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Neurofilaments contain α-melanocyte-stimulating hormone (α-MSH)-like immunoreactivity

(intermediate filaments/retina/pituitary)

URSULA C. DRÄGER*, D. LOUISE EDWARDS*, AND JOCHEN KLEINSCHMIDT†

*Department of Neurobiology, Harvard Medical School, Boston, MA 02115; and †Department of Neurobiology and Behavior, State University of New York at Stony Brook, Stony Brook, NY 11794

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ABSTRACT **An antiserum to α-melanocyte-stimulating hormone (α-MSH) was found to contain antibodies to at least two types of determinants on the α-MSH peptide: one is present only on the free peptide, the other is shared with neurofilaments. Immunoblots from mouse brain showed the neurofilament crossreactivity to be located on proteins in the M_r 140,000 range. The neurofilament-crossreactive portion of the antiserum could be selectively absorbed out with a cytoskeletal preparation, which abolished all affinity of the antiserum to the retina but did not affect the labeling pattern in the pituitary. Absorptions with desacetyl-α-MSH and corticotropin seemed to indicate that the determinant shared with neurofilaments is not located at either end of the α-MSH peptide, but somewhere in between. The immunohistochemical labeling of the retina with the α-MSH antiserum was compared to the labeling with monoclonal antibodies against M_r 200,000 neurofilaments. In the adult retina the α-MSH-like immunoreactivity was found to be slightly more widespread; most consistently it was detectable in cell bodies of large ganglion cells, whereas the heavy neurofilament subunit was absent from somata and proximal axons of these cells. In the developing mouse brain, expression of the heavy subunit was found to lag 2–3 wk behind expression of the M_r 140,000 proteins. This confirms previous reports of a more restricted distribution and late expression of high molecular weight neurofilaments as compared to the lower subunits.**

α-Melanocyte-stimulating hormone (α-MSH) consists of the first 13 amino acids of corticotropin (ACTH), acetylated on the NH_2-terminal and amidated on the COOH-terminal ends; in addition, desacetylated and diacetylated forms have been found (1–4). It is one of several pro-opio-melanocortin-derived peptide hormones, the melanotropins, named originally for their effects on skin pigmentation: stimulation of pigment migration in cold-blooded vertebrates and increase of melanin synthesis in mammals (5–9). More recently pronounced effects of α-MSH and related peptides on complex nervous functions are being stressed, including reactions that involve arousal, visual attention, and memory, in addition to growth hormone-like effects in the embryo (8, 10–12).

The localization of α-MSH in the central nervous system has been studied with biochemical and immunohistochemical methods (1, 8, 13–20). The immunohistochemical descriptions seem to fall into two main categories, presumably reflecting two main types of α-MSH antisera. In the first type α-MSH-like immunoreactivity is found primarily in the pituitary intermediate lobe, in addition to a few cell bodies in the arcuate nucleus of the hypothalamus and sparse fibers throughout the brain stem (e.g., see ref. 16). In the second type of studies α-MSH antisera are found to label fibers and cell bodies throughout the central nervous system (14, 17, 19, 20) in a distribution that reminded us of the labeling pattern with neurofilament antisera (21, 22).

In mammalian neurons, neurofilaments consist of a triplet of intermediate-filament polypeptides having M_r values of approximately 68,000, 145,000, and 200,000 (23). In the present study we examined the relationship of the α-MSH-like immunoreactivity to neurofilaments by comparing the retinal labeling with an α-MSH antiserum of the second type to the labeling with monoclonal antibodies directed against the heavy subunit of the neurofilament triplet. The histological labeling in the retina was indicative of an affinity of the α-MSH antiserum to lower M_r neurofilament subunits, and immunoblots showed a crossreactivity with intermediate M_r neurofilament proteins.

METHODS

Antibodies. Antisera to α-MSH and ACTH were purchased from Immuno Nuclear (Stillwater, MN; lot numbers 31261 and 11151). As neurofilament marker we used mainly the monoclonal antibody R3 that reacts strongly with high M_r neurofilaments (unpublished data). For retinas other than of the mouse we used also the monoclonal antibody RT97 of Anderton *et al.* (24) in conjunction with the α-MSH antiserum. Like R3 this antibody recognizes strongly the heavy neurofilament subunit, but it shows a wider crossreactivity between species. The Pruss hybridoma line generating an antibody against a common determinant on all intermediate filaments (25) was purchased from American Type Culture Collection.

Histology. C57BL/6J mice were perfused through the heart with 4% buffered paraformaldehyde. The retinas, brains, and pituitaries were cut at 7 or 10 μm on a cryostat. Sections were mounted on gelatinized slides and incubated with antibodies in 0.5% Triton X-100. Supernatants of R3 were used straight; ascites of R3 and RT97 were diluted 1:50 to 1:100. The α-MSH antiserum was usually diluted 1:200, but its histological staining, both in the retina as in the pituitary, was still visible at dilutions of more than 1:50,000. Antibody binding in the tissue was made visible with fluorescein isothiocyanate (FITC)- and rhodamine isothiocyanate (RITC)-labeled secondary antibodies (Boehringer Mannheim; Cappel Laboratories, Cochranville, PA).

Immunoblots. Either total mouse brain or a cytoskeletal extract of mouse cerebellum was used for immunoblots. Cytoskeletal preparations were made following the protocol of Chiu *et al.* (26). Proteins were denatured in $NaDodSO_4$ and separated according to M_r on 5–15% linear gradient $NaDodSO_4$/polyacrylamide gels (27). The gels were transferred electrophoretically onto nitrocellulose filters (28), and the filters were

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Abbreviations: FITC, fluorescein isothiocyanate; RITC, rhodamine isothiocyanate; MSH, melanocyte-stimulating hormone; ACTH, corticotropin.

reacted for antibody binding by using horseradish peroxidase-conjugated secondary antibody (Cappel Laboratories). The α-MSH antiserum was diluted 1:500.

Absorptions. Absorptions were done at 4°C overnight. One microliter of α-MSH antiserum was incubated with the following synthetic peptides at several concentrations: α-MSH peptide (Sigma), 5–50 μg; desacetyl-α-MSH (Peninsula Laboratories, San Carlos, CA), 5–200 μg; monkey β-MSH (Peninsula Laboratories), 10–100 μg; γ-MSH (Peninsula Laboratories), 10–100 μg; ACTH, 10–40 μg. For the absorption of the α-MSH antiserum with the cytoskeletal preparation an excess of protein was used (500 μg with 2 μl of antiserum) and the mixture was stirred in a homogenizer because of the insolubility of the preparation.

RESULTS

Fig. 1 shows a section of mouse retina, double-labeled with the α-MSH antiserum and the monoclonal antibody R3 that is directed against the heavy neurofilament subunit; only neurons are labeled. In the outer plexiform layer both antibodies labeled horizontal processes identically, as could be best seen in horizontal sections through this layer, examined at high magnifications (not shown). Previously we identified these processes as the dendrites of axonless horizontal cells (ref. 29 and unpublished data). In the inner plexiform layer the two antibodies labeled a largely different subset of processes; those containing α-MSH-like immunoreactivity formed a plexus in the middle of the layer, whereas the R-3 positive fibers were not arranged in a clear laminar fashion. Only some of the processes in this layer were double-labeled with both antibodies. In the inner row of cells of the inner nuclear layer a few cell bodies stood out, some of which resembled amacrine cells in shape and distribution and others displaced ganglion cells (30, 31). Amacrine cells could be labeled by either of the antibodies and some by both, but displaced ganglion cells, only by the α-MSH antiserum (see arrows in Fig. 1).

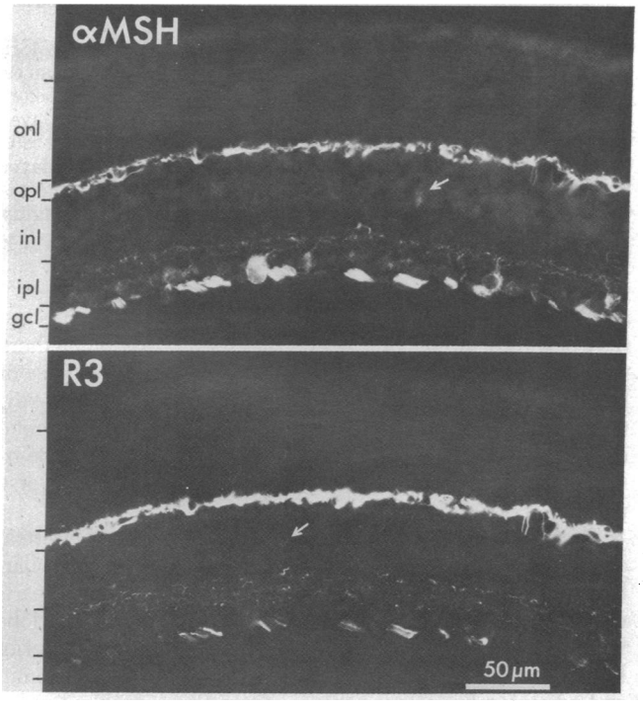

FIG. 1. Transverse section through adult mouse retina double-labeled with α-MSH antiserum and R3. Binding of the antiserum was made visible with RITC-labeled secondary antibody, and binding of R3, with FITC-labeled antibody. Retinal layers are indicated as: onl, outer nuclear layer; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer. Note that the cell bodies in the ganglion cell layer are only labeled by the antiserum but not by R3. Arrows point to a displaced ganglion cell labeled by the antiserum and to an amacrine cell labeled by R3.

In the ganglion cell layer usually only the α-MSH antiserum, but not R3, labeled cell bodies; most cells were lightly labeled, except for the largest ganglion cells, which showed heavy labeling. Optic axons were labeled by both antibodies, but to a different extent. Close to the optic disk the axonal labeling was rather similar. In more peripheral retinal locations many axons were still well labeled with the α-MSH antiserum, but the R3 labeling decreased in intensity and disappeared from all but a few axons. A horizontal section of the ganglion cell layer from an intermediate retinal location, double-labeled with both antibodies, is shown in Fig. 2. The difference in axonal labeling was most pronounced with respect to rare fibers projecting to the retina from elsewhere, the efferent fibers (unpublished data). Both antibodies labeled these fibers, but only with R3 did they stand out appreciably in intensity over optic axons (not shown here). Although the axonal labeling with the two antibodies was rather similar close to the optic disk, it was never identical, as could be best seen in cross sections through the optic tract (Fig. 3). R3 consistently accentuated the large axons, whereas the α-MSH antiserum seemed to label most or all axons with similar intensity.

The difference between the two antibodies was even more pronounced in the developing brain than in the adult. At embryonic day 12, the earliest age tested, fibrillar neuronal components were labeled throughout the nervous system by the α-MSH antiserum but very little by R3. In newborn mice the retina showed bright fibrillar α-MSH-like immunoreactivity but still only very faint traces of the R3 antigen. Sections of a retina from a 1-day-old mouse are shown in Fig. 4. The α-MSH antiserum labeled heavily the developing axonless horizontal cells

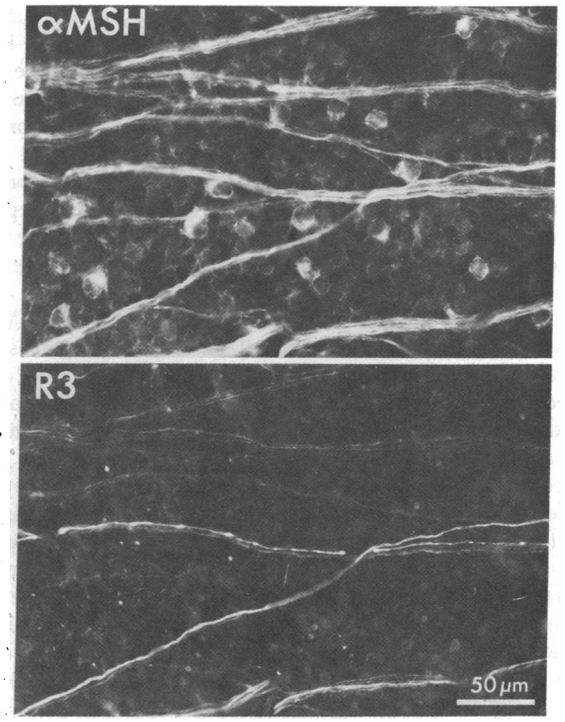

FIG. 2. Horizontal section of the ganglion cell layer double-labeled with α-MSH antiserum and R3. Cell bodies are only recognized by the antiserum; optic axons are labeled by both antibodies, but to a different extent.

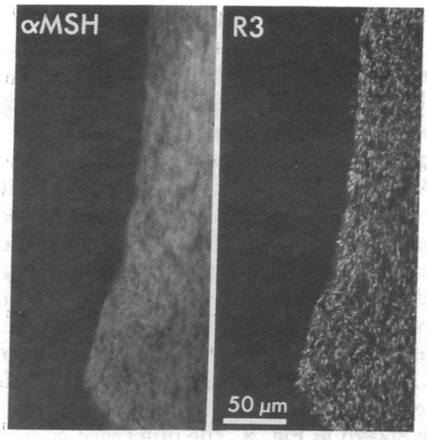

FIG. 3. Section through optic tract double-labeled with α-MSH antiserum and R3. The flat appearance of the tract in the α-MSH-labeled aspect reflects homogeneous labeling of most or all optic axons.

in the outer retina (arrows) and cell bodies and optic axons in the inner retina. In the R3-labeled section the axonless horizontal cells can barely be distinguished (arrows); in addition, but not very obvious here, optic axons close to the disk, as well as a few cell bodies in the optic fiber layer and in the ventricular zone, were weakly labeled. Only about 10 days to 2 wk later did the R3 labeling in the retina reach intensity levels comparable to those in the adult.

For a general comparison, the α-MSH-like immunoreactivity was expressed earlier in development than the M_r 200,000 neurofilaments recognized by R3. In the adult it was somewhat more abundant than the R3–antigen, and it was distributed more uniformly throughout the extent of some cells, whereas the R3–antigen tended to be absent from proximal regions of cells. Most structures positive for R3 were also labeled with the α-MSH antiserum, except for the ventricular cells in the perinatal retina and some fibers in the inner plexiform layer of the mature retina, some of which resembled the dendrites of wide-field amacrine cells (31). The distribution of the α-MSH-like immunoreactivity resembled the staining pattern with neurofibrillar methods in the retina (32–34). The Bodian neurofibrillar method has been shown to stain all three neurofilament subunits (35). The more restricted distribution of the R3 labeling and its later ontogenetic appearance is consistent with reports on a more restricted distribution and late expression of the high M_r neurofilament protein, as compared to the two lower M_r subunits (36, 37).

In addition to the retina of the mouse, we tested the α-MSH antiserum on retinas from a variety of vertebrate species, including chicken, ox, rat, rabbit, cat, and monkey, and compared it to the labeling with the monoclonal neurofilament antibody RT97 (24). In all cases an α-MSH-like immunoreactivity was associated with filamentous components of neurons reminiscent of neurofibrillar staining patterns. In the monkey the nuclei of some cells, in particular of cones, were also lightly labeled. The most consistent difference between the α-MSH-like immunoreactivity and the labeling patterns with the monoclonal neurofilament antibodies was with respect to large ganglion cells; as in the mouse the α-MSH antiserum, but not the monoclonal antibodies, labeled the soma region of these cells. This difference was most pronounced for a small population of giant ganglion cells in the bovine retina; it was quite prominent with respect to the large displaced ganglion cells in the bird retina, but it was relatively subtle in the rat retina. In the rabbit the most conspicuously labeled cell bodies in the inner retina were those of the class of amacrine cells studied by Vaney *et al.* (38). However, these amacrine cells were labeled by both

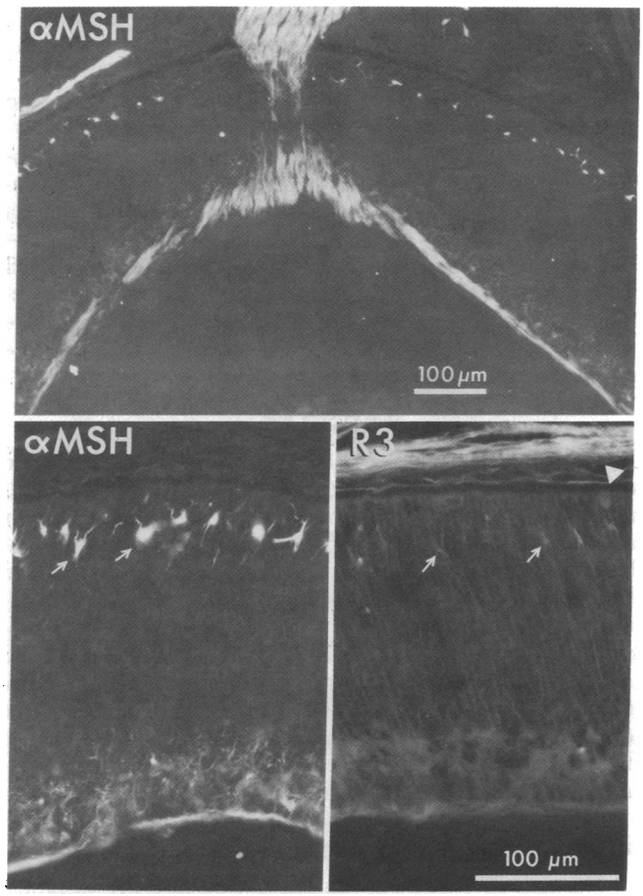

FIG. 4. Sections of newborn mouse retina labeled either with α-MSH antiserum or R3. The labeling of optic axons with the antiserum can be best seen in the low-power view (*Upper*). Higher magnification (*Lower Left*) shows the labeling with the antiserum of the inner plexiform layer that is demarcated at this age; the immature axonless horizontal cells (arrows) are still mostly radially oriented, as the outer plexiform layer is not segregated yet (*Lower Left*). In the R3-labeled section the axonless horizontal cells can be barely distinguished (arrows). A faintly labeled ventricular cell is indicated by a triangle. The heavy scleral labeling is an artifact due to binding of secondary antibody.

antibodies, whereas the ganglion cells, as in the other species, were only recognized by the peptide antiserum. In addition there were substantial differences between species in the details of labeling; for instance, in the outer retina of bird and monkey, no horizontal cell processes were labeled; in rat the horizontal cells were well labeled with RT97, but very poorly with the α-MSH-antiserum, whereas the bovine retina showed the reverse pattern.

Though all labeling with the α-MSH antiserum in the retina, as in most of the brain of the mouse, appeared neurofibrillar-like, a very different pattern was seen in the pituitary and parts of the hypothalamus. All cells in the intermediate pituitary lobe were almost solidly stained, and scattered cells in the anterior lobe contained labeled granules (Fig. 5). In the hypothalamus and adjoining brainstem regions sparse fibers with a beaded appearance were heavily stained by the α-MSH antiserum (not shown). These fibers were readily distinguished from those with continuous neurofilamentous-like labeling. Both types of labeling, the neurofibrillar-like as well as the peptide-like type, were totally prevented by absorption of the α-MSH antiserum with synthetic α-MSH at the lowest concentrations of peptide used.

To distinguish the components of the α-MSH antiserum responsible for the two types of histological staining, we made a

FIG. 5. Section through the mouse pituitary, labeled with α-MSH antiserum that had been absorbed with a cytoskeletal preparation. Untreated antiserum gave an identical labeling pattern. Note the heavy labeling of the intermediate lobe and scattered labeled cells in the anterior lobe (left side).

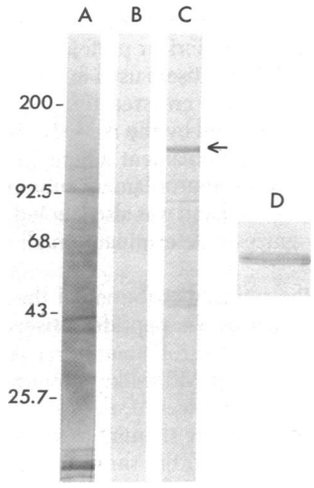

FIG. 6. Immunoblots of cytoskeletal proteins prepared from mouse cerebellum. Lanes: A, proteins stained with amido black; B, immunoperoxidase staining with α-MSH antiserum preabsorbed with α-MSH peptide; C, immunoperoxidase staining with α-MSH antiserum; D, enlargement of M_r 140,000 region marked by arrow in lane C. M_r standards are indicated on left: myosin, 200,000; phosphorylase b, 92,500; bovine serum albumin, 68,000; ovalbumin, 43,000; α-chymotrypsinogen, 27,500.

Triton X-100-insoluble cytoskeletal preparation from mouse brain (26). With this preparation it was possible to absorb out all of the neurofibrillar-like affinities from the α-MSH antiserum, while the labeling of the pituitary and hypothalamus was only slightly reduced in overall intensity. The section of the pituitary shown in Fig. 5 was in fact labeled with such an absorbed antiserum. Such differential absorption can be explained by the presence of two types of antibodies in the α-MSH antiserum against two types of determinants on the α-MSH peptide. One type is present both on the free α-MSH peptide as well as on neurofibrillar components; the other is present only on the free peptide.

To narrow down the location of the site on the α-MSH peptide that is recognized by the antibodies crossreactive with neurofibrillar components, we studied several related peptides: ACTH, desacetyl-α-MSH, β-MSH, and γ-MSH. ACTH contains the amino acid sequence of α-MSH, but it lacks the NH_2-terminal acetyl group; desacetyl-α-MSH consists of the first 13 amino acids of ACTH with an amidated COOH-terminal; β-MSH and γ-MSH share a core sequence with α-MSH (8). An antiserum to ACTH did not label neurofibrillar components in retina or brain. The labeling in the pituitary was very similar to that with the α-MSH antiserum, except that the scattered cells in the anterior lobe were slightly more pronounced. Absorption of the ACTH antiserum with synthetic ACTH (10 μg/μl of ACTH antiserum) eliminated all labeling, whereas absorption with synthetic α-MSH (40 μg/μl) had little histological effect. Absorption of the α-MSH antiserum with synthetic ACTH at relatively high concentrations of peptide (40 μg/μl of antiserum) had similar effects as absorption with the cytoskeletal preparation: the neurofibrillar staining in the retina was abolished, but the pituitary labeling both in the anterior and intermediate lobes was hardly affected. For synthetic desacetyl-α-MSH very high peptide concentrations were needed to obtain a differential absorption effect: with 100–200 μg of peptide per μl of α-MSH antiserum the retinal affinity could be eliminated; however, the pituitary labeling was substantially reduced in intensity, and it appeared to have become more selective for particular sites. Absorptions of the α-MSH antiserum with β-MSH or γ-MSH had no obvious effect. (Because of the lack of any effect these experiments lacked an internal control; however, we did verify with the *Anolis* skin test that the β-MSH and γ-MSH preparations used had melanotropic activity; ref. 39.) We interpret the absorption experiments to indicate that the antigenic determinant on the peptide shared with neurofibrillar components is probably not located at either end of the peptide, but somewhere in between, and it does not appear to involve the core sequence common to all melanotropic peptides. It is present on ACTH and it is also accessible to antibodies, because the neurofibrillar-crossreactive antibodies could be absorbed out from the α-MSH antiserum with ACTH, but it is probably not very immunogenic in the context of the ACTH molecule.

To determine the neurofibrillar components recognized by the peptide antiserum, we separated total brain proteins, as well as cytoskeletal preparations of mouse brain, on $NaDodSO_4$/polyacrylamide gels, transferred them onto nitrocellulose paper, and tested for antibody binding. On such blots the antiserum bound heavily to a region in the M_r 140,000 range, at the same M_r as a band labeled by the antibody of Pruss *et al.* (25) against a common determinant on all intermediate filaments (not shown). In addition, when cytoskeletal preparations were used, a lighter band around M_r 90,000 and several faint bands between M_r values of 43,000 and 55,000 were stained (Fig. 6); these additional bands were not visible on blots of whole brains. α-MSH peptide, which was not present in the cytoskeletal preparations, has a M_r of 1,665 and would not have been detected with the gel system used. The labeling in the M_r 140,000 region consisted of three bands, which correspond in M_r and spacing to the three neurofilament proteins in this region described in the mouse brain (40). The fainter bands at M_r 90,000 and M_r 43,000–45,000 probably represent degradation products, because similar bands are labeled with an antiserum specific for intermediate M_r neurofilaments (41). All labeling on the blots was prevented by preabsorption of the antiserum with synthetic α-MSH.

DISCUSSION

We have shown evidence that a commercial α-MSH antiserum contains antibodies against at least two types of determinants, both of which are present on synthetic α-MSH peptide. One

type is present only on free peptide; the other type is shared with determinants on neurofibrillar proteins in the mouse brain. Some, but not all, α-MSH antisera used in previous studies seem to contain the neurofibrillar crossreactive portion (14, 16, 17). The determinant recognized by the α-MSH antiserum is probably on the M_r 140,000 neurofilament subunit in the mouse brain, because this was the only neurofilament subunit labeled on immunoblots, and the same band was also labeled by a monoclonal antibody that recognizes a determinant on all intermediate filaments (25).

We compared the retinal distribution of the intermediate M_r neurofilaments labeled by the peptide antiserum with the distribution of M_r 200,000 neurofilaments recognized by monoclonal antibodies. The α-MSH-like immunoreactivity confirmed the distribution of lower M_r neurofilament subunits, as predicted from neurofibrillar staining patterns and histological comparisons with antisera to the three subunits of the neurofilament triplet (32–34, 37). Whereas the heavy neurofilament subunit recognized by the monoclonal antibodies was largely absent from cell bodies and proximal axons of ganglion cells, the α-MSH antiserum stained some of these, in addition to most of the structures recognized by the monoclonal antibodies. However, there were also processes that were labeled by antibodies to the heavy subunit but did not contain α-MSH crossreactivity. This observation seems at odds with the general notion that all neurofilaments contain the two lower M_r subunits and that the heavy M_r subunit is more restricted in distribution and is never found without the two lower subunits (22, 36, 37, 42). We do not know how to explain this discrepancy. Possibly the site recognized by the α-MSH antiserum is inaccessible in some locations, or it is missing on some of the intermediate M_r proteins, which would be in agreement with Sternberger's findings of an unexpected diversity in histological labeling patterns with monoclonal antibodies against neurofilaments (43).

The crossreactivity of the α-MSH antiserum with sites on neurofilaments appears to be evolutionarily conserved through all warm-blooded vertebrate species that we tested, and probably even further. The question remains whether there is a functional significance to this strange crossreactivity. For the rabbit retina, Bauer and Ehinger (44) reported a pronounced and specific effect of α-MSH peptide on the release of dopamine and γ-aminobutyric acid. In their test system α-MSH was an excitant two orders of magnitude stronger than the strongest excitants known. However, we found that all α-MSH-like immunoreactivity in the retina could be abolished by preabsorption of the antiserum with a cytoskeletal preparation (we also tested this in the rabbit) and appears to be neurofilament-associated. With radioimmunoassay, Pourcho (19) found an α-MSH concentration four times that of serum in the retina, which is very low, presumably too low to be detected with the immunohistochemical techniques used. In many cases in which a pronounced physiological action of a compound has been observed, a natural ligand was eventually found. That in the retina not much free α-MSH appears to be present despite its pronounced physiological effects makes one wonder whether the crossreactivity of the α-MSH antiserum with neurofilaments may be of significance. However, it is difficult to see how large intracellular proteins as the neurofilaments, for which only a structural role is being considered, could relate to the communication between cells.

Note Added in Proof. Additional absorption experiments showed that the neurofilament-crossreactive portion of the α-MSH antiserum could be selectively removed by $ACTH_{1-10}$ and $ACTH_{1-4}$, but not by $ACTH_{4-11}$. Hence, the crossreactive site on the α-MSH peptide may be in its NH_2-terminal amino acid sequence.

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