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Original Article

Colocalization of Oxytocin and Phosphorylated Form of Elongation Factor 2 in the Rat Hypothalamus

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Oxytocin (OT) is one of the neuropituitary hormones and is synthesized in the neurons of the paraventricular nucleus (PVN) and supraoptic nucleus (SON). Previous studies have shown that the mRNAs encoding OT are delivered from the soma to both dendrites and axons of the neurons in the PVN and SON. However, it has not been elucidated whether a translational regulation mechanism to enable local synthesis of the hormone exists in the axons of the neurons of PVN and SON. Elongation factor 2 (EF2) is essential for polypeptide synthesis during protein translation. Moreover, phosphorylation of EF2 by EF2 kinase enhances the translation of certain mRNA species. In the present study, in order to shed light on the mechanisms involved in the translational regulation of OT synthesis, we investigated the localization of phosphorylated EF2. Phospho-EF2 was localized in the soma of the neurons in PVN and SON, and in the swellings of the median eminence where axonal tracts of the neurons in the PVN and SON exist. The phosphorylated form was also observed in the rat hypophysis. Moreover, phospho-EF2 and OT were colocalized in a part of the neurons in the PVN and SON. These results suggest that OT may be partially translated in the axons of neurons in the PVN and SON, and then secreted from the pituitary.

Key words: oxytocin, PVN, SON, elongation factor 2, local translation

The neurohypophysial nonapeptide oxytocin (OT) is a highly conserved molecule synthesized as preprooxytocin in particular neurons in the hypothalamus [1]. The processed OT peptide is released into the peripheral circulation in response to a variety of physiological stimuli such as suckling, parturition, or certain kinds of stress [2]. The peripheral functions of OT induce normal labor through uterine contraction and normal development of offspring through lactation [1, 2]. OT is also released from the dendrites and axons into the central nervous system, where it presumably plays a role as a neurotransmitter or as a modulator of synaptic plasticity [3, 4].

It has been thought that the precursor of OT is subject to modification and processing in the soma of neurons in the PVN and SON and is then transported to nerve terminals in the posterior pituitary [1]. However, recent studies have shown that OT mRNA is sorted to both dendrites and axons [4, 5]. These results suggest that there must be translational machinery and regulatory mechanisms to

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enable local synthesis of OT in response to stimuli in the nerve terminals at the posterior pituitary, and OT mRNA is locally translated there.

Dendrites are equipped with components of the translation machinery [6–8]. However, it is unclear whether the axons of the oxytocinergic neurons in the PVN and SON have such machinery. Evidence for the axonal translation of OT has not been obtained. Elongation factor 2 (EF2) is essential for protein translation. EF2 catalyses the movement of peptidyl-tRNA from the A site to the P site on the ribosome, thereby regulating polypeptide synthesis during translation [9]. Moreover, EF2 is phosphorylated by a Ca^{2+}/calmodulin-dependent protein kinase, EF2 kinase [10]. The phosphorylation of EF2 inhibits its activity and generally leads to an inhibition of total protein synthesis, but increasing evidence also indicates that EF2 phosphorylation selectively enhances translation of certain mRNA species [11, 12].

To investigate the occurrence and mechanism of the local translation of OT, in the present study we examined the localization of OT and phospho-EF2 in the neurons of PVN and SON.

Materials and Methods

Animals. Male Wistar rats (Japan SLC, Hamamatsu, Japan) weighing 200–230 g were used. They were housed individually in plastic cages on a standard light and dark cycle at 23 ± 1°C with free access to tap water and laboratory chow (Charles River, Wilmington, MA, USA). All animal use procedures were approved by the Okayama University Animal Care committee and were in strict accordance with the Japan Physiological Society's Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences.

Immunohistochemistry. The animals were deeply anesthetized with sodium pentobarbital (40 mg/kg i.p.) and perfused transcardially with 100 ml of physiological saline followed by 900 ml of 4% paraformaldehyde and 0.2% picric acid in 100 mM phosphate buffer (pH7.4). The brains were carefully excised and immersed in the same solution for 24 h at 4°C and then cryoprotected overnight with 30% sucrose in 100 mM phosphate buffer (pH7.4). The brains were cut crosswise into several blocks and the blocks containing the hypothalamus were coronally and serially cut into sections of 30-μm thickness using a freezing microtome. After sectioning, the slices were immersed in 100 mM Tris-buffered saline (pH7.4) (TBS). Every fourth section was used to detect phospho-EF2 and the next section was used to detect OT. The sections were washed with TBS containing 0.1% Tween 20 (T-TBS) containing 0.3% hydrogen peroxide to reduce the background and preincubated for 3 h at 25°C with T-TBS containing 5% dried nonfat milk. Free-floating sections were gently rocked for 18 h at 4°C with the primary antibody solution either T-TBS containing anti-phospho-EF2 antibody (1: 5,000) or polyclonal OT antibody (1: 10,000, Pierce, Rockford, IL, USA). The production and characterization of the anti-phospho-EF2 antibodies were described previously [11]. The sections were washed and bound IgG was detected using biotinylated antisera and avidin-conjugated peroxidase [13] using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). As a control for OT immunostaining, some sections were incubated with OT antisera preadsorbed with OT. Several sections were counterstained with Cresyl Violet.

Some of the sections immunostained with anti-phospho-EF2 were then gently rocked for 18 h at 4°C with the solution containing polyclonal OT antibody (1: 5,000, Pierce). The sections were washed and bound IgG was detected using biotinylated antisera and avidin-conjugated alkaline phosphatase using the Vectastain ABC kit (Vector Laboratories).

Western blotting analysis. The animals were decapitated under nitrous oxide anesthesia. The removed brains were dipped into ice-cold physiological saline. The hypophysis and median eminence were excised from 2 rats and sonicated for 10 sec with 4 volumes of lysis buffer using a microtip sonicator. The lysis buffer contained 10 mM Tris (pH7.4), 1% Triton X-100, 1.0% sodium lauryl sulfate, 5 mM EDTA, 1 mM phenylmethyl sulphonyl fluoride, 0.28 U/ml aprotinin, 50 μg/ml leupeptin, 1 mM benzamidine and 7 μg/ml pepstatin. Protein content was determined by the method of Bradford using a kit from Bio-Rad (Hercules, CA, USA). After centrifugation (16,000 *g* for 5 min), aliquots containing approximately 10 μg protein were separated by SDS-PAGE (12% polyacrylamide).

The proteins were transferred electrophoretically to nitrocellulose sheets (Hybond-C, GE Healthcare, Piscataway, NJ, USA). The blots were washed once with 100 mM Tris-buffered saline, pH7.4 (TBS), and then incubated for 3h with TBS containing 0.1% Tween 20 (T-TBS) plus 5% dried nonfat milk (Difco, Becton and Company, Sparks, MD, USA). The blots were then transferred to fresh TBS-T/milk containing polyclonal rabbit anti-phospho-EF2 antibody (1: 500) or polyclonal rabbit anti-total EF2 antiserum (1: 500). The production and characterization of these antibodies were described previously [14, 15]. After incubation for 18h at 4°C, the blots were washed 3 times in TBS and then immunoreactivity was detected using the enhanced chemiluminescence method (ECL, GE Healthcare).

Results

Distribution of phospho-EF2 and OT in the diencephalon. The distribution of phospho-EF2 and OT in the diencephalon was investigated by immunohistochemistry. Total EF2 and EF2 kinase were ubiquitously distributed in the central nervous system including the PVN and SON (data not shown, manuscript in preparation). Interestingly, high levels of phospho-EF2 were detected in the lateral, medial, and periventricular zones of the hypothalamus (Fig. 1). In particular, the highest levels of phospho-EF2 were detected in the neurons of PVN and SON (Fig. 1). Staining of phospho-EF2 was also detected in the inner layer of the median eminence, which is an axonal tract of neurons from the PVN and SON to the hypophysis [14] (Fig. 2). The staining was scattered in the inner layer, and the shape was spherical (arrows in Fig 2A & B). The distribution of phospho-EF2 and total EF2 in the median eminence was further assessed by immunoblotting analysis, and both total EF2 and phospho-EF2 were detected in this region (Fig. 2C). Furthermore, immunoblot analysis revealed that both total EF2 and phospho-EF2 were distributed in the hypophysis (Fig. 2C).

Colocalization of phospho-EF2 and OT in the hypothalamus. Double immunostaining analysis indicated that phospho-EF2 and OT were colocalized in a part of the neurons in the PVN and SON (Fig. 3). OT-positive neurons were a subset of neurons positively stained for phospho-EF2 (Fig. 3). Phospho-EF2 was detected predominantly in the perykarya and was weakly detected in the axons, while OT was strongly detected in both the perykarya and axons of the paraventriculo-hypophysial tract (Fig. 3).

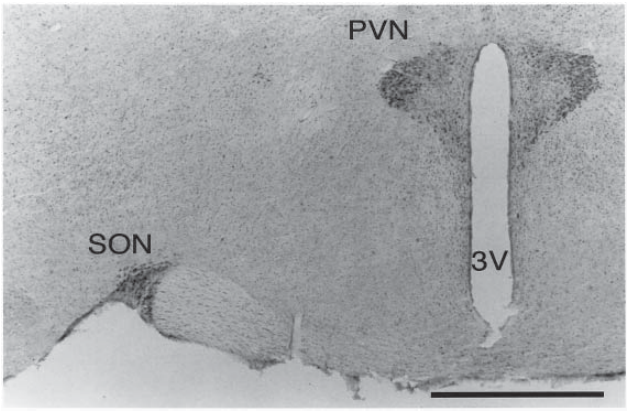

Fig. 1 Localization of phospho-EF2 in rat diencepahalon. Sections from the hypothalami of male rats were dissected and phospho-EF2 was detected by immunohistochemistry. High levels of phospho-EF2 were detected in the lateral, medial, and periventricular zones of the hypothalamus. In particular, phospho-EF2 was enriched in the magnocellular and parvocellular neurons of the paraventricular nucleus (PVN) and in the magnocellular neurons of the supraoptic nucleus (SON). 3V, third ventricle. Bar = 1mm.

Discussion

The results of the present study showed that the phosphorylated form of elongation factor 2 (phospho-EF2) was distributed in the axons of the neurons of the PVN and SON. Considering the functional role of EF2 in protein or peptide synthesis, these results suggest that the axons may be equipped with components of the translation machinery. Double immunostaining analysis of phospho-EF2 and OT showed that OT-positive neurons were a subset of phospho-EF2-positive neurons, indicating that OT coexists with phospho-EF2 in the neurons of the PVN and SON. Some of the phospho-EF2-positive neurons in the nuclei did not contain OT. OT and vasopressin (VP) and their respective neurophysins are synthesized in different neurons in the 2 nuclei [15, 16]. Moreover, VP mRNA is delivered to axons and dendrites of hypothalamic magnocellular

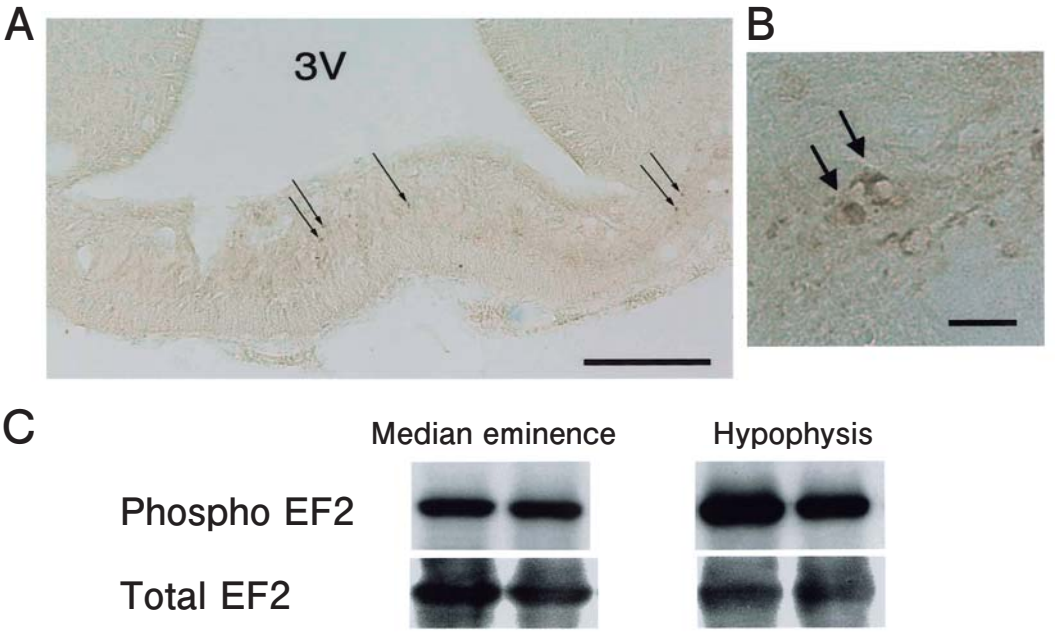

Fig. 2 Localization of phospho-EF2 in the median eminence and hypophysis. **A**, Phospho-EF2 was detected within axonal swellings in the internal layer of the median eminence (arrows). 3V indicates the third ventricle. Bar = 200 μm; **B**, High magnification of internal layer of the median eminence. Arrows, axonal swelling bodies. Bar = 20 μm; **C**, Western blotting analysis of phospho-EF2 and total EF2 in the median eminence and hypophysis. Phospho-EF2 was detected in both regions. The hypophysis and median eminence were taken from 2 male rats and the homogenized samples from each rat were loaded in different lanes.

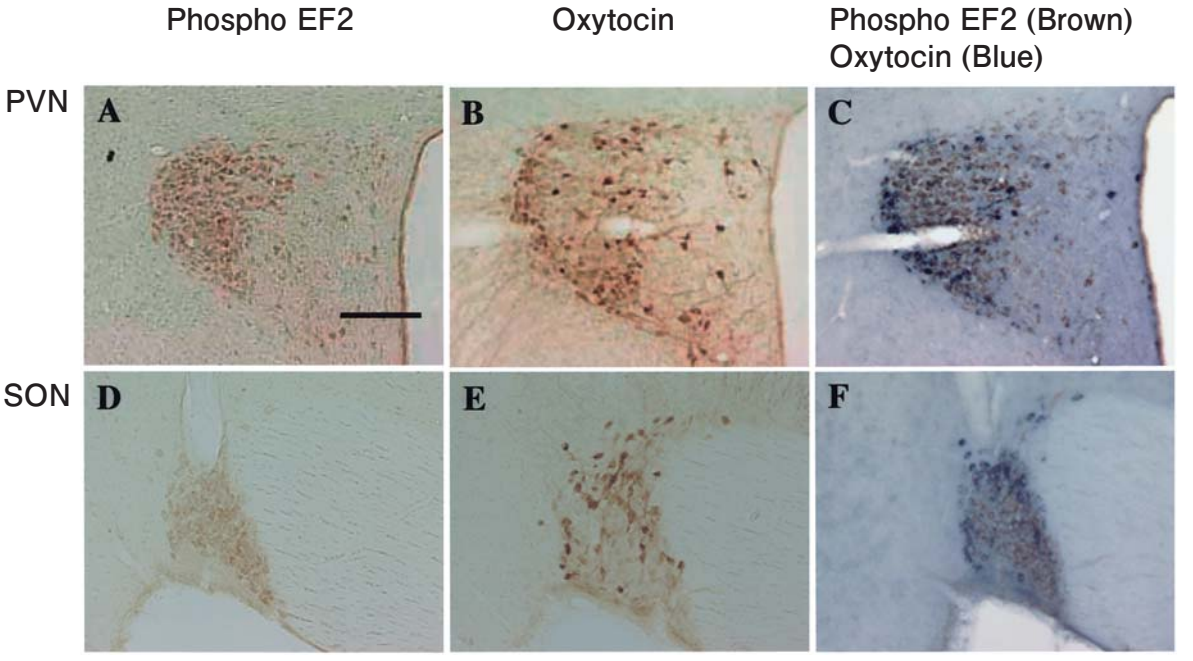

Fig. 3 Immuno-staining of phospho-EF2 and oxytocin in PVN and SON. **A** and **D**, phospho-EF2; **B** and **E**, OT; **C** and **F**, double-immunostaining for phospho-EF2 and OT. **A–C**, PVN; **D–E**, SON. Phospho-EF2 and oxytocin were detected in the sections from the hypothalamus by immunohistochemistry. Oxytocin-positive neurons (blue) were a subset of phospho-EF2-expressing neurons (brown). Bar =100 μm.

neurons [5]. Their results suggest that the phospho-EF2-positive neurons lacking OT may be VP neurons, and some neurohypophysial hormones may be secreted from the hypophysis through local protein translation.

Phosphorylation of EF2 by EF2 kinase inhibits the ability of EF2 to support peptide elongation and the resultant protein synthesis during translation [10, 17, 18]. However, recent studies have shown that the phosphorylation of EF2 increases the synthesis of a specific protein, *i.e.*, alpha Ca^{2+}/calmodulin dependent kinase II in synaptoneurosomes after synaptic activation [12]. Other proteins specifically enhanced like this kinase have not been completely identified, but there must be other proteins whose synthesis is specifically increased through enhanced translation mediated by phospho-EF2 in neurons. The present results suggest that OT may be one of these proteins.

Subcellular localization of mRNA species is observed in many species of animal and plants [19–21]. In neurons, some RNAs are transported from the perykarya to dendrites or axons [5, 8]. Increasing evidence is being accumulated about the transport machinery of mRNAs in neurons. A cis-acting element within the 3' untranslated region of the mRNAs that binds a trans-acting RNA binding protein controls dendritic mRNA localization [22–24]. Hematopoietic zinc finger was identified as a trans-acting factor [25]. Although the mechanism of the translocation of OT mRNA in axons remains unclear, the present results suggest that the transport machinery may exist in the neurons of the PVN and SON. OT synthesis and release from the pituitary are induced in response to a variety of physiological stimuli, such as suckling, parturition, or certain kinds of stress [1]. During such physiological stimuli, EF2 kinase may be activated, resulting in an increase of phospho-EF2 and OT translation in axons of the neurons in the PVN and SON.

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