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Involvement of neuromedin S in the oxytocin release response to suckling stimulus

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ABSTRACT

We recently identified neuromedin S (NMS) from the rat hypothalamus as an endogenous ligand for the FM-4/TGR-1 receptor distinct from neuromedin U. In the present study, we examined the role of NMS in the oxytocin release response to suckling stimulation by rat pups. Intracerebroventricular (icv) injection of NMS induced cFos expression in the paraventricular nucleus and supraoptic nucleus. Double immunohistochemical analysis revealed induction of cFos expression in a proportion of oxytocinergic neurons in both nuclei. In addition, icv injection of NMS stimulated oxytocin release dose-dependently in intact rats, and increased milk secretion in lactating rats. On the other hand, icv injection of anti-NMS antiserum into lactating rats significantly suppressed suckling-induced milk ejection. These results suggest that, in the rat, endogenous NMS plays an important role in the oxytocin release response to the suckling stimulus.

In 2000, neuromedin U (NMU) was identified as an endogenous ligand for the orphan receptors FM-3/GPR66 and FM-4/TGR-1 using a reverse-pharmacological technique. Therefore, these receptors were designated neuromedin U receptor 1 (NMU-1R) and 2 (NMU-2R), respectively [1–6]. Recently, we identified neuromedin S (NMS), consisting of 36 amino acid residues, from rat brain as another distinct endogenous ligand for these receptors. NMS shares seven amino acid residues of the carboxyl terminal core structure with NMU, and binds to both NMU receptors with an affinity almost equal to that of NMU. However, the NMS and NMU genes have been mapped to separate chromosomes, and the distributions of the two peptides largely differ [7–9]. Several studies have demonstrated that although both NMS and NMU share common physiological roles in circadian rhythm and food intake in rats, the strength and duration of their action largely differ [7,10,11]. Therefore, the possibility that there may be a specific receptor for NMS remains.

NMU-2R is expressed in various brain regions, such as the suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN), supraoptic nucleus (SON), arcuate nucleus (ARC), along the wall of the third ventricle in the hypothalamus and the CA1 region of the hippocampus [5,6,12,13]. Consistent with the distribution of this receptor, icv injection of NMS stimulates the expression of cFos in these areas [7,10]. Recently, it has been shown that NMU-2R mRNA is expressed in oxytocinergic neurons [14]. We have also confirmed in a preliminary study that cFos is expressed in oxytocin immunostained cells in the PVN, indicating the possible involvement of NMS in oxytocin release. In this study, therefore, we examined whether cFos expression is present in oxytocinergic neurons in the SON as well as the PVN, and whether icv injection of NMS increases the plasma oxytocin concentration.

If NMS is involved in oxytocin release, it is important to investigate the physiological significance of the NMS-oxytocin axis. It is well known that suckling-induced oxytocin release is very important for milk ejection in mammals. Mice lack oxytocin or its receptor shows a deficient milk ejection response to the suckling stimulus, and their pups die of malnutrition within a few days after birth [15]. However, the mechanism of oxytocin release in response to suckling has not been clarified. In this study, therefore, we focused on the role of NMS in suckling-induced oxytocin release in the lactating rat.

Materials and methods

Animals and icv injection. Adult female Wistar rats were housed individually in Plexiglas cages in an animal room maintained under a constant light-dark cycle (lights on from 7:00–19:00 h) and temperature (22 ± 1 °C). Food and water were provided *ad libitum*. A proportion of female rats were mated on the day of proestrus at approximately three months of age. The day of delivery was

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considered to be day 0 of lactation. The average number (±SEM) of pups per dam at delivery was 13.40 ± 1.48 (n = 112).

A 27-gauge intracerebroventricular (icv) cannula was implanted into the lateral cerebral ventricle of each rat under pentobarbital anesthesia using a method described previously [16]. During 4 days of postoperative recovery, the rats became accustomed to the handling procedure. Then 10 μl of NMS (Peptide Inc., Osaka, Japan) or saline was injected through the cannula from a 50-μl Hamilton syringe into each free-moving rat. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Plasma oxytocin measurement. To examine the effect of NMS on oxytocin release, 0.02, 0.2 or 2 nmol NMS, or saline, was injected icv into intact rats. In addition, the same doses of NMU (Peptide Inc., Osaka, Japan) were also injected to compare the effects with those of NMS. Whole blood was collected by decapitation at 5 and 60 min after treatment into a tube containing EDTA and the proteinase inhibitor aprotinin (Sigma–Aldrich, St. Louis, USA). After centrifugation at 4 °C, the plasma was stored at −80 °C until measurement of oxytocin concentration using an enzyme immunoassay (EIA) kit (Assay Designs Co., Ann Arbor, MI, USA). The intra- and interassay coefficients of variation were 4.6% (n = 4) and 8.2% (n = 5), respectively.

Double immunostaining for cFos and oxytocin. Double immunohistochemical staining for oxytocin and cFos was performed using a modification of a method described previously [11] on frozen brain tissue, which was removed from each rat 90 min after icv injection of 0.5 nmol NMS. Sections were cut at a thickness of 18 μm with a cryostat at a temperature of −20 °C. The sections were then fixed with 4% paraformaldehyde for 20 min and blocked for 1 h in 5% normal donkey serum in PBST, followed by incubation overnight at 4 °C with rabbit antiserum against rat oxytocin (Progen Biotechnik, Inc., Germany) together with goat antiserum against rat cFos (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the sections were incubated with a second antibody solution of Alexa-488-labeled anti-rabbit IgG antibody and Alexa-555-labeled donkey anti-goat IgG antibody solution for 30 min. The samples were observed using a fluorescence microscope (Axioskop 2plus: Zeiss, MA, USA). Digital images were contrasted and color-adjusted using Adobe Photoshop 7.0 for Windows.

Preparation of anti-rat NMS antibody. A polyclonal antibody was raised against the specific N-terminal portion of rat NMS, because the seven-residue C-terminal amidated sequence of rat NMS is identical to that of rat NMU [7]. Antiserum was obtained using the protocol reported previously [17]. In brief, a synthetic peptide, (Cys^0)-rat NMS (1–20), was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (Pierce). A New Zealand white rabbit was immunized by subcutaneous injection of this conjugate emulsified with Freund's complete adjuvant. The specificity of this antibody was confirmed by radioimmunoassay [17], and anti-rat NMS antibody did not cross-react with rat NMU. The neutralizing activity was verified by the calcium-mobilization assay using CHO cells stably expressing NMU-1R,-2R receptors [7].

Measurement of milk ejection volume. To examine whether icv injection of NMS or anti-NMS antiserum affects milk ejection in lactating dams after suckling stimulation by pups, we measured the increase of pup body weight due to milk intake during 15 min after initiation of suckling after treatment of the dams with NMS or anti-NMS antiserum. Normal rabbit serum was used as a control for anti-NMS antiserum. In this experiment, litter size was adjusted to 12 and 8 after birth for dams treated with NMS and anti-NMS antiserum, respectively, as it has been generally confirmed that a large litter size results in a smaller individual pup milk intake, and vice versa. Therefore, if treatment of dams with NMS and anti-NMS antiserum increases and decreases milk ejection, respectively, pup body weight is likely to increase and decrease during 15 min after the start of suckling, respectively. On day 10 of lactation, all pups were removed from the dam for 8 h, and then 15 min before they were returned, the dam was icv-injected with 0.2 nmol NMS, diluted (50×) normal rabbit serum, or the same dilution of anti-NMS antiserum.

Statistical analysis. The data (means ± SEM) were analyzed statistically by ANOVA with the *post hoc* Fisher's test, and differences at $P < 0.05$ were considered statistically significant.

Results

Cells immunoreactive for oxytocin were abundant in the medial magnocellular part of the PVN and the dorsal magnocellular part of the SON. A few cells immunoreactive for oxytocin were also observed in the medial parvicellular part of the PVN and ventral part of the SON (Fig. 1A and D). Icv injection of NMS induced cFos expression predominantly in the medial magnocellular part of the PVN and dorsal magnocellular part of the SON (Fig. 1B and E). Treatment with saline did not induce cFos expression in these regions (data not shown). A proportion of cells immunoreactive for oxytocin also expressed cFos (Fig. 1C and F).

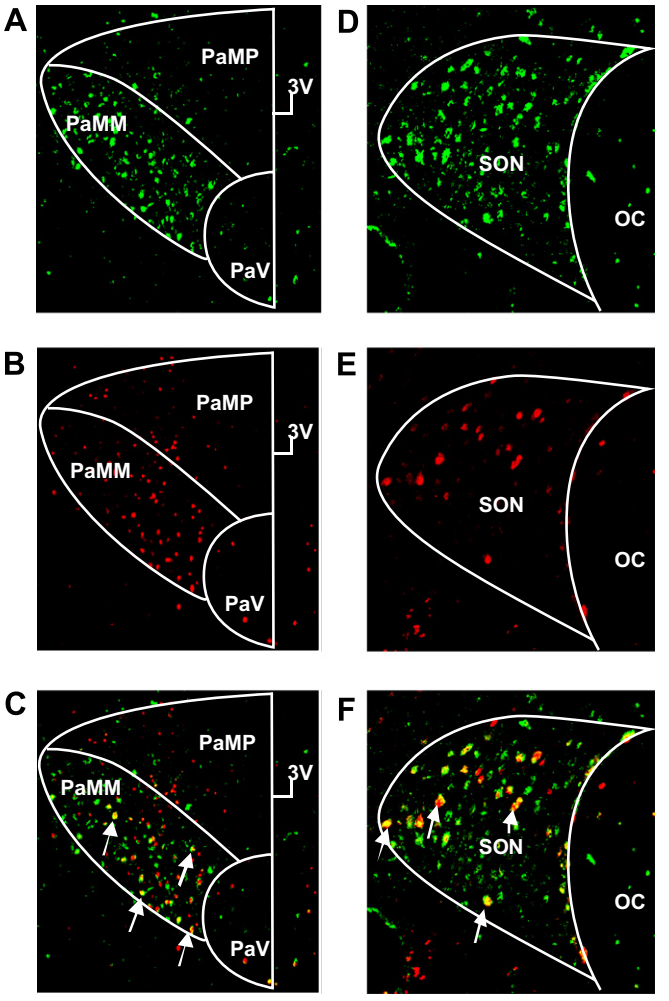

Fig. 1. Immunofluorescence staining for oxytocin (A, D) and for cFos (B, E) in the PVN and SON regions of the hypothalamus. cFos expression was determined 90 min after icv injection of 0.5 nmol NMS. C and F represent double immunostaining for cFos (red) and oxytocin (green) in the PVN (C) and SON (F). Arrows indicates typically co-stained cells. OC: optic chiasma, PaMM: paraventricular hypothalamic nucleus, medial magnocellular part. PaV: paraventricular hypothalamic nucleus, ventral part. PaMP: paraventricular hypothalamic nucleus, medial parvicellular part 3V: third ventricle.