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α-Melanocyte stimulating hormone: Immunohistochemical identification and mapping in neurons of rat brain

(immunofluorescence/hypothalamus/arcuate nucleus/α-melanotropin)

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ABSTRACT **α-Melanocyte stimulating hormone (α-melanotropin) immunofluorescence was observed in rat brain by means of a highly specific and well-characterized antibody. The hormone was contained in arcuate nucleus cell bodies and in varicose fibers. Dense populations of hormone-containing fibers were present in the septum, the nucleus interstitialis stria terminalis, and the medial preoptic, anterior hypothalamic, dorsomedial, and periventricular nuclei. Moderate numbers of fibers were seen in the paraventricular and arcuate nuclei, the amygdala, the region of the tractus diagonalis, the mammillary body, the central gray, the cuneiform nucleus, and the nucleus of the solitary tract. There is an interesting correlation of α-melanocyte stimulating hormone fibers with regions of noradrenergic axonal projections and terminal fields.**

α-Melanocyte stimulating hormone (α-MSH, α-melanotropin), a basic acetyltridecapeptide amide, was isolated from pituitary glands and characterized over 20 years ago (1–3). Although the presence of melanotropic activity has long been known within the brain (4, 5), only recently have significant quantities of immunoreactive α-MSH been shown to be present in the mammalian brain (6–8). Although there is no clearly defined physiological function for α-MSH in the brain, many behavioral and electrophysiological effects of this peptide have been described (9–11). The present investigation describes the immunohistochemical localization of α-MSH-containing processes and cell bodies, in addition to a detailed mapping of the distribution of these fibers in the rat brain.

MATERIALS AND METHODS

The antibody to α-MSH was generously provided by M. C. Tonon and H. Vaudry (Laboratoire d'Endocrinologie, Mont-Saint-Aignan, France). The antiserum (81-0103) to α-MSH was raised in rabbits against synthetic α-MSH (Ciba–Geigy, Summit, NJ) conjugated to bovine serum albumin by carbodiimide (7, 12). Vaudry *et al.* (7) have demonstrated the high specificity of this antibody to α-MSH because there is little or no cross-reactivity with human or bovine β-MSH, ovine β-lipotropin, natural porcine corticotropin (ACTH), or the synthetic ACTH fragments, ACTH 1–16, ACTH 1–24, ACTH 1–19, ACTH 1–10, ACTH 17–39, and ACTH 11–19, in their radioimmunoassay system. They have also presented evidence suggesting that the antigenic determinant for this antibody is the region Gly^{10}-Lys^{11}. In our immunocytochemical system preabsorption controls were performed with α-MSH and related peptides. Aliquots of 50 μl of diluted α-MSH antiserum (1:75) were incubated overnight at 4°C with 1, 0.01, 0.001, or 0.0001 μg of α-MSH (Boehringer Mannheim), monkey β-MSH (generously provided by D. Liu, Bethesda, MD), ACTH 1–10 (Peninsula Labs, San Carlos, CA), or porcine ACTH (Sigma). Addition of 1 or 0.01 μg of α-MSH completely prevented the immunoreactivity in rat brain, whereas 0.001 μg decreased immunoreactivity by approximately 25%. No decrease in immunoreactivity was noted with any of the other peptides as preabsorbants.

The indirect immunohistochemical procedure of Coons (13) and Hökfelt *et al.* (14) was used with minor modifications. Male Sprague–Dawley rats (150–300 g) were perfused through the ascending aorta with 100 ml of cold phosphate-buffered saline followed by 500–750 ml of ice-cold 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). Rats that received vinblastine (20 μg in 10 μl of saline) into the lateral ventricle and were killed 1, 2, or 6 days after injection were also processed as above. The descending aorta was clamped off. The brains were placed in a brain slicer and 3-mm slices were fixed in 4% paraformaldehyde (4°C) for 90 min and washed in 5% sucrose in phosphate-buffered saline (24 hr at 4°C). The tissue slices were frozen on metal chucks and 20-μm sections were cut in a cryostat (−15°C) and mounted on chrome/alum-coated slides. The slides were dipped into 0.2% Triton X-100 in phosphate-buffered saline for 15 min and incubated in a humidity box for 60 min at 37°C with α-MSH antiserum (diluted 1:75 with 0.3% Triton X-100 in phosphate-buffered saline). The slides were washed three times for 5 min each time in 0.2% Triton X-100 in phosphate-buffered saline (4°C) and then incubated for 15 min with fluorescein-labeled goat antiserum (Cappel, Downington, PA) to rabbit IgG (0.04 mg/ml) diluted 1:90 with phosphate-buffered saline containing 0.3% Triton X-100. They were washed for a fourth time in phosphate-buffered saline and subsequently mounted in glycerine/phosphate-buffered saline (3:1). Sections were examined under a Leitz Orthoplan fluorescence microscope equipped with a Ploem illuminator.

RESULTS

α-MSH immunoreactivity was observed within discrete varicose nerve fibers (Fig. 1), giving the typical beaded appearance observed with the catecholamine histofluorescence technique. α-MSH was primarily located in the hypothalamus, preoptic and septal areas, mammillary body, and periaqueductal central gray. A mapping of the localization of the α-MSH immunoreactive fibers is presented in Figs. 2 and 3. The fibers are shown as bold dashed lines or groups of dots. The relative densities (sparse, moderate, or dense) of α-MSH fibers are indicated by the separation of dots and dashes, a convention for which allowance must be made for illustration of small anatomical structures. Frequently, a distinct directionality of fiber movement was observed and is indicated on the maps by the orientation of the dashed lines. The actual direction of fiber projections (e.g., rostro-caudal or medio-lateral) cannot be stated with certainty.

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Abbreviations: α-MSH, α-melanocyte stimulating hormone (α-melanotropin); ACTH, corticotropin.

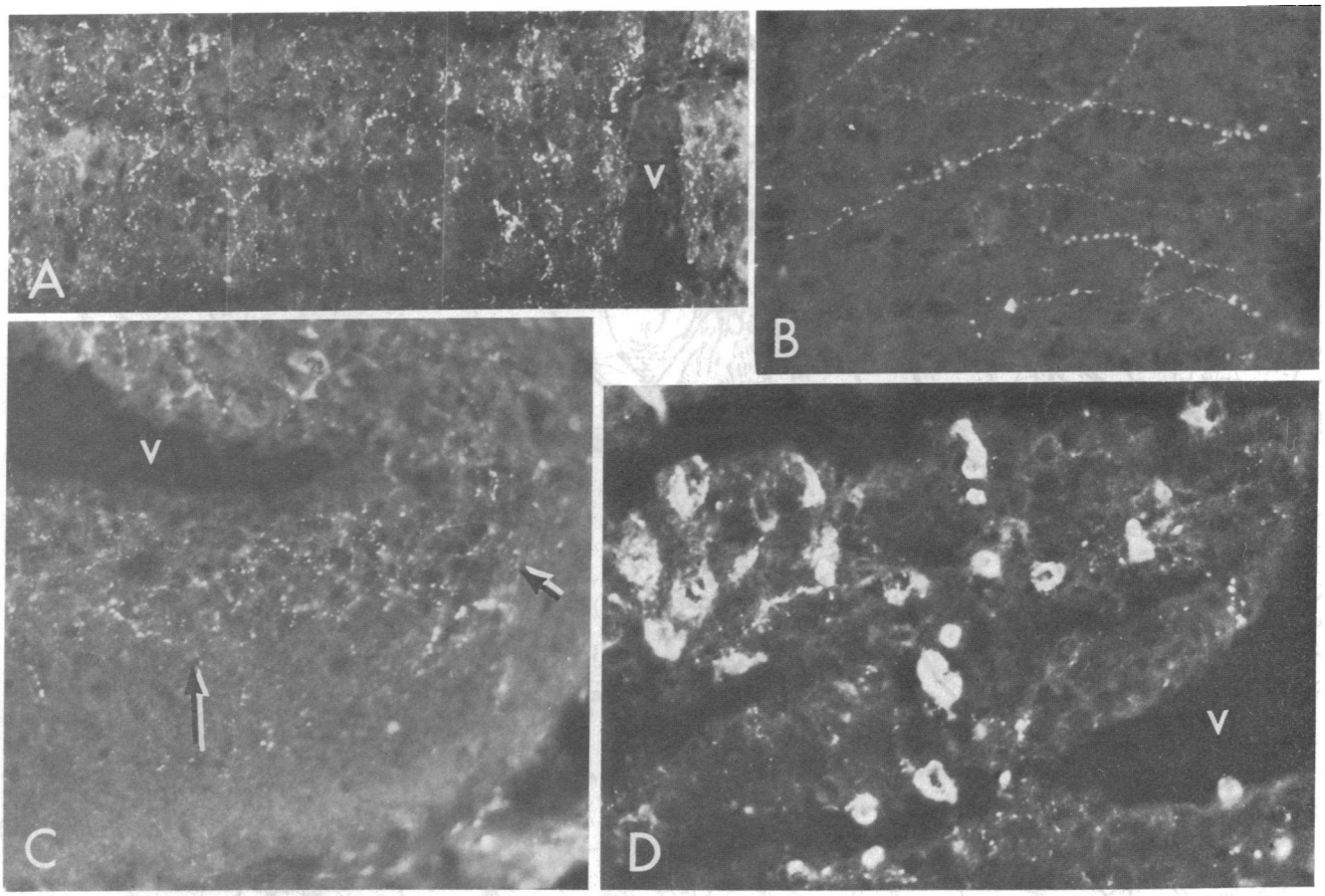

FIG. 1. (*A*) Heavy concentration of α-MSH varicose fibers in the anterior hypothalamic nucleus. Periventricular nucleus adjacent to the third ventricle (v) also shows dense accumulation of fibers (approximate coordinate A6000). (×180.) (*B*) Long varicose α-MSH-containing processes in the supramammillary decussation just medial to the medial forebrain bundle (level A2500). (×320.) (*C*) Median eminence. α-MSH fibers are noted (arrows) in the internal layer ventral to the lateral aspect of the third ventricle (v). (×320.) (*D*) α-MSH-containing perikarya in the arcuate nucleus after vinblastine treatment (20 μg, 1 day) (level A4800, v-third ventricle). (×350.)

Forebrain

Within the septal region, a moderate number of α-MSH-containing fibers was observed medial to the nucleus accumbens in the tractus septohypothalamicus and tractus diagonalis that seem to terminate in the lateral septal nuclei (Fig. 2 *a–c*). These projections appeared to emanate more caudally from the medial forebrain bundle (Figs. 2 *b* and *c*). A heavy fiber distribution was present in the dorsal and ventral part of the nucleus interstitialis stria terminalis (Fig. 2 *c–e*) and appeared to contribute or receive fibers from the stria terminalis (Fig. 2 *d* and *e*).

In the preoptic area and hypothalamus a dense accumulation of α-MSH fibers was observed in the medial preoptic, periventricular, and anterior hypothalamic nuclei (Fig. 1*a*) and the nucleus dorsomedialis (Fig. 2 *d–i*). Moderate numbers of fibers were observed in the lateral preoptic nucleus (Fig. 2*d*) and the caudal level of the medial forebrain bundle (Fig. 2 *h–l*). The orientation of fibers suggested a medio-lateral or dorso-ventral projection system via the medial forebrain bundle. Another system of fibers appeared to course within the dorsal supraoptic commissure (CSDV) towards the medial forebrain bundle (Fig. 2 *h–m*). The fibers at this level were oriented in a dorso-medial and ventro-medial direction. A large tract-like formation of fibers was also seen in the ansa lenticularis (Fig. 2 *e–g*). This formation appeared to project medial to the internal capsule towards the strial terminalis (Fig. 2*e*) and paraventricular nucleus (Fig. 2*g*), which contained a moderate number of fibers.

At the level of the posterior hypothalamus (Fig. 2 *j–l*) and the mammillary body (Fig. 2*m*) a rostro-caudal projection of fibers was noted along the substantia grisea centralis (central gray) and periventricular area adjacent to the third ventricle (Fig. 2 *j–m*). This projection seems to be continuous with more ventral fibers that pass through the supramammillary decussation (Figs. 1*B* and 2*m*), between the crus cerebri and zona incerta at the level of the Forel's field h2 (Fig. 2 *k* and *l*) and the ventral and medial aspect of the medial forebrain bundle (Fig. 2 *j–m*). This major projection extends rostrally through the thalamus, medial and ventral to the fasciculus retroflexus (Fig. 2 *j* and *k*), and appears to project through the nucleus subparafascicularis and the nucleus reuniens into the dorsomedialis and periventricular nuclei (Fig. 2 *h* and *i*). In addition, a dense projection of fibers was observed within and ventral to the zona incerta (Fig. 2 *h* and *i*), which appears to be continuous with the dorsomedial and periventricular nuclei.

In the amygdala, numerous fibers appeared to project ventrally from the stria terminalis along the lateral aspect of the medial amygdaloid nucleus and the medial part of the central amygdaloid nucleus to the basal amygdaloid nucleus (Fig. 2 *h–k*).

The thalamus contained a heavy concentration of varicosities in the nucleus preventricularis thalami (rotundocellularis) (Fig. 2 *e–k*) and a moderate density of fibers in the ventro-medial part of the nucleus rhomboideus (Fig. 2*h*).

In the median eminence, moderate numbers of α-MSH fibers were located in the internal layer (Figs. 1*C* and 2 *h–j*), with

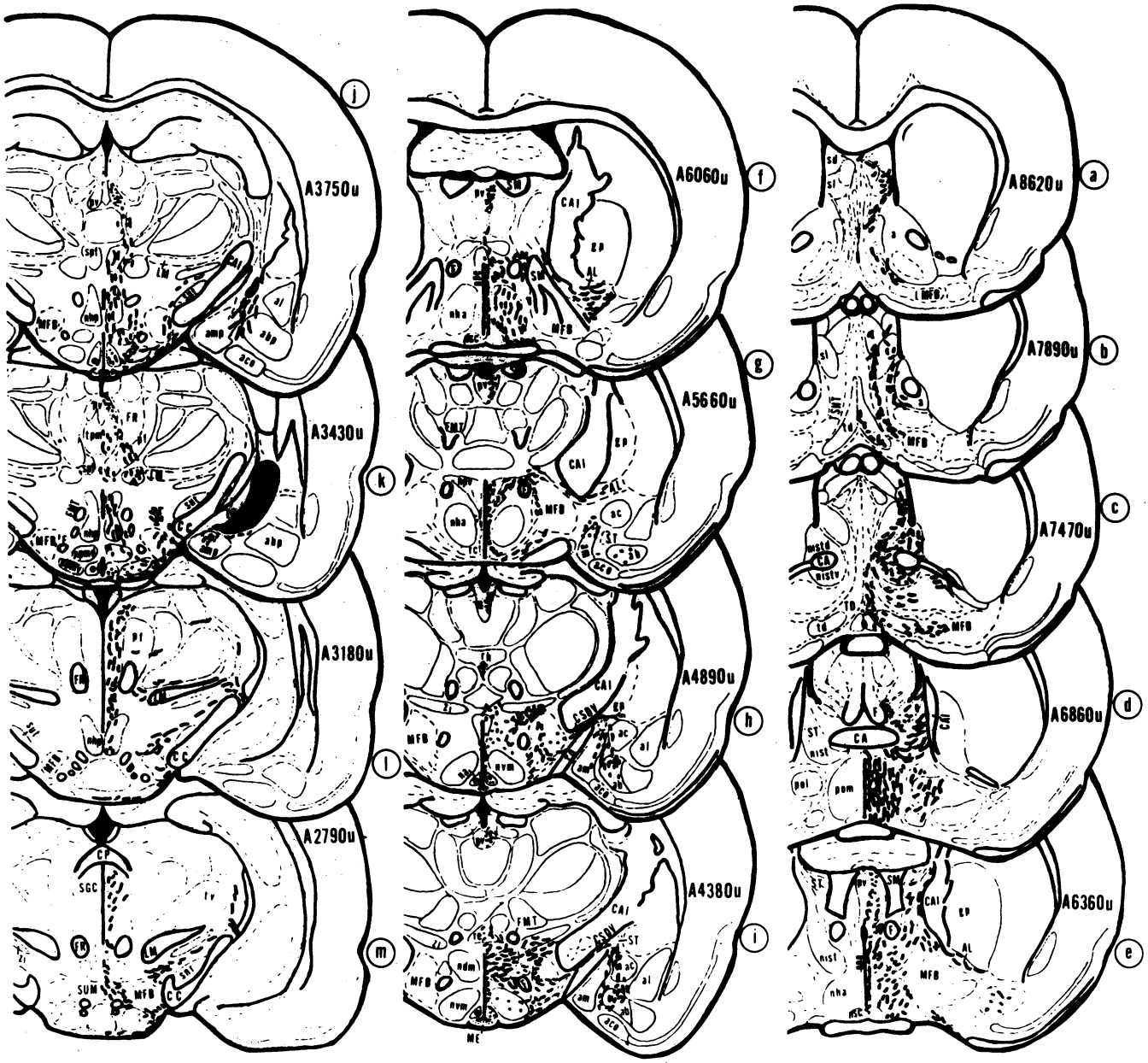

FIG. 2. Schematic frontal sections of α-MSH-containing fibers. Coordinates (right side of drawings) are taken from the topographic atlases of König and Klippel (15) and Jacobowitz and Palkovits (16), which should also be consulted for undesignated areas. α-MSH fibers are shown as dark dashed lines or dotted accumulations. Cell bodies are filled circles. a, n. accumbens; ab, n. amygdaloideus basalis; abp, n. amygdaloideus basalis posterior; ac, n. amygdaloideus centralis; aco, n. amygdaloideus corticalis; AL, ansa lenticularis; al, n. amygdaloideus lateralis; am, n. amygdaloideus medialis; amp, n. amygdaloideus medialis posterior; CA, commissura anterior; CAI, capsula interna; CC, crus cerebri; CP, commissura posterior; CSDD, commissura supraoptica dorsalis, pars dorsalis (Ganser); CSDV, commissura supraoptica dorsalis, pars ventralis (Meynert); F, fornix; FMT, fasciculus mamillothalamicus; FR, fasciculus retroflexus; gp, globus pallidus; LM, lemniscus medialis; ME, median eminence; MFB, fasciculus medialis prosencephali (medial forebrain bundle); na, n. arcuatus; ndm, n. dorsomedialis; nha, n. hypothalamicus anterior; nhp, n. hypothalamicus posterior; nist, n. interstitialis striae terminalis; nistd, n. interstitalis striae terminalis pars dorsalis; nistv, n. interstitialis striae terminalis pars ventralis; npe, n. periventricularis hypothalami; npmd, n. premamillaris dorsalis; npmv, n. premammillaris ventralis; npv, n. paraventricularis; nsc, n. suprachiasmaticus; nvm, n. ventromedialis; pf, n. parafascicularis; pol, n. preopticus lateralis; pom, n. preopticus medialis; pv, n. periventricularis thalami; rc, area retrochiasmatica; re, n. reuniens; rh, n. rhoboideus; sd, n. dorsalis septi; SGC, substantia grisea centralis; sl, n. lateralis septi; SM, stria medullaris thalami; snr, substantia nigra zona reticularis; spf, n. subparafascicularis; ST, stria terminalis; SUM, decussatio supramamillaris; sut, n. subthalamicus; TD, tractus diagonalis Broca; td, n. tractus diagonalis Broca; TO, tractus opticus; tpm, n. posteromedianus thalami; TSHT, tractus septohypothalamicus; zi, zona incerta.

occasional fibers that penetrated into the external layers. A more heavy concentration of fibers was contained in the lateral part of the median eminence just ventral to the arcuate nucleus.

Normally only a sparse number of faint fluorescent cell bodies were seen in the arcuate nucleus. After vinblastine treatment, however, numerous intense fluorescent cell bodies were observed in the entire rostro-caudal extent of the arcuate nucleus (Figs. 1*D* and 2 *h–k*).

Hindbrain

At the level of the mesencephalon and pons, many fibers were observed in the substantia grisea centralis adjacent to the cerebral aqueduct (Fig. 3 *a–e*). There is a medio-lateral projection of fibers through the nucleus cuneiformis (Fig. 3 *b* and *c*) towards a region just ventral to the medial geniculate body (Fig. 3 *a* and *b*) and the red nucleus (Fig. 3 *b* and *c*). Some fibers were

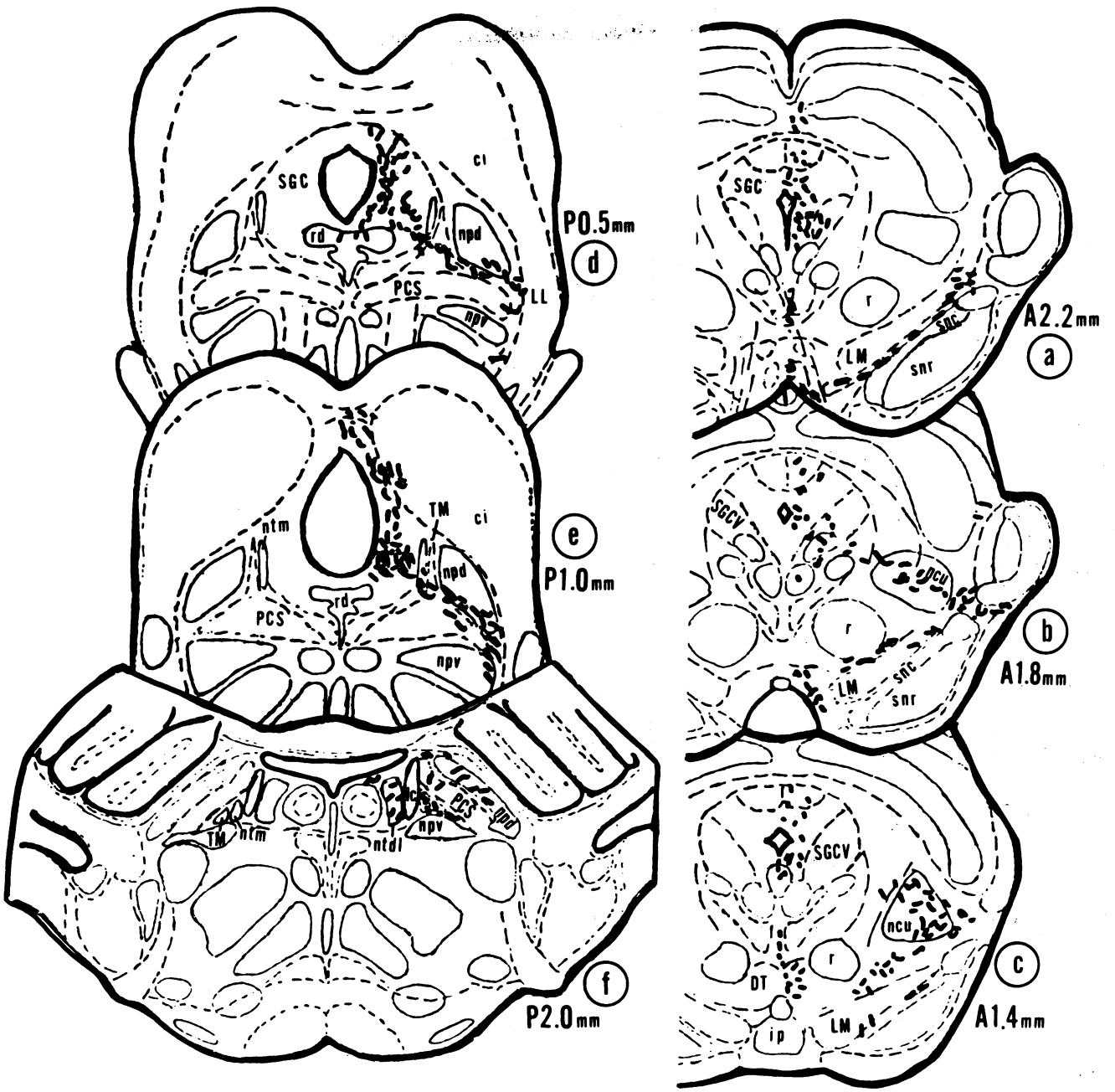

FIG. 3. See legend for Fig. 2. Coordinates are taken from the atlas of Palkovits and Jacobowitz (17). ci, Colliculus inferior; DT, decussationes tegmenti; ip, n. interpeduncularis; lc, locus coeruleus; LL, lemniscus lateralis; LM, lemniscus medialis; ncu, n. cuneiformis; npd, n. parabrachialis dorsalis; npv, n. parabrachialis ventralis; ntd, n. tegmenti dorsalis Gudden; ntm, n. tractus mesencephali; PCS, pedunculus cerebellaris superior; r, n. ruber; rd, n. raphe dorsalis; SGC, substantia grisea centralis; SGCV, substantia grisea centralis ventralis; snc, substantia nigra zona compacta; snr, substantia nigra zona reticularis; TM, tractus mesencephalicus nervi trigemini.

found above the substantia nigra zona compacta (Fig. 3 *a–c*) and in the midline above and lateral to the interpeduncular nucleus (Fig. 3 *b* and *c*). At the level of the inferior colliculus, the large medio-lateral projection is located between the nucleus parabrachialis dorsalis and the superior cerebellar peduncle (Fig. 3 *d* and *e*).

The locus coeruleus contained very few, if any, immunoreactive fibers. However, the region surrounding the locus coeruleus (nucleus tegmenti dorsalis lateralis, nucleus parabrachialis dorsalis, nucleus tractus mesencephali, tractus mesencephalicus nervi trigemini, and superior cerebellar peduncle) contained moderate numbers of fibers (Fig. 3*f*). In the medulla a moderate density of fibers was observed in the nucleus tractus solitarii. Only very sparse numbers of fibers were seen in the reticular formation. No fibers were found in the spinal cord.

DISCUSSION

A degree of caution is warranted in interpreting results of immunohistochemical studies (14). Characterization of the α-MSH antiserum used in this study revealed a high specificity for synthetic α-MSH with essentially no crossreactivity with ACTH or β-lipotropin. However, the presence of an unknown α-MSH-like substance cannot be ruled out. Therefore, for purposes of simplicity we refer to α-MSH immunoreactive neurites as "α-MSH fibers."

This study describes a restrictive distribution of α-MSH-containing fibers throughout the brainstem, with greatest concentration in the hypothalamus and preoptic area. No nerves were observed in the cerebral cortex, cerebellum, hippocampus, and spinal cord.

There is a remarkable similarity between the localization of α-MSH fibers and β-lipotropin-containing processes described by Watson *et al.* (18). Differences, however, were observed in the locus coeruleus and substantia nigra zona compacta, where heavy and moderate concentrations of β-lipotropin fibers were found, respectively, in contrast to essentially none in the present study. In the above report, crossreactivity studies were not described for α-MSH. If no such crossreactivity exists, it remains an intriguing possibility that both β-lipotropin and α-MSH are contained within identical neurons.

As previously noted for β-lipotropin fibers (18), a remarkable coincidence of α-MSH fibers and noradrenergic axonal projections exists (16, 17, 19). Likewise, the proximity of α-MSH and noradrenergic fibers suggests a possible α-MSH- aminergic interaction. Further, dopaminergic–α-MSH interaction at the pituitary level has been described (20). A noradrenergic–α-MSH interaction has been suggested from stereochemical molecular comparison of norepinephrine and α-MSH (21), where a serine-tyrosine catecholamine-like configuration at the amino terminus of the α-MSH molecule was noted. It was therefore proposed that a competitive or agonist relationship may exist between the catecholamine molecule and peptidergic nerves or cells.

α-MSH-containing cell bodies were observed only in the arcuate nucleus. Localization of cell bodies in the confines of the arcuate nucleus was also reported with immunoreactive β-lipotropin (18). α-MSH was also recently identified in vesicles within arcuate nuclei cell bodies and a few nerve fibers of the hypothalamus by using the peroxidase-antiperoxidase procedure for identification of the peptide at the ultrastructural level (22).

The extensive distribution of α-MSH in the brain suggests that it is involved in significant neuronal circuitry, supports the notion of a neuroregulatory role for this neuropeptide, and lays the groundwork for a rational approach for further study of possible interactions of α-MSH with other neuronal systems.

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