IN VITRO BIOSYNTHESIS AND PROCESSING OF COMPOSITE COMMON PRECURSORS CONTAINING AMINO ACID SEQUENCES IDENTIFIED IMMUNOLOGICALLY AS NEUROPHYSIN I/OXYTOCIN AND AS NEUROPHYSIN II/ARGININE VASOPRESSIN

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1. Introduction

In the mammalian pituitary and hypothalamus an interesting class of precursors to polypeptide hormones has been reported that have the unusual feature of comprising more than one functionally distinct polypeptide [1–6]. These composite precursors bear a close resemblance to viral polyproteins [7] which also comprise several distinct functional products. To date, direct evidence for such cellular polyproteins [8], in the form of amino acid sequence, is only available for pro-opiocortin [1,9] which consists of corticotropin, β-lipotropin, endorphins and melanotropins. However, indirect evidence points to the existence of similar composite precursors in the hypothalamus, namely the common precursors to arginine vasopressin/neurophysin II and oxytocin/neurophysin I.

A tentative glycopolypeptide precursor with app. $M_r > 20\,000$ was isolated from mice and rat hypothalami and cross-reacted with antibodies raised against one of the cysteine-rich neurophysins [10,11] known to transport the nonapeptide hormones oxytocin and arginine vasopressin to their storage site in the posterior pituitary [12]. An AVP-like peptide as well as one of the neurophysins could be released from the precursor by trypsin-treatment [13]. Another precursor has been extracted from hypothalami which may be the pro-form to oxytocin and another neurophysin species; this precursor is not glycosylated and has M_r 15 000–17 000 [13,14]. Here we report the synthesis of the composite common precursors to OT/Np I and to AVP/Np II by translation of hypothalamic poly(A)(+) RNA in cell-free systems of reticulocyte lysate and wheat germ. We also demonstrate that the immunologically identified precursors can be processed either in the presence of microsomal membranes or in oocytes *(Xenopus laevis)* after micro-injection of hypothalamic poly(A)(+) RNA.

Abbreviations: OT, oxytocin; AVP, arginine vasopressin; Np I, bovine neurophysin I; Np II, bovine neurophysin II; LHRH, luteinizing hormone releasing hormone; SDS, sodium dodecyl sulfate; app. M_r, apparent relative molecular mass

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2. Methods

2.1. *Antisera*

Rabbit antisera to bovine Np I and Np II were purchased from Bioproducts (Brussels). According to this company anti-Np I was purified by affinity chromatography on Np II–Sepharose columns which apparently improves the specificity of anti-Np I. Anti-Np II was purified in a similar way using Np I–Sepharose. Bioproducts claims this procedure to reduce cross-reactivity of the neurophysin antisera to <0.5% as judged by radioimmunoassay. Rabbit antisera to AVP or OT were obtained from Ferring, Kiel. According to Ferring there is no cross-reaction between anti-AVP and OT or lysine vasopressin and <30% with vasotocin; anti-AVP does not discriminate between oxidized or reduced AVP. Also, little cross-reaction was observed between anti-OT and arginine vasopressin or lysine vasopressin [15]. All antisera were precipitated with 28 g ammonium sulfate/100 ml to produce the IgG fractions.

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2.2. *Preparation of bovine hypothalamic poly(A)(+) RNA and its translation in cell-free systems*

Bovine hypothalamic poly(A)(+) RNA was isolated from detached polysomes and further purified by oligo(dT)-cellulose chromatography [16]. The poly(A)(+) RNA was translated either in cell-free systems from reticulocyte lysate (New England Nuclear or Amersham) or wheat germ [16,17]. In general the reticulocyte system was favoured because of its high translation efficiency. The translation products were identified using the specific IgG fractions. The immunoreactive material was precipitated with *Staphylococcus aureus* immonoabsorbant [16,17] and analyzed by SDS–polyacrylamide gel electrophoresis. For other details see [16,17].

3. Results and discussion

In [17], rabbit antiserum raised against porcine neurophysins was used for identification of the cell-free translation products; this antiserum did not discriminate between Np I and Np II, yielding 3 immunoreactive products with M_r 21 000 (21 k), 18 000 (18 k), and 16 500 (16.5 k) (fig.1, lane 2). Because of the cross-reactivity of the antiserum with porcine Np I and Np II the translation products could not be correlated with either of the two neurophysins. Using more specific antibodies to bovine Np I or Np II the different polypeptides could be identified: anti-Np I immunoprecipitated the 16.5 k while anti-Np II reacted with the 18 k and 21 k products (fig.1, lanes 3,4,7,8). As shown below the 16.5 k polypeptide is the composite common precursor to OT and Np I, the 21 k product to AVP and Np II. The 18 k precursor shows immunological cross-reaction with anti-Np II but not with anti-AVP; it is virtually absent in the wheat germ (fig.1, lanes 6,8) and oocyte systems (fig.3, lane 8) which may suggest that the 18 k product is a premature 21 k polypeptide derived from a pre-termination event. The relationship of the 18 k with the 21 k precursor is discussed later.

3.1. *The composite precursor to oxytocin and neurophysin I*

The in vitro product with M_r 16.5 k could be immunoprecipitated using either antibodies to Np I (fig.1, lanes 3,7; fig.2, lane 5) or to OT (fig.2, lane 1). Competition experiments indicated that anti-Np I or anti-OT specifically recognized amino acid sequences

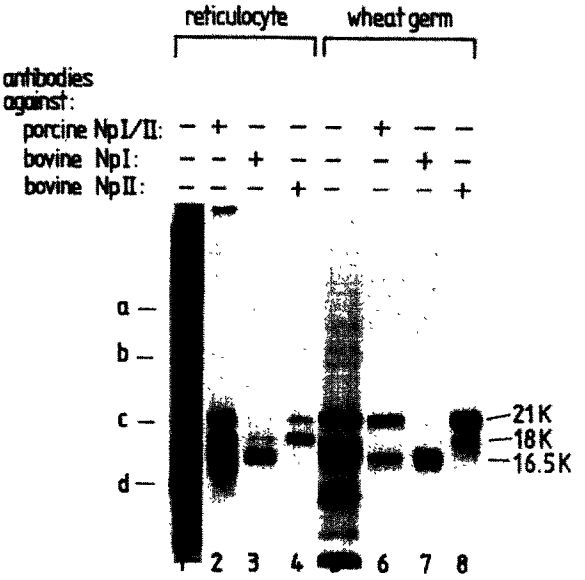

Fig.1. Translation of 0.5 μg bovine hypothalamic poly(A)(+) RNA. Incorporation of [^{35}S]cysteine in the reticulocyte lysate (Amersham or New England Nuclear) or wheat germ system (total vol. 25 μl), identification of the translation products by immunoprecipitation and analysis by SDS–polyacrylamide (15%) gel electrophoresis was done as in [16,17]. For immunoprecipitation 10 μg respective purified antibodies were used. For porcine anti-Np I/Np II see [17]. Lanes 1 and 5 represent the total translation products prior to immunoprecipitation. Gels were dried and fluorographed [17]. In this and subsequent fluorograms lanes are derived from different gels and aligned according to the position of marker proteins; (a) ovalbumin, 43 000; (b) carbonic anhydrase, 30 000; (c) soy bean trypsin inhibitor, 20 000; (d) α-lactalbumin, 14 000.

within the 16.5 k precursor which are immunologically identical to Np I or OT. Excess of OT (fig.2, lane 2) but not of Np I (lane 3), LHRH (lane 4) or BSA (lane 1) effectively competed with the 16.5 k precursor for anti-OT. With excess of AVP (not shown) some competition was observed which may be due to the low immunological cross-reactivity of anti-OT with AVP [15]. When competition experiments were performed using anti-Np I only excess of Np I (fig.2, lane 6) but not of OT (lane 7) competed in binding of anti-Np I to the 16.5 k product.

That both anti-Np I and anti-OT recognized the same precursor was supported by sequential immunoprecipitation studies. The immunoprecipitate obtained with anti-Np I (fig.2, lane 12) was sedimented and the supernatant fraction now supplemented with anti-OT (fig.2, lane 13). The results show

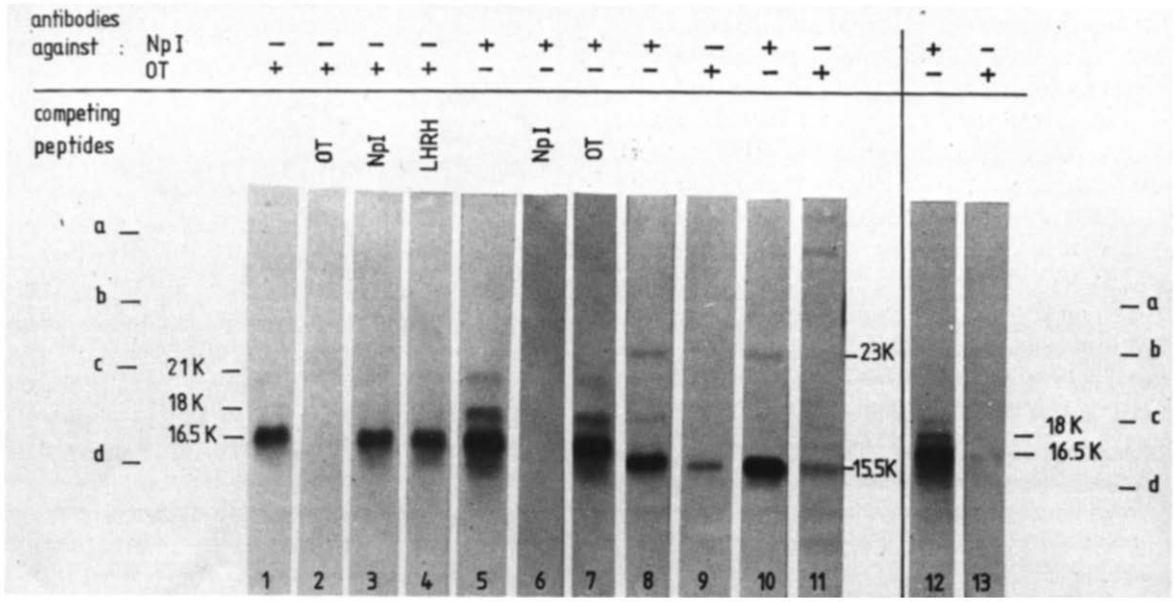

Fig.2. Synthesis, processing and sequential immunoprecipitation of the Np I/OT precursor in the reticulocyte lysate and the oocyte *(X. laevis)* system. Bovine hypothalamic poly(A)(+) RNA was translated in the reticulocyte lysate system (total vol. 25 μl; lanes 1–9,12,13) using 20 μCi [^{35}S]cysteine as radioactively labeled amino acid. Lanes 10,11 show experiments where each oocyte (stage VI) was injected with 30–50 ng poly(A)(+) RNA from bovine hypothalami and preincubated for 9 h in Barth's medium at 20°C. The oocytes were then transferred to Barth's medium containing 333 μCi/ml of [^{35}S]cysteine and incubation was continued for 11 h at 20°C. Five oocytes were homogenized and immunoprecipitated with 10 μg respective antibody. Where indicated the immunoprecipitation was carried out in the presence of excess (10 μg/25 μl assay) unlabeled peptide (OT, Np II or LHRH). Lanes (8,9): 5 A_{260} units of dog pancreas microsomal membranes [17] per ml assay mixture were added prior to incubation. Lanes (12,13) sequential immunoprecipitation: 25 μl reticulocyte lysate assay mixture was subjected to an initial immunoprecipitation step with anti-Np I. The immunoprecipitate was collected by centrifugation (lane 12); the supernatant fraction of this initial step was supplemented with anti-OT (lane 13). Experiments shown in lane (12) and (13) were carried out under slightly different electrophoretic conditions and therefore the radioactive products and the standard proteins (see legend to fig.1) run at different positions.

that the first immunoprecipitation step had already precipitated the 16.5 k polypeptide. Similar results were obtained in the reverse experiment where the sequential immunoprecipitation was first started with anti-OT followed by a second step with anti-Np I (not shown). The data strongly suggest that the 16.5 k polypeptide is a composite common precursor containing amino acid sequences immunologically identical to OT and Np I.

3.2. *Processing of the OT–Np I precursor*

The precursors to Np I/Np II can be processed by a microsomal membrane fraction [17]. Using the wheat germ system we found that the 16.5 k precursor could be converted into a product with M_r 15.5 k. These data are now confirmed and extended by using the more specific antiserum to Np I as well as anti-OT. Fig.2 (lanes 8,9) demonstrates that in the presence of microsomal membranes from dog pancreas the 16.5 k precursor was processed to yield a 15.5 k product, which could be identified by antibodies to Np I as well as to OT. By analogy to the signal hypothesis [18] the 16.5 k composite precursor apparently represents the pre-pro-form to OT and Np I, which in the presence of membranes is converted into the 15.5 k pro-form. This is confirmed by experiments where *N*-formy [^{35}S]methionyl-tRNA$_i^{Met}$ is incorporated into the 16.5 k pre-pro-form (not shown).

The assumption that the 15.5 k product is the processed pro-form to the common OT–Np I precursor is also supported by experiments where hypothalamic poly(A)(+) RNA had been micro-injected into oocytes from *Xenopus laevis*. The immunoreac-

tive material identified either by anti-Np I (fig.2, lane 10) or anti-OT (fig.2, lane 11) migrated at the same position as the 15.5 k product obtained from the microsomal membrane experiments. The 18 k and 21 k as well as the 23 k polypeptides (fig.2, lane 10) visible as minor bands (fig.2, lane 8) are evidence of the low level of cross-reactivity towards Np II found with anti-NP I.

Experiments with tunicamycin-treated ascites cell membranes as well as chromatography of the 15.5 k polypeptide on concanavalin A–Sepharose columns gave no indication that the processed precursor to Np I and OT was core glycosylated, in contrast to the AVP–Np II common precursor. These findings agree with [13,14] that a precursor to Np I and OT extracted from hypothalamic tissue is not glycosylated.

3.3. *Biosynthesis and processing of the composite common precursor to AVP and Np II after micro-injection of hypothalamic poly(A)(+) into oocytes* (X. laevis)

Translation of bovine hypothalamic poly(A)(+) in cell-free systems from reticulocyte lysate or wheat germ yields a composite common precursor, the pre-pro-form to AVP and Np II [19]. In the presence of a microsomal membrane fraction this pre-pro-form with M_r 21 k was processed to a pro-form with M_r 19 k and core-glycosylated to yield a glycopolypeptide with M_r 23 k ([19], fig.3, lanes 1,2,5,6). Another immunoreactive polypeptide with app. M_r 18 k cross-reacted with anti-Np II but not with anti-AVP. In contrast to [17] the 18 k product is not processed by microsomal [19]; the discrepancy in the interpretation may be explained by the less specific antibodies used in those studies; these antibodies were unable to discriminate between Np I and Np II. We have injected hypothalamic mRNA into oocytes *(X. laevis)* to study the processing of the composite AVP/Np II precursor in an in vivo system. The major product was the 23 k core-glycosylated pro-form essentially confirming the in vitro experiments using added membranes (fig.3, lanes 4,8). Additional bands were observed at 25 k, 20 k and 14 k which were only evident in the oocyte system. These products were presumably the results of further modifications and/or proteolytic cleavages of the precursor. Whether the 25 k product has additional oligosaccharide chain(s) attached to the AVP/Np II precursor and whether the 14 k polypeptide is an intermediate derived by processing of the glycosylated pro-form is under investigation.

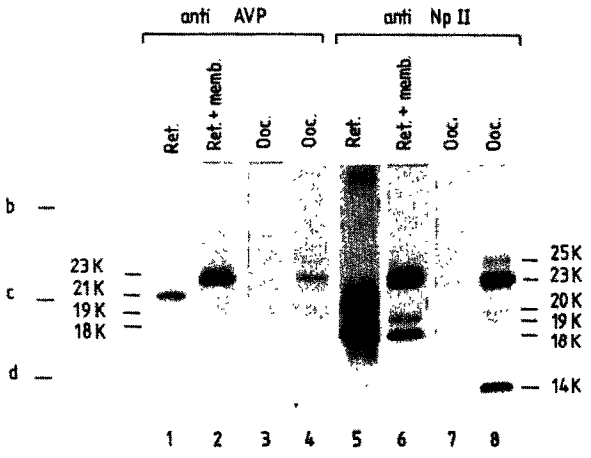

Fig.3. Synthesis and processing of the AVP/Np II precursor. In the experiments shown in lanes 3,4,7,8,30–50 ng poly(A)(+) RNA were micro-injected into oocytes and incubated as outlined in the legend to fig.2. Lanes 4 and 8 show the products identified with anti-AVP and anti-Np II, respectively; immunoprecipitation in the presence of: anti-AVP and 10 μg/25 μl assay of unlabeled AVP (lane 3); anti-Np II and 10 μg/25 μl assay of unlabeled Np II (lane 7). For other conditions see legends to fig.1 and 2.

These indicate that two hypothalamic oligopeptide hormones are made via composite common precursors [8,16] which in one case includes OT and Np I, in the other AVP and Np II. The two precursors are synthesized as pre-pro-forms with a signal sequence cleaved off when microsomal membranes are present during translation. The AVP–Np II but not the OT–Np I precursor is a glycopolypeptide. That the two hypothalamic precursors are polyproteins has been documented here by immunological means; their further characterization by tryptic mapping as well as precise location of the peptides within the respective precursors are presently being studied.

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