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RAT HYPOTHALAMIC PROOPIOMELANOCORTIN MESSENGER RNA IS UNAFFECTED BY ADRENALECTOMY

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The negative feedback control of hypothalamic cortocotrophin releasing factor (CRF) and anterior pituitary proopiomelanocortin (POMC) by corticosteroids is well understood. However, less is known about the mechanisms that regulate POMC gene expression in the arcuate nuclei in the medial basal hypothalamus (MBH). Using a sensitive and specific S1 endonuclease protection assay, we have examined the effect of adrenalectomy on POMC mRNA in the rat MBH and pituitary. Our results show that adrenalectomy does not change POMC mRNA levels in the MBH at 7 or 14 days post surgery. The neurointermediate lobe of the pituitary was similarly unaffected by adrenalectomy, while in the anterior lobe, POMC mRNA increased 7-10 fold at both time points, effects that were prevented by dexamethasone treatment. We conclude that while POMC mRNA in the anterior lobe of the pituitary is regulated by plasma glucocorticoids, in the MBH and neurointermediate lobe, it is not.

The hypothalamo-pituitary-adrenal axis can be considered the prototype physiological negative feedback control system, and as such has been extensively studied. Briefly, CRF (and AVP), released from the paraventricular nucleus of the hypothalamus (PVN) into the hypophyseal portal system, act upon the anterior lobe of the pituitary to enhance the production of POMC. POMC is then processed to form mainly ACTH, which is released into the circulation and stimulates the production and release of glucocorticoids by the adrenals. Both CRF and ACTH are under negative feedback control by glucocorticoids, a mechanism that has been shown to act both at the level of secretion and of gene expression (1). In the intermediate lobe of the pituitary, POMC-expressing cells do not appear to be regulated by glucocorticoids, and the main products of the POMC gene appear to be the smaller peptides α-MSH and ß-endorphin. Much less is known about the mechanisms that regulate POMC gene expression in a third population of POMC-producing cells: the neurons of the arcuate nucleus in the medial basal hypothalamus

Abbreviations: ACTH: adrenocortico trophic hormone; ADX: adrenalectomized; AL: anterior lobe of the pituitary; ANOVA: analysis of variance; AVP: arginine vasopressin; CRF: corticotrophin releasing hormone; Dex: dexamethasone; MBH: medial basal hypothalamus; NIL: neurointermediate lobe of the pituitary; POMC: proopiomelanocortin; PVN: paraventricular nucleus; TCA: trichloroacetic acid.

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(MBH). These nuclei have afferent and efferent connections with many brain areas and may be of great importance in neuroendocrine regulation. Studies performed thus far suggest that POMC mRNA in the MBH may be modulated by dopamine (2), by CRF (3), and by sex steroids (4). However, the fundamental question of feedback regulation of MBH POMC mRNA by glucocorticoids remains in dispute. Birnberg et al (5) reported that adrenalectomy did not affect POMC mRNA in rat hypothalamus at 14 days post surgery, while Beaulieu et al (6) found that adrenalectomy resulted in an increased concentration of rat hypothalamic POMC mRNA at 7 days post surgery, an effect that could be prevented by dexamethasone treatment. While seemingly contradictory, these two studies may indicate that hypothalamic POMC mRNA is regulated in a time-dependent, biphasic manner.

An impediment to the study of hypothalamic POMC mRNA is the fact that it is present in amounts 1-2 orders of magnitude less than in the pituitary (1), making quantitative asssay difficult. We have developed a sensitive and specific solution hybridization S1 endonuclease protection assay capable of detecting POMC mRNA in the attomole range. Using this assay, we have reexamined the effect of adrenalectomy and dexamethasone treatment on POMC gene expression in the MBH and in the anterior (AL) and neurointermediate (NIL) lobes of the pituitary.

MATERIALS AND METHODS

Animals: Bilaterally adrenalectomized (adx) or sham operated male Sprague-Dawley rats, 200-250g, were obtained from Charles River (Kingston, NY). In the first set of experiments, the adx animals were divided into two groups at seven days post surgery: one group received saline (0.9% NaCl) to drink, while the other group was maintained on saline containing 2μg/ml dexamethasone 21-phosphate (dex, Sigma) (7). Sham operated animals received tap water. After a further seven days, the animals were sacrificed. In the second set of experiments designed to uncover any possible biphasic effects, a group of sham operated rats and a group of adx rats was sacrificed at seven days post surgery. All animals received rat chow ad lib.

Tissue preparation: Animals were sacrificed by decapitation between 9:00 am and 11:00 am. Trunk blood was collected from a sample of animals for plasma corticosterone assay (Nichols Institute, River Edge, NJ). The brain and pituitary were removed from the skull and the anterior lobe (AL) of the pituitary was freed from the remaining intermediate and posterior lobes which were then handled together as the neurointermediate lobe (NIL). The mediobasal hypothalamus (MBH) was removed by inverting the brain and making two transverse razor cuts at the level of the optic chiasm and mamillary bodies. Two sagittal cuts were then made through the fornices, followed by a coronal cut halfway along the fourth ventricle which freed the MBH, a block of approximately 2x2x3 mm which includes the entire arcuate nuclei, as well as the median eminence.

RNA extraction: Immediately following dissection, tissues were subjected to a modified guanidine isothiocyanate RNA extraction (RNAzol B, Cinna/Biotecx, Friendswood, TX). Yields were: AL, 36.6 ± 2.8 μg/gland; NIL, 14.4 ± 1.2 μg/gland; MBH, 37.3 ± 1.9 μg/pair. There were no significant differences in yield from each tissue between the treatment groups (ANOVA, $P<0.05$).

Solution hybridization probe synthesis: The gene sequence for rat POMC (8) was analyzed using Stemloop software (Genetics Computer Group, University of Wisconsin, Madison, WI) to find a region of suitable length and minimal internal complementarity against which to direct the

probe. The chosen sequence, a 93 nucleotide region comprising bases 569-661 which code for ß-endorphin, was assembled from synthetic oligonucleotides and force-cloned into M13mp18 phage replicative form DNA as previously described (9). The single-stranded M13mp18 DNA containing the 93 nucleotide insert was purified from the phage, and the correct sequence and orientation of the insert verified by dideoxy sequencing. Full homology with the chosen sequence ensures that hybrids between the probe and the relevant region of POMC mRNA are fully protected from S1 endonuclease.

The construct was used as a template from which to prepare a single-stranded uniformly labeled probe as previously described (9).

Solution hybridization assay: POMC mRNA was quantified by incubating radiolabeled probe (40-50000 cpm) with total RNA extracted from MBH (25-50µg), AL (2-10µg), or NIL (0.15-0.9µg), and with increasing amounts of template DNA for construction of a standard curve as previously decribed (9). S1 endonuclease (BRL) was then added to all tubes to degrade all single-stranded RNA and probe, and after 90 min S1-resistant hybrids were precipitated with TCA (7.5%), collected by vacuum filtration over Whatman GF/B filters, and their radioactivity measured by liquid scintillation spectrometry. The steady state levels of POMC mRNA were determined from the standard curve.

Northern analysis: Messenger RNA from rat pituitary and MBH was analyzed by Northern blot using standard procedures. Briefly, markers and tissue mRNA were size fractionated on a 1% agarose gel containing 2% formaldehyde and transferred to Nytran membranes (Schleicher and Schuell, NH) with 10X SSC overnight, and then linked to the membrane by baking under vacuum at 80°C for 2 hours. Following a 2 hour prehybridization at 42°C in Hybrisol I (Oncor) containing 100µg/ml salmon sperm DNA, hybridization to the POMC probe, and to a ß-actin probe (Oncor) was performed by incubation overnight at 42°C in the same solution. The following day, the blots were washed twice for 15 minutes with 2X SSC, 0.1% SDS at room temperature and once for 10 minutes in 1x SSC, 0.1% SDS at 37°C, then visualized by autoradiography or by a phosphorimager (Molecular Dynamics).

Statistical analyses: All statistical comparisons were made using analysis of variance (ANOVA). When the ANOVA was significant, post tests were performed using the Bonferroni method to generate a P value for differences between groups. Differences were considered to be significant when $P<0.05$.

RESULTS

Figure 1 shows a typical standard curve from an S1 endonuclease protection assay. Recovered radioactivity rises linearly with template from 12.5 to 400 amole. The insert shows the result of Northern blots demonstrating that the POMC probe hybridizes to a single RNA species of approximately 1100 bases in both the pituitary and the MBH, corresponding to full length POMC mRNA (1).

While sham operated animals had a mean plasma corticosterone concentration of 15.6 ± 2.5 µg/dl, adrenalectomized animals had a dramatically reduced concentration of only 1.5 ± 0.5 µg/dl. The group of rats that had adrenalectomy and dexamethasone treatment had corticosterone levels below the detection limit of the assay. We observed that dexamethasone treatment of adrenalectomized rats caused increased urine output and decreased body weight (not shown), in agreement with the findings of others (10, 11).

Figure 2 illustrates the steady state tissue levels of POMC mRNA 14 days after adrenalectomy with or without seven day dex treatment in the MBH, AL, and NIL. A

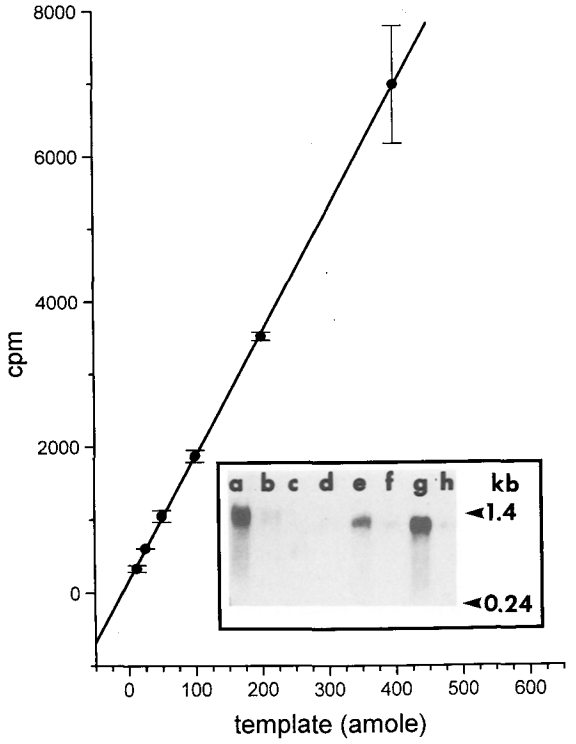

Figure 1. Standard curve from S1 endonuclease protection assay.

Radiolabeled probe was incubated with 12.5 to 400 amole of template, and then S1-resistant hybrids were collected and counted as described in Materials and Methods. Each point is the mean and standard error of two determinations. Insert: Northern blot of RNA from MBH and AL using POMC probe. RNA from MBH and AL was extracted and separated, then hybridized to the POMC probe as described in Materials and Methods. Lanes a and b, 30 and 8.5μg MBH RNA respectively; lanes c-e, 0.2, 0.6, 3.0 μg AL RNA freshly extracted from one animal and, lanes f and g, 0.6 and 3.0 μg from a second animal. Lane h, 0.6 μg AL RNA after storage at -70°C for one week.

comparison of values for the sham-operated animals confirms that the POMC mRNA content of the MBH is 1-2 orders of magnitude less than the AL (1) and almost 3 orders of magnitude less than the NIL. In both the NIL and MBH, adrenalectomy produced no significant difference in mean POMC mRNA compared to sham operated animals, neither did dexamethasone treatment of adrenalectomized animals produce a significant difference. In the AL, in contrast, adrenalectomy resulted in an almost seven-fold increase in POMC mRNA compared to sham operated animals, while treatment of adrenalectomized animals with dexamethasone reversed this increase. The changes in POMC message seen in the AL are specific: ß-actin mRNA, as determined by Northern blots, was not affected (not shown).

Figure 3 demonstrates that the effect of adrenalectomy on POMC mRNA from the MBH, NIL, and AL of animals sacrificed at seven days post surgery is essentially the same as at 14 days. While no significant change is evident in MBH and NIL POMC mRNA content of adx compared to sham-operated animals, in the AL, the adx animals show an approximately ten-fold increase compared to the sham group.

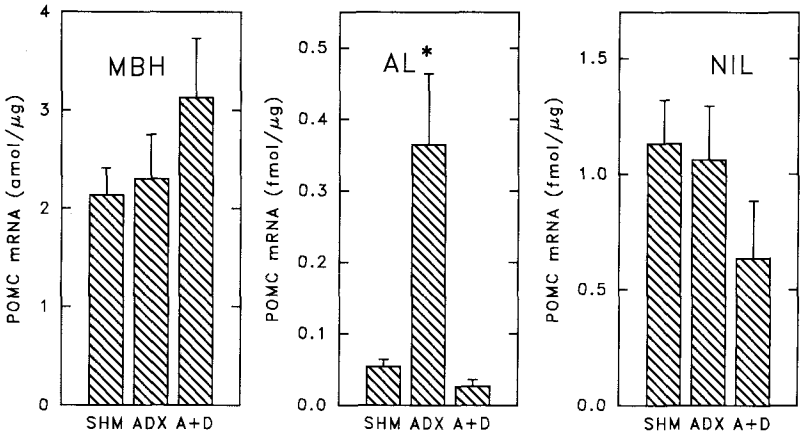

Figure 2. Steady state tissue levels of POMC mRNA in the MBH, AL, and NIL 14 days after surgery with or without 7 day dex treatment.

Columns and bars represent means and standard errors. MBH: SHM=sham operated day 0, n=9; ADX=adrenalectomized day 0 n=9; A+D=adx treated with dexamethasone days 7-14 post surgery, n=8. AL: SHM, n=8; ADX, n=8; A+D, n=7. NIL: SHM, n=6; ADX, n=7; A+D, n=7. Results were analysed by ANOVA and the Bonferroni method. Differences between groups ($P<0.05$) are indicated by the asterisk.

DISCUSSION

The major finding of this study is that adrenalectomy in rats does not increase POMC mRNA in the MBH or in the NIL, but does significantly upregulate POMC mRNA in the AL. This is true at both seven and 14 days post surgery. Since adrenalectomy causes a dramatic fall in the plasma

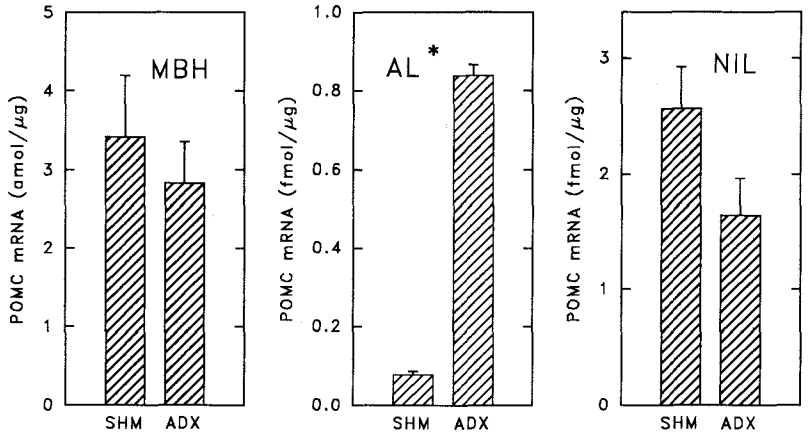

Figure 3. Steady state tissue levels of POMC mRNA in the MBH, AL, and NIL 7 days after surgery.

Columns and bars represent means and standard errors. MBH: SHM=sham operated day 0, n=6; ADX=adrenalectomized day 0, n=7. AL: SHM, n=6; ADX, n=6. NIL: SHM, n=6; ADX, n=6. Results were analysed by ANOVA and the Bonferroni method. Differences between groups ($P<0.05$) are indicated by the asterisk.

levels of corticosterone, we conclude that it is the removal of the negative feedback effect of this steroid on the AL (1), and on hypothalamic CRF (1), that brings about the increase in POMC mRNA. This is supported by the observation that steroid replacement of adrenalectomized animals with dexamethasone reverses the increase in POMC mRNA in the AL. Since no change in POMC mRNA is seen in the NIL and MBH between sham and adrenalectomized animals, we can extend this argument to conclude that POMC mRNA in these sites is not under negative feedback control by glucocorticoids.

Our results in the pituitary confirm the earlier studies of others (1). However, this is one of the few studies to examine the regulation of POMC mRNA in the hypothalamus. In the first such study, Birnberg et al (5), obtained results similar to those described here. However, in a later study, Beaulieu et al (6), using Northern and dot blot analysis of material pooled from 21 animals, found that rat hypothalamic POMC mRNA increased more than 3-fold following adrenalectomy, and that this increase was prevented by dex or corticosterone. They concluded that hypothalamic POMC mRNA was under negative control by glucocorticoids, and suggested that the earlier study may have used methods of insufficient sensitivity to accurately measure the small amount of POMC mRNA present in the hypothalamus. We have avoided such problems in the present experiments by using a method with a sensitivity of 12.5 amole of POMC mRNA (the rat MBH contains 40-60 amole POMC mRNA). In addition, while Birnberg et al studied animals sacrificed at 14 days after adrenalectomy, and those studied by Beaulieu et al were sacrificed at only 7 days post surgery, in the present study animals were studied at both 14 and 7 days after adrenalectomy, with no qualitative difference in outcome. This argues against a possible biphasic effect of glucocorticoids on POMC mRNA in the MBH which could have explained the conflict in the previous studies, and indicates that hypothalamic POMC is not regulated by glucocorticoids.

Another approach to the study of POMC regulation in the hypothalamus is the determination of peptide content or secretion from hypothalamic explants or from hypothalamic cell culture. Peptide content is influenced by both synthesis and release, and therefore is not an ideal indicator of the activity of POMC-producing neurons. Nevertheless, most studies to date have demonstrated no change in hypothalamic immunoreactive ß-endorphin content following adx or dex, although an increase following dex has also been reported (for review, see 12, 13).

Assay of ß-endorphin-like immunoreactivity released from explants or culture of hypothalamic tissue isolates a single factor, but introduces potential artifacts arising from the *in vitro* nature of these studies. Kapcala et al (14) were unable to demonstrate an inhibitory effect of dex on basal or on potassium-stimulated immunoreactive ß-endorphin release from dissociated fetal hypothalamic cell culture, nor were they able to show a CRF stimulation of release. However, in a fetal hypothalamic explant, CRF did stimulate release. Measures of steady state mRNA level are thought to provide a more reliable indicator of peptidergic activity (1).

The POMC gene is present in a single copy per haploid genome. The question of the mechanism of tissue-specific expression and regulation of this gene by glucocorticoids is intriguing. Drouin et al (15), using the corticotroph tumor cell line AtT20, have demonstrated that a 543 base pair fragment of the 5'-flanking region confers cell-specific expression, and have identified a negative glucocorticoid response element in a proximal POMC promoter which

interacts with glucocorticoid receptor in a unique fashion that correlates with glucocorticoid repression of POMC. Thus, the presence or absence of certain regulatory transcription factors or mechanisms in the arcuate nucleus might partly explain the differential regulation of the POMC gene. A further role of different glucocorticoid receptor subtypes is unlikely: there is good evidence that type II glucocorticoid receptors mediate a direct inhibition of CRF-induced ACTH release at the anterior pituitary (16), and the arcuate nucleus also appears to contain type II receptors (17).

Increased CRF levels following adrenalectomy are thought to contribute greatly to the observed increase in POMC mRNA in the anterior pituitary (1). A lack of response to CRF at the arcuate nucleus, or the lack of direct innervation of POMC-producing neurons by CRF-containing axon terminals, might explain both the lack of effect of adrenalectomy on these neurons, as well as the relatively low basal levels of POMC mRNA found in the MBH. Though Kapcala et al (14) were unable to demonstrate an effect of CRF on ß-endorphin release from fetal hypothalamic cell cultures, in other studies, they demonstrated CRF receptors and CRF-induced ß-endorphin release from hypothalamic explants (see 14), thus the explanation for the lack of glucocorticoid regulation of MBH POMC gene expression is likely to be found at a level beyond the receptor.

In conclusion, our results indicate that POMC mRNA in the MBH is not regulated by glucocorticoids, and in this respect, the MBH resembles the NIL. Given the strategic location of the MBH and the large amounts of POMC mRNA present in the NIL, further study to identify the roles and regulation of the POMC system in each could prove of great value in expanding our knowledge of neuroendocrine regulation.

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