Postnatal Development of ACTH and α-MSH in the Medulla Oblongata of Rat: α-MSH Is the Predominant Peptide

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ALESSI, N. E. AND P. QUINLAN. *Postnatal development of ACTH and α-MSH in the medulla oblongata of rat: α-MSH is the predominant peptide.* PEPTIDES 6: Suppl. 2, 137–141, 1985.-ACTH and α-MSH levels were measured by radioimmunoassays in extracts of the caudal medulla oblongata of developing rats on postnatal (P) days 1–42 at 7 day intervals, and in adult rats. From P1 to adulthood, ACTH increased >11-fold from 7.2±1.9 fmol to 82.4±12.6 fmol per medulla section (mean±S.E.M.). In comparison, α-MSH increased >7-fold from 68.75±11.0 fmol to 491±97.8 fmol during this time period. ACTH/μg of soluble protein decreased during postnatal development from 0.006±0.01 to 0.005±0.001 fmol/μg of protein and α-MSH increased from 0.06±0.01 fmol/μg of protein to 0.11±0.009 fmol/μg of protein between P1 and P7, decreased to 0.015±0.003 fmol/μg of protein by P42 and increased to 0.03±0.006 fmol/protein per unit protein by adulthood. These data indicate a significant shift in the levels of α-MSH detected during development with a decrease in the concentration of material occurring from early postnatal development (P1–P7) to adulthood, which does not appear to be solely related to a regional increase in protein. These studies, as well as radioimmunoassays for ACTH and α-MSH in combination with sizing chromatography of pooled extracts at P1, P7 and the adult, demonstrated the predominance of α-MSH at all ages.

ACTH   α-MSH   Medulla oblongata   Postnatal development   Nucleus tractus solitarius

ALPHA-MELANOCYTE stimulating hormone (α-MSH) and adrenocorticotrophin hormone (ACTH) are derived from the pro-opiomelanocortin (POMC) molecule. In the anterior lobe of the adult rat pituitary, ACTH and 16K are the predominant forms produced along with β-lipotropin related peptides; whereas in the intermediate lobe ACTH, as well as 16K and β-lipotropin, are biosynthetic intermediates, with essentially all ACTH cleaved to yield α-MSH [4]. Until recently the only anatomical site within the central nervous system thought to have neurons with POMC-derived peptides was the hypothalamus (arcuate nucleus). Recently, perykarya of neurons containing POMC related peptides, Beta-endorphin (β-END), (ACTH), and 16K were identified in the nucleus tractus solitarius (NTS) during development and in the adult rat [9, 10, 16, 18]. Biochemical studies of the postnatal ontogeny of β-END immunoreactivity demonstrated the continued presence of β-END immunoreactivity in this area throughout postnatal development despite earlier immunohistolential studies that demonstrated a "drop out" of identifiable neurons at day 14 postnatally [1,2]. Early studies demonstrated the presence of ACTH and α-MSH immunoreactivity in fibers of the medulla using immunocytochemical techniques [15,20]. Biochemical studies of this area clearly demonstrate the predominance of α-MSH as compared to ACTH in this area of the adult rat [7]. In this study, ACTH and α-MSH are measured in the caudal medulla during postnatal development to determine their developmental patterns and their relationship through devel-

opment. Further, radioimmunoassays are used in combination with sizing chromatography to determine the patterns of processing in the caudal medulla oblongata during postnatal development of the rat.

METHOD

Tissue Preparation

Postnatal (P) rats, taken at 7 day intervals (P1 . . . P42), as well as adults (70–80 days of age), were sacrificed by decapitation. The caudal medulla oblongata was dissected as a block, placed on dry ice, and stored at −80°C. At the time of processing each tissue sample was placed in 1 ml of tissue extraction solution containing 0.1 N HCl and acetone (1:3). The sample was homogenized for 10 seconds. The homogenizer probe was then washed with 1 ml of extraction solution which was combined with the previous aliquot of suspension. Two hundred microliters of this suspension was removed for later protein determination. The remainder of the suspension was centrifuged at 10,000 rpm for 20 minutes. The supernatant was decanted and saved. The pellet was resuspended in 1 ml of extraction solution and centrifuged again at 10,000 rpm for 20 minutes. The supernatant was decanted into the previous wash and the pellet was discarded. The supernatant was then lyophilized and stored at −80°C. To measure individual time point levels the extracted tissue was resuspended in 1 ml of 1% formic acid. The tissue from six animals were used per time point. The values are
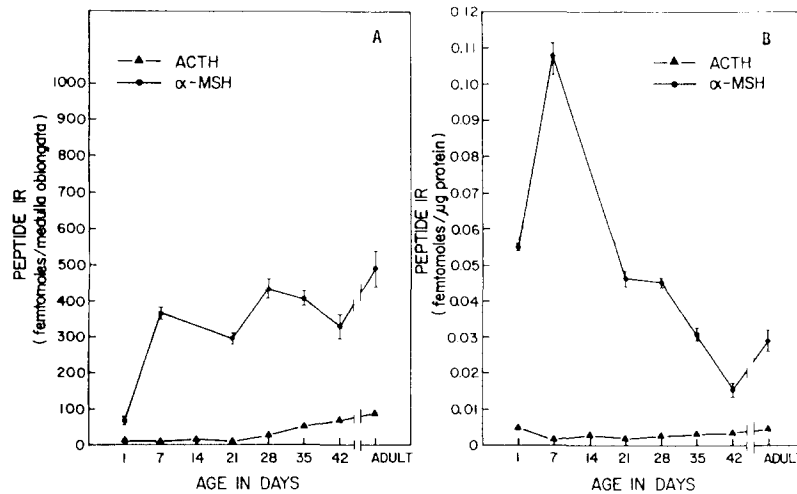
FIG. 1. (A) Patterns of ACTH and α-MSH immunoreactive material per medulla oblongata of developing rats. Results are expressed as mean±S.E.M. for 6 animals. (B) Patterns of ACTH and α-MSH immunoreactive material per μg of soluble protein in each medulla oblongata region analyzed. Results are expressed as mean±S.E.M. for 6 animals per time point.

expressed as the mean±standard error of the mean (S.E.M.) per time point. Tissue to be chromatographed was resuspended in 1% formic acid, combined, relyophilized and stored at −80°C.

Radioimmunoassays

ACTH. The radioimmunoassay (RIA) was similar to that described previously [13]. The anti-ACTH serum "IgG-ACTH-1" was obtained from the IgG Corporation, Nashville, TN 37211. The peptide used for all standards was ACTH (1–39) human (cat. No. 8740, Peninsula Laboratories, Belmont, CA 94002). The IC_{50} averaged 2.0–2.5 fmol per tube over a number of assays. The specificity of the assay was as follows: ACTH (1–24), (1–39)—100%, α-MSH (1–13)—0.00%; Desacetyl α-MSH—0.01%, Deamindated α-MSH—0.01%, Human β-END (61–91)—0.0%, and Human β-lipotropin—0.0%.

α-MSH. This RIA for α-MSH is similar to that previously described [14]. The peptide used for all standards and as well as for iodination was α-MSH (cat. No. 7251, Peninsula Laboratories, Belmont, CA 94002). The α-MSH antibody (Number 23) was donated by Thomas C. O'Donohue. The IC_{50} for the assay over a number of times was 40–45 fmol per tube. The assay demonstrated the following specificity: α-MSH, Desacetyl α-MSH, Deamindated α-MSH—100%; ACTH (1–24), Human ACTH (1–39), Human β-END (61–91), Human β-lipotropin (β-LPH)—0.0%; ACTH (1–10), (4–10), (4–11)—0.0%.

Levels of soluble protein were determined by the Lowry technique [12].

Chromatography

A 9×560 mm column was prepared with G-50 superfine gel (cat. No. G50-50, Sigma Chemical Co., St. Louis, MO 63178). The eluent buffer was 10% formic acid, 0.1% BSA, and 0.1% 2-mercaptoethanol. The flow rate was 0.40 ml per minute with 1.1 ml of eluent collected per fraction. Collected samples were lyophilized and stored at −80°C. At the time of

the assay, all samples were resuspended in 1 ml of 1% formic acid.

RESULTS

Postnatal Developmental Patterns

Figure 1 depicts the concentrations of ACTH and α-MSH in the caudal medulla of rats during postnatal (P) development and in the adult rat. When compared per total tissue region ACTH increased 11-fold from 7.2±1.9 fmol to 82.4±12.6 fmol from P1 to adulthood, whereas α-MSH increased 7-fold from 68.5±11.0 fmol to 491±97.8 fmol (Fig. 1A). α-MSH remained the predominant form throughout postnatal development with its sharpest increase of >5-fold occurring from P1 to P7. When expressed per μg of soluble protein both ACTH and α-MSH demonstrated significant alterations in their levels during postnatal development (Fig. 1B). From P1 to P7 the levels of ACTH decreased significantly from 0.006±0.001 fmol/μg protein to 0.001±0.0002 fmol/μg protein which remained stable until P42 at which time levels increased to adult levels (ANOVA, $p<0.0004$). α-MSH demonstrated an equally significant yet opposite response (ANOVA, $p<0.0000$). The most significant alterations occurred from P1 to P7 at which time levels increased 20-fold from 0.05±0.01 fmol/μg protein to 0.1074±0.009 fmol/μg protein followed by a comparable decrease at P14. These levels continued to decrease throughout postnatal development until reaching adult levels 0.03±0.006 fmol/μg protein α-MSH remains the predominant form present throughout postnatal development varying from 6 to 80 times more than the amount of ACTH.

Chromatography

Figure 2 compares the molecular weight patterns of ACTH (left) and α-MSH (right) in caudal medulla oblongata sections from postnatal (P) day 1 (top), P7 (middle) and adult rats (bottom). ACTH immunoreactive material runs in the vicinity of the ACTH marker at each time point. At P1 there
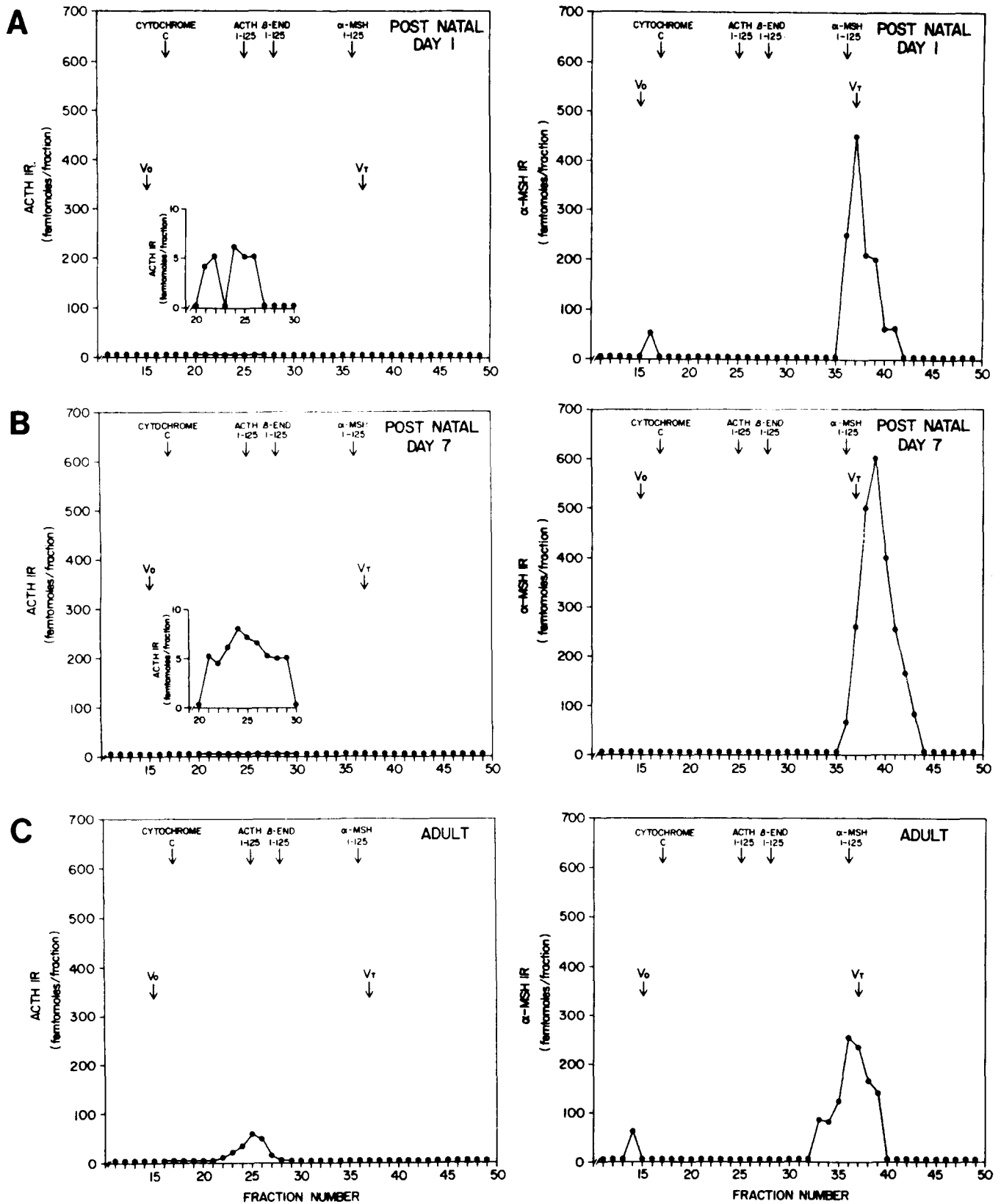
FIG. 2. Mol. wt. pattern of ACTH (left) and α-MSH (right) in the caudal medulla oblongata of Postnatal (P) day 1 (A), P7 (B), and adult rats (C). Aliquots from the extracts of 10 P1 male, 10 P7 male and 4 adult male caudal medulla were chromatographed on a Sephadex G-50 superfine column and aliquots of the 1.1 ml fractions, collected from the column, assayed for ACTH or α-MSH. For further details see the Method section. Void volume was determined with blue dextran; and total volume was determined with cobalt chloride.

are two peaks identified in this area. These two peaks, as well as the single peaks identified at P7 and adulthood, are from the same fractions suggesting that the material identified from these time points is the same. The greatest portion of α-MSH immunoreactivity runs at the location of the α-MSH marker. The exception is with high molecular weight material identifiable with α-MSH assay which runs with the void volume. Table 1 describes the quantity of ACTH and α-MSH identified at each age. α-MSH increases at P7 as compared to P1. Also, the ratio of α-MSH to ACTH changes from being greater than 40 to approximately 5 during development indicating an alteration in the ratio of production of these materials. These chromatographs support the finding that α-MSH is the predominant form throughout postnatal development yet in the adult the ratio of products shifts.

DISCUSSION

The results reported provide strong evidence of α-MSH being the predominant peptide originating from the ACTH portion of the pro-opiomelanocortin molecule in the caudal medulla oblongata. This supports an earlier study which reported its predominance in the adult rat and further demonstrates its predominance from the newborn period throughout the postnatal (P) period as well [5]. Of particular note is the significant shifts which occur in the levels of α-MSH throughout postnatal development. First is the sharp rise in α-MSH concentration at P7 and second is the subsequent marked decrease to adult levels. The P7 peak appears to coincide with a time during which the postnatal brain is undergoing a growth surge. α-MSH has been proposed to be of importance for the maturation of several organ systems [8,19]. Further, changes in the ratio of α-MSH/ACTH have been suggested to be of importance in the maturation of physiological alterations of the fetal adrenal gland [3, 9, 11]. The role of α-MSH in the maturation of the medulla and its associated nuclei remains a question requiring further investigation.

If alterations in α-MSH levels are to be used as a basis for inference concerning its role in the development and maturation of the central nervous system, then an important question needs answering, i.e., are the alterations in α-MSH levels during postnatal development "true" or "relative." In Fig. 1A, the levels of α-MSH and ACTH are expressed as the total quantity per caudal medulla oblongata, whereas in Fig. 1B the levels are expressed as the concentration of α-MSH and ACTH per unit protein. Although the total quantity of α-MSH increases from P1 to adulthood (Fig. 1A), the concentration increases from P1 to P7 and peaks (Fig. 1B). Subsequently, the concentration decreases significantly. If the rise and drop in concentration of α-MSH is "true" representing alterations in the production of α-MSH, one could speculate as to its importance during development and its possible role as a trophic hormone. Whereas, if this decrease is "relative," representing a steady state level of production of α-MSH throughout development with a decrease resulting from a marked increase in proteins during maturation, the argument for α-MSH as a trophic hormone would be less persuasive. A comparison of the alterations in α-MSH concentration to both ACTH and β-Endorphin concentrations during postnatal development sheds light on this question. The results of similar time point studies during the postnatal period of β-Endorphin and ACTH are quite different than that of α-MSH [1]. First, neither ACTH nor β-Endorphin concentrations have peaks at P7, and, second,

TABLE 1

DISTRIBUTION OF ACTH AND α-MSH IN CAUDAL MEDULLA OF RAT AT POSTNATAL (P) DAY 1, P7 AND IN THE ADULT

Period	Number of areas pooled	α-MSH (fmol)	ACTH	Ratio α-MSH/ACTH
P1	10	1237.5	26.6	46.5
P7	10	2330.0	53.9	43.2
Adult	4	1090.0	205.4	5.3

Values are calculated from corresponding integrated peptide peaks of G-50 column separation.

their concentrations do not decrease as markedly as that of α-MSH during the postnatal period. The stability of the postnatal patterns of ACTH and β-Endorphin alterations in concentration would suggest these substances have a greater steady state, possibly reflecting a balance of production, utilization and/or degradation throughout development. In comparison, the marked alteration in the concentration of α-MSH suggests variable turnover rates which is age dependent, strengthening a hypothesis of its being important as a trophic hormone. Needless to say, further studies in which α-MSH and its associated POMC-related peptides are simultaneously studied will strengthen and clarify its role during development.

A question of importance is the determination of the site of origin of the α-MSH measured in this study. Neurons of the hypothalamus and the medulla contribute to the α-MSH immunoreactivity identified. Monosodium glutamate (MSG) induced lesions of the hypothalamus have been shown to result in not only a 90% reduction in the hypothalamic levels of α-MSH, but reductions of α-MSH levels in extrahypothalamic areas as well [6]. Levels of α-MSH in the medulla oblongata of adult rats following MSG treatment of neonates decreased by 50% from 205.0 ± 23.7 to 104.0 ± 10.6 pg per region. From this data one can conclude that only a portion of the α-MSH in the medulla originates from the hypothalamus. The percentage of either α-MSH or ACTH material originating from either source contributing to the immunoreactive material identified remains unknown not only in the adult, but throughout postnatal development as well.

Further, of importance is the question of what forms the α-MSH measured are in, and whether there are alterations in forms during the postnatal period studied. Two major forms, α-MSH and deacetylated α-MSH, naturally exist in the pituitary as well as brain [14]. Behavioral experiments have clearly demonstrated that the α-MSH, due to its resistance to peptidase degradation, was more potent in facilitating behavioral performance. Consequently, depending on the form of α-MSH present its physiological effects might be greatly increased or decreased. This question would be complicated further if the ratio of forms of α-MSH vary during postnatal development, as has been reported in the anterior pituitary of the rat [17]. Although there are studies demonstrating the trophic effects of α-MSH none have attempted to determine if this effect could be modified by this posttranslational processing event. Consequently, given these studies and their results, it is obvious that knowing the levels of α-MSH present is only the first step and that subsequent studies will have to concentrate on the identification of forms and their ratios.

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