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THE HUMAN HYPOTHALAMIC LHRH PRECURSOR IS THE SAME SIZE AS THAT IN RAT AND MOUSE HYPOTHALAMUS

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The synthesis of the decapeptide luteinizing hormone releasing hormone (LHRH) in human, rat and mouse brain has been investigated by studying the in vitro translation products of Poly A^+ mRNA extracts from the hypothalamus. The translation products of all three species contained a single 28000 MW polypeptide which immunoprecipitated with a specific anti-LHRH serum. This polypeptide was not present in the translation products of Poly A^+ mRNA extracts from the hypothalamus of the hypogonadal mouse, a mutant strain totally deficient in LHRH. These results show that in the human, rat and normal mouse, LHRH is synthesized as a component of a precursor peptide with a molecular weight of 28000.

Many biologically active peptides are synthesized as part of larger precursor molecules (1,2,3,4), and, recently, we demonstrated the presence in rat hypothalamus of a 28000 precursor of LHRH (5). The aim of the present study was to determine whether the in vitro translation products of hypothalamic mRNA extracts from human (post-mortem) and mouse brain also contained a polypeptide which incorporated an amino acid sequence immunologically similar to that of biologically active LHRH. In addition to normal mice we studied mRNA extracts from the hypothalamus of hypogonadal (hpg) mice, a mutant strain totally deficient in LHRH (6,7).

MATERIALS AND METHODS

Total RNA was extracted from the hypothalamic tissue of 60 adult female rats (Wistar-Cob), 60 adult normal mice (both sexes), 60 adult hpg mice (both sexes) and one human female aged 67 (three hours post-mortem) and one human male aged 72 (12 hours post-mortem) by the guanidinium thiocyanate procedure (8). Neither the human female nor male suffered from either a neurological or psychiatric disorder. In the mouse and rat the block of tissue extended from rostral to the optic chiasm to the mamillary body, laterally to the lateral extent of the median eminence, and about as deep as the anterior commissure. In the human, the block of tissue extended from the lamina terminalis to the mamillary bodies, laterally to the optic tracts and as deep as the hypothalamic sulcus. These dissections ensured the incorporation of most of the hypothalamic LHRH cell bodies. The Poly A^+ enriched fractions were

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isolated by oligo (dT)-cellulose chromatography (9). The mRNA samples were translated in an amino-acid depleted rabbit reticulocyte lysate system (Amersham International) supplemented with 1 μCi/μl of a ^{3}H-amino acid mix consisting of leucine (130 Ci/mmol), lysine (76 Ci/mmol), phenylalanine (84 Ci/mmol), proline (100 Ci/mmol) and tyrosine (50 Ci/mmol) and all other amino acids at 50 μM. All other components of the translation mix were as described previously (5). The incorporation of labelled amino acids into protein was monitored by trichloroacetic acid precipitation of a small aliquot of the translation mix after incubation for 60 min. Approximately 70000 acid precipitable counts of each sample were loaded (generally 5-10 μl) in SDS-β-mercaptoethanol sample buffer. Electrophoresis was carried out at 15 mA constant current for 15 hours on 8-20% SDS polyacrylamide gradient slab gels with a 4.75% stacking gel according to the method of O'Farrell (10). The gels were fluorographed (11,12) and exposed to film (Kodak X-omat R) for 10 days.

Aliquots of the translation mixes containing a minimum of 10^{6} acid precipitable counts (typically 80-150 μl) were taken for treatment with the specific LHRH antiserum. This antiserum (HC6), which was raised in rabbits against a LHRH-haemocyanin conjugate, did not cross react with either nine different analogues of LHRH modified at either the N or C terminus, or pituitary hormones, but did cross react 100% with the free acid of LHRH. The translation products from all four sources were immunoprecipitated as described previously (5) using donkey anti-rabbit IgG (Wellcome Laboratories, Dartford) as the second antibody. In a second immunoprecipitation procedure, applied to the rat hypothalamic translation products, Staphylococcal protein A (Miles Yeda Ltd.) replaced the second antibody in the precipitation of antigen-antibody complexes. The lyophylised Staphylococcal protein A was suspended in immunoprecipitation buffer (5) containing 0.2% bovine serum albumin (Sigma, ultra pure). Three volumes of the same buffer were added to the translation mixes with 50 μl of the protein A suspension. In control experiments, an excess (10 μg) of cold LHRH was added at this stage. The mixes were inverted gently to ensure thorough mixing of the suspension for 30 min at 4°C, and centrifuged, and 10-20 μl of the LHRH anti-serum were added to the supernatants. The supernatants were incubated at room temperature for 30 min and then at 4°C for 16 h. A further 50 μl of protein A suspension was added and mixed by gentle inversion at 4°C for 4 h. The precipitate was washed four times with 1.5 ml of immunoprecipitation buffer by gentle homogenisation and centrifugation. The final precipitate was boiled for 5 min in SDS-β-mercaptoethanol sample buffer (5) and the supernatant was electrophoresed on an 8-20% gradient gel as described above. The gels were fluorographed (11,12) and exposed to film (Kodak X-omat R) for between 10 days and 1 month. Autoradiograms from separate gels were aligned according to the positions of the ^{14}C-molecular weight standards which were electrophoresed in parallel.

RESULTS AND DISCUSSION

Figure 1 shows that the translation products of human hypothalamus spanned a similar molecular weight range to those of the rat and mouse and were of comparable specific activity despite the delay between death and RNA extraction. This confirms (13) that the *in vitro* translation of mRNA occurs with similar efficiency irrespective of whether the original tissue was fresh or had been stored at 4°C for several hours.

Among the translation products of the human and normal mouse and rat hypothalamic mRNA was a single major band with an apparent molecular weight of

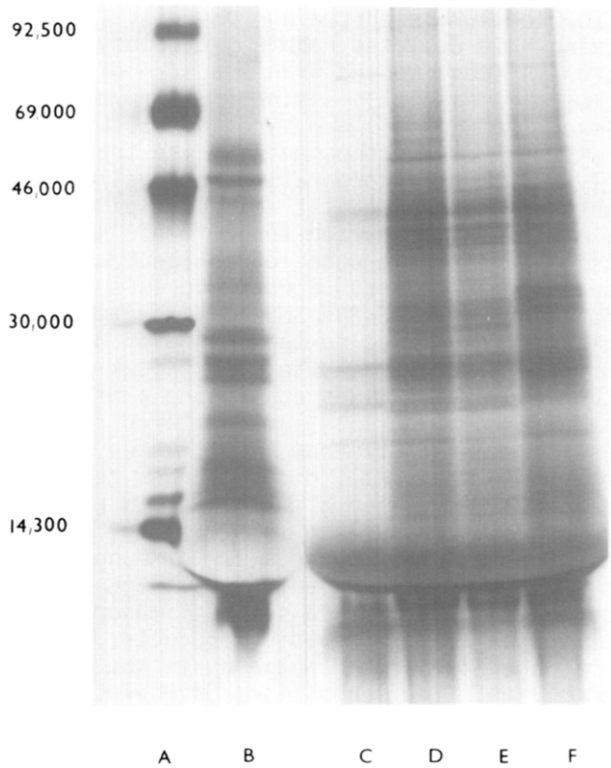

Figure 1

This figure shows the ^{3}H-labelled translation products of hypothalamic messenger RNA samples of human (lane B), normal mouse (lane D) hpg mouse (lane E) and rat (lane F) following polyacrylamide gel electrophoresis and autoradiography. Lane C shows a similar translation mix to which no exogenous mRNA was added and lane A shows 2 µl of a ^{14}C-labelled molecular weight standard mixture: phosphorylase b, 92500; bovine serum albumin, 69000; ovalbumin, 46000; carbonic anhydrase, 30000; and lysozyme, 14300.

28000, which was detected by immunoprecipitation of the radioactively labelled translation products with a specific anti-LHRH serum (Figs. 2 and 3). This same polypeptide was immunoprecipitated using both the second antibody and the protein A immunoprecipitation techniques from the rat hypothalamic mRNA translation products (Fig. 2). The polypeptide could not be detected, however, amongst the translation products of the rat, human (Fig. 2) or normal mouse (Fig. 3) mRNA when an excess of cold LHRH was added before immunoprecipitation, showing that the decapeptide is able to compete for the antiserum and block its binding with the precursor comnpletely. The 28000 molecular weight band was also not detected by the identical immuno-precipitation of a translation mix to which no exogenous mRNA was added or in which normal rabbit serum replaced the anti-LHRH serum (5).

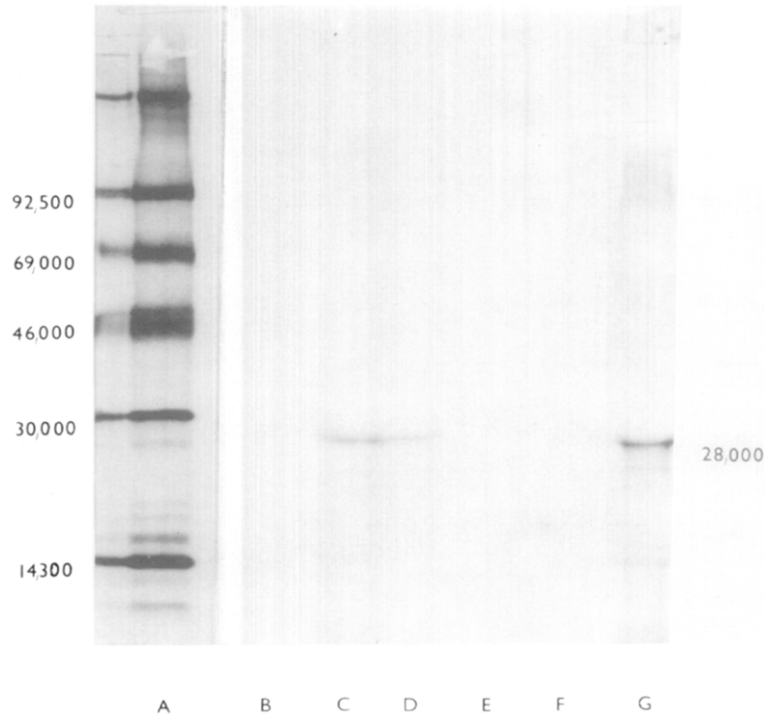

Figure 2

Polyacrylamide gel electrophoresis and autoradiography following immunoprecipitation of rat and human hypothalamic mRNA translation products with an anti-LHRH serum. Lanes B, C and D show the rat hypothalamic translation products immunoprecipitated in the presence of excess cold LHRH (lane B), using either the second antibody (lane C) or the Staphylococcal protein A immuno-precipitation technique (lane D). A 28000 molecular weight polypeptide was immunoprecipitated by both methods. Lane E shows that no polypeptides were immunoprecipitated when a translation mix without exogenous mRNA was treated in an identical manner. Lane G shows that the main immunoprecipitation product in an human translation mix was a 28,000 MW polypeptide; no immunoprecipitation was seen in the presence of excess cold LHRH (lane F). Lane A shows 2 μl of a ^{14}C-labelled standard molecular weight mixture identical to those detailed in Fig. 1.

When the translation products from the hypothalamic mRNA of the hpg mouse were treated in an identical manner with the anti-LHRH serum no polypeptides were immunoprecipitated (Fig. 3). Though the precise mechanism of the inherited defect in the hpg mouse is unknown, these results suggest that it is unrelated to the processing mechanism of a large precursor molecule since the precursor appears to be either completely absent, or present in a mutated form which is not recognised by the anti-LHRH serum. It is unlikely that the polypeptide is present at levels below our limits of detection since the genetics of the hpg condition clearly show that the primary defect represents

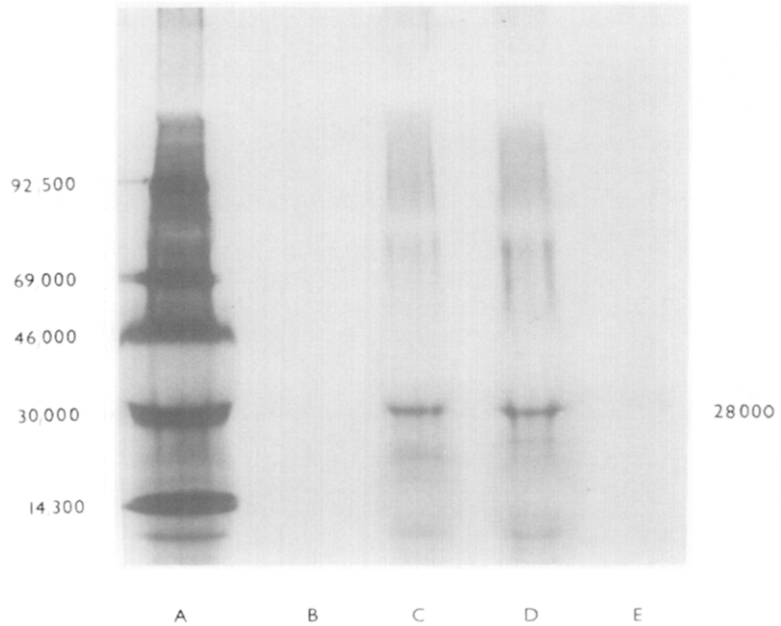

Figure 3

Polyacrylamide gel electrophoresis and autoradiography following immunoprecipitation of normal mouse and hpg mouse hypothalamic mRNA translation products with an anti-LHRH serum.

Lane C shows the normal mouse translation products immuno-precipitated using the second antibody immunoprecipitation technique and lane D shows a similar immunoprecipitation of rat hypothalamic translation products. The only immunoprecipitated polypeptide migrated with an apparent molecular weight of 28000. Lane E shows the hpg mouse translation products treated by an identical procedure and demonstrates the absence of any immuno-precipitate. Lane B shows a no-message blank treated by the same procedure using the anti-LHRH serum. Lane A shows the ^{14}C-labelled standard molecular weight mixture as detailed in Fig. 1.

an 'all or none' situation (6). These results do not allow us to speculate further on the nature of the primary defect in the hpg mouse; the presence of a defective, non-translateable mRNA, or its complete absence due to a transcriptional defect remains to be determined with the aid of specific LHRH nucleic acid probes.

The identification of a large 28000 molecular weight LHRH immunoreactive polypeptide within the hypothalami of three different mammalian species, and the demonstration of its complete absence from the hpg mouse hypothalamus shows that LHRH is initially synthesized by way of a precursor molecule which is twenty times larger than the active peptide in a manner similar to other hypothalamic peptides such as vasopressin (3), oxytocin (4) and somatostatin

(14,15). Gel filtration studies of hypothalamic extracts suggest that the precursor is probably processed to LHRH by way of intermediates which range in size from 26000 to 1800 (16,17). The precursor may also contain more than one sequence of LHRH and/or other biologically active peptides. However, unless the hpg condition is due to a point mutation, the fact that the hpg mouse appears to be free of any other obvious endocrine or non-endocrine abnormalities suggests that active components of the precursor, other than LHRH, are unlikely to be crucial for the normal function of the animal.

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