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FURTHER CHARACTERIZATION OF THE EXTRA-ARCUATE ALPHA-MELANOCYTE STIMULATING HORMONE-LIKE MATERIAL IN HYPOTHALAMUS: BIOCHEMICAL AND ANATOMICAL STUDIES

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ABSTRACT

Previous studies had shown the existence of an extra-arcuate cell group in lateral hypothalamus which contains alpha-melanocyte stimulating hormone (a-MSH)-like immunoreactivity, but no other pro-opiomelanocortin (POMC) immunoreactivity. The question we have attempted to address in this series of studies is whether the material is indeed a-MSH or a cross-reacting material. Chromatographic studies failed to detect any material which is different from a-MSH or des-acetyl-a-MSH, suggesting that either the material is authentic a-MSH/des-acetyl-a-MSH, or that it is not detected by our RIAs. A series of manipulations including dissections of arcuate vs. extra-arcuate hypothalamic areas, treatment with colchicine, lesions with monosodium glutamate and knife cuts were aimed at isolating the extra-arcuate region and showing that it contains an excess of a-MSH over beta-endorphin (B-END), presumably deriving from the extra-arcuate group. However, all studies showed parallel changes in a-MSH and B-END, suggesting that we were not detecting a non-POMC derived a-MSH in these studies. This led to the tentative conclusion that the material was not a-MSH and was not being detected by our RIA's. This hypothesis was tested by further characterizing the material immunohistochemically. These studies led to the conclusion that the extra-arcuate material had a carboxy-terminal homology with a-MSH but differed from it in the midregion, since antisera directed at the 4-10 region of a-MSH failed to stain this non-POMC cell group. Finally, the anatomy of this extra-arcuate group is described, particularly the projections to the striatum, hippocampus, neocortex and olfactory bulb.

INTRODUCTION

Pro-opiomelanocortin (POMC) derived peptides, adrenocorticotrophic hormone (ACTH), beta-lipotropin (B-LPH), alpha-melanocyte stimulating hormone (a-MSH) and beta-endorphin (B-END), are found in only two cell groups in the central nervous system, the arcuate nucleus and the nucleus tractus

solitarious (see 1). Several years ago, Watson and Akil (2,3) described the existence of an extra-arcuate, lateral hypothalamic cell group which appeared to contain an a-MSH-like immunoreactivity (a-MSH-LI). These observations were also extended by others in the rat (4-6) as well as the cat (7) and rhesus monkey (our observation). We had previously named these cells the alpha-2 (a-2) group to distinguish it from the arcuate a-MSH cells (8). Since a-MSH precursor is a POMC product, one would expect other peptides derived from this to be co-stored with it. However, it is clear that antisera directed against other domains of the precursor, including against full ACTH, B-END, 16K amino-terminal peptide, or even gamma-MSH (g-MSH) (see 9-11) fail to stain this group. This has led to the suggestion that the material in the a-2 cell group may be a-MSH derived from a unique non-POMC precursor. Alternatively, the a-2 system may not contain authentic a-MSH, but rather a peptide with structural similarity. While there is evidence that the material in these cells behaves similarly to a-MSH on HPLC (12), the possibility of molecular similarities between an unidentified peptide and a-MSH, which may result in antibody cross-reactivity under immunocytochemical but not under RIA conditions, cannot be ruled out (13).

Biochemical characterization of a-MSH-LI have revealed that only a-MSH or des-acetyl-a-MSH (i.e., ACTH(1-13)-amide) are stored in the hypothalamus (14; and our own observation). This has led most investigators to conclude that the a-2 system must, indeed, contain one of those two peptides. In this paper, we have pursued this issue further, in an attempt to distinguish between the two hypotheses. We have used a combination of surgical, biochemical and immunohistochemical techniques in an attempt to further characterize the a-MSH-LI in the two hypothalamic cell groups. Taken together our results suggest that the immunoreactive material in the lateral hypothalamic cell group is not authentic a-MSH but an amidated peptide with carboxy-terminal homology to a-MSH. This unknown peptide has a widespread distribution to several brain target areas including the striatum, hippocampus and cortex. Its importance is further underscored by the fact that we can detect it in monkey brain.

METHODS

RATIONALE OF STUDIES

A. The biochemical strategy consisted of the following steps:

1) Study with a number of antisera the a-MSH-LI in total rat hypothalamus. Using coupled HPLC/RIA techniques, do any unique peaks emerge?

2) Dissect the arcuate region and the lateral hypothalamic region (a-2 cells) separately, and determine whether we can detect a different ratio of a-MSH-LI to B-END. The arcuate POMC cells should have an a-MSH:B-END ratio close to 1. In contrast the a-2 cell areas, which do not contain B-END or other POMC peptides, should have high a-MSH:B-END ratio. One problem to keep in mind is that the a-2 cell areas also contain arcuate POMC fibers (with both a-MSH and B-END). Thus, much of our effort is toward separating the cell and fiber systems. Given that the anatomical studies have shown that the cells are more numerous in the a-2 cell group, we expected a substantially higher a-MSH:B-END ratio in that area as opposed to the ratio in the arcuate region. These studies were carried out with and without colchicine.

3) Produce lesion of the arcuate cell group with monosodium glutamate (MSG). Study the relative drop in a-MSH versus B-END in hypothalamus and other projection areas.

4) Use knife-cuts to isolate the arcuate cell group and study a-MSH:B-END ratios as above.

B. The characterization of the material being visualized immunohistochemically employed the following approaches:

1) Use a large number of a-MSH antisera which are either amino- or carboxy-terminally directed. Determine which of them would preferentially demonstrate the arcuate versus a-2 cell group.

2) Use N-acetyl-ACTH(1-13), diacetyl-ACTH(1-13)-amide, ACTH(1-13)-amide, ACTH(1-13) as well as a-MSH (N-acetyl-ACTH(1-13)-amide) in systematic blocking studies to determine whether the visualization depends on the amino-terminal modification, the carboxy-terminal modification or the midportion of the peptide.

3) Use an antiserum directed against the midportion of a-MSH, i.e., ACTH(4-9) to determine whether the a-2 cell group contains a mid-region identical to authentic a-MSH.

C. The general anatomical description of the distribution and projectional pathways of the extra arcuate cell group and its comparison to the arcuate projection was carried out using the following approaches:

1) General but more detailed description using classical immunohistochemical tools in normal and colchicine treated rats.

2) Description in arcuate-lesioned or hypothalamic knife-cut rats to eliminate the POMC cell group and study the a-2 system.

3) Cell counts of the two groups under normal and lesioned conditions.

TECHNIQUES

A. Chromatography and RIA's:

Tissue extraction is carried out in acetone:0.2N HCl (3:1). The extracts are pooled, evaporated in a Savant vacuum evaporator, and resuspended in 1% formic acid in preparation for molecular sieving (cf. 15).

B. Molecular Sieving:

Molecular sieving is carried out on a 1.5 X 90 cm Sephadex G-50 superfine column in 1% formic acid. This procedure, adopted from Jackson and Lowry (16), is useful in that good recovery (in excess of 70%) is obtained without addition of protecting proteins such as bovine serum albumin. This is important since large amounts of protein have been found to diminish the life of the HPLC column used in the subsequent step. With this molecular sieving, we separate POMC, B-END, B-LPH and smaller fragments. Most importantly, this column can separate B-END(1-31) from B-END(1-27). The column is precalibrated with blue Dextran, Cytochrome C, [^{3}H]B-END (courtesy of Dr. C.H. Li), and cobalt chloride. After extensive washing and sample application, approximately 1 ml fractions are collected, evaporated and subjected to RIA.

C. HPLC Separation:

Multiple forms of a-MSH-like immunoreactivity were separated using a reverse phase high performance liquid chromatographic system with an Ultrasphere Octyl Column. The mobile phase had the following composition: Buffer A = 40 mM of potassium phosphate (pH 2.4) with 0.1% TEA. Buffer B = 15% methanol in acetonitrile. The flow rate was 0.8 ml/min. The gradient began at 19% B and was raised to 137% B over a period of 33 minutes. Standards were monitored with a Hitachi Variable UV detector set at 210 nm.

Tissue extracts were monitored by collecting 0.4 ml fractions, evaporating them to dryness and subjecting them to a-MSH RIA's.

With this procedure we are able to separate the 3 endogenous forms of a-MSH (non-acetylated, mono-acetylated and diacetylated) in both their non-oxidized and oxidized forms. Their order of appearance, from shortest to longest retention time, is: Des-acetyl-a-MSH sulfoxide (ACTH(1-13)-amide oxidized), a-MSH sulfoxide, des-acetyl-a-MSH, diacetyl-a-MSH sulfoxide, a-MSH, and diacetyl-a-MSH.

D. Alpha-MSH RIAs:

The RIA's for a-MSH use several antisera specific to a-MSH (see Table 1). Two were used routinely - one raised by us (final dilution 1:2,000) and one raised by Drs. Voigt and Martin (University of Ulm, West Germany) at final concentration of 1:30,000. With [^{125}I]a-MSH tracer, the antigenic determinant of both antisera is primarily the amidated carboxy-terminus with a secondary determinant at the N-acetylated amino-terminus as evidenced by total lack of cross-reactivity with ACTH(1-13) or ACTH(4-10) and a 70% cross-reactivity with ACTH(1-13)-amide. The total final volume per assay tube is 0.25 ml. The assay buffer is 150 mM of sodium phosphate with 1% NaCl and 0.3% human serum albumin, pH 8.2. The assay is incubated at equilibrium at 4°C for 24 hours. It is then separated with a slurry of 0.6 ml dextran-coated charcoal. The tubes are centrifuged and the supernatant which contains the bound tracer is counted. The IC_{50} using our antiserum is 10 to 15 fmol/ml. The range of sensitivity of the assay is from 2 fmol per assay tube at 10% inhibition to 300 fmol per assay tube at 90% inhibition.

E. Immunocytochemistry:

Adult male Sprague-Dawley rats were used. Brain sections were prepared for immunocytochemistry as described previously (17). Rats were anesthetized with sodium pentobarbital (50 mg/kg) given intraperitoneally, and placed on crushed ice. The cardiovascular system was flushed with 0.9% saline followed by perfusion with ice-cold 4% formaldehyde (pH 7.4) in 0.1 M phosphate buffer, for 30 minutes. The brains were removed promptly and placed in the same fixative for an additional hour, after which they were transferred into 15% sucrose in phosphate-buffered saline (PBS) and refrigerated overnight. Tissue blocks were frozen onto brass cryostat chucks with liquid nitrogen and sectioned in a cryostat at -20°C. Sections of 10-20 um thickness were thaw-mounted onto gelatin-coated slides and stored at -70°C.

Primary rabbit antisera (Table 1) were diluted with 0.3% Triton X-100 in 0.02 M PBS, added onto the sections, allowed to incubate for 1 hour at 37°C, and overnight at 4°C. After 3 X 10 minutes PBS washes, the sections were incubated with goat anti-rabbit serum (Arnel) for 30 minuutes at 37°C, and then at 4°C overnight. The sections were again washed in PBS, and incubated in successive steps with horseradish peroxidase (HRP) antiserum, followed by PBS wash, and HRP enzyme (Sigma, Type VI) for 40 minutes each, at 37°C. The slides were then washed in PBS and immersed in a solution of 0.03% H_2O_2 and 0.125 mg/ml diaminobenzidine (Sigma) for 15 minutes with constant stirring. The sections were washed in distilled water, briefly osmicated, washed, dehydrated through ethanols and xylenes and mounted in Permount. Observations and photography were perfomred using a Leitz Orthoplan Microscope.

F. Colchicine pretreatment:

To enhance the visualization of neuronal perikarya, adult rats wre injected intracerebroventricularly with varying doses of colchicine (50-400 ug

in 10 ul 0.9% saline) (17). The main action of colchicine is inhibition of microtubule synthesis, resulting in retardation of axonal transport and an accumulation of synthesized material in neuronal perikarya (18). For injection, each animal was anesthetized with diethyl ether or sodium pentobarbital, the skull was exposed, and the colchicine was injected into the lateral ventricle. The skull opening was filled in with bone wax and the scalp was closed using suture or wound clips. Animals were allowed to recover for 24 to 48 hours, and were then sacrificed as described.

G. Monosodium Glutamate treatment:

Monosodium glutamate (MSG), when administered in large doses to neonatal animals, produces widespread destruction of neurons within the medial-basal hypothalamus (see 19). To induce neuronal damage in the arcuate nucleus, MSG (1 g per 1.5 ml 0.9% saline) was administered intraperitoneally to rat pups on postnatal days 1,3,5,7 and 9 in doses of 4 mg/g body weight. Their littermates were injected with normal saline for control purposes. With these high doses of the excitatory amino acid, only about two-thirds of treated animals survived to adulthood. Some of the surviving rats were also treated with colchicine (50 ug per rat) prior to immunocytochemistry or RIA.

H. Hypothalamic islands:

Another method of separating the arcuate nucleus from the rest of the hypothalamus, including the a-2 neurons, is to surgically isolate the medial-basal hypothalamus using a knife, resulting in the so-called "hypothalamic island" preparation. Complete deafferentation of the arcuate nucleus can be achieved using a Halasz knife (20) with a 1.4 mm horizontal and 2.0 mm vertical blade. The blade is lowered stereotaxically to the retrochiasmatic area at the base of the hypothalamus and rotated 360 degrees. The resulting "island" includes the arcuate nucleus, the ventromedial nucleus, and the more ventral regions of the dorsomedial nucleus (21). Some of these rats were treated with 50 ug of colchicine prior to sacrifice.

I. Cell body counts:

The numbers of immunoreactive perikarya both within the arcuate nucleus as well as the a-2 system of MSG-treated and control rats were determined as follows: with the aid of an ocular grid, immunoreactive perikarya were counted in every tenth section through the entire right side of the hypothalamus, both in the frontal and parasagittal planes. The numbers thus obtained were multiplied by ten to obtain a statistically valid estimate (22) of immunoreactive perikaryal numbers on the right side of the brain.

RESULTS

BIOCHEMICAL STUDIES

A. Characterization of a-MSH-LI in total hypothalamus:

Molecular sieving results from Sephadex G-50 revealed that a-MSH-LI emerged at the correct retention time for a 13 amino acid peptide. No material of a different molecular weight could be reliably detected.

Comparison of three antisera--Watson/Akil, Eskay, and Voigt/Martin (Table 1)--showed parallel results, although the absolute values differed, sometimes by a factor of 8. We were able to determine that this was due to spontaneous

Table 1. List of POMC Antisera. Indicated titers are under immunocytochemical conditions. N/W = not with, Aff. = affinity purified.

Antibody	Source	Titer	Key Cross-reactivities
B-END	Watson/Akil	1:1000	B-LPH; No other peptides
ACTH	Watson/Akil	1:2000	ACTH(20-24); No other peptides
ACTH(4-10)	Voigt	1:300	Human ACTH(1-13), ACTH(4-10), a-MSH; N/W a-MSH(1-7), ACTH(11-19)
a-MSH (aff.)	Watson/Akil	1:100	a-MSH
a-MSH	Watson/Akil	1:1000	a-MSH, des-, di-Ac-a-MSH; N/W ACTH(1-13), N-Ac-a-MSH(1-13)
a-MSH (Ann) (aff.)	Mains/Eipper	1:100	N/W a-MSH, a-MSH(1-7), ACTH(1-13), (11-24), (1-24), (11-19), N-Ac-a-MSH(1-13), des-, di-Ac-a-MSH
a-MSH (Patti)	Mains/Eipper	1:500	a-MSH, des-, di-Ac-a-MSH; N/W ACTH(1-13), N-Ac-a-MSH(1-13)
a-MSH	Voigt/Martin	1:750	a-MSH, des-, di-Ac-a-MSH; N/W ACTH(1-13), N-Ac-a-MSH(1-13)
a-MSH	Eskay	1:500	Not studied
a-MSH	Van Leeuwen	1:500	Not studied
a-MSH	Vaudry	1:500	a-MSH, des-, di-Ac-a-MSH; N/W N-Ac-a-MSH(1-13), a-MSH(1-13)
a-MSH	Pelletier	1:500	a-MSH, des-, di-Ac-a-MSH; N/W N-Ac-a-MSH(1-13), a-MSH(1-13)
a-MSH (#373)	Immunonuclear	1:500	a-MSH, des-, di-Ac-a-MSH; N/W a-MSH(1-13), N-Ac-a-MSH(1-13)
a-MSH (#379)	Immunonuclear	1:200	a-MSH, des-, di-Ac-a-MSH; N/W N-Ac-a-MSH(1-13), a-MSH(1-13)
g-MSH (Gerti)	Mains-Eipper	1:500	g-MSH; N/W a-, B-, des-, di-Ac-a-MSH, ACTH(1-13), N-Ac-a-MSH(1-13)
CLIP(1-13)	Mains/Eipper	1:100	Not studied
CLIP (Kathy)	Mains/Eipper	1:1000	Not studied
16K Peptide	Mains/Eipper	1:3000	<<1% B-LPH, ACTH

oxidation of a-MSH during tissue handling, resuting in changes in cross-reactivity (e.g. our antiserum prefers oxidized forms by a factor of 3, whereas the other two show a preference for the non-oxidized forms). When these issues were controlled, approximately equivalent amounts of a-MSH-LI were read in the three assays.

The results with reverse-phase HPLC confirmed previous findings that the main immunoreactive peptide in hypothalamus is des-acetyl-a-MSH and its oxidized form (14). Authentic a-MSH could be detected but constituted less than 10% of the total material. There is no reliable evidence for a unique non-a-MSH peptide. Thus, it appeared that the a-2 group may, indeed, contain either a-MSH or des-acetyl-a-MSH.

B. Content in arcuate versus a-2 region:

As indicated below, the number of cells in the a-2 region is large compared to the true POMC cells in the arcuate nucleus. If these cells were to contain authentic a-MSH or des-acetyl-a-MSH, we would expect large readings with the a-MSH RIA in the a-2 versus arcuate region. Furthermore, the ratio of a-MSH:B-END immunoreactivity would be expected to vary in the two areas,

Table 2. Effect of colchicine on B-END and a-MSH-LI (pmoles/tissue)

Experiment 1: Total hypothalamus

	Control	Post-Colchicine	% Control
B-END-LI	4.6 ± 0.5	3.4 ± 0.4	74%
a-MSH-LI	2.5 ± 0.3	2.1 ± 0.3	84%
a-MSH:B-END	1:1.8	1:1.6	

Experiment 2: Arcuate vs. a-2, post-colchicine

	Arcuate	a-2
B-END-LI	2.36 ± 0.25	1.31 ± 0.21
a-MSH-LI	1.6 ± 0.07	1.81 ± 0.16
a-MSH:B-END	1:1.5	1:0.7

Table 3. Effect of MSG lesions on arcuate vs. a-2 groups (pmoles/tissue)

		Control	MSG	% Control
Arcuate:	B-END-LI	2.2 ± 0.4	0.7 ± 0.1	32%
	a-MSH-LI	1.5 ± 0.2	0.4 ± 0.1	27%
	a-MSH:B-END	1:1.5	1:1.7	
a-2:	B-END-LI	1.9 ± 0.3	0.6 ± 0.1	31%
	a-MSH-LI	1.6 ± 0.2	0.4 ± 0.7	25%
	a-MSH:B-END	1:1.2	1:1.5	

Table 4. Hypothalamic islands. Total hypothalamus

	Control	Lesioned	% Control
B-END-LI	4.33 ± 0.5	1.23 ± 0.2	31%
a-MSH-LI	2.16 ± 0.2	0.46 ± 0.6	21%
a-MSH:B-END	1:2.0	1:2.7	

i.e., it should be close to 1:1 in the arcuate, and larger than that in the a-2 region.

Total a-MSH immunoreactivity in total hypothalamus (including both a-MSH regions) ranged between 1.1-3.5 pmoles/animal across several experiments. However, dissection of hypothalamus into arcuate and a-2 regions (n=8) revealed approximately equal distribution of the immunoreactivity in the two areas (arcuate:a-2 ratio = 1:1.1). This ratio was identical to that observed for B-END across these two hypothalamic areas.

Since we failed to obtain differential distributions or a-MSH:B-END ratios in the two areas, we reasoned that the fibers from the arcuate nucleus flowing in the vicinity of the a-2 cell group may be obscuring the picture. In order to approximate the conditions of the immunohistochemical demonstration, we treated rats (n=6) with colchicine at the same dose and assessed a-MSH versus B-END content. As can be seen in Table 2, the first experiment was concerned with the effect of colchicine on total hypothalamic content. Colchicine treatment resulted in a drop to 74-84% of control in hypothalamic peptide content. However, the overall a-MSH:B-END ratio did not

shift substantially (1:1.8 in controls, versus 1:1.6 in colchicine treated). In a second experiment (Table 2) we dissected the hypothalami of colchicine treated rats into arcuate and a-2 regions and subjected the extracts to a-MSH RIA. The arcuate region continued to show an a-MSH:B-END ratio larger than unity (1:1.5), whereas the a-2 region now exhibited a 1:0.7 ratio, showing, for the first time a higher a-MSH reading compared to B-END. This provided some evidence for redistribution of the immunoreactivity and offered partial support to the notion that we can detect a-MSH and not B-END in the a-2 cell group. Note, however, that the loss of B-END in the a-2 region was not complete making it difficult to interpret this result unequivocally. Note also that the distribution of a-MSH-LI in the arcuate versus a-2 region continued to approximate 1:1.1, thereby failing to reflect the large number of cells seen anatomically in the a-2 region.

C. Results of monosodium glutamate lesions and hypothalamic islands:

Since colchicine treatment did not lead to a substantial redistribution of the B-END sufficient to study the non-B-END-containing system, we sought other methods to minimize the input of the arcuate system in the a-2 region. We therefore produced arcuate lesions with two methods: MSG and hypothalamic islands. Following these treatments, we examined the relative losses in B-END versus a-MSH. Here again, we expected a substantial increase in a-MSH:B-END ratio following the treatments, since we would spare the a-2 system which would express a-MSH but not B-END. Furthermore, following the treatments, we dissected the hypothalami into an arcuate and an extra-arcuate (including a-2) region, and examined the distribution of a-MSH immunoreactivity, expecting substantially more a-MSH-like material in the a-2 than in the arcuate punches.

The results of this series of experiments are shown in Tables 3 and 4. In general, both types of lesions produced significant losses in total B-END content within the hypothalamus. MSG lesions (Table 3) revealed similar drops in both B-END and a-MSH immunoreactivity (down to 25-32% of control). More importantly, this treatment did not lead to a redistribution of a-MSH-like material in the arcuate versus a-2 region. As usual, the two areas contained approximately the same amount of a-MSH, and showed the same degree of loss.

Similarly, the hypothalamic islands study revealed a parallel drop in a-MSH and B-END following the lesion (Table 4). Molecular sieving studies in these animals confirmed an almost total loss of a-MSH-sized material in the lesioned animals.

To Summarize, the biochemical studies showed the following:

1) We cannot detect a substance other than des-acetyl-a-MSH or authentic a-MSH in rat hypothalamus.

2) Specific dissections of the arcuate versus a-2 regions in control, colchicine treated, or arcuate-lesioned rats failed to yield evidence for a substantial amount of a-MSH in the a-2 region beyond what is derived from POMC.

This combination of results would lead to one of two conclusions: a) the a-2 system contains a very small amount of authentic a-MSH/des-acetyl-a-MSH. In that case, assay variance may not allow us to detect substantial shifts in proportions following our treatments; or b) the material in the a-2 system is not authentic a-MSH, and is only seen to a small extent under our RIA conditions. Hence, we do not detect a uniquely different form upon chromatography, but we also see little shifts in patterns following various treatments. In favor of the second hypothesis is our knowledge that there are

approximately twice as many cells in the a-2 system as compared to the POMC-containing arcuate group. Given this latter possibility, it became critical to return to the immunohistochemical studies, which reveal the a-2 system reliably, and determine with great care the nature of the immunoreactivity.

CHARACTERIZATION OF THE HISTOCHEMICAL IMMUNOREACTIVITY IN THE a-2 GROUP

Biochemical anatomy of a-2 neurons:

The general purpose of the following two studies was to characterize the immunoreactive material within the a-2 neurons. Specifically, we used multiple antisera against POMC peptides, including several directed against a-MSH, ACTH, CLIP, and g-MSH (Table 1). The first study involved the staining of both the arcuate POMC cells and the a-2 cells in the same section in colchicine treated rats. All POMC antisera were characterized by blocking studies and were found to be specific for the peptide core (or one of its fragments) used as original antigen. All POMC antisera, except for those directed against a-MSH (see below) stained only the arcuate POMC cell group; none stained the a-2 cell group. These data are consistent with our initial reports and several other papers since (2-5).

The focus of the second study was to evaluate the "nature" of the a-2 immunoreactivity by studying: several a-MSH antisera, an a-MSH(4-10) antiserum, as well as g-MSH (Table 5). In the a-MSH antisera study, five MSH variant peptides (see Table 6 for their structure) were used to describe the antigenic site critical to the staining of each a-MSH antiserum. The results of this study can be summarized as follows:

1) Among the eight MSH antisera, six were thought to be carboxy-terminal directed, one amino-terminal directed and one midregion directed (Table 5). All of the carboxy-terminal directed antisera stained both the arcuate POMC cells and the a-2 cells. When these antisera were studied under 10 uM blocking by each of the five MSH peptide analogs (a-MSH, diacetyl-a-MSH, des-acetyl-a-MSH, a-MSH(1-13), N-acetyl-a-MSH(1-13)), it was found that seven were actually carboxy-terminal amide oriented for both cell groups (excluding the midregion antiserum from Voigt). While true a-MSH blocked the staining of both cell groups for all antisera, removal of the carboxy-terminal amide (i.e. use of a-MSH(1-13) or N-acetyl-a-MSH(1-13)) removed the ability of these peptides to block the staining. In contrast, alteration of the amino-terminus (diacetyl-a-MSH, des-acetyl-a-MSH) did not alter the ability of the peptide to block the staining (Table 5). It was concluded that the a-2 antigen was similar to the amidated carboxy-terminus of true a-MSH.

2) A serum directed against g-MSH peptide which has a sequence homology to the ACTH/MSH(4-10) region (see Table 7), only stained the arcuate POMC cells (but not the a-2 cells; Table 5). It was concluded that there was at least some key antigenic change in the a-2 antigen, from the ACTH/MSH(4-10) core sequence.

3) Most telling were findings with the antibody raised against ACTH/MSH(4-10) region. This antiserum is specific to the middle core of a-MSH (Table 7). It was unable to stain the a-2 cells, but stained the arcuate POMC cells in the same section (Table 5).

These studies taken as a whole suggest that the antigen in the a-2 cells is very similar to the amidated carboxy-region of a-MSH, but differs from the middle core of a-MSH, since antisera which recognize this core do not stain the a-2 cell group.

Table 5. "MSH" immunocytochemistry of arcuate (a-1) and "a-2" cells. The designation +/+ (or -/-) indicates that the immunoreactivity in both the a-1/a-2 cell groups are blocked (or not blocked) by that peptide.

			Peptide blocking: a-1/a-2				
Antiserum	Source	Area stained	a-MSH	di-Ac-a-MSH	des-Ac-a-MSH	a-MSH (1-13)	N-Ac-a-MSH (1-13)
a-MSH	Mains/Eipper	a-1/a-2	+/+	+/+	+/+	-/-	-/-
a-MSH	Voigt/Martin	a-1/a-2	+/+	+/+	+/+	-/-	-/-
a-MSH	Watson/Akil	a-1/a-2	+/+	+/+	+/+	-/-	-/-
a-MSH #379	Immunonuclear	a-1/a-2	+/+	+/+	+/+	-/-	-/-
a-MSH #373	Immunonuclear	a-1/a-2	+/+	+/+	+/+	-/-	-/-
a-MSH	Vaudry	a-1/a-2	+/+	+/+	+/+	-/-	-/-
a-MSH	Pelletier	a-1/a-2	+/+	+/+	+/+	-/-	-/-
a-MSH(4-10)*	Voigt	a-1 only	+/	+/	+/	+/	+/
g-MSH**	Mains/Eipper	a-1 only	-/	-/	-/	-/	-/

*Also blocks with a-MSH(4-10) but not with a-MSH(1-7) or a-MSH(11-19)
**Also is not blocked by B-MSH; but is blocked by g-MSH

Table 6. a-MSH peptide forms.

Name	Structure
a-MSH = N-Ac-ACTH(1-13)-amide	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
des-Ac-a-MSH = ACTH(1-13)-amide	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
di-Ac-a-MSH = di-Ac-ACTH(1-13)-amide	(Ac,Ac)-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
MSH(1-13)= ACTH(1-13)	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val
N-Ac-a-MSH(1-13) = N-acetyl-ACTH(1-13)	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val

Table 7. a-MSH, B-MSH, g-MSH sequences in rat.

Name	Sequence
a-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 ("4-10" Core: Met-Glu-His-Phe-Arg-Trp-Gly)
B-MSH	Ala-Asp-Gly-Pro-Tyr-Arg-Val-Glu-His-Phe-Arg-Trp-Gly-Asn-Pro-Pro-Lys-Asp
g-MSH	Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly

DISTRIBUTION OF a-2 NEURONS AND THEIR PROJECTIONS

A. Distribution of perikarya and effects of lesions:

a-MSH immunoreactive perikarya of the a-2 system are scattered throughout the lateral hypothalamic area, zona incerta, and other nuclei (Figure 1). These perikarya are seen from middle to posterior hypothalamus. The nuclei containing a-MSH-LI include the periventricular, dorsomedial, and dorsal ventromedial (Figure 2).

Perikaryal counts were performed on normal rats pretreated with colchicine, and on rats treated neonatally with MSG followed by colchicine prior to sacrifice. Perikarya were counted in the right arcuate nucleus (B-END) and the right a-2 region (a-MSH). The results are presented in Table 8. The a-2 perikarya outnumber the arcuate perikarya by an almost 2:1 margin. Neonatal MSG treatment resulted in a loss of approximately 75% in the number of arcuate perikarya, while the a-2 perikarya were not affected (Figure 3). Neuron loss was greatest in the rostral two-thirds of the arcuate nucleus. Some neurons in the periarcuate regions appeared to be spared as were some situated more caudally within the nucleus. Additionally, in rats with hypothalamic knife-cuts, no arcuate perikarya survived, while a-2 neurons remained apparently unaffected (Figure 4). Cell counts were not performed in the latter rats.

B. Fiber projection systems:

Projections of the arcuate POMC neurons are extensive; fiber immunoreactivity is seen in numerous limbic forebrain, hypothalamic, periaqueductal, and brain stem nuclei (see 1). The a-2 neurons, on the other hand, appear to project to the striatum, hippocampal formation, cerebral cortex, and the olfactory bulb.

Immunoreactive a-MSH fibers are seen scattered throughout the caudate-putamen complex. In the hippocampal formation, immunoreactive fibers are seen in the dentate gyrus stratum molecular and the hilus. In the hippocampus proper, immunoreactive fibers appear in the stratum oriens, within the mossy fiber layer, as well as throughout stratum radiatum (Figure 5B). Furthermore, these fibers are often seen traversing several strata, e.g., through stratum granulosum and stratum pyramidal. Fibers are also seen in the fimbria. In the cerebral cortex, a-MSH immunoreactive fibers are scattered in most cortical laminae of the frontal, parietal, occipital, cingulate, entorhinal, and piriform regions (Figure 5C, D). These fibers often traverse several laminae, ending in lamina I. In the cingulum bundle, fibers are seen oriented in parallel with the corpus callosum, presumably projecting within the bundle. In the olfactory bulb, scattered immunoreactive fibers are visualized in the anterior olfactory nucleus, in the intermediate olfactory tract, and the internal plexiform, mitral cell, external plexiform, and glomerular layers.

There appear to exist several possible pathways for the projections of the a-2 neurons (Figure 6). In particular, a-MSH immunoreactivity is present in the following pathways:

1) The medial forebrain bundle and its rostral extension, the cingulum bundle, through which a-2 fibers could reach both cortical and hippocampal targets.

2) The fornix, which might carry fibers from the surrounding a-2 neurons to the hippocampus (see also Figure 5A).

3) Lateral projections of the a-2 neurons into the temporal cortex, which might gain access to both the amygdala and hippocampus. However, it is

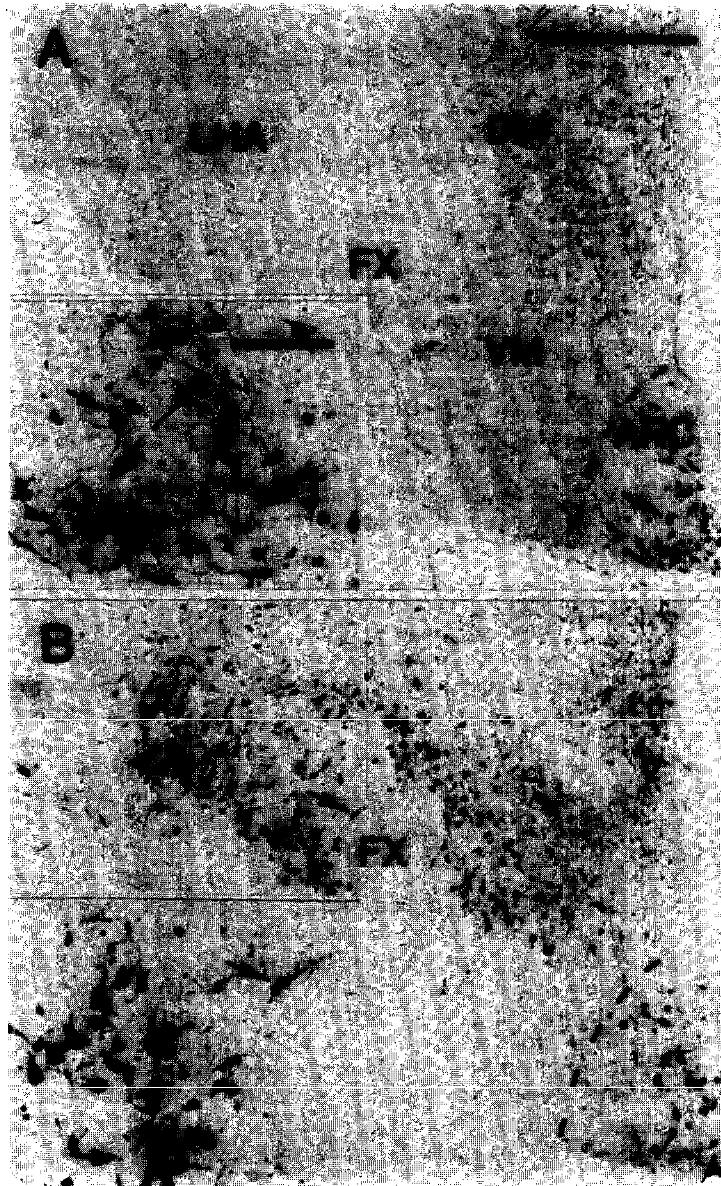


Figure 1. Normal rat: A and B are two adjacent 20 um sections (montage) stained with antisera to B-END and a-MSH, respectively. Note that B-END cells are confined to the arcuate nucleus (ARC), demarcated by dashed lines, while a-MSH perikarya are present in both the arcuate nucleus as well as the ventromedial (VM), dorsomedial (DM) and lateral hypothalamic area (LHA). In the inset of each panel, respective arcuate B-END (A) and lateral hypothalamic a-MSH (B) neuronal perikarya are magnified. In each panel and inset, the arrows point to common cells. FX: fornix. Bar (A, B) = 0.5 mm. Bar (insets) = 100 um.

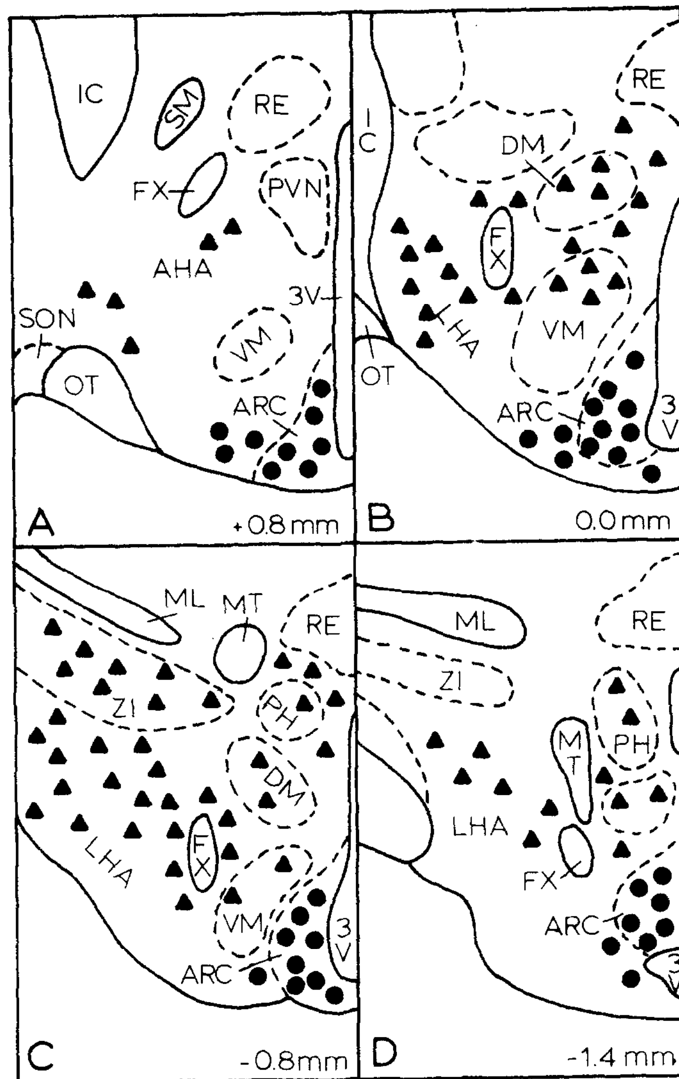


Figure 2. Schematic representation of the distribution of the arcuate (solid circles) versus a-2 (solid triangles) perikarya throughout the hypothalamus, from rostral to caudal (A-D). The coordinates in the lower right hand corner of each panel are with respect to Bregma (32). AHA: anterior hypothalamic area, ARC: arcuate nucleus, DM: dorsomedial nucleus, FX: fornix, IC: internal capsule, LHA: lateral hypothalamic area, MFB: medial forebrain bundle, ML: medial lemniscus, MT: mammillothalamic tract, OT: optic tract, PH: posterior hypothalamus, PVN: paraventricular nucleus, RE: reuniens nucleus of thalamus, SM: stria medularis, SON: supraoptic nucleus, VM: ventromedial nucleus, ZI: zona incerta, 3V: third ventricle.

Table 8. Counts of immunoreactive perikarya in the arcuate (B-END) and a-2 (a-MSH) neuronal groups in "normal" and "MSG-treated" rats. Two animals were used from each group.

Animal	Normal Colchicine (#1)	Normal Colchicine (#2)	Normal Mean	MSG Colchicine (#1)	MSG Colchicine (#2)	MSG Mean
Arcuate (B-END)	4233	5240	4736	1740	620	1180
a-2 (a-MSH)	6189	10,540	8364	9260	12,080	10,670

not clear whether these latter fibers arise from the arcuate or a-2 neurons.

4) the intermediate olfactory tract, which might carry fibers into the olfactory bulb.

In the MSG lesioned rats, without colchicine treatment, the projections of the arcuate POMC system appear to be diminished as indicated by a decrease in the intensity of B-END immunoreactivity in periventricular thalamus, amygdala and periaqueductal gray, areas which normally receive input from these neurons. By contrast, no loss in a-MSH immunoreactivity was noted in the hippocampus or cortex of the MSG-treated rats, indicating the possible origin of these fibers from the a-2 neurons. Thus, it seems possible to separate, although not completely, the arcuate and a-2 systems by means of neonatal MSG-induced lesions.

In rats with knife-cut isolations of the medial-basal hypothalamus, also without colchicine treatment, the projections of the arcuate POMC neurons were completely abolished. As expected, no drop in a-MSH immunoreactivity was noted in the a-2 projection areas. Apparently, surgical isolation of the arcuate nucleus results in a more complete depletion of peptide immunoreactivity in the projections of this system, perhaps due to anterograde degeneration of the axons of POMC neurons. This procedure leaves the a-2 neurons intact, thereby providing a means for the complete isolation of the arcuate and a-2 neuronal systems.

DISCUSSION

A. Biochemical and immunohistochemical studies:

The results of the combined biochemical and immunohistochemical studies lead us to suggest that a unique antigen is present in the a-2, extra-arcuate cell group which is commonly labeled with antisera directed against a-MSH. This antigen appears to share with authentic a-MSH a carboxy-terminal homology, including the amidation. However, it appears to differ from authentic a-MSH in the ACTH/MSH(4-10) region, and possibly beyond. These conclusions have been reached in part because of our inability to detect a substantial amount of a-MSH immunoreactivity, beyond the B-END amounts, in the region of the a-2 group following such varied treatments as MSG, colchicine and hypothalamic islands. We now believe that under RIA conditions, where the antigenic determinants of the assay appear more complex, we have only partial cross-reactivity with the a-2 antigen. If we were to suppose that the cross-reactivity is in the range of 15-25% of the a-MSH cross-reactivity, depending on the sera, one can understand why variations in the assay conditions, and in the chemical modification which occur during extraction and chromatography, would lead to variations in our ability to detect the antigen and quantitate its presence following the treatments. Hence, we now conclude

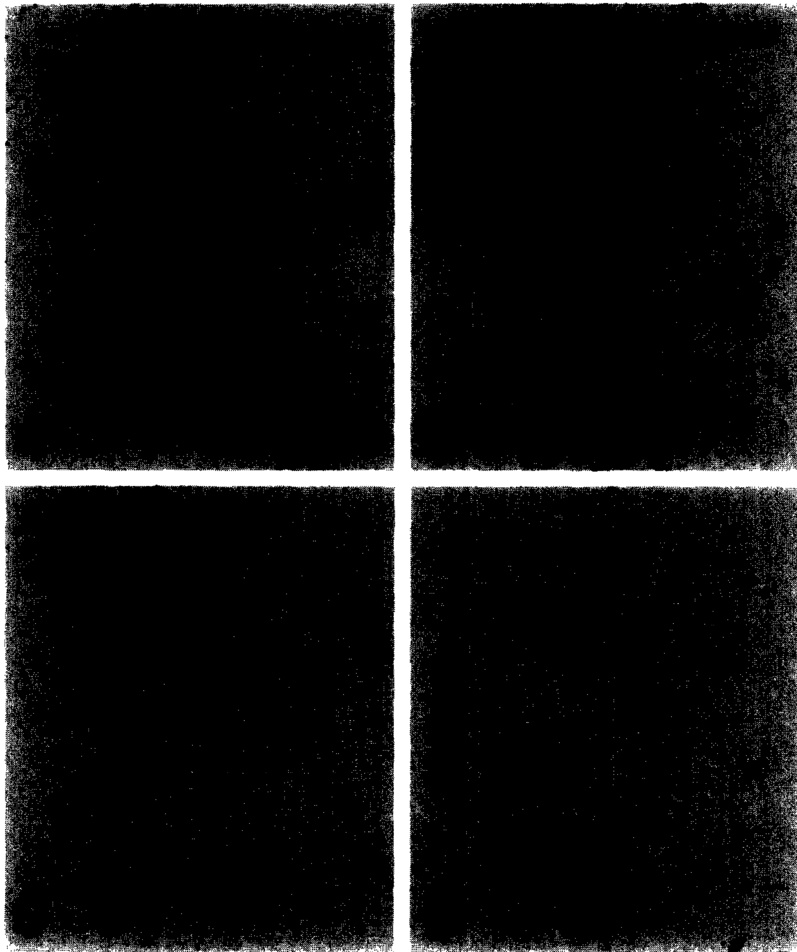
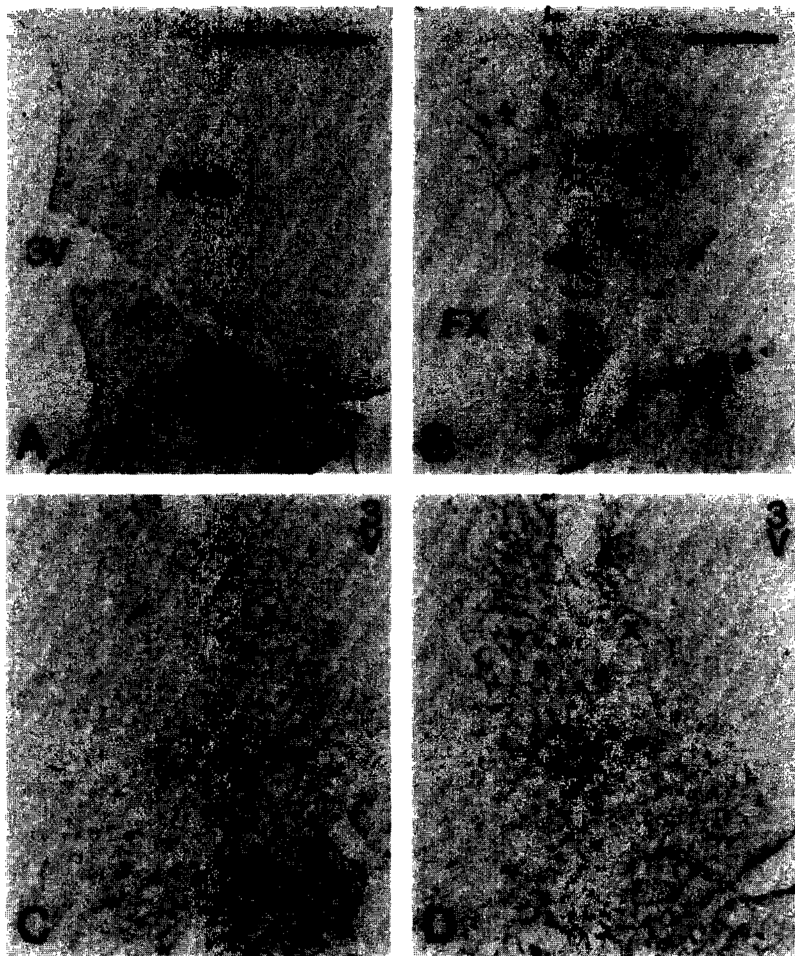


Figure 3. MSG-lesioned rat: Panel A shows hypothalamus at the level of the arcuate nucleus (demarcated by the dashed line) stained with a-MSH. Note that a-2 neurons are apparently unaffected following MSG treatment (A, and magnified area in B; the position of a common vessel is marked by x). Conversely, there is a significant loss in arcuate perikarya, as seen in panels C (a-MSH) and D (B-END). Cell loss in the arcuate nucleus following MSG is approximately 75% (see Table 8). FX: fornix, 3V: third ventricle. Bar (A) = 0.5 mm. Bar (B-D) = 100 um.



<u>Figure 4.</u> <u>Knife-cut rat</u>: Panel A shows a section through the anterior hypothalamic area (AHA), stained with B-END, showing the position of the knife cut (arrows) which completely separates the medial-basal hypothalamus from the rest of the brain. In more caudal sections, it can be seen that a-2 neurons survive this operation (B), while the arcuate neurons stained for both B-END (C) and a-MSH (D), do not. A common vessel is indicated by x in the two adjacent sections C and D. FX: fornix, 3V: third ventricle. Bar (A) = 0.5 mm. Bar (B-D) = 100 um.

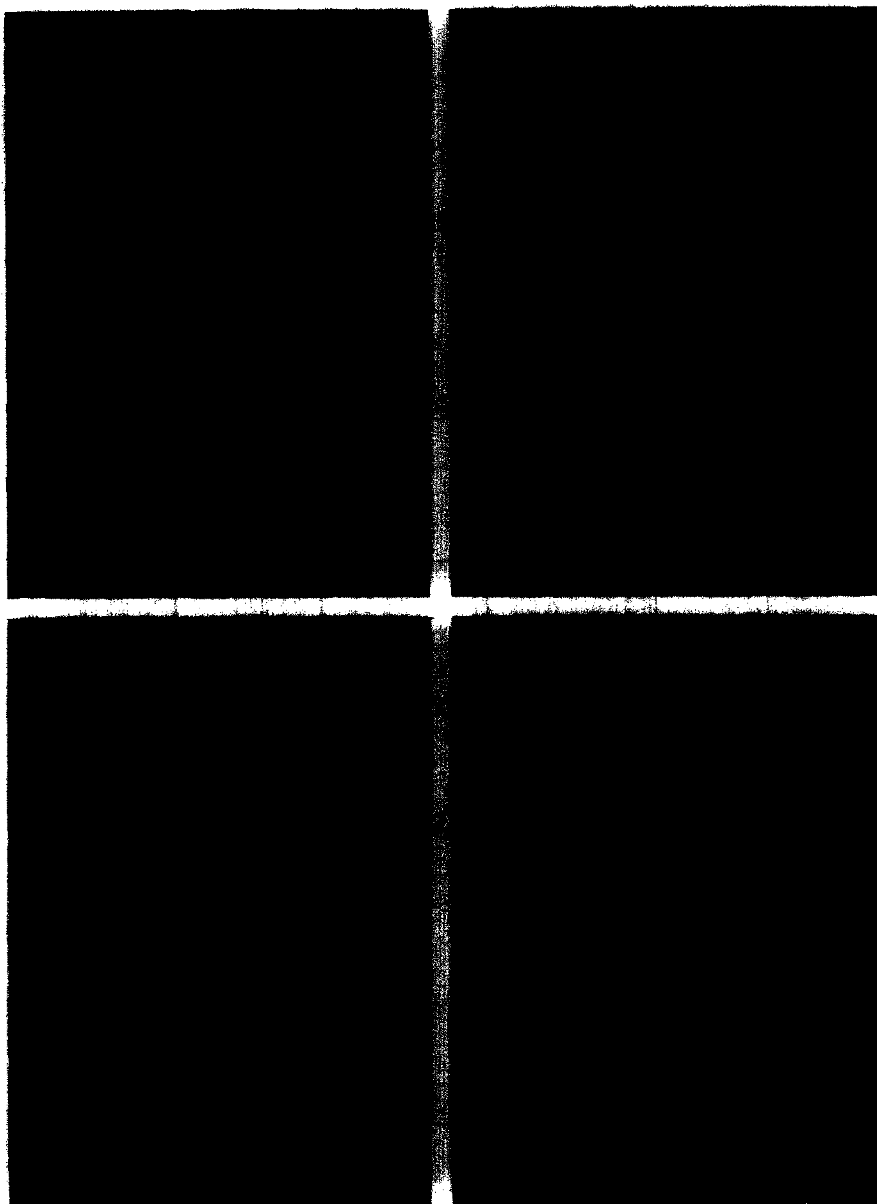


Figure 5. Normal rat: These four darkfied photomicrographs show a-MSH immunoreactive fibers in several locations throughout the forebrain. In panel A, fibers can be seen in the lateral septum (LS) and in fornix (FX) ventral to the corpus callosum (CC). In B, fibers are seen in the hippocampal mossy fiber layer (MF), adjacent to the pyramidal cell layer (PYR), as well as in stratum radiatum (RAD). In C and D numerous fibers can be seen in the different laminae of the frontal (C) and parietal (D) cortex. Bar (A-D) = 100 um.

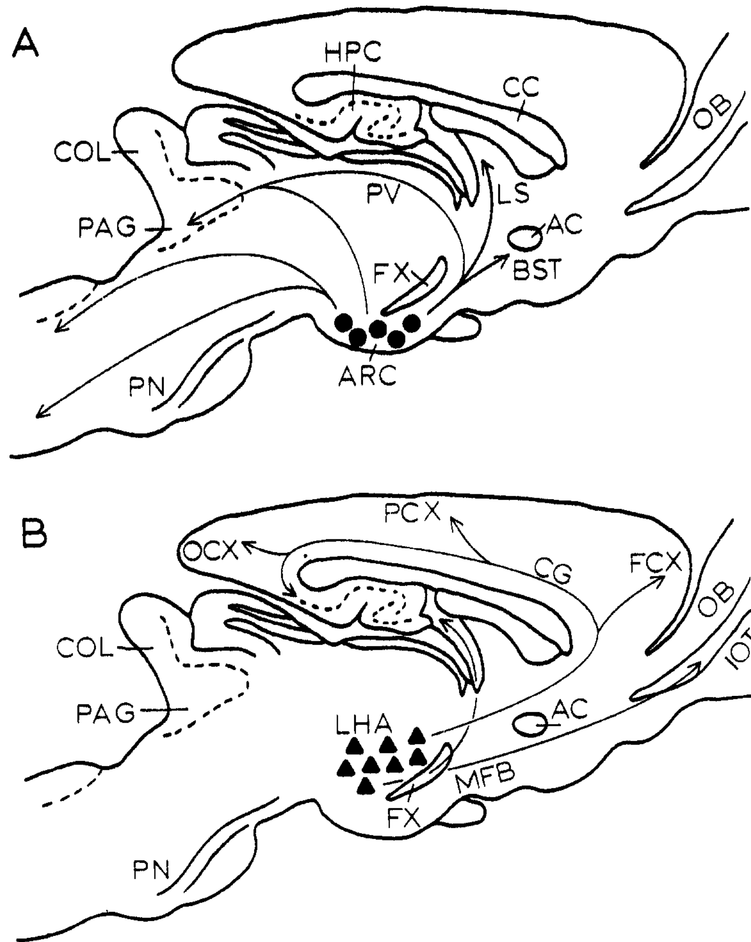


Figure 6. Schematic representation of the possible projection pathways of the arcuate versus lateral hypothalmić a-2 neurons. Arcuate B-END/a-MSH (i.e., POMC) projections are shown in parasagittal section A, while a-2 (a-MSH alone) projections are shown in section B. AC: anterior commissure, ARC: arcuate nucleus, BST: bed nucleus of stria terminalis, CC: corpus callosum, CG: cingulum, COL: colliculus, FCX: frontal cortex, FX: fornix, HPC: hippocampus, IOT: intermediate olfactory tract, LHA: lateral hypothalamic area, LS: lateral septum, MFB: medial forebrain bundle, OB: olfactory bulb, OCX: occipital cortex, PAG: periaqueductal gray, PCX: parietal cortex, PN: pons, PV: periventricular nucleus of thalamus.

that the chromatographic studies, in showing only a-MSH and des-acetyl-a-MSH in the hypothalamus, underscore not the identity between the a-2 antigen and authentic a-MSH, but rather their substantial difference. On the other hand, the antigenic determinant under immunohistochemical conditions appear consistant. All antisera seemed to depend almost exclusively on the carboxy-terminus amidated region. Using other amidated peptides did not cause blockade in the demonstration suggesting that the a-2 antigen has an amidated tri- or tetra-peptide homologous to the a-MSH terminal sequence. The most critical finding in these studies is the fact that a midportion antibody directed at the ACTH/MSH(4-10) region failed to stain the a-2 group. Neither was this group stained with g-MSH antisera (although its antigenic determinants are not as clear cut as that of the MSH(4-10) antibody). This evidence is key to our conclusion that the a-2 antigen differs from either a-MSH or des-acetyl-a-MSH found in the arcuate POMC neurons.

B. Anatomical studies:

In this study, we have also shown that the a-2 neurons are distributed widely throughout several hypothalamic regions, numbering almost twice as many as the arcuate POMC neurons. Neither MSG treatment, nor knife-cut isolations of the medial-basal hypothalamus appeared to affect the a-2 neurons, while drastically diminishing (MSG) or completely abolishing (knife-cuts) the arcuate POMC neurons. The MSG findings are also consistent with the observations of significant reduction in radioimmunoassayable or immunocytochemically detectable levels of POMC-derived peptides in the arcuate nucleus after neonatal MSG treatment (23-26). Quantitative perikaryal counts confirmed the qualitative observations at least in the case of MSG-treated animals. However, in the knife-cut hypothalami, one would not expect to detect perikaryal loss outside of the "island" with the possible exception of those neurons that might normally be projecting "into" the island (e.g., to the median eminence).

Immunoreactive a-MSH fibers (other than POMC projections) were noted throughout the nucleus caudate-putamen, in all areas of the cerebral cortex, in the hippocampal formation, and in the olfactory bulb. Studies using combined tract-tracing and immunocytochemistry have shown that a major source of some of these fibers are neurons in the lateral hypothalamus and zona incerta which also contain a-MSH-LI (27-30). Nevertheless, previous immunocytochemical studies of the a-2 projections have not speculated on the possible projection pathways of the a-2 neurons, although, Kohler et al. (27) have provided evidence for some specific projections (see below). Using 20 um brain sections immunostained with a-MSH antisera, we have noted several probable projection pathways:

1) Immunoreactive fibers were noted in the fornix and hippocampal fimbria. This route could provide a direct link between the lateral hypothalamic a-2 neurons (e.g., those surrounding the fornix) and the hippocampus.

2) Immunoreactive fibers were also noted in the amygdala region and the surrounding cortical areas. Some of these caudally projecting fibers may gain access to the hippocampus. In support of these findings, Kohler et al. (27) have demonstrated two possible routes of a-2 projections into the hippocampal formation; dorsally through the septum and fimbria, and ventrally through the candul amygdala and entorhinal area.

3) Furthermore, immunoreactive fibers were noted within the medial forebrain bundle (coursing through lateral hypothalamus), the septal projections of this bundle as well as its rostral extensions into the cingulum

bundle. These fibers could possibly represent the cortical innervation pathway. Saper et al. (30) have demonstrated a direct link between the a-2 neurons and the frontal cortex, using retrograde tracers and immunofluorescence. Immunoreactive fibers in the cingulum bundle could also project into the temporal lobe area, where a-MSH-LI was detected in the entorhinal cortex, presubiculum and subiculum. This route could provide yet another projectional link between the a-2 neurons and the hippocampus.

4) Lastly, immunoreactive a-MSH fibers could gain access to the olfactory bulb areas via the intermediate olfactory tract. Fibers in this tract cross at the anterior commissure, within which numerous a-MSH fibers can be seen. Conversely, these a-MSH fibers might originate from the olfactory bulb itself, since perikarya containig a-MSH immunoreactivity have been described in the mitral cell layer (31).

C. Conclusions:

We are in the unusual position of knowing a great deal of the brain anatomy of an unknown substance. It is now necessary to determine the full sequence of the unique a-2 antigen. Nevertheless, the anatomical distribution of this system serves to underscore its possible functional importance. The studies in both intact and arcuate-lesioned animals show a wide-spread distribution with projections into striatal, cortical and hippocampal target areas. This distribution, coupled with the fact that the system has been found in cat by Micevych and Elde (7) and in monkey as shown here, suggest the possible presence of a functionally important peptide. Now that we recognize the need for a "pure" carboxy-terminal assay for biochemical studies, it is conceivable that a purification scheme can be elaborated to purify and sequence this unique and interesting antigen.

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