in the brain with higher concentrations in the septum, the medial basal hypothalamus and the paraventricular nucleus of the thalamus[7,11,27]. In the frog, a rich plexus of immunoreactive nerve fibers directed towards the ventral telencephalic area was detected. These rostral projections seemed to be homologous to those recently described in the lizard[16]. The abundant telencephalic processes suggest that α-MSH may play an important role in the brain of these animals. Also in support of a possible role of α-MSH-related peptides as neurotransmitters in non-mammalian vertebrates is the observation that ACTH modifies the turning behavior in the toad[10].

Previous studies have shown that the frog hypothalamus contains a substance immunologically related to α-MSH. On the basis of gel filtration experiments, it was proposed that brain α-MSH was

similar, if not identical, to synthetic mammalian α-MSH[14,34]. However, recent studies clearly indicated the presence of both desacetylated and acetylated forms of α-MSH in the rat brain[13,20]. Our present results demonstrating that des-Nα-acetyl α-MSH and authentic α-MSH co-exist in the frog brain are in line with the studies performed in mammals[13,21]. The sulfoxide derivatives of α-MSH and des-Nα-acetyl α-MSH were probably artifactually generated during the acetic acid extraction procedure, since the methionine residue is highly susceptible to oxidation[21]. At the pituitary level it has been shown that, in the frog, pars intermedia cells contain only the desacetyl form of α-MSH[35,36]. In these animals, as in toad[18], acetylation of α-MSH is a late step which is strictly linked to exocytosis. Since the antibodies used for immunocytochemical detection of α-MSH-containing neurons are directed against the C-terminal region of the molecule[12,33] and thus recognize both desacetyl and authentic α-MSH, it is not possible at the moment to determine whether, in the frog brain, desacetyl α-MSH is preferentially located in the cell bodies.

Two recent studies indicate that dopamine can influence the acetylation of POMC-derived peptides in the pars intermedia of the rat[8] and frog[15]. Since acetylation of β-endorphin and α-MSH profoundly modifies the intrinsic activities of these peptides[7,21,28], further experiments will be conducted to investigate a possible role of dopamine in the posttranslation processing of α-MSH in the frog brain.

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