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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).

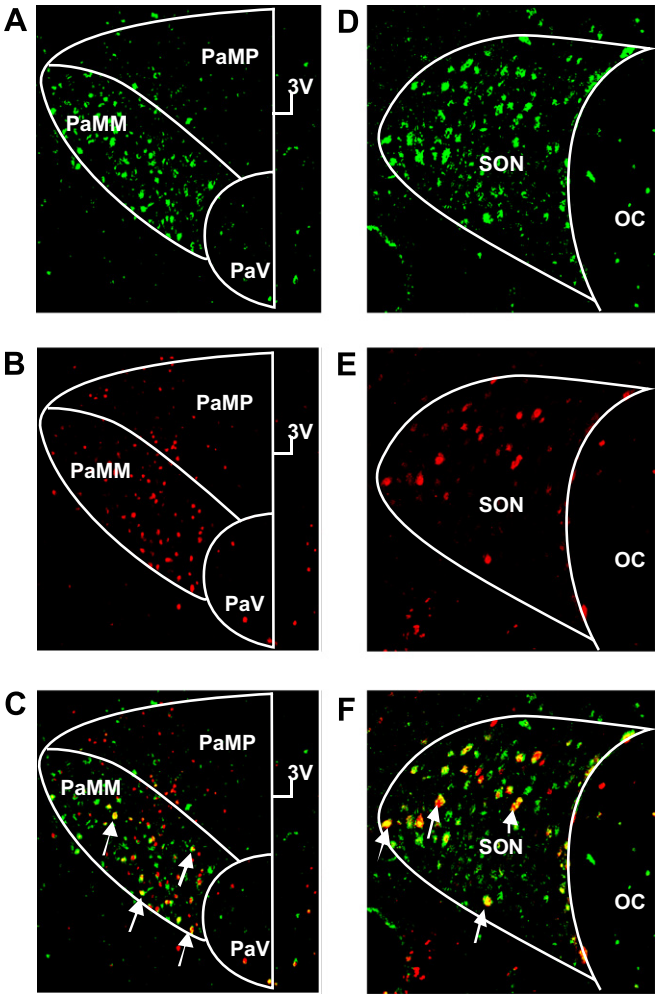

Fig. 1. Immunofluorescence staining for oxytocin (A, D) and for cFos (B, E) in the PVN and SON regions of the hypothalamus. cFos expression was determined 90 min after icv injection of 0.5 nmol NMS. C and F represent double immunostaining for cFos (red) and oxytocin (green) in the PVN (C) and SON (F). 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.

Icv injection of 0.2 and 2 nmol, but not 0.02 nmol, of NMS into intact rats significantly increased the plasma oxytocin concentration at 5 min after treatment in comparison with rats treated with saline ($P < 0.01$) (Fig. 2). This significant increase was also observed at 60 min after treatment with 2 nmol NMS. On the other hand, when 2 nmol NMU was injected, a significantly increased oxytocin concentration was observed at 5 min, but not at 60 min after injection (Fig. 2). There was a significant difference in oxytocin concentration between 0.2 nmol of NMS and NMU at 5 and 60 min after treatment.

When we measured milk intake during 15 min after initiation of suckling in individual pups that had been removed from the dam for 8 h, less than 1 ml and over 1 ml of milk intake per pup was observed for control dams nursing 12 and 8 pups, respectively (Fig. 3A and B). Icv injection of 0.2 nmol NMS into lactating dams nursing 12 pups resulted in a larger increase of pup body weight gain than that observed in the saline-treated group (Fig. 3 A). On the other hand, icv injection of anti-NMS antiserum into lactating dams nursing 8 pups resulted in a larger decrease of body weight gain than that observed in the group treated with normal rabbit serum (Fig. 3B). In the case of treatment with normal rabbit serum, milk intake in individual pups showed little variation among litters (Fig. 3B1–4). For treatment with anti-NMS antiserum, however, there was a large variation of milk intake by individual pups (Fig. 3B1–4).

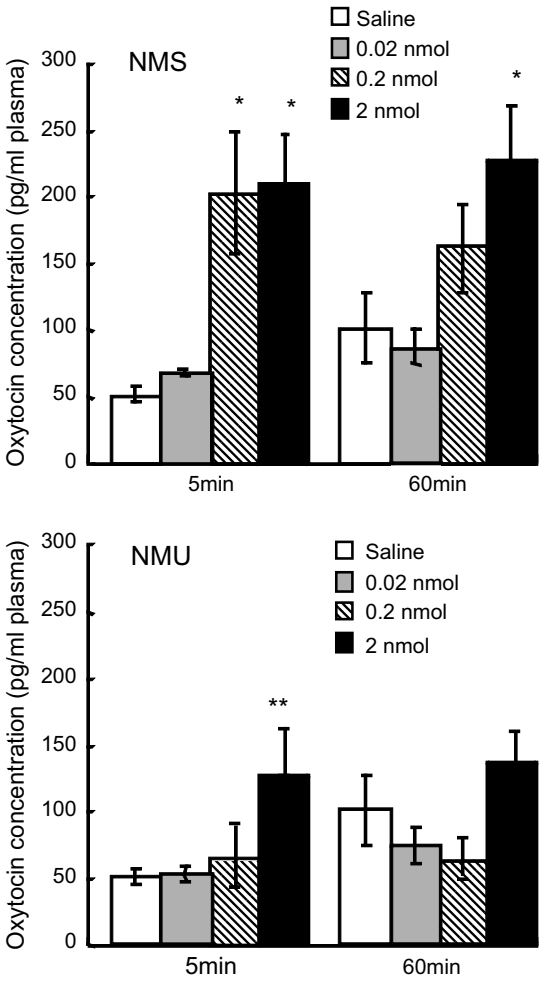

Fig. 2. Effect of icv injection of NMS and NMU on plasma oxytocin concentration in rats. Whole blood was collected by decapitation at 5 and 60 min after icv injection of 0.02, 0.2 or 2 nmol of NMS and NMU, or saline. Each bar and vertical line represent the mean ± SEM ($n = 5$). Asterisks indicate significant differences for saline group ($^{*}P < 0.01$, $^{**}P < 0.05$).

Discussion

In the present study using double immunohistochemical analysis, we confirmed that icv injection of NMS activates a proportion of oxytocinergic neurons in the PVN and SON. This result is consistent with other studies demonstrating that NMU-2R and oxytocin mRNA are co-expressed in PVN neurons [14], and that NMU-2R protein is distributed in PVN and SON neurons [11]. These findings suggest the possible involvement of NMS in oxytocin synthesis and/or release by acting directly on oxytocinergic neurons. Although cFos expression induced by NMS was also observed in neurons other than oxytocinergic neurons in the PVN and SON, a proportion of them were probably vasopressin neurons, as described previously [11].

In the present study, we demonstrated that icv injection of NMS increased the plasma oxytocin concentration significantly within 5 min after treatment with maximum effect caused by 0.2 nmol NMS. This rapid release of oxytocin into circulation may indicate that NMS may mimic the suckling-induced oxytocin release. On the other hand, high dose of NMU was required in order to stimulate the oxytocin release. This stimulating effect of NMU on oxytocin release is in agreement with a previous study that demonstrated a significant increase of plasma oxytocin concentration from 15 min after injection of 3.0 nmol NMU [18]. We suspect that 2 nmol or more NMU and NMS are pharmacological, and not a physiological dose, since icv injection of over 2 nmol NMU and NMS has been shown to frequently cause abnormal behavior, such as grooming, barrel rolling, and so on [19].

Oxytocin plays an important role in suckling-induced milk ejection. Therefore, we evaluated whether endogenous NMS is involved in this mechanism. To prevent any influence of endogenous NMS, we first used a specific antibody against NMS. A calcium-mobilization assay using CHO cells expressing recombinant NMU-1R, -2R receptors revealed that the anti-NMS antibody inhibited calcium mobilization by NMS, but not NMU, in a dose-dependent manner (data not shown). Icv injection of the anti-NMS antiserum into lactating dams nursing 8 pups resulted in a lower increase of milk intake by individual pups than that observed in a group of dams treated with normal rabbit serum. This indicates that icv injection of anti-NMS antiserum decreased the oxytocin release in response to suckling by pups, followed by a decrease of milk ejection. In pups that were returned to dams that had been treated with anti-NMS antiserum, there were large variations in milk intake among individuals, indicating that only pups with strong suckling force could take the milk, because milk ejection might have become weakened due to the decreased concentration of oxytocin. These results suggest that endogenous NMS is involved in the milk ejection mechanism through release of oxytocin in response to the suckling stimulus. If this is the case, then icv injection of NMS may conversely increase suckling-stimulated milk ejection. In fact, the present study showed that icv injection of 0.2 nmol NMS into lactating dams nursing 12 pups caused a larger increase of pup body weight gain than that observed in the saline-treated group.

Although the mechanism of NMS involvement in oxytocin release induced by suckling is still unknown, several possibilities can be considered. The suckling stimulus induces distinctive electrical activity consisting of brief high-frequency discharges of action potentials in oxytocinergic neurons [20]. This activity induces bolus oxytocin release every 5–15 min [21], increasing the oxytocin concentration above the threshold necessary to contract the mammary gland, followed by ejection of milk. We

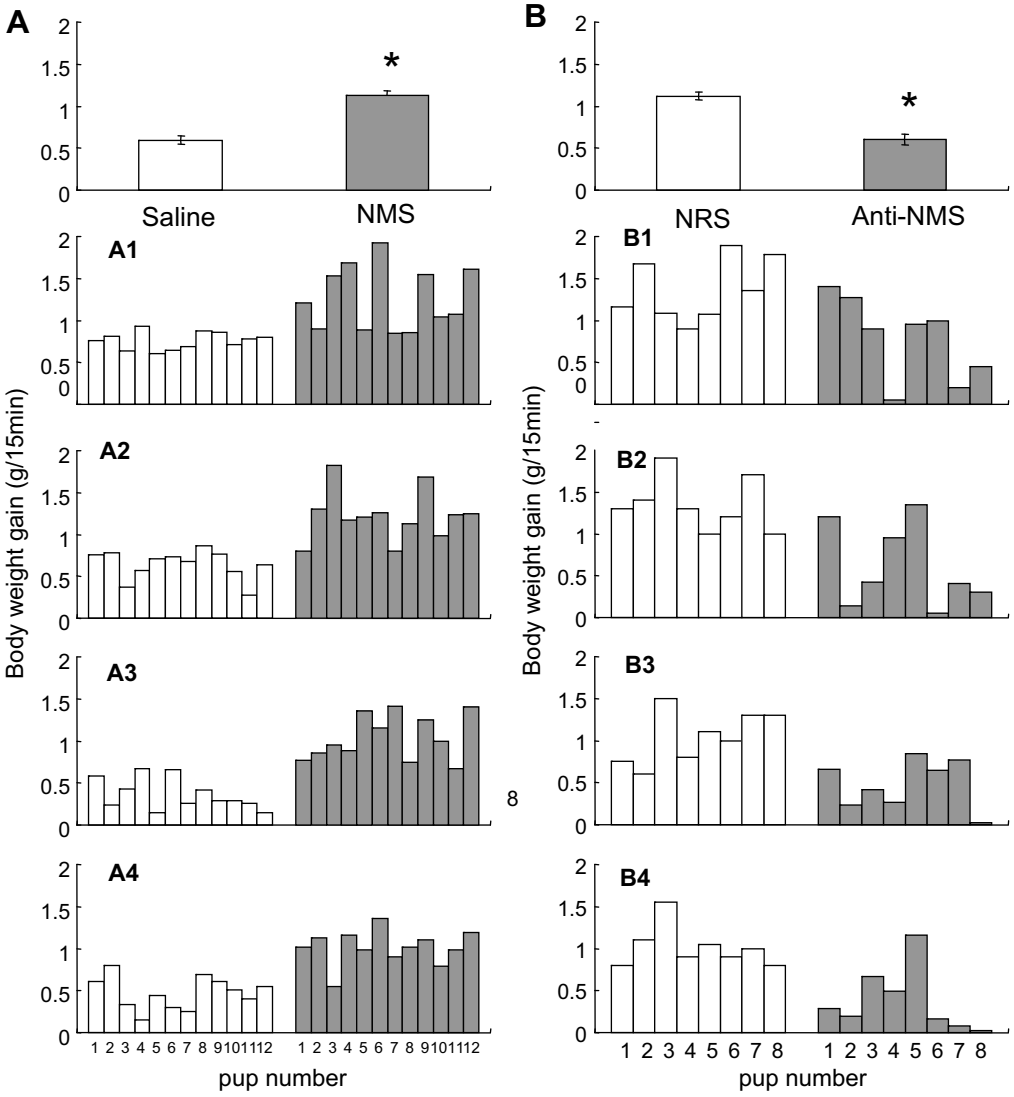

Fig. 3. Effects of treatment of dams with NMS (A) and anti-NMS antiserum (B) on milk intake by individual pups during 15 min from initiation of suckling. Pups had been removed from the dam for 8 h, and thereafter returned. NMS (0.2 nmol) and diluted (50×) anti-NMS antiserum were injected icv 15 min before the pups were returned. Saline and diluted normal rabbit serum were used as controls, respectively. Milk ejection volume was estimated from pup body weight gain during 15 min of suckling. (A) and (B) represent the mean body weight gain in individual pups. Each bar and vertical line represent the mean ± SEM ($n = 6$). Asterisks indicate significant differences from each control group ($^{*}P < 0.05$). A1–4 and B1–4 are samples comparing pup body weight gain between dams treated with NMS and those treated with saline (A group), and between dams treated with normal rabbit serum and those treated with anti-NMS antiserum (B group), respectively. Groups A1–4 and B1–4 consisted of dams with 12 and 8 pups each, respectively. Each bar represents the individual pup body weight gain during 15 min of suckling.

previously showed that icv injection of NMS increased the neuronal firing rate in the PVN [10]. Therefore, NMS may directly affect the change of electrical activity in oxytocinergic neurons, as is the case for other neural transmitters involved in the generation of brief high-frequency action potentials in these neurons [22–24].

Recently, autocrine regulation of oxytocin release by oxytocin itself has been demonstrated. Centrally released oxytocin from dendrites of oxytocinergic neurons initiates this electrical activity by binding to the oxytocin receptor (OXT-R) expressed on the dendrites [25,26]. Indeed, bilateral injection of an OXT-R antagonist into the SON significantly decreases the peripheral oxytocin concentration, followed by a reduction of milk secretion [24]. Therefore, the suckling stimulus might initially stimulate the secretion of NMS, which in turn elicits oxytocin release, and consequently this oxytocin induces the further release of more oxytocin. OXT-R is a receptor coupled with the G-protein Gαq/11, and oxytocin stimulates the initiation of this receptor's electrical activity, and consequently its own receptor-mediated release [26]. Interestingly, NMU-2R is also coupled with Gαq/11 [27]. Therefore, the signal pathway for oxytocin release from NMU-2R may be the same as that from OXT-R, and this common signal pathway may also contribute to the large release of oxytocin necessary for milk ejection.

In our previous study, we showed that endogenous NMU may be involved in nociceptive reflexes, since the reflex responses to heat and pain were significantly decreased in NMU-knockout mice. Conversely, icv injection of NMU into wild-type mice dose-dependently stimulated such nociceptive reflexes [28]. These findings suggest a role of spinal NMU in these reflexes. Unlike NMU, however, neither NMS nor its mRNA was detected in the spinal cord, and icv injection of NMS did not induce cFos expression in the spinal cord or medulla (unpublished observation). Therefore, it is assumed that NMS is not involved in spinal or medullary reflexes after the suckling stimulus.

In conclusion, the present study has demonstrated that the novel peptide NMS may play an important role in milk ejection

through release of oxytocin in response to the suckling stimulus in lactating rats. Although it is assumed that NMS may act on oxytocinergic neurons like a neurotransmitter, further studies will be required to elucidate the mechanism of action of NMS in oxytocin release in response to suckling.

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