DISTRIBUTION OF NEURONAL ALPHA MSH IN *Spea multiplicata*: EFFECTS OF STRESS

by

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ABSTRACT

It has been previously demonstrated in *Bufo* sp. that stress induces alterations in neuronal and pituitary melanocortins. Here I examined the effects of an acute stressor on regional brain alpha-melanocyte-stimulating hormone (α-MSH) content in the New Mexican spadefoot toad, *Spea multiplicata*. First the distribution of α-MSH neurons in *Spea* was seen using immunocytochemistry combined with radioimmunoassay (RIA). α-MSH-ir cells were observed in the preoptic nucleus and ventral infundibulum of the hypothalamus. Ascending immunoreactive fibers projected to the nucleus accumbens and olfactory nucleus of the telencephalon. Some fibers reach the amygdala and the optic tectum. High concentrations of α-MSH were detected in the hypothalamus, the location of the largest population of α-MSH-ir cell bodies, with lower amounts in the preoptic area, telencephalon, brainstem, and optic tectum as determined by RIA. Exposure to a brief stressor elevated α-MSH levels in the preoptic area and optic tectum 60-min after exposure.

I conclude that the distribution of neuronal melanocortins in *Spea* resembles the pattern observed in *Bufo*, with an exception of the preoptic cell group, which has not been observed in latter species. As in *Bufo*, the activity of melanocortin neurons is altered during stress. Melanocortins have been implicated in the control of visual learning in toads. This work suggests that the stress-induced alteration in neuronal melanocortin activity may influence the behavior of the toad and how they gather information about their environment

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LIST OF ABBREVIATIONS

Ab	antibody
ABC	avidin biotinyted-peroxidase complex
ACTH	adrenocorticotropin
ANOVA	analysis of variance
AL	anterior lobe
CNS	central nervous system
CLIP	corticotropin like intermediate peptide
CRF	corticotropin releasing factor
CSF	cerebrospinal fluid
DAB	diaminobenzidine
END	endorphin
GARGG	goat anti-rabbit gamma globulin
GnRH	gonadotropin releasing hormone
-ir	-immunoreactive
IL	intermediate lobe
LH	luteinizing hormone
LPH	lipotropic hormone
MCH	melanin concentrating hormone
MSH	melanocyte stimulating hormone
NEI	neuropeptide glutamic acid isoleucine
PBS	phosohate-buffered saline

PC	proconvertase
POMC	proopiomelanocortin
PRL	prolactin
RIA	radioimmunoassay
RT-PCR	reverse transcriptase polymerase chain reaction
S.E.M	standard error of mean
TRH	thyrotropin releasing hormone

CHAPTER I

INTRODUCTION

Discovery of Melanocyte Stimulating Hormone (MSH)

Classical bioassays involving surgical removal of a tissue and then observing the physiological changes have led to the discovery of several hormones. Such experiments in 1916 resulted in the discovery of a skin-darkening compound. Striking color change was observed in hypophysectomized frog larvae as compared to controls (Smith, 1916). Similar work done in the same year by Allen (1916) confirmed these results. Surgical removal of the hypophyseal anlage in tadpoles led to their discoloration, in contrast to the control tadpoles which retained their dark green body color (Allen, 1916). The exact part of the pituitary responsible for skin darkening was determined in 1921 by the work of Swingle (Swingle, 1921). Transplantation of the pituitary intermediate lobe (IL) of adults to larvae caused them to turn dark. The control animals that were grafted with other brain tissues remained pale (Swingle, 1921). This experiment confirmed that the IL regulates skin pigmentation. The hormone was named 'intermedin' by Zondek, owing to its site of synthesis and Harris and Lerner later changed the name to melanocyte stimulating hormone (MSH) in 1957 (Zondek, 1932).

Isolation and Structure of MSH

Melanocyte stimulating hormone was first isolated from hog posterior pituitary gland by Lee and Lerner (1956). They isolated two distinct compounds each possessing MSH like activity. The more active one was named alpha-melanocyte -stimulating hormone (α-MSH) and the other one was called β-MSH (Lee and Lerner, 1956).

The amino acid sequence of α-MSH was deciphered in 1957 by Harris and Lerner (Harris and Lerner, 1957). It was found to be composed of thirteen amino acids (tridecapeptide) with protected carboxy and amino termini. An amide group substituted the C-terminus and an 'unidentified' group on the N-terminus was later shown to be an acetyl group. It was found that corticotropins, α-MSH, and β-MSH all had a common core heptapeptide sequence (Harris and Lerner, 1957). The complete sequence of β-MSH (18 AA) was elucidated in 1956 (Harris and Roos, 1956).

The identical core heptapeptide sequence in ACTH, α-MSH, and β-MSH and the identical first 13 amino acids of ACTH and α-MSH suggested a common precursor.

Pro-Opiomelanocortin (POMC)

Compounds having similar amino acid sequence but having different molecular weights (size heterogeneity) have been an indicator of the existence of precursors for several peptide hormones. It was true in this case too. Plasma, pituitary, and tumor extracts indicated the presence of two compounds of different molecular weights but possessing ACTH activity (Yalow and Berson, 1971). The larger of the two peptides was identified as a 31KD polypeptide, called 'big ACTH'. This polypeptide was later found to be a precursor of corticotropins, melanotropins, lipotropins, and endorphins (Chretien et al., 1979). The precursor was named pro-opiomelanocortin (POMC) (Eipper and Mains, 1981). Proopiomelanocortin has 8 pairs of basic amino acids and one sequence of 4 basic amino acids that are the sites of proteolytic cleavage (Bertagna, 1994). The enzymes that breakdown POMC are pro-convertases 1 and 2 (PC-1 and PC-2). PC-1 is expressed by corticotropes of pituitary anterior lobe (AL) and both PC-1 and PC-2 are

expressed by melanotrophs of pituitary intermediate lobe (IL) (Zhou et al., 1993). PC-1 breaks POMC into ACTH and β-LPH. PC-2 cleaves POMC into β-endorphin, CLIP, and α-MSH (Benjannet et al., 1991) Fig 1.1.

Melanotropins in Extra Pituitary Areas

The pituitary gland is not the only source of melanotropins. Early biological data showed that hypothalamic extracts of pig and dog contained α-MSH, β-MSH and ACTH activities (Guilleman et al., 1962; Schally et al., 1962). Other than posterior and anterior pituitary, melanotropic-lipolytic activity was seen in different regions of bovine, simian and human brains (Rudman et al., 1973).

POMC containing neurons have been identified using immunocytochemistry in every vertebrate examined so far. In rainbow trout α-MSH-immunoreactive (ir) cells were seen in medial portion of the nucleus lateralis tuberis. α-MSH-ir fibers were seen in the ventral thalamus and the floor of hypothalamus (Vallarino et al., 1989).

Radioimmunological data showed the presence of α-MSH-ir in the brain of hypophysectomized frog (Vaudry et al., 1978). Both des-acetyl and acetylated α-MSH neurons were observed in the infundibular region of the ventral hypothalamus nuclei in frogs. α-MSH-ir fibers were seen to project rostrally towards the telencephalon (Benyamina et al., 1986). In the frog, *Rana esculenta*, α-MSH-ir cells were detected in the preoptic area, in the nucleus infundibularis ventralis of the posterior hypothalamus and in median eminence (Vallarino, 1987).

Proopiomelanocortin(POMC)

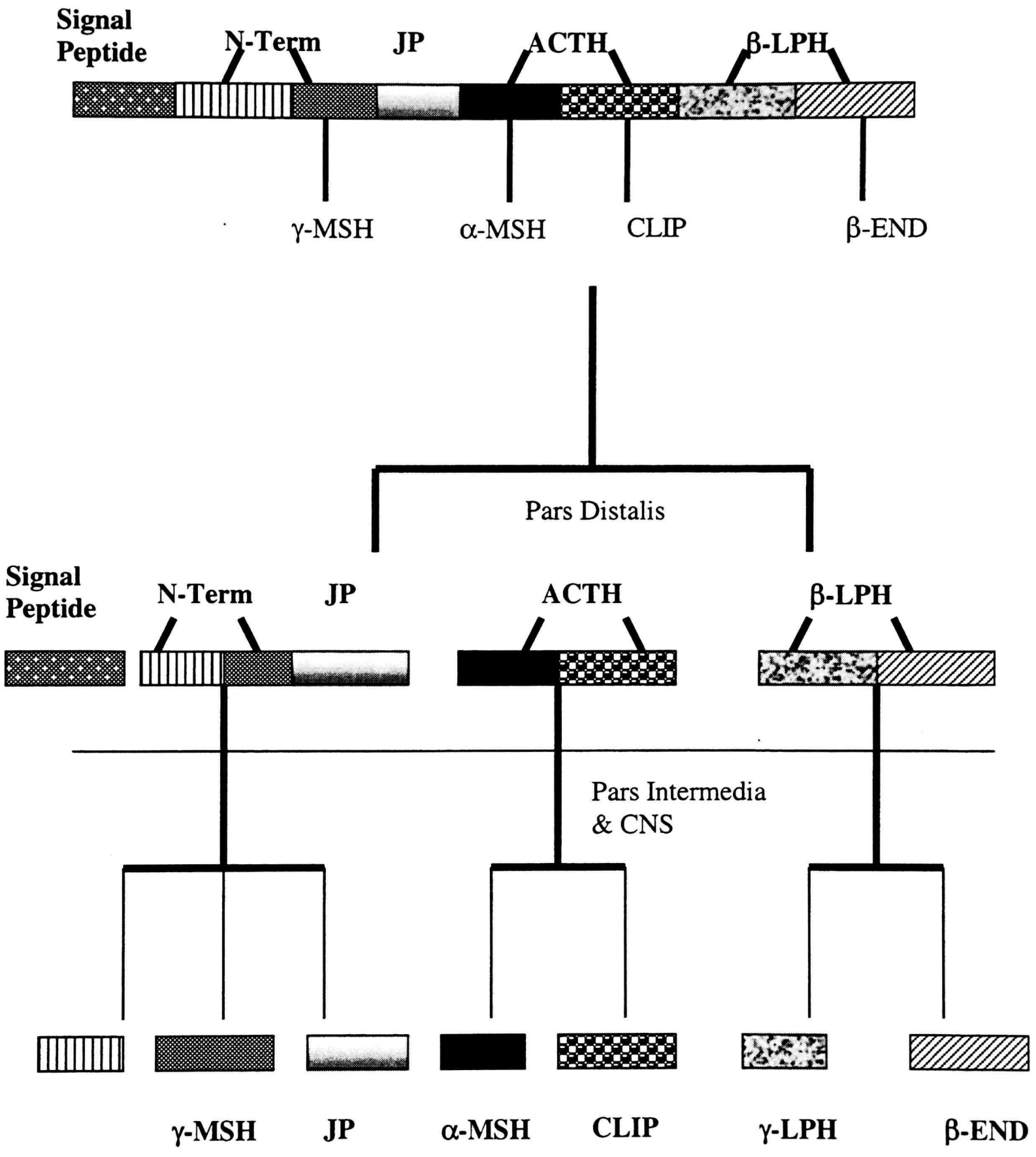


Figure 1.1. Proteolytic breakdown of POMC. Adapted from Castro et al., 1997

In *Bufo cognatus,* α-MSH immunoreactive cell groups were detected only in the infundibular hypothalamus. Fibers were seen to project to nucleus olfactory, nucleus accumbens, septum, caudal thalamus, optic tectum, and brain stem (Kim and Carr, 1997). In *Xenopus* brain, using in-situ hybridization and immunocytochemistry, POMC, α-MSH, γ-MSH, non-acetylated endorphins, and ACTH were found in neuronal cell bodies in the ventral hypothalamic nuclei and in the neurons of the anterior preoptic area. The supra-chiasmatic nucleus was found to have α-MSH, non-acetylated endorphins and ACTH. The neurons of posterior tubercle, olfactory bulb, medial septum, medial, and lateral parts of amygdala, the ventro-medial and post-thalamic nuclei, optic tectum, and antero-ventral tegmental nuclei had α-MSH alone. Locus ceruleus was found to have POMC and ACTH (Tuinhof et al., 1998).

In the lizard *Lacerta muralis*, immunoreactive cell bodies were seen in the ventrolateral part of preoptic area. Cell bodies were also seen in the caudal part of supraoptic and paraventricular areas of the hypothalamus. Some cells were also seen in the mesencephalo-diencephalic boundary region. Fibers were seen in the internal zone of the median eminence (Vallarino, 1984). In the lizard *Anolis carolinensis,* immunoreactivity to β-endorphin, ACTH and α-MSH was seen in a cell group in mesencephalic tegmental area. This cell group sent projections to mesencephalic central gray and other brain stem structures. Other cell body group was in medial basal hypothalamus with projections to ventral telencephalic and diencephalic areas (Khachaturian et al., 1984).

Early studies indicated 2 sources of MSH in rat brain. Hypophysectomy reduced the hypothalamic MSH content and concentration but it did not alter MSH concentration

in pineal gland and CNS. This suggested that MSH was locally synthesized within the CNS (Vaudry et al., 1978). Since then, extensive work has been done to localize the distribution of melanocortins in the mammalian brain. Immunocytochemical studies combined with RIA showed the presence of α-MSH cells in the arcuate nucleus of the hypothalamus (Donohue and Jacobowitz, 1980; Donohue et al., 1979). It was confirmed that arcuate region is responsible for the bulk of extrahypothalamic α-MSH by surgically isolating the arcuate region of hypothalamus from the rest of the brain and estimating the concentration of α-MSH (Eskay et al., 1979).

Other brain regions that contained high concentrations of hormone containing fibers were the septum, nucleus interstitialis stria terminalis, medial preoptic area, anterior hypothalamic area, dorsomedial, and periventricular nucleus. Moderate α-MSH concentration was noted in amygdala, septal area, central gray, dorsal raphae of nucleus of solitary tract (Donohue and Jacobowitz, 1980; Jacobowitz and Donohue, 1978).

Later experiments showed the presence of two α-MSH-ir cell groups. The first group was the previously known arcuate nucleus. The cells in this nucleus also stained for ACTH and the 16K fragment. The second group of nucleus was the dorsolateral region of hypothalamus, the cells of which only stain for α-MSH (Guy et al., 1980).

To see if the projections from the two cell groups are similar, arcuate nucleus was destroyed by treatment with monosodium glutamate in neonatal rats. α-MSH-ir cell bodies in the arcuate nucleus were absent in adults while the dorsolateral cell group was still present (Guy et al., 1981). Similarly, treatment with colchicine decreased ACTH, β-END fibers and content in dorsolateral hypothalamus, but it did not alter the α-MSH,

ACTH, and β-END fibers and content in the arcuate region (Jegou et al., 1983). These studies indicated that these two cell groups had different fiber projections.

The dorsolateral region of hypothalamus in rat has been suggested to correspond to the preoptic region of frog hypothalamus while the arcuate region of rat hypothalamus corresponds to the ventral infundibular nucleus in frog (Anderson et al., 1987). Coexistence of melanin concentrating hormone (MCH) and α-MSH like peptide was first reported in dorsolateral hypothalamus of rat brain (Naito et al., 1986). Immunohistochemical localization of MCH in frog brain showed it to be colocalized with α-MSH in the preoptic cells while the ventral infundibular nucleus had POMC/ MSH specific cells (Anderson et al., 1987). It was later on shown that α-MSH antiserum directed against the amidated C-terminus of α-MSH also recognizes a peptide called neuropeptide (N) glutamic acid (E) isoleucine (I) amide, produced from the MCH prohormone. The dipeptide in amidated C-terminus of NEI is –Proline-Isoleucine-amide that is closely related to the dipeptide at the C-terminus α-MSH which is Proline- Valine-amide, leading to cross reactions (Nahon et al., 1989).

Recently POMC gene has been found to be expressed in human caudal medulla by RT-PCR (Grauerholz et al., 1998). The distribution of POMC peptides in monkey brain is seen to be similar to that of rat brain (Khachaturian et al., 1984).

The presence of melanotropins within the CNS suggested that these peptides might act within the CNS as neuromodulators (Delbende et al., 1985).

Physiological Roles of Neuronal Melanocortins

Neuronal melanocortins, ACTH and MSH play important physiological roles. Intracerebroventricular injection of α-MSH caused a dose related increase in grooming behaviors in male rats. In addition, α-MSH also caused stretching and yawning syndrome (Hirsch and Donohue, 1986). Only α-MSH treated group exhibited improved learning on day 3 of training period when groups of rats were treated with different solutions like TRH, α-MSH, and vasopressin and tested for spatial learning using environmental cues (Yehuda, 1987). α-MSH influences learning and memory through visual information processing (Handelmann et al., 1983). The ability of different melanocortin peptides to influence habituation of prey catching behavior in toads was tested. It was found out that ACTH $_{(4\text{-}10)}$ and α-MSH facilitated acquisition behavior, while ACTH $_{(1\text{-}39)}$ was the only peptide that delayed extinction of the habituation behavior (Carpenter and Carr, 1996). In addition to influencing behavior, neuronal melanocortins have a significant role to play in energy metabolism. Melanocortins play a role in reducing body weight by decreasing food intake and increasing energy expenditure and they do it by activation of leptin (Satoh et al., 1998). Neuronal melanocortins act via MC-4 receptor located in different parts of the brain (Lu et al., 1994). Intracerebroventricular injection of leptin and MSH antagonist reversed the leptin induced decreased food intake and body weight gain (Satoh et al., 1998). In response to overfeeding, POMC mRNA in arcuate region increases relative to the control rats. Rats who were injected with MSH antagonist following overfeeding, the regulatory hypophagia of MSH was reversed and intake of overfed rats increased with respect to

control rats. This suggested that POMC derived melanocortins promotes weight loss and anorexia by hypophagic responses to overfeeding (Hagan et al., 1999).

Neuronal Melanocortins and Stress

Stress was first described in detail in 1936 (Selye, 1936). Earlier during the second half of nineteenth century, Claude Bernard had emphasized the ability of all living organisms to maintain a constant internal environment despite changes in external environment. Walter Cannon called this homeostasis (Selye, 1956). Selye borrowed the engineering term "stress" to describe the physiological response of body towards the non-specific stimuli and referred to the agents causing stress as "stressors" (Szabo, 1996).

Among various stress-induced responses, Selye demonstrated the participation of the hypophyseal-adrenal axis (Selye, 1956). External stimuli, like noxious chemicals, when presented to an organism, are perceived by the nervous system. This triggers the release of ACTH from the AL, stimulating the adrenal cortex to produce glucocorticoids that facilitate energy metabolism allowing organisms to cope with external stress (Autelitano, 1998). Analysis of the distribution pattern of corticotropin releasing factor (CRF) and opiomelanocortin systems has shown their innervation and distribution in brain areas associated with homeostatic centers, pain modulation, and stress. This has led to the suggestion that these peptide systems have a role in the integration of certain autonomic, visceral and homeostatic mechanisms (Joseph et al., 1985).

Response of the POMC systems to several kinds of stressors has been studied in different vertebrate species. In fishes, mild stress like handling and confinement caused an activation of corticotropes of AL causing an increase in plasma ACTH (Sumpter et al.,

1985; Sumpter et al., 1986). Severe stress like thermal shock and removal from water causes an activation of melanotrophs and increases plasma α-MSH levels in addition to an increase in plasma ACTH levels (Sumpter et al., 1985; Sumpter et al., 1986).

Extensive work has been done on rats to see the response to stress. Acute stress causes an increase in POMC derived peptides from pituitary to systemic circulation. Recurrent stress actually caused an increase in POMC mRNA in the AL (Holtt et al., 1986). Stress induced α-MSH release was measured and its effect on plasma prolactin and LH in ovariectomized rats was studied (Khorram et al., 1985). It was found that α-MSH levels increased in the hypothalamus, AL, IL, and median eminence. It was suggested that α-MSH of the brain origin increases in response to stress and it causes a decrease in the levels of PRL and LH in plasma (Khorram et al., 1985). Brief ether stress followed by supine restraint caused an increase in plasma concentrations of α-MSH in female rats (Berkenbosch et al., 1984; Goudreau et al., 1993). Acute restraint stress caused an elevation of α-MSH, and β- endorphin from the IL (Carr et al., 1990). Stress induced activation of α-MSH was suggested to be partly due to decrease in the activity of tuberohypophyseal dopaminergic neurons in the IL (Lookingland et al., 1991).

Objective of Current Study

Little is known about how neuronal melanocortins respond during stress in non-mammalian vertebrates. The effect of administering an ether stressor and measuring the levels of α-MSH in brain areas and plasma of *Bufo speciosus* has been done in our lab. Ether stress caused a decrease in the levels of α-MSH in telencephalon and preoptic area,

but had no effect in optic-tectum, thalamus/hypothalamus and brainstem (Olsen et al., 1999). Although these findings suggest that neuronal melanocortins play a role in amphibian stress response, they represent data from one genera of a single family of anurans (Bufonidae). To expand our knowledge of how neuronal melanocortins respond to stress in amphibians, we studied the distribution of neuronal melanocortins in a previously unstudied group of toads, the spadefoot toads, *Spea multiplicata.* In addition the effects of ether stressor on the levels of brain α-MSH was also tested.

CHAPTER II

MATERIALS AND METHODS

Animal Collection and Maintenance

Male spadefoot toads (9-11 gm), *Spea multiplicata,* were collected from breeding ponds in and around Lubbock, TX during May 1998-1999. Animals were housed in a 50 L-aquarium containing 6-7 cm topsoil bed. The animals were maintained at a constant temperature of 19-21 ^{0}C with 12 hr light/12 hr dark phases. Animals had free access to water and were fed mealworms once every week and crickets every 2 wks.

Tissue Collection

For immunocytochemical studies, toads were rapidly decapitated, brains removed and placed in Bouin's fixative for subsequent paraffin embedding. For determination of α-MSH content by radioimmunoassay (RIA), brains were rapidly removed, and placed in ice-cold amphibian saline (0.6%), and dissected into 5 regions (Kim and Carr, (1997): telencephalon (Tel), preoptic area (PO), optic-tectum (OT), caudal thalamus and retrochiasmatic hypothalamus (T/HT) and brainstem (BS). The samples were immediately immersed in ice-cold 0.5 ml 1N acetic acid. The tissues were sonicated for 30 sec, the homogenates centrifuged at 16,000 X g for 15 min at 4^{0}C and the supernatants collected and lyophilized. Pellets were analyzed for protein content using a modification (Markwell et al., 1981) of Lowry's method (Lowry et al., 1951).

Immunocytochemistry

α-MSH-, ACTH-, β-END-, and MCH-ir neurons were visualized in paraffin sections of brain tissue using the avidin biotinylated-peroxidase method employing an avidin biotinylated-peroxidase complex (ABC) kit from Vector Laboratories. Brains were processed for routine paraffin embedding, sectioned at 7 μm and sections mounted on ethanol-cleaned slides for 7 days in an oven set at 37^0C.

The immunocytochemistry has been previously described (Kim and Carr, 1997). The slides were deparaffinized in xylene and processed in different concentrations of ethanol (100, 95 and 70 % v/v). Sections were rinsed in 0.1M phosphate buffered saline (PBS, pH 7.2) for 5 minutes followed by treatment with H_2O_2 solution (0.9% in PBS) for 10 min. The slides were then placed into normal goat serum (NGS, 2% in PBS). Trypsin (0.25mg/ml in 0.5M Tris buffer) was applied for 3 minutes. Following serial rinses in PBS, tissue sections were incubated with α-MSH antiserum diluted in PBS (1:2000 dilution for α- MSH cells, 1:1000 for α-MSH fibers, 1:2000 for MCH cells, 1: 500 for ACTH cells, and 1:500 for β-END cells) for 48 hrs at 4^0C. After washing in PBS, biotinylated goat anti-rabbit gamma globulin (GARGG) was applied for 2 hrs at room temperature (22^0 C) at a dilution of 1: 500. Sections stained for α-MSH/ ACTH/ β-END/ MCH were incubated in ABC for an additional 2 hours at 21^0 C. Immunoreactive cells were visualized with 0.08% DAB (diaminobenzidine tetrachloride) in 0.05 M Tris buffer containing 0.002% H_2O_2. Following several rinses in PBS, sections were stained lightly with toluidine blue, dehydrated and coverslips mounted with permount. To check if α-MSH, ACTH, and β-END cells are present in the similar regions of the brain areas, alternate sections were stained with the respective antibodies.

The α-MSH antiserum (Ab#97, donated by Dr. K. Knigge, University of Rochester, school of Medicine and Dentistry, Division of Neuroendocrinology) has been previously characterized (Melrose and Knigge, 1988). The following synthetic peptides were ineffective in reacting with the α-MSH antiserum: ACTH 1-39, β-endorphin (camel), β-endorphin (human), β-MSH, gonadotropin-releasing hormone (GnRH), thyrotropin-releasing hormone (TRH), substance P, somatostatin, oxytocin, and Met-enkephalin. The specificity of the antiserum was tested by preadsorbing the antiserum with 10 μg synthetic α-MSH and NEI for 18 hrs at 4 ^{0}C. The antiserum to human ACTH (hACTH-IC2) was donated by Dr. A.F. Parlow, Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center. The β-endorphin antiserum (a gift from Dr. Bob Dores, Department of Biological Sciences, University of Denver) recognizes the mid portion of β-END between residues 16-26. The salmon MCH antiserum (a gift from Dr. P.Y.Risold, Laboratoire d' Histologie' Embryologie Cytogenetique, Faculte' de Medecine, Besancon, France) was raised against synthetic salmon MCH coupled to bovine serum albumin with glutaraldehyde. This antiserum was preabsorbed with MCH, human GRF_{1-37}, human GRF_{29-37}, and α-MSH. It was found that MCH antiserum does not crossreact with human GRF_{1-37}, human GRF_{29-37}, and α-MSH (Fellmann et al., 1987).

The nomenclature and grouping of the nuclei in the brain of *Spea multiplicata* are based on the papers of Wada et al (1980) and Neary and Northcutt (1983).

Effect of Stress on α-MSH Content in Brain Regions

Animals were divided into 5 groups; control and four experimental. Toads were exposed to ether vapors for 45 sec and then returned to their home aquaria. Toads were sacrificed at 30, 60, 90, and 120 min following termination of ether treatment and brain tissue was collected. Tissue from control animals was collected within 30 sec following removal of subjects from home aquaria. The transportation from the aquarium to the ether jar was done quickly to prevent any stress caused due to handling the animals.

Radioimmunoassay (RIA)

Radioimmunoassay for α-MSH was performed using a disequilibrium assay (Khorram et al., 1984) as described by Kim and Carr (1997). α MSH was iodinated with chloramine-T and purified by gel filtration (Sephadex G-10). α-MSH was measured in acid extracts of toad brain. Each lyophilized brain sample was reconstituted in 500 µl distilled water and sonicated for 30 sec. Duplicates were prepared with 200 µl of reconstituted brain sample. The antiserum was diluted to 1:48,000 in a final assay volume of 600 µl. Standards were assayed in duplicate using synthetic α-MSH at concentrations of 8-2000 pg/tube. Standards and samples were incubated with antiserum for 24 hr, followed by addition of 10,000 cpm ^{125} I-labeled α-MSH. Assay was terminated by incubating with GARGG as a secondary antibody for 24 hrs at 4^0 C. To separate bound antibody from the free hormone, 2 ml of 0.05M phosphate buffer was adder to each tube, and tubes were centrifuged at 2800 X g for 30 min. The supernatants were then aspirated, and pellet counts taken by γ-counter.

α-MSH antiserum (H-50, donated by Dr. William Millington, Division of Structural and Systems Biology, University of Missouri, Kansas, MO and Dr. G. Mueller of the uniformed services University of Health Science, Bethesda, MD) recognizes the C-terminus of α-MSH and therefore detects multiple acetylated isoforms of α-MSH, but not deamidated α-MSH, α-MSH fragments, β-END or β-lipotropin (Pettibone and Mueller, 1984).

Data Analysis

To account for slight differences in brain dissection from animal to animal, brain α-MSH concentrations were expressed as a function of the tissue protein content. Variance around the group means was analyzed by one-way analysis of variance (ANOVA). Data from different groups was compared by Tukey-Kramer multiple comparison test. 5-8 animals were used in the control and the experimental groups. The α-MSH content was expressed as ng/mg of total protein content. The type-I (α) error level was set up as .05. Values are reported as mean ± S.E.M.

CHAPTER III

RESULTS

Distribution of α–MSH in the Brain of *Spea multiplicata*

α-MSH-, ACTH-, β-END-, and MCH- ir cells and fibers in *Spea* brain were detected and localized using immunocytochemistry. Coronal sections of the brain were analyzed with antibodies raised against α-MSH, ACTH, β-END, and MCH. Table 3.1 summarizes the distribution and relative abundance of α-MSH-, ACTH- and β-END-ir perikarya and fibers. The schematic distribution of α–MSH, ACTH, and β-END- ir cells and fibers is shown in Fig. 3.1 and 3.2.

Telencephalon

Intensely stained α-MSH, ACTH and β-END fibers were seen in the ventral telencephalic region. Many immunoreactive fibers terminated in the nucleus accumbens and some in the olfactory nucleus. Moderate fiber staining was seen in the dorsal and ventral striatum. Some fibers were also observed in the septum area, mainly nucleus medialis septi and nucleus lateralis septi. Pallial area was devoid of fibers. All the fibers that were seen had a beaded varicose appearance and they were close to the ependymal layer surrounding the lateral telencephalic ventricles (Fig. 3.1).

Diencephalon

Two distinct immunoreactive cell groups were observed in the diencephalon. The first cell group was found in the ventral area of the preoptic nucleus. These cells were mainly round or oval in shape, stained dark brown and they were close to the third ventricle (Figs. 3.1 and 3.2). Projections from these cells were mainly unipolar but some were also bipolar. The fibers from this cell group were beaded in appearance and they were close to the ventricle penetrating the ependyma and appearing to contact the cerebrospinal fluid. Fibers from the preoptic area projected anteriorly to the lateral forebrain bundle. Some fibers crossed the anterior commissure and reached the amygdala.

A second neuronal cell group was seen in the ventral infundibular area. These cells were intensely stained and were mainly bipolar cells. These cells were also close to the ependyma and some fibers seemed to touch the cerebrospinal fluid. There were many fibers that were seen in the dorsal infundibular area (Figs. 3.1 and 3.2). Some fibers were seen projecting towards the median eminence from the nucleus infundibularis ventralis.

Other Parts of the Brain

Fiber projections in other parts of the brain could not be traced in coronal sections. However in sagittal sections some fiber staining in the optic tectum and habenular nucleus was observed (Fig. 3.3). Lot of non-specific staining was seen in the brain stem.

Comparison of the Distribution of α-MSH and MCH Neurons

MCH-ir cells were seen in the caudal portion of the preoptic area and in the dorsal infundibular area. These areas were not similar to the areas that stained positive for α-MSH, ACTH and β-END.

Controls

No immunostaining was observed when α-MSH antiserum was substituted with normal rabbit serum. Preincubation of the α-MSH antiserum with 10 μg synthetic α-MSH eliminated specific immunoreactivity (Fig. 3.4). Preadsorption of α-MSH antiserum with 10 μg NEI had no effect on immunostaining in the ventral infundibular area or preoptic area. Preadsorption of the β-END antiserum with 10 μg endorphin peptide eliminated immunoreactivity in the preoptic area and the infundibular area (Table 3.2 and Fig. 3.4).

Effect of Ether Stressor on Regional Brain α-MSH Levels

Figure 3.5 shows regional brain α-MSH levels in control animals. Of the five regions, the greatest level of α-MSH was seen thalamus/hypothalamus, followed by the preoptic area. Figure 3.6 shows brain α-MSH levels from animals 30, 60, 90 and 120 min following the stressor. α-MSH levels increased significantly in the preoptic area and optic tectum 60-min following the stress. α-MSH levels in telencephalon and thalamus/hypothalamus followed the same trend but the result was not statistically significant (Fig. 3.6).

Table 3.1. Distribution and relative density of α-MSH/ACTH-ir perikarya and fibers in the brain of the toad ***Spea multiplicata.***

Structure	Perikarya	Fibers
Telencephalon		
Nucleus olfactorius anterior (NOA)	-	++
Bulbous of olfactorius accessorius (BOA)	-	-
Pallium dorsalis (PD)	-	-
Pallium mediale (PM)	-	-
Pallium laterale, pars dorsalis (PLd)	-	-
Pallium laterale, pars ventralis (PLv)	-	-
Nucleus medialis septi (NMS)	-	+
Nucleus accumbens septi (NAS)	-	+++
Nucleus lateralis septi (NLS)	-	+
Striatum, pars dorsalis (Std)	-	+
Striatum, pars ventralis (Stv)	-	++
Medial forebrain bundle (MFB)	-	+
Nucleus diagonal band of Broca (NDB)	-	-
Amygdala, pars lateralis (Al)	-	+
Amygdala, pars medialis (Am)	-	+
Nucleus entopeduncularis (NEP)	-	++
Diencephalon		
Epiphysis (E)	-	-
Habenular commissure (HC)	-	-
Nucleus habenularis dorsalis (NHD)	-	+
Nucleus habenularis ventralis (NHV)	-	+
Area ventralis anterior thalami (AVA)	-	-
Area ventrolateralis thalami (AVL)	-	+
Nucleus dorsomedialis anterior thalami (NDMA)	-	-
Nucleus dorsolateralis anterior thalami (NDLA)	-	-
Corpus geniculatus laterale (CGL)	-	-
Lateral forebrain bundle (LFB)	-	++
Nucleus posteriocentralis thalami (NPC)	-	+
Nucleus posteriolateralis thalami (NPL)	-	-
Nucleus preopticus (NPO)	+++	+++
Nucleus infundibularis dorsalis (NID)	-	+++
Nucleus infundibularis ventralis (NIV)	+++	+++
Median eminence (ME)	-	+
Posterior commissure (PC)	-	-
Optic chiasma (OC)	-	+

Table 3.1. Continued

Structure	Perikarya	Fibers
Mesencephalon		
Nucleus mesencephalicus nervi trigemini (NMNT)	-	-
Stratum album superficiale tecti (SAS)	-	-
Stratum griseum superficiale tecti (SGS)	-	-
Stratum album centrale tecti (SGC)	-	-
Stratum album periventriculare tecti (SGP)	-	-
Nucleus of the film (NF)	-	-
Nucleus profondus mesencephali (NPM)	-	-
Nucleus anterodorsalis tegmenti mesencephali (NAD)	-	-
Nucleus anteroventralis tegmenti mesencephali (NAV)	-	-
Nucleus posterodorsalis tegmenti mesencephali (NPD)	-	-
Nucleus posteroventralis tegmenti mesencephali (NPV)	-	-
Nucleus of the trochlear nerve (NTRO)	-	-
Trochlear nerve (TrN)	-	-
Nucleus of the occulomotor nerve (NOM)	-	-
Torus semicircularis (TS)	-	-
Nucleus reticularis isthmi (NRIS)	-	-
Nucleus interpeduncularis (NIP)	-	-
Nucleus isthmi (NI)	-	-
Nucleus reticularis superior (NRS)	-	-
Nucleus cerebelli (NCER)	-	-
Metencephalon		
Granular layer of cerebellum	-	-
Purkinje cell layer of the cerebellum (C)	-	-
Molecular layer of the cerebellum	-	-
Rhombencephalon		
Griserum centrale rhombencephali (GC)	-	-
Sulcus limitans (SL)	-	-
Medialis longitudinal fascicle (MLF)	-	-
Choroid plexus (PCh)	-	-
Nucleus vestibularis (NV)	-	-
Nucleus cochlearis (NC)	-	-

+, scarce; ++, moderate; +++, dense; -, no immunoreactive fibers or cells.

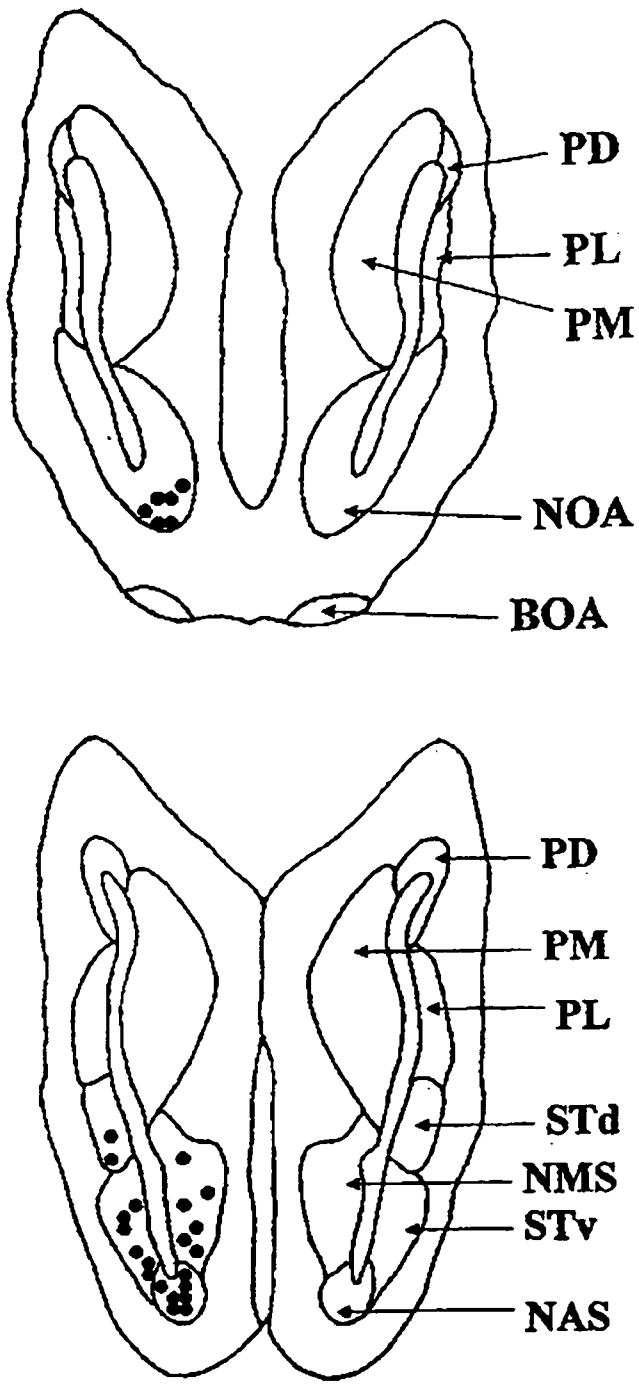
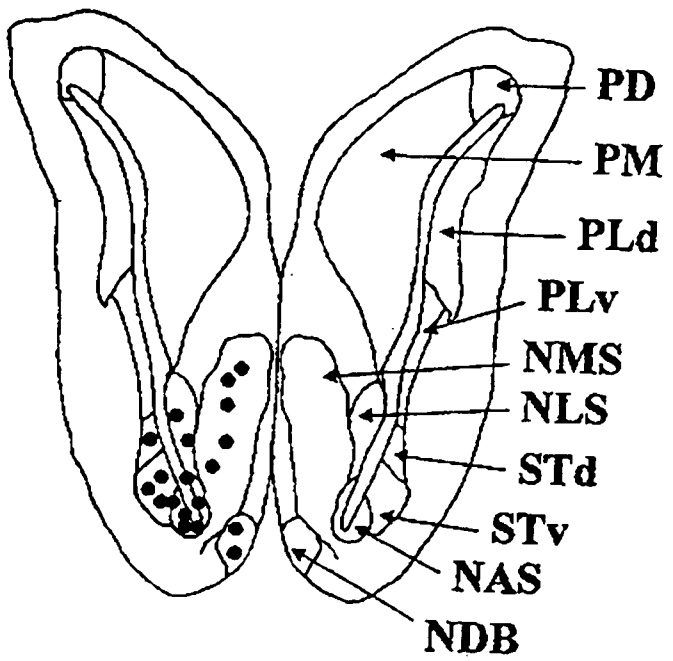


Figure 3.1. Camera lucida drawings of representative transverse sections through the brain of *Spea* illustrating distribution of α-MSH/ ACTH/ β-END-ir perikarya (stars) and fibers (closed circles). See Table 3.1 for abbreviations.



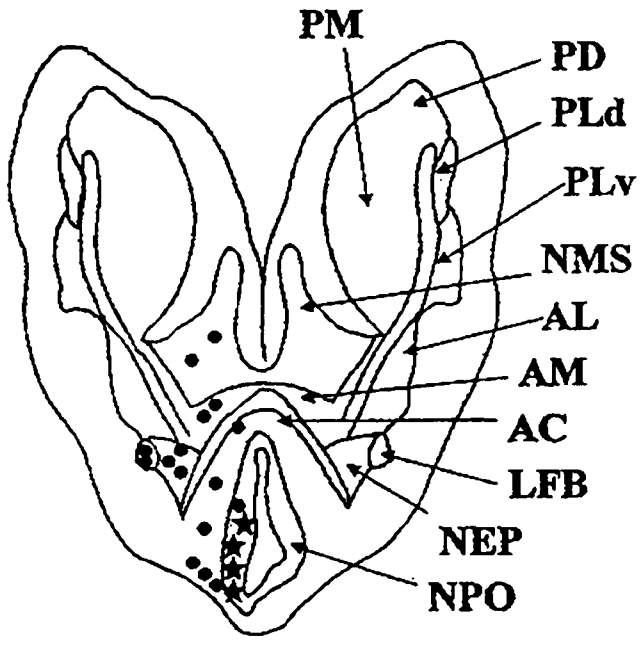


Figure 3.1. Continued

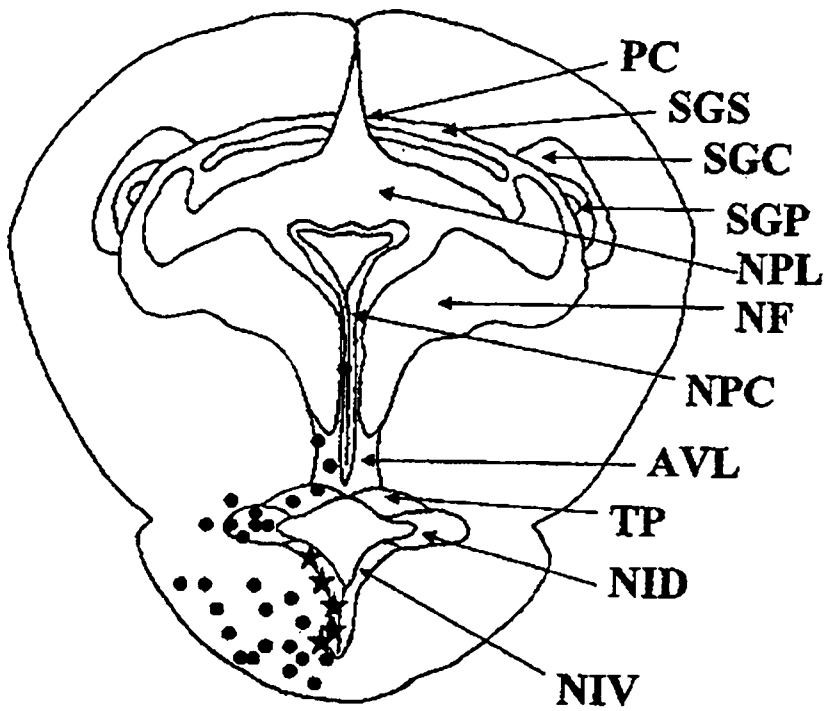
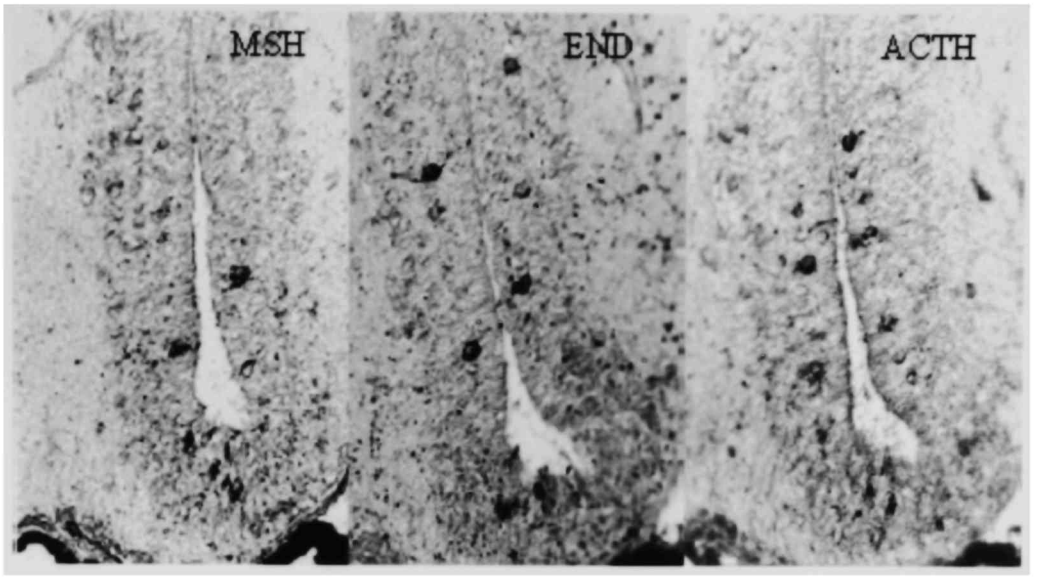


Figure 3.1. Continued

NPO



NIV

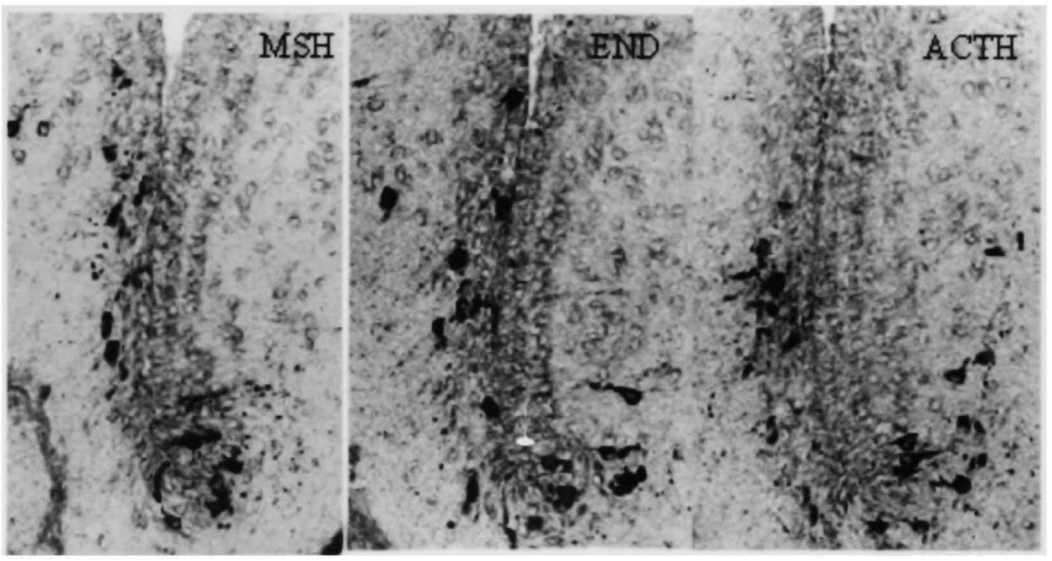


Figure 3.2 Photomicrographs showing immunoperoxidase staining for α-MSH/ACTH/ β-END immunoreactivity in the brain of *Spea*. Coronal sections demonstrating the immunoreactive cells and fibers in the preoptic area (NPO) and ventral infundibulum (NIV) Final magnification, 600X

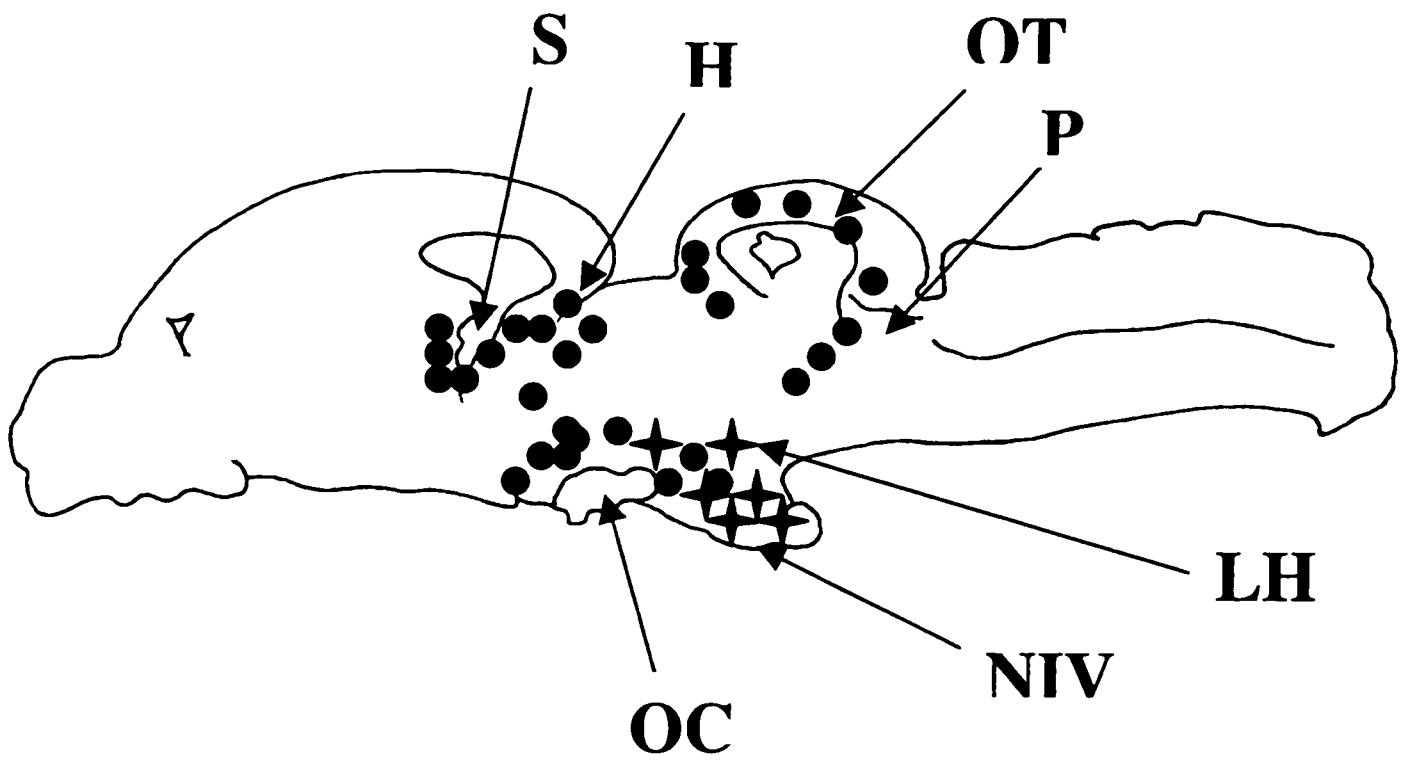


Figure 3.3. Schematic sagittal section through the brain of *S.multiplicata* depicting the distribution of α-MSH containing perikarya and axons. H, habenular nucleus; LH, lateral hypothalamus; NI, nucleus isthmi; NIV, ventral infundibulum; NPO, nucleus preopticus; OC, optic chiasma; OT, optic tectum; P, posterior thalamus; S, septum.

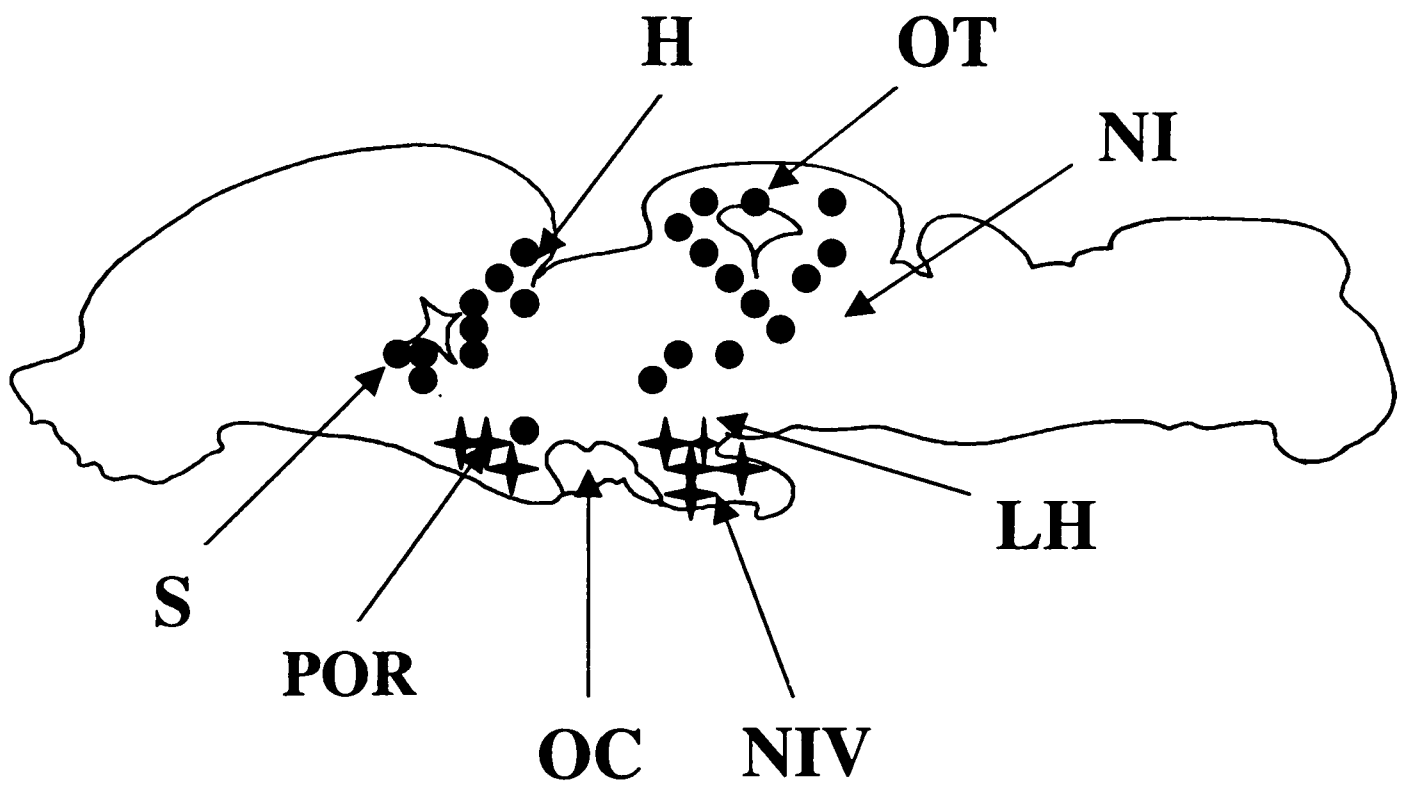


Figure 3.3. Continued.

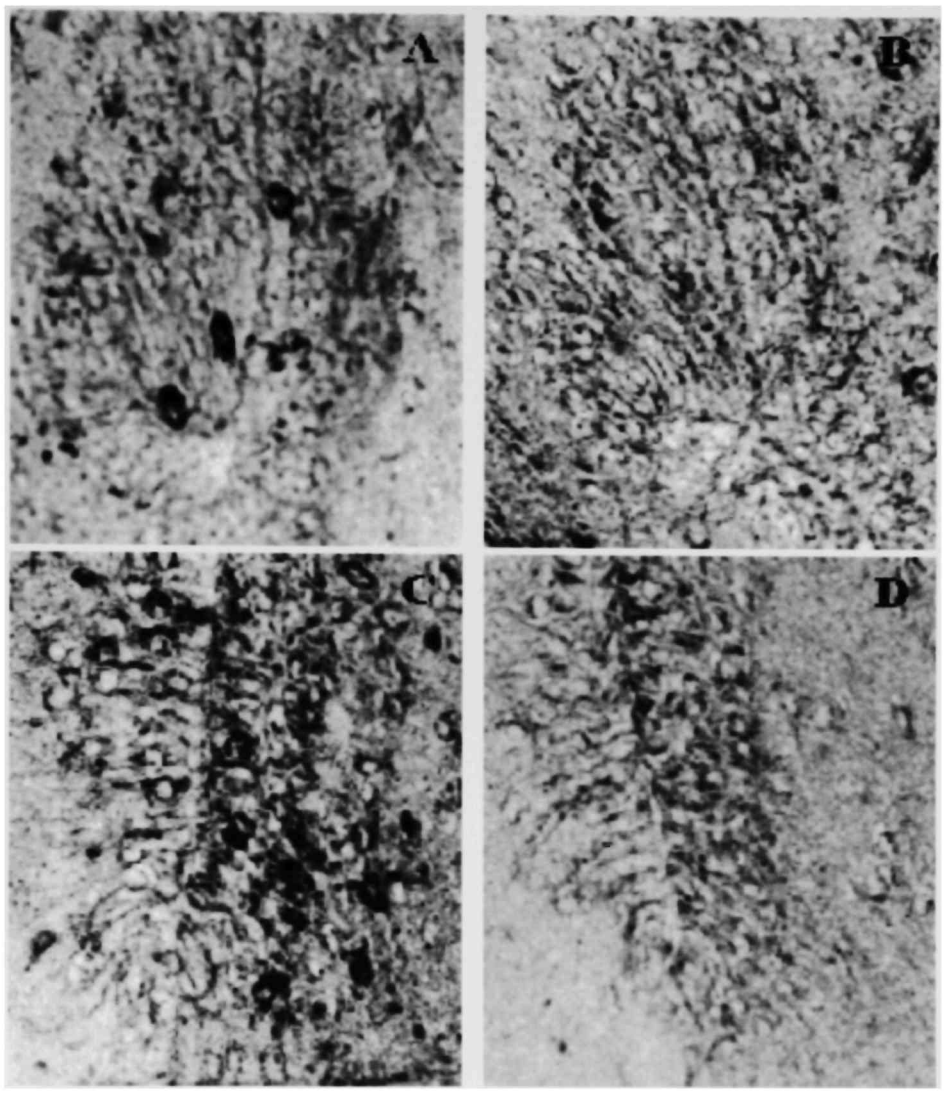


Figure 3.4 Photomicrographs showing immunoperoxidase staining for preincubation studies in the coronal sections of *Spea* brain
A α-MSH ab. preincubated with NEI peptide in NPO
B. α-MSH ab. preincubated with MSH peptide in NPO.
C α-MSH ab preincubated with NEI peptide in NIV
D α-MSH ab preincubated with MSH peptide in NIV
Refer to Table 3.1. for abbreviations and Table 3.2. for details. Final magnification, 1300 X.

Table 3.2. The Control experiment of immunopeoxidase staining for α-MSH and β-END antiserum

Primary ab / **Brain Region**	α-MSH/ β-END/ ACTH	α-MSH ab+α-MSH	α-MSH ab+ NEI	β-END ab +β-END	Normal Rabbit serum
Nucleus preopticus (NPO)	+	-	+	-	-
Ventral Infundibulam (NIV)	+	-	+	-	-

+ ; positive staining.
- ; negative staining.
See the text for more detail.

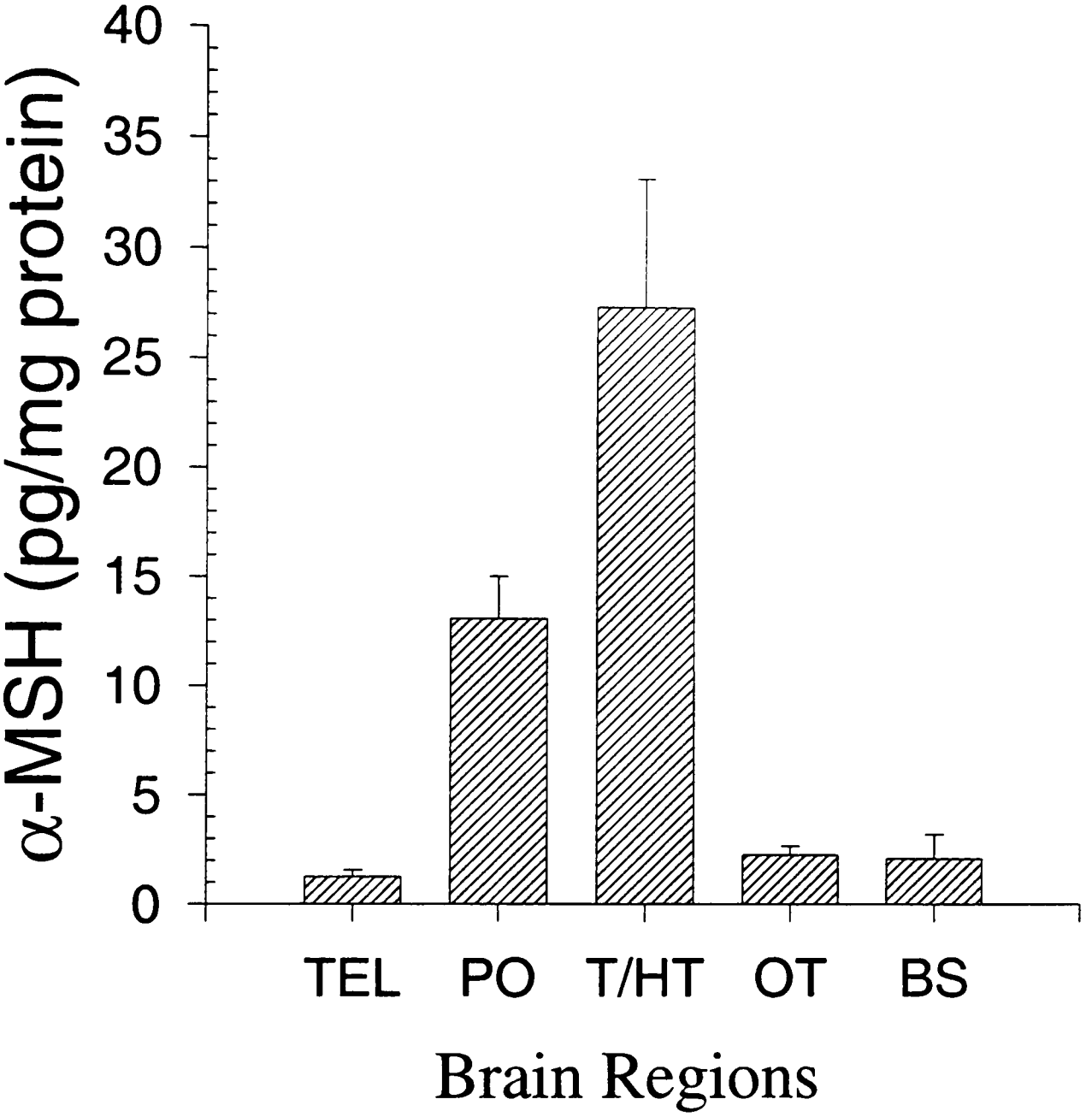


Figure 3.5. α-MSH levels in brain regions of unstressed toads.

Bars represent mean + SEM of 5-8 animals per group. Tel- Telencephalon, PO - Preoptic area, T/HT - Thalamus/Hypothalamus, OT- Optic-Tectum, BS - Brain Stem.

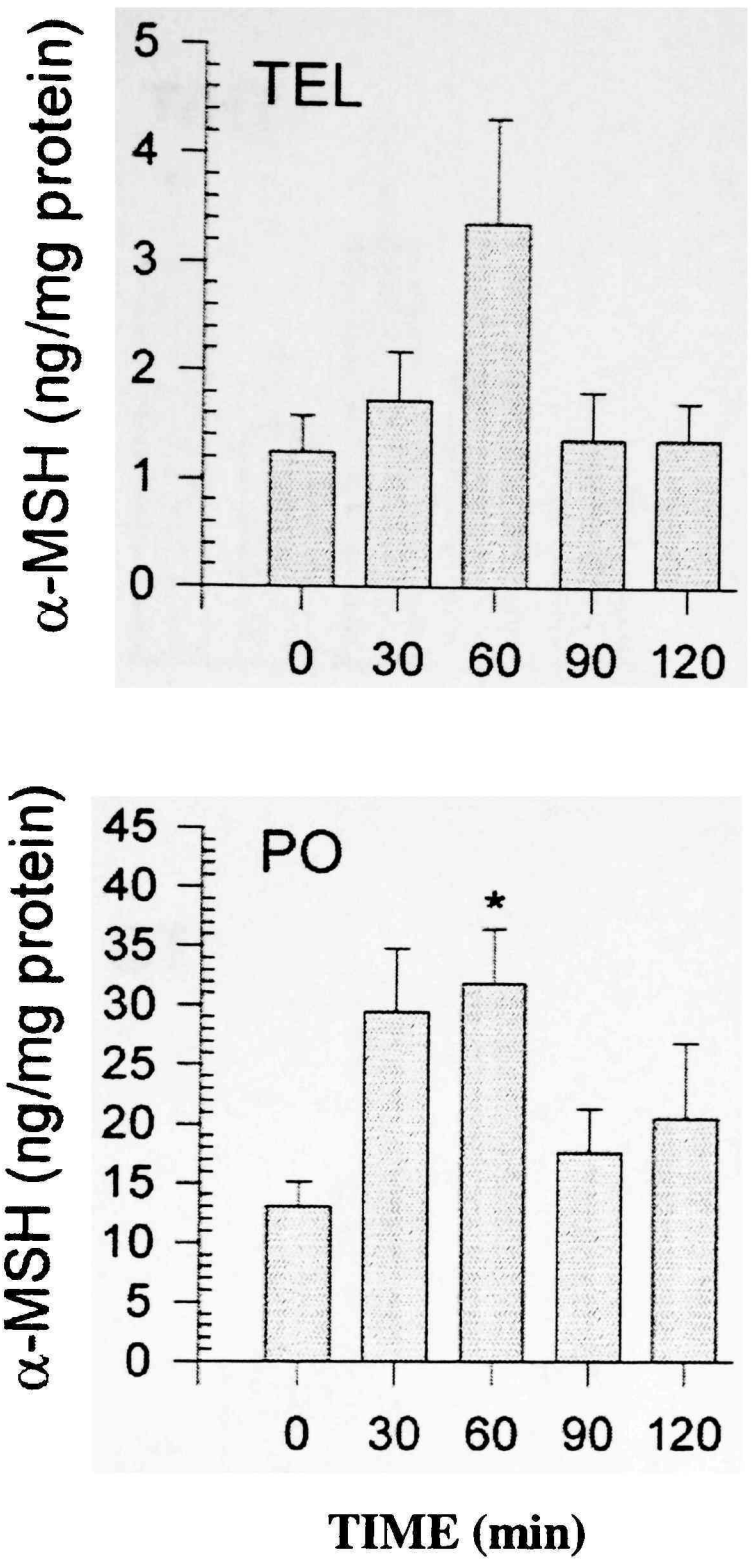
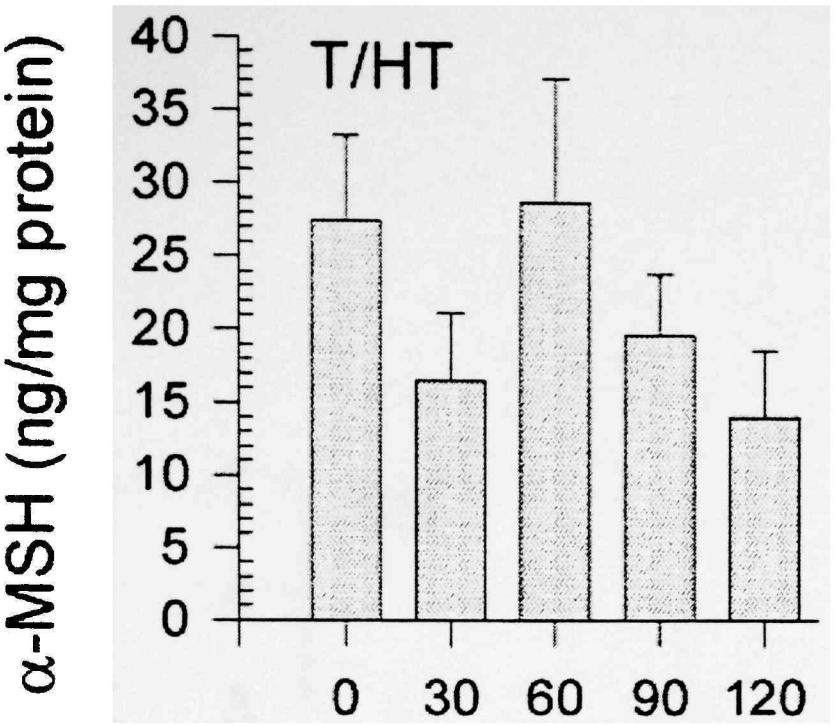


Figure 3.6. α-MSH levels in brain regions following ether stress.

Toads were sacrificed 30, 60, 90 and 120 min following ether exposure. 0min bar represents the control α-MSH level indifferent brain areas of unstressed toads. Bars represent mean + SEM of 5-8 animals per group. Bars with asterisk were significantly different ($p<0.05$) by Tukey-Kramer multiple comparison test.



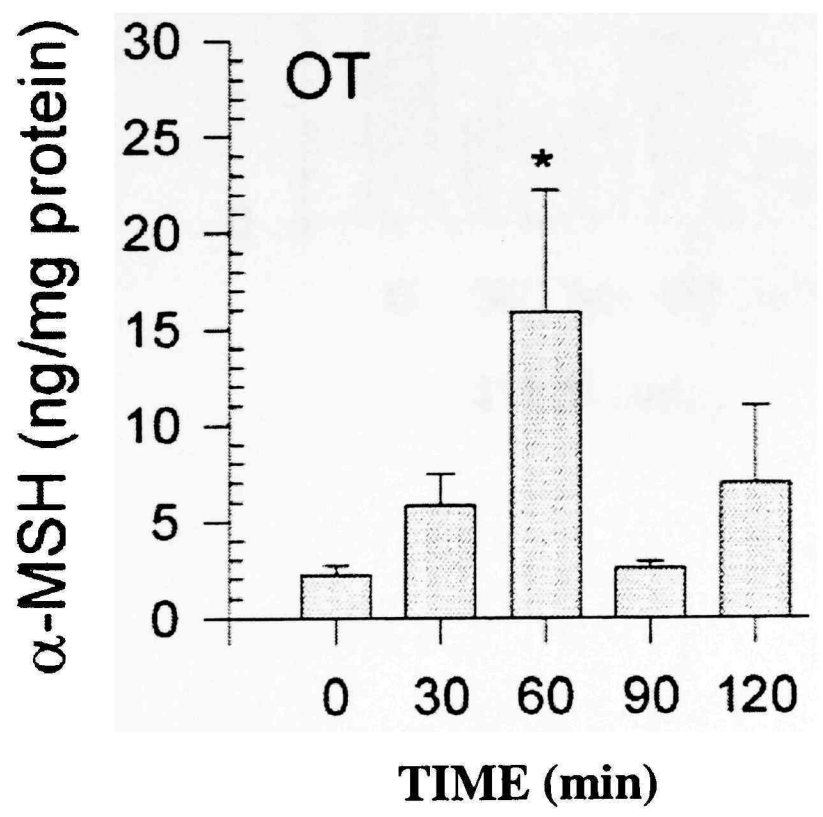


Figure 3.6. Continued.

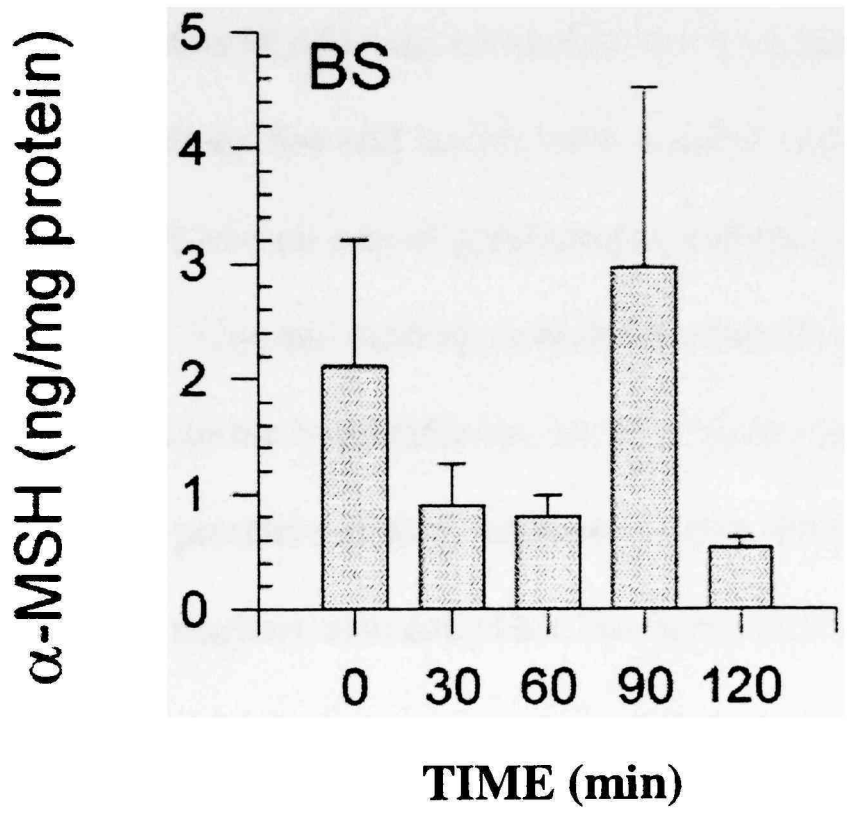


Figure 3.6. Continued.

CHAPTER IV

DISCUSSION

The distribution of neuronal melanocortins has been examined in only a handful of the 4277 of anuran species: *Rana* (Benyamina et al., 1986; Vallarino, 1987), *Bufo* (Kim and Carr, 1997) and *Xenopus* (Tuinhof et al., 1998). In the present study we describe the distribution of neuronal melanocortins in a representative of the family Pelobatidae. Immunoreactive cell bodies were found in two distinct areas. The first cell group was seen in the ventral part of preoptic area and the second cell group was located in the hypothalamus. Coronal sections revealed the presence of cell bodies in the ventral infundibular nucleus of the hypothalamus, while a closer examination of the parasagittal sections showed the presence of some immunoreactive cells in the dorsal area of the ventral infundibular nucleus, also called the lateral hypothalamic nucleus by Neary and Northcutt (1980).

The immunoreactive distribution is in general agreement with previous studies done in frogs (Benyamina et al., 1986; Vallarino, 1987). Distribution of α-MSH cells and fibers in *Spea* resemble the general distribution in *Bufo speciosus* (Kim and Carr, 1997), except that, α-MSH-ir cells were observed in the nucleus preopticus. This is not a trivial finding. The cell group thought to produce POMC peptides in the rat dorsolateral hypothalamus was later shown to actually contain only MCH producing neurons and not POMC-producing neurons (Nahon et al., 1989).

α-MSH and MCH neurons have been shown to be colocalized in the preoptic area (Naito et al., 1986; Anderson et al, 1987). It was later shown that α-MSH antiserum recognizes a C-terminus dipeptide of NEI, a proteolytic product of MCH prohormone (Nahon et al, 1989). It was shown in the present study that the α-MSH cells in the preoptic area of *Spea* are POMC cells, as these cells stained positive for three major biosynthetic end products, α-MSH, β-END, and ACTH. Conclusive evidence was found when the MCH neurons were found to be located in the caudal part of the preoptic area adjacent to the third ventricle, whereas POMC peptide neurons were located to the rostral preoptic area surrounding the preoptic recess (Fig. 4.1). Further evidence was obtained by the demonstration that NEI failed to block specific α-MSH-ir immunostaining in the preoptic area (Fig. 3.3).

Several major projections from POMC cell groups were seen to innervate the telencephalon, diencephalon and mesencephalon. The most striking projection was to the ventral telencephalic area to the nucleus accumbens and some fibers to the olfactory nucleus (Fig. 3.1). These fiber projections were very similar to that found in *Bufo* (Kim and Carr, 1997).

The nucleus accumbens has been described in the ventral wall of the telencephalic hemisphere (Marin et al., 1997) in anurans. The nucleus accumbens is known to be composed of two large territories that are neurochemically and cytoarchitecturally complex, which have been called the shell and the core (Meredith, 1999; Freedman and Cassell, 1994). These two regions have varying concentrations of different peptides and peptide receptors. The shell shows a higher concentration of substance P, dynorphin, dopamine D_3 receptors, 5-HT_4 receptors, and calretinin (Zahm, 1999; Meredith, 1999).

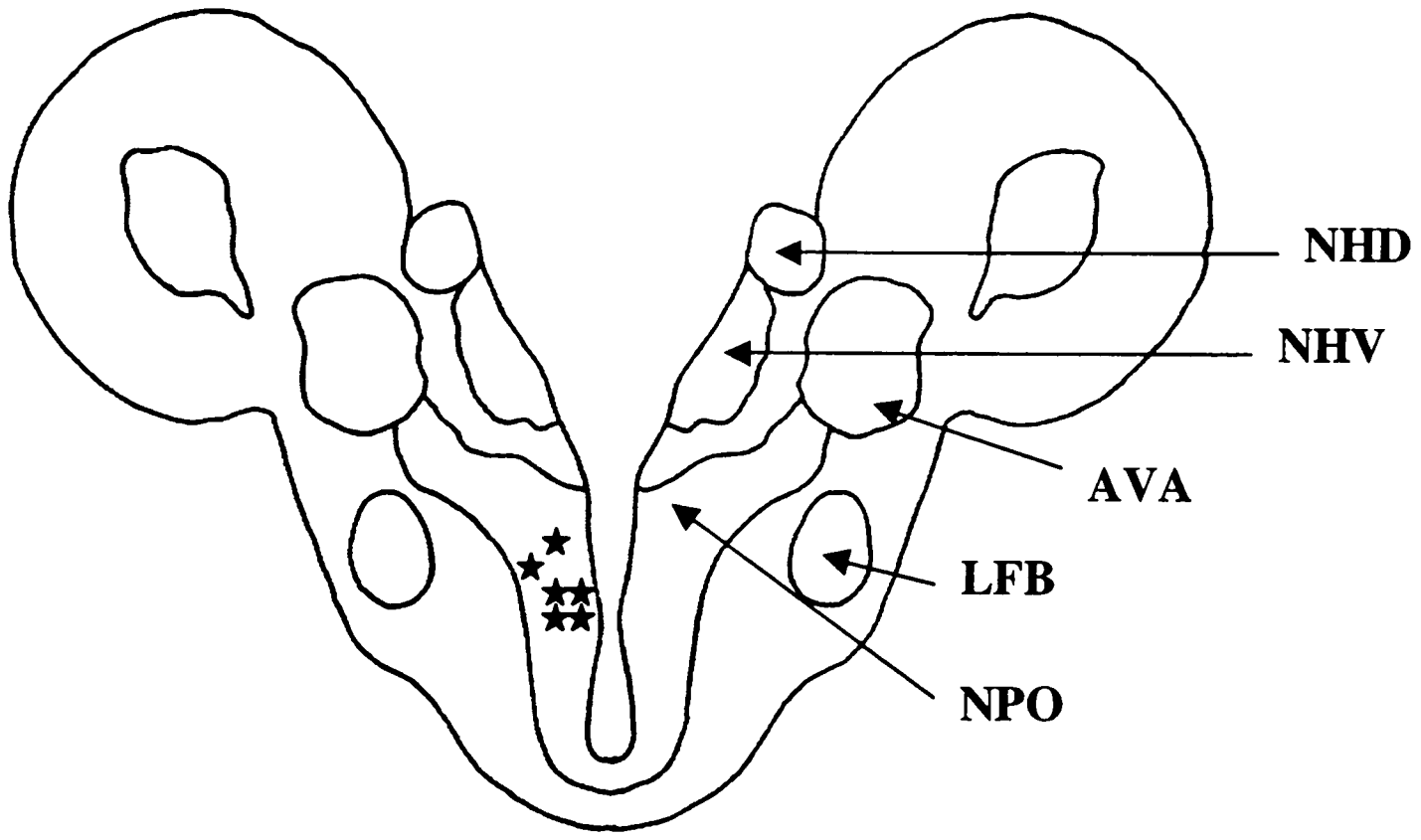


Figure 4.1. Camera lucida drawing showing the MCH immunoreactive cells (stars) in the caudal preoptic area. Refer to Table 3.1 for abbreviations.

Some substances like calbindin-D 28kDa, enkephalin, and the $GABA_A$ receptors are present in high concentrations in the core area (Zahm, 1999; Meredith, 1999). The organization of the basal ganglia, that is composed of the ventral striatum and the nucleus accumbens, has been studied in amphibians by anterograde and retrograde tracing studies. These studies have shown the afferent and efferent connections of the nucleus accumbens. This region has inputs from the olfactory bulb, medial pallium, preoptic area, ventral thalamus, ventral hypothalamic nucleus, posterior tubercle, locus ceruleus and the nucleus of solitary tract (Marin et al., 1997). Efferent projections of the nucleus accumbens project to the medial amygdala, preoptic area, the ventral hypothalamic nucleus, dorsomedial posterior tubercle, medial tegmental area, pontomesencephalic reticular formation, and the raphae (Marin et al., 1997).

Functionally, the part of the ventral striatum that merges with the core region of nucleus accumbens associates multiple pleasurable stimulus with rewarding properties and relates negative stimulus like pain, fear, and anxiety with aversive properties (Gray, 1999). It has been suggested that in anurans olfactory and vomeronasal information may reach the striatum and nucleus accumbens via the lateral amygdala (Marin et al., 1997). Moreover the portion of anterior thalamus that projects to nucleus accumbens has been called a multimodal sensory area (Marin et al., 1997) because it receives several inputs from other prominent limbic structures and probably links these messages to the nucleus accumbens (Marin et al., 1997). It has also been suggested that in anurans during prey-catching behavior, the stimulus-response mediating circuit is influenced by the forebrain and ventral striatum and with these two areas lateral thalamic nucleus also participates in this behavior (Ewert et al., 1999). α-MSH has been known to modulate prey-catching

behavior in anurans (Olsen et al., 1999). Since there are α-MSH projections to the forebrain of *Spea* brain, it could be speculated if this peptide has any role in prey-catching behavior of this anuran.

α-MSH has been used in this study as a marker to locate the different POMC products like β-END and ACTH in the brain regions. Endogenous opioid peptides are opiates produced in brain and pituitary. Opioid peptides (endorphins, enkephalins and dynorphins) are all proteolytic products of precursor hormones, POMC, proenkephalin, and prodynorphin respectively (Simon and Hiller, 1989). Endogenous opioids are involved in stress-induced analgesia which is mediated by the μ and δ receptors and the opiate mediated analgesia is blocked by anatagonists like naloxone and naltrexone (Olson et al., 1984). Several studies have shown the presence of opioid peptides and receptors in the basal forebrain region. The medial parts of nucleus accumbens shell is rich in dynorphin and enkephalin while the rostral and caudal zone of core of nucleus accumbens is rich in opioid receptors (Meredith, 1999). The function of opiates in the basal forebrain has been shown in mammals. Dopaminergic connections, opioid peptides, GABA, glutamate, and serotonin interacting with the ventral tegmental area and basal forebrain are involved in the drug-reward circuit and in reinforcement behavior (Koob, 1999). Stress has shown to cause an increase in β-END levels in plasma, cerebrospinal fluid (CSF), nucleus accumbens and ventral tegmental area (Olson et al., 1984). It has also been shown that stress induces an increase in corticotropin releasing factor (CRF) secretion which causes a dose-dependent behavior activation (Koob, 1999). Apart from the main analgesic function, it has been suggested that opiates are involved in modulating learning and memory by interacting with ACTH (Olson et al., 1984).

Amphibians appear to have a well-developed endogenous opiate system and have shown to exhibit a dose dependent and naloxone sensitive analgesia (Stevens et al., 1995). Immobilization stress activates the endogenous opioid system in frogs (Stevens and Pezella, 1989). This study showed intense innervation of β-END fibers in the nucleus accumbens. This suggests that the endogenous opioid system might be activated during stress in *Spea* and may play a role in stress induced behavioral responses of the animal in response to the stressor.

Other α-MSH fibers that reached the medial and lateral septal area appeared to originate from the preoptic area. Neuronal melanocortins have been suggested to have an antipyretic role in mammals (Hadley et al., 1999). Concentration of α-MSH and CRF increased within the septal region of the brain in febrile rabbits (Holdeman et al., 1985). The increase in α-MSH was noticed to be pulsatile and the greatest increase in concentration and greatest number of increases occurred when the temperature rised rapidly (Bell and Lipton, 1987). POMC fiber projections to the septal region in *Spea*, suggest an antipyretic role in amphibians. Some fibers from the preoptic area projected laterally to the lateral forebrain bundle and some fibers crossed the anterior commissure to reach the amygdala and to the habenular region.

The preoptic area has been shown to have peptidergic neurosecretory cells, neurons, and glia cells in frogs (Chetverukhin and Polenov, 1993). In frogs, the preoptic area and hypothalamus are involved in secretion of gonadotropin releasing hormone (GnRH) in response to environmental cues transmitted by the sensory pathways (Wilczynski et al., 1993). GnRH secretion and reproductive activity goes down due to an increase in CRF and ACTH, in response to stress (Chrousos, 1998). The functions of

preoptic area POMC peptide secretion on reproductive activity in *Spea,* while under stress needs to be addressed. In frogs, dopamine has been to shown to inhibit the release of POMC-related peptides in a dose dependent manner (Tonosaki et al., 1995; Jenks et al., 1985) and in addition to inhibiting α-MSH release from IL, it also affects the acetylation of des-acetyl α-MSH to α-MSH (Jenks et al., 1985). Knowing the inhibitory role of dopamine on POMC peptides, the significance of locating POMC cells in the preoptic area in *Spea* needs to be explored further.

There were some perikarya reported in the lateral amygdala region in *Rana ridibunda* by Benyamina and coauthors in 1986, but these cells were not found in *Rana esculenta* by Vallarino in 1987 and *Bufo speciosus* by Kim and Carr in 1997. No immunoreactive cells were seen in the amygdala region in the *Spea* brain.

The projections to the mesencephalon appear to originate from the infundibular area. There are fiber projections to the optic tectum from the lateral hypothalamic region, which are in agreement to studies in frog (Benyamina et al., 1986) and toad (Kim and Carr, 1997). No immunoreactivity was seen in the posterior part of *Spea* brain that is tegmentum, cerebellum and medulla oblongata.

The immunocytochemical localization of α-MSH immunoreactivity in the brain of *Spea multiplicata* was confirmed by biochemical studies. Radioimmunoassay was done on the brain extracts. α-MSH concentration was found to be highest in the caudal thalamus and retrochiasmatic hypothalamus, followed by preoptic area (Fig. 3.5). This is in agreement with the immunocytochemical studies as the thalamus/hypothalamus and the preoptic areas were the two areas that showed the largest population of α-MSH-ir cells.

Previous studies in mammals have shown a relationship between stress and increase in plasma α-MSH content (Carr et al., 1990; Goudreau et al., 1993; Iturriza and Eberle, 89) and increase in brain α-MSH content (Khorram et al., 1985). It has been shown that catecholamines from the adrenal medulla facilitate stress-induced secretion of α-MSH and β-END from the IL in rats (Kvetnansky et al., 1987). This study in a non-mammalian vertebrate attempts to see a relationship between α-MSH content in different parts of the brain in response to ether stressor.

In response to ether exposure, the α-MSH content increased significantly 60 min after ether administration in the caudal thalamus/retrochiasmatic hypothalamus and optic tectum. There was an increase α-MSH content in telencephalon and preoptic area 60 min following the stressor but this result was not very significant (Fig. 3.6).

One interpretation could be that there is an enhanced synthesis of α-MSH in *Spea* brain in response to a stressor. The enhanced synthesis further leads to a time dependent delivery of this peptide to the various regions that has the fiber projections. It has been known that α-MSH potentiates learning, attention and memory in humans and in other animals (Olsen et al., 1999; Hadley and Haskell-Luevano, 1999; Datta and King, 1982). Animals treated with MSH or the heptapeptide core sequence of MSH/ACTH showed delayed extinction of both active and passive conditioned avoidance behavior (Carpenter and Carr, 1996; Bohus and DeWied, 1967; DeWied, 1977). α-MSH facilitates a number of behaviors that might be adaptive during stress. For example, α-MSH facilitates acquisition of habituation caused by a non-rewarding stimulus (Olsen et al., 1999) in *Bufo*, reducing the toad orienting movements to a non-relevant prey stimulus, potentially

reducing exposure to predators. The elevation of α-MSH levels in brain areas of *Spea* following an exposure to stress may play an adaptive role in this species. Behavioral studies on spadefoot toads need to be done to test for this hypothesis.

In summary, this work is the first one to report POMC-ir cells in the preoptic nucleus in the brain of a toad species. Other than this, the POMC-ir cells and fibers in *Spea* brain is similar to that found in *Bufo*. It was also found that there is an alteration of α-MSH content in response to a physiological stress, suggesting a neuromodulatory role of α-MSH in this species.

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