Vol. 94, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 16, 1980 Pages 916-923

α MSH-LIKE PEPTIDES IN RAT BRAIN: IDENTIFICATION AND CHANGES IN LEVEL DURING DEVELOPMENT

Y. Peng Loh+, Robert L. Eskay* and Michael Brownstein*

Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development+ and Laboratory of Clinical Science National Institute of Mental Health*, National Institutes of Health, Bethesda, Maryland 20205

Received March 28,1980

SUMMARY: MSH-like peptides were extracted from rat brains and separated by high pressure liquid chromatography. Using C and N terminally directed antibodies, and a bioassay for melanotropic activity, two major melanotropic peptides were detected. One peptide was identified as αMSH and the other as des-acetyl αMSH, a form which has not been previously reported in brain. Analysis of the level of αMSH-like peptides in brain and pituitary gland during development, showed steady increases of pituitary αMSH from day 18 fetuses to adults, whereas in brain, significant increases were not observed until one day post partum. This difference in the time of onset of αMSH production in the two tissues suggests the presence of biosynthetically independant pools of αMSH-like peptides in pituitary and brain.

Alpha melanocyte stimulating hormone (αMSH) was discovered in high concentrations in the intermediate lobe of the pituitary gland; more recently, an extrahypophysial source in the brain has been reported, (16). Most of the brain αMSH-like peptides have been reported to originate in the arcuate nucleus of the hypothalamus (7-10) and subcellularly localized in synaptosomes (2,11,12), within secretory vesicles, (13,11). These peptides are released from synaptosomes with high K^+ stimulation (14), and they have been shown to have profound effects on the central nervous system (15,19). It has therefore been proposed that α MSH-like peptides may play an important role in interneuronal communication, at the synaptic level.

Characterization of the α MSH-like peptides in the brain (2,11) has revealed the presence of a peptide with electrophoretic, chromatographic and immunological properties, indistinguishable from synthetic α MSH (N Ac $ACTH_{1-13}$ NH_2). Loh et al (2), have reported the presence of another major melanotropic peptide,

Abbreviations: αMSH = melanocyte stimulating hormone, ACTH = adrenocorticotropic hormone, HPLC = high pressure liquid chromatography, TCA = trichloroacetic acid, RIA = radioimmunoassay, Ab = antibody, SEM = standard error of the mean

0006-291X/80/110916-08$01.00/0

in addition to αMSH, in rat brain. This peptide shared many properties with αMSH but differed in others. In this communication, we show the separation of two major melanotropic peptides from rat brain, using HPLC, and identify them as αMSH and des-acetyl αMSH. Differences in the level of these peptides in brain and pituitary gland during development is also reported.

MATERIALS AND METHODS:

Synthetic des-actyl αMSH ($ACTH_{1-13}$ NH_2), $ACTH_{1-10}$, $ACTH_{4-10}$ were kindly supplied by Dr. H. M. Greven (Organon International B.V., Oss, The Netherlands). Synthetic αMSH was a gift from Dr. W. Rittel (Ciba-Geigy Ltd., Basel, Switzerland). Rats of the Osborne-Mendel strain (250-300g) were supplied by the National Institutes of Health, Bethesda, Maryland. The lizard, Anolis carolinensis used in the bioassay was purchased from Rand McNally Inc., Somerset, Wisconsin. Solvents for HPLC and the C_{18} Sep-Pak cartridges were purchased from Waters Associates, Inc., Milford, Ma.. The C-terminal antibody against αMSH was kindly provided by Dr. Charles Oliver, (UER Medecine Nord, Marseille, France).

Rats were killed by decapitation and the brains removed and dropped into boiling 0.1N HCl for 10 mins. The brains were then homogenized in 0.1N HCl. Extraction of αMSH-like peptides and initial purification of these peptides on a Sephadex G_{10} column were as described previously (2). The αMSH-like peptides eluted from the Sephadex G_{10} column were purified on a Sep-Pak C_{18} cartridge. After the sample was applied on the cartridge, it was washed with 2ml of 1% acetic acid. The αMSH peptides were then eluted from the cartridge with 2ml of 80% methanol in 1% acetic acid. The methanol-acetic acid eluant was dried under N_2, resuspended in 1% acetic acid, and separated by HPLC, using a μBondapak C_{18} reversed-phase column (Waters Associates Inc., Milford, Ma.). The column was eluted with a linear gradient of 16% to 40% methanol in 1% acetic acid. The flow rate was 2ml/min. and 1ml fractions of eluant were collected.

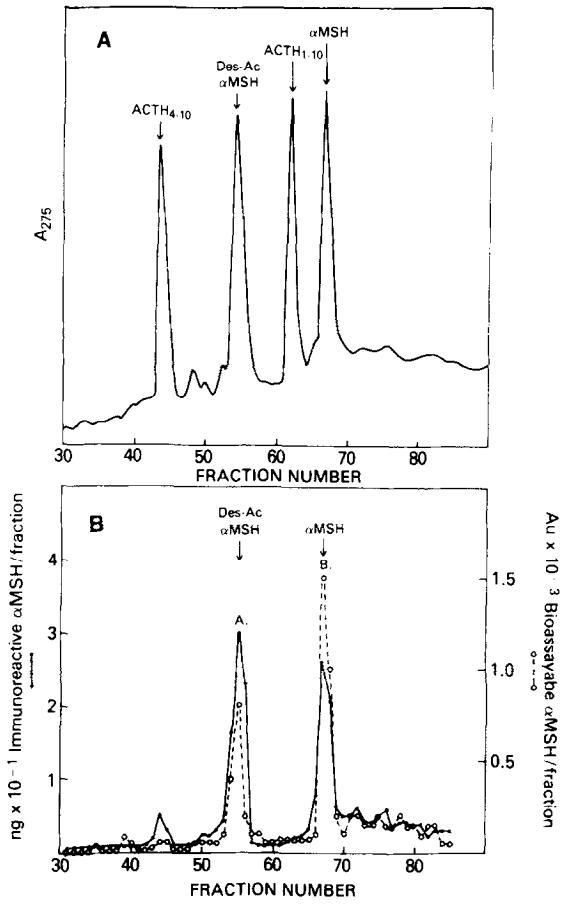
The αMSH-like peptides eluted from the C_{18} column were detected by radioimmunoassay (RIA), using two different antibodies to αMSH, and by a bioassay for melanotropic activity. The RIA procedure was as described previously (20) with several modifications (7). One antibody used was specific for the C-terminal end of MSH (Lys-Pro-Val NH_2), and showed similar cross-reactivity for synthetic α MSH and des-acetyl αMSH. The other antibody recognized the N terminal end of αMSH, specifically requiring the presence of the N acetyl group. This antibody showed a 15% cross-reactivity with des-acetyl αMSH relative to 100% cross-reactivity with αMSH. The Anolis skin bioassy (21) provides a probe for identifying the presence of the mid-portion (αMSH_{6-10}) of the αMSH molecule since this sequence is necessary for melanotropic activity (22).

For the developmental studies, brains and pituitary glands from 18-21 day old fetuses and 1-40 day post partum rats were examined. Treatment of the tissues and extraction of the αMSH peptides with 0.1N HCl were as described previously (2). The TCA precipitation and Sephadex G_{10} purification steps omitted. The levels of αMSH in the 0.1N HCl soluble extract from the brain and pituitary glands of the developing animals were determined by RIA, using the C terminal specific αMSH antibody. The wet weight of the brains were also recorded.

RESULTS AND DISCUSSION:

Identification of αMSH-like peptides in brain.

Fig. 1A depicts the HPLC separation of a number of αMSH and ACTH related peptides. The procedure used successfully resolved the peptides as indicated,



ig. 1. A) HPLC separation of synthetic $ACTH_{1-10}$, $ACTH_{4-10}$, αMSH and des-acetyl αMSH using a linear gradient with initial conditions of 16% methanol in 1% acetic acid and increasing to final conditions of 40% methanol in 1% acetic acid over 30 mins. The abscissa shows the fraction number and the ordinate the absorbance at 275 nm (in arbitary units).

B) HPLC separation of whole brain extract using conditions described in A). Each fraction was immunoassayed using a C terminal antibody and bioassayed. The solid line (•——•) shows the profile of radio-immunoassayable activity and the dotted line (o--o) the bioassayable activity. The left ordinate shows the ng of immunoreactive αMSH/ fraction and the right ordinate, the bioreactive αMSH in Anolis units (1 Anolis unit = 10^{-12} M αMSH). The abscissa shows the fraction number. The arrows indicate the elution position of des-acetyl αMSH and αMSH.

two of which differed only in the acetylation of the N terminal end of the molecule (αMSH and des-acetyl αMSH). Analysis of the TCA soluble extract of 50 rat brains by the HPLC procedure showed two major immunoreactive (C terminal antibody) MSH peaks, designated A and B (Fig. 1B, solid line). Peak A co-eluted with synthetic des-acetyl αMSH and peak B co-eluted with synthetic

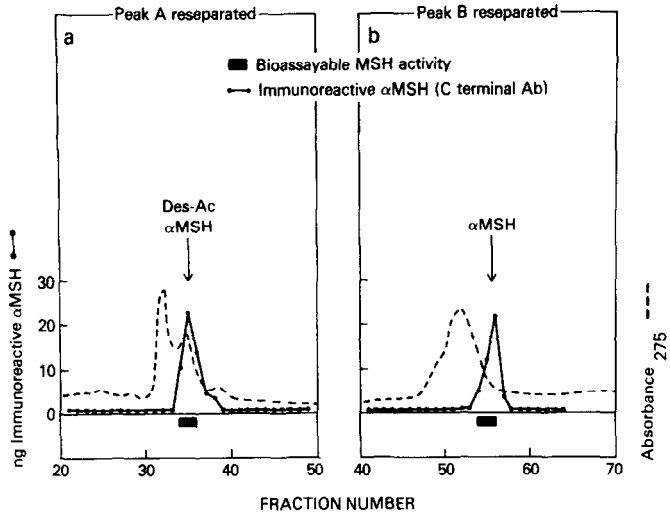


Fig. 2. HPLC reseparation of peak A and peak B from the first elution, using the same linear gradient conditions as in Fig. 1 except that final conditions were achieved in 60 mins.
a) Reseparated peak A showing purification of a immunoreactive αMSH peak (•—•) from other peaks shown by the 275 nm absorbance profile (---). The solid bar (▬) indicates fractions with bioassayable activity.

b) Reseparated peak B showing purification of a immunoreative αMSH peak (•—•) as evidenced by the 275 nm absorbance profile (---). The solid bar (▬) indicates fractions with bioactivity.
The left ordinate shows ng of immunoreative αMSH/fraction and the abscissa the fraction number. The arrows indicate the elution position for αMSH and des-acetyl αMSH.

αMSH. Bioassay of the fractions revealed two major melanotropic peaks (Fig. 1B, dotted line) which corresponded to the two immunoreactive αMSH peaks (A and B).

Peaks A and B eluted from the first HPLC run were reseparated using a linear gradient with a more gentle slope than the first separation (Fig. 2). The 275 nm absorbance profile of peak A (Fig. 2a) revealed the presence of several peptides within this peak. Only the peak which co-ran with synthetic des-acetyl αMSH showed immunoreactivity (using the C-terminal antibody) and bioassayable activity. Fig. 2b shows the reseparation of peak B. The peak detected spectrophotometrically at 275 nm showed no αMSH immunoreactivity or bioassayable activity. However, a peak with both immunoreactivity and bioassayable activity which co-eluted with synthetic αMSH was observed.

TABLE I
Immunological Identification of Des Ac αMSH and αMSH From Rat Brain
Cross-Reactivity (pg αMSH equiv.)

Reseparated Peak	C-Terminal Ab.	N-Terminal Ab.
(A) Des Ac αMSH	150	55
(B) αMSH	214	260

Thus further purification of the putative αMSH and des-acetyl αMSH peaks was achieved by the second separation on the C_{18} column.

The fractions, co-eluting with des-acetyl αMSH from reseparated peak A and with αMSH from reseparated peak B, were tested for their cross-reactivity with the N-terminal specific antibody to αMSH, (Table 1). The putative des-acetylαMSH peak (Fig. 2a) showed only 30% cross-reactivity with the N terminal antibody relative to the C-terminal antibody. This is consistent with the identification of this peak as des-acetyl αMSH, since it reacted poorly with the N terminal antibody. The cross-reactivity of this peak with the N terminal antibody was a little higher than expected when compared to cross-reactivity of this antibody with synthetic des-acetyl αMSH (see methods). This may be due to a minor contaminating peptide, which cross reacts with the N terminal antibody, even after the second reseparation on the HPLC column. In contrast, the putative αMSH peak, reseparated from peak B (Fig. 1B), showed very similar cross-reactivity with both the C and N terminal specific antibodies (Table 1).

In conclusion, by the use of HPLC, N and C terminal specific antibodies to αMSH, and a bioassay specific for the mid-portion of αMSH (residues 6-10), two major melanotropic peptides in the brain have been detected. One of these peptides has been identified as αMSH, because it 1) co-elutes with synthetic αMSH, in a highly selective HPLC procedure (see Fig. 1A); 2) cross-reacts with C and N terminally directed antibodies for αMSH, and 3) pocesses potent melanotropic activity. The second peptide has been identified as des-acetyl αMSH, since it 1) co-elutes with synthetic des-acetyl αMSH by HPLC, 2) cross

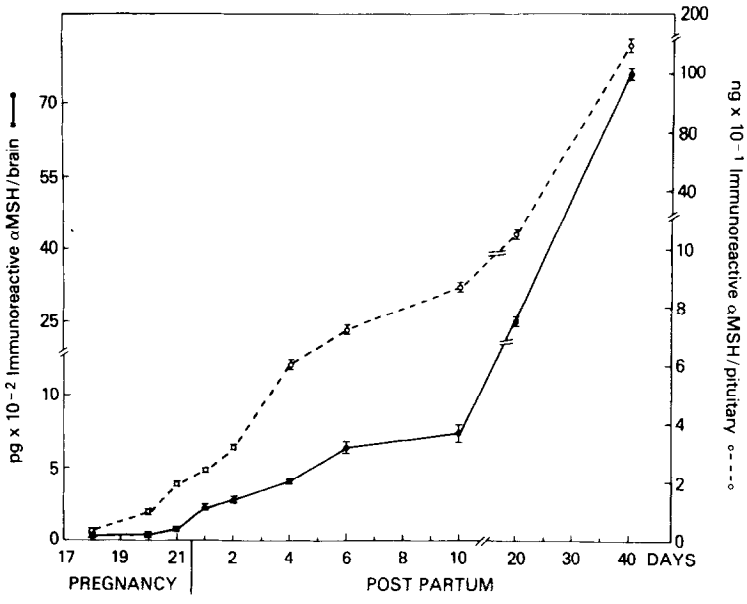


Fig. 3. Time course of increase of levels of immunoreactive αMSH (C-terminal antibody) in brain (•—•) and pituitary (o--o) during development. The SEM for each point is indicated by the bars. The left ordinate shows the pg immunoreactive αMSH/brain and the right ordinate the ng immunoreactive αMSH/pituitary. The abscissa shows the days of pregnancy and post partum. The wet weight (mg) of the brains of 18,20,21 day old fetuses and 1,2,4,6,10,20 and 40 day old animals were 109 ± 2.5 (mean ± SEM), 193 ± 2.6, 241 ± 2.3, 332 ± 5.9, 357 ± 3.7, 532 ± 10.3, 711 ± 9.3, 1010 ± 21.9, 1480 ± 16.4 and 1741 ± 9.9 respectively.

reacts with a C terminal antibody to αMSH, but showed poor cross-reactivity with the N terminal antibody and 3) displays melanotropic activity. Alpha-MSH and des-acetyl αMSH have been isolated, from pituitary, purified and sequenced (23). However, because there is considerably less of these peptides in brain and CSF (24), it has proven more difficult to purify enough for sequencing. Brain αMSH-like peptides have been partially characterized, and the existence of different forms of immunoreactive and melanotropic αMSH-like peptides, including the possibility of a des-acetylated form of αMSH, has been postulated (2,6,25,26). Our study identifies αMSH and a des-acetyl αMSH, as the two major forms of melanotropic peptide, in brain. Recently acetylated and des-acetylated forms of another peptide common to brain and pituitary gland, β endorphin, have been reported (27). The significance of the post-translational modification of these peptides with respect to their neurobiological function has yet to be determined.

Changes in level of immunoreactive αMSH during development.

The ontogenesis of αMSH in brain and pituitary gland was examined. Fig. 3 (closed circles) indicates that there was <30 pg. of immunoreactive αMSH/brain in day 18 fetuses and the level remained low until one day after birth. The MSH levels increased steadily from day 1 to 7654 pg. ± 305/brain in 40 day old adults. In contrast, pituitary immunoreactive MSH increased rapidly from 3.2 ng ± 0.35/pituitary in day 18 fetuses to 63.5 ng ± 8.6/pituitary in 40 day old adults. Similar results were reported on the basis of immunocytochemical studies (28,29) of fetal pituitaries. Very little αMSH was present in 17 day old fetuses but the level increased significantly at day 18 and continued to rise during fetal development (26). The ontogenesis of bioassayable melanotropic activity in the pituitary (data not shown), was similar to that shown for immunoreactivity. The transient rise of bioassayable αMSH level in day 19 and 20 fetal pituitary, followed by a decrease until 4 days after birth reported by Tilders et al (28) was not observed in our studies.

The difference in the onset as well as the time course of αMSH production in brain and pituitary gland suggest that the biosynthesis of αMSH in brain is independant of the pituitary gland. The onset of αMSH production in brain appears to occur at a time when most neurons have differentiated and are beginning to express their phenotypic properties (30).

ACKNOWLEDGEMENT: We would like to thank Robert Long for his excellent technical assistance, and Mrs. N. Garvey for typing the manuscript.

REFERENCES

1. Loh, Y. P. and Gainer, H. (1977) Brain Res. 130, 169-175.
2. Loh, Y. P., Zucker, L., Verspaget, H., and van Wimersma Greidanus, Tj. B. (1979) J. Neurosci. Res. 4, 147-156.
3. Oliver, C. and Porter J. C. (1978) Endocrinol. 102, 697-705.
4. Schally, A. V., Lipscomb, H., Long, J. M., Dear, W. E. and Guilleman, R. (1962) Endocrinol. 60, 478-480.
5. Krieger, D. T., Liotta, A. and Brownstein, M. J. (1977) Proc. Natl. Acad. Sci. USA, 74, 648-652.
6. Rudman, D., Scott, J. W., Del Rio, A.E., Houser, D. H. and Sheen, S. (1962) Am. J. Physiol. 266, 682-686.
7. Eskay, R. L., Giraud, P., Oliver, C., and Brownstein, M. (1979) Brain Res. 178, 55-67.
8. Jacobowitz, D. M. and O'Donohue, T. L. (1978) Proc. Natl. Acad. Sci., USA 75, 6300-6304.

9. Eskay, R. L., Brownstein, M. and Long, R. T. (1979) Science 205, 827-829.
10. O'Donohue, T. L. and Jacobowitz, D. M. (1979) Brain Res. 176, 101-109.
11. Oliver, C., Barnea, A., Warberg, J., Eskay, R. L. and Porter, J. C. (1977) Front Hormone Res. 4, 162-166.
12. Barnea, A., Oliver, C., and Porter, J. C. (1977) J. Neurochem. 29, 619-624.
13. Barnea, A., Neaves, W. B., Cho, G. and Porter, J. C. (1978) J. Neurochem. 30, 937-948.
14. Warberg, J., Oliver, C., Eskay, R. L., Parker, C. R., Barnea, A. and Porter J. C. (1977) Front. Hormone Res. 4, 167-169.
15. Gispen, W. H., Wiegant, V. M., Greven, H. M. and deWied, D. (1975) Life Sci 17, 645-652.
16. Kastin, A. J., Sandman, C. A., Stratton, L. O., Schally, A. V., and Miller, L.H. (1975) Prog. Brain Res. 42, 143-150.
17. Van Wimersma Greidanus Tj. B. (1977) Front. Hormone Res. 4, 129-139.
18. Nicolov, N. (1967) Folia Med. Plovdiv, 9, 249-255.
19. Strand, F.L., Stoboy, H., and Cayer, A. (1973) Neuroendocrinol. 13, 1-20.
20. Usategui, R., Oliver, C., Vaudry, G., Lombardi, G., Rosenberg, I., and Mourre, A. M. (1976) Endocrinol. 98, 189-196.
21. Tilders, F. J. H., van Delft, A. M. L., and Smelik, P.G. (1975) J. Endocrinol. 66, 165-175.
22. Schwyzer, R. and Eberle, A. (1977) Front. Hormone Res. 4, 18-25.
23. Li, C. H., Danho, W. O., Chung, D. and Rao, A. J. (1975) Biochem. 14, 947-952.
24. Thody, A. J., DeRotte, A. A., Van Wimersma Greidanus, Tj. B. (1979) Brain Res. Bulletin 4, 213-216.
25. Swaab, D. F. and Visser, M. (1977) Front. Hormone Res. 4, 170-178.
26. O'Donohue, T. L. and Jacobowitz, D. M. (1980) Polypeptide Hormones, in press, Raven Press, New York.
27. Zakarian, S. and Smyth, D. (1979) Proc. Natl. Acad. Sci. USA 76, 5972-5976.
28. Swaab, D. F., Visser, M. and Tilders, F. J. H. (1976) J. Endocrinol. 70, 445-455.
29. Chatelain, A., Dupouy, J. P. and Dubois, M. P. (1979) Cell and Tiss. Res. 196; 409-427.
30. Coyle, J. T. (1974) Dynamics of Degeneration and Growth in Neurons, pp. 425-434, Pergamon Press, New York.