

# Comparison of feeding suppression by the anorexigenic hormones neuromedin U and neuromedin S in rats

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## Abstract

We compared the central mechanisms of feeding suppression by the anorexigenic hormones neuromedin U (NMU) and neuromedin S (NMS) in rats. I.c.v. injection of either NMU or NMS dose dependently decreased 3-h food intake during the first quarter of a dark period. Pretreatment involving i.c.v. injection of a specific anti-NMS IgG blocked the suppression of food intake induced by i.c.v.- and i.p.-injected leptin, but anti-NMU IgG elicited no blockade. Quantitative PCR analysis revealed that i.c.v. injection of NMU or NMS caused a dose-dependent increase in CRH and proopiomelanocortin mRNA expression in the paraventricular nucleus (PVN) and

arcuate nucleus (Arc) respectively. In tissue cultures of the Arc, secretion of  $\alpha$ -melanocyte-stimulating hormone was stimulated by NMU and NMS, with more potent stimulation by NMS. The time-course curves of the increase in neuronal firing rate in Arc slices in response to NMU and NMS showed almost the same pattern, with a peak 10–15 min after treatment, whereas the time-course curves for the PVN slices differed between NMU and NMS. These results suggest that NMS and NMU may share anorexigenic effects, depending on physiological conditions.

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## Introduction

Neuromedin U (NMU) is a highly conserved brain-gut peptide that was first isolated from the porcine spinal cord (Minamino *et al.* 1985) and later from the brain, spinal cord, and intestine of other species (Domin *et al.* 1986, 1989, O'Harte *et al.* 1991, Austin *et al.* 1995). Thus far, the only known physiological role of NMU is contraction of smooth muscles in blood vessels, the uterus, and the gastrointestinal tract (Minamino *et al.* 1985). Two G-protein-coupled receptors (NMUR1 and NMUR2) are thought to bind to NMU and mediate its physiological effects (Fujii *et al.* 2000, Hedrick *et al.* 2000, Hosoya *et al.* 2000, Howard *et al.* 2000, Kojima *et al.* 2000, Raddatz *et al.* 2000, Szekeres *et al.* 2000). NMUR1 (formerly FM-3/GPR66) is located in a wide range of peripheral tissues, such as intestine, testis, pancreas, uterus, lung, and kidney. In contrast, expression of NMUR2 (formerly FM-4/TGR-1) is limited to areas of the brain such as the paraventricular nucleus (PVN) along the wall of the third ventricle in the hypothalamus and the CA1 region of the hippocampus (Howard *et al.* 2000, Raddatz *et al.* 2000, Guan *et al.* 2001, Graham *et al.* 2003).

Neuromedin S (NMS), consisting of 36 amino acids, has been identified in the rat brain by a reverse-pharmacological

technique as another endogenous ligand for FM-3/GPR66 and FM-4/TGR-1 (Mori *et al.* 2005, 2008). This neuropeptide is expressed mainly in the suprachiasmatic nucleus (SCN). Although NMS shares a C-terminal core structure (7-amino acid residues) with NMU and activates recombinant NMUR1 and NMUR2 expressed in Chinese hamster ovary cells with almost the same affinity as NMU, NMS is not a splice variant of NMU because the NMS and NMU genes have been mapped to discrete chromosomes (Mori *et al.* 2005, 2008). In addition, although NMU mRNA has been detected in peripheral and central organs (Howard *et al.* 2000, Yu *et al.* 2003, Ivanov *et al.* 2004), the distribution of NMS is limited to the testis, spleen, and SCN (Mori *et al.* 2005).

Evidence accumulated over the last decade has suggested that NMU and/or NMS are involved in the central regulation of feeding, energy homeostasis, stress, and circadian rhythms, probably through NMUR2 (Nakazato *et al.* 2000, Hanada *et al.* 2001, Ivanov *et al.* 2002, Nakahara *et al.* 2004a,b, Ida *et al.* 2005, Mori *et al.* 2005, Novak *et al.* 2006, 2007, Zeng *et al.* 2006, Novak 2009, Peier *et al.* 2009). I.c.v. administration of NMU or NMS suppresses both dark-phase food intake and fasting-induced feeding (Howard *et al.* 2000, Kojima *et al.* 2000, Ida *et al.* 2005).

Transgenic overexpression of NMU promotes leanness and hypophagia in mice (Kowalski *et al.* 2005). Conversely, disruption of the *NMU* gene in mice (producing NMU *KO* mice) results in severe obesity by increasing food intake (Hanada *et al.* 2004). Central administration of leptin decreases body weight in NMU *KO* mice, suggesting that NMU-induced anorexia is independent of the leptin signaling pathway (Hanada *et al.* 2004). In contrast, immunoblockade of endogenous NMU by anti-NMU IgG partly inhibits leptin-induced suppression of feeding, suggesting that NMU is located downstream in the leptin signaling pathway (Jethwa *et al.* 2005). Although the reason for the discrepancy between these findings is unknown, the anti-NMU IgG used in the above immunoblockade experiment might have bound to NMS as well as to NMU because it would have recognized NMU-8, which shares an almost common C-terminal region with NMU and NMS (Wren *et al.* 2002, Jethwa *et al.* 2005, Mori *et al.* 2005).

Although the mechanism of NMU- and NMS-induced suppression of food intake is known to involve the hypothalamic anorexigenic peptides proopiomelanocortin (POMC) in the arcuate nucleus (Arc) or corticotrophin-releasing hormone (CRH) in the PVN, or both, several incongruous problems related to this food intake suppression remain to be solved. NMU *KO* mice develop obesity characterized by a decrease in the expression of mRNA for POMC in the Arc and CRH in the PVN. However, i.c.v. injection of NMU in rats does not affect *POMC* mRNA expression in the Arc but augments *CRH* mRNA expression in the PVN (Hanada *et al.* 2004). In contrast, i.c.v. injection of NMS into rats also induces anorexia by increasing the expression of *POMC* mRNA in the Arc and *CRH* mRNA in the PVN, and pretreatment with  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) and CRH antagonists blocks NMS-induced suppression of food intake (Ida *et al.* 2005). Hanada *et al.* (2003) reported that CRH *KO* mice showed no reduction in food intake after NMU injection. Thompson *et al.* (2004) demonstrated that chronic administration of NMU into the PVN stimulated the hypothalamus–pituitary–adrenal axis but did not influence food intake or body weight.

Here, therefore, we compared the molecular and electrophysiological mechanisms involved in the effects of NMU and NMS on feeding suppression in the Arc and PVN, and we examined which of NMU and NMS was involved in leptin-induced suppression of food intake.

## Materials and Methods

### Animals

Male Wistar rats weighing 260–300 g were maintained in individual cages under controlled temperature (21–23 °C) and light (lights on from 0700 to 1900 h) conditions with food and water made available *ad libitum*. All procedures were

performed in accordance with the Japanese Physiological Society's guidelines for animal care for studies involving the use of laboratory animals.

### Preparation of anti-rat NMU and NMS antibodies

Polyclonal antibodies were raised against the specific N-terminal portions of rat NMU and NMS because the 7-residue C-terminal amidated sequences of rat NMS and NMU are identical (Mori *et al.* 2005). Antiserum was obtained by using a protocol reported previously (Hosoda *et al.* 2000). In brief, a synthetic peptide, (Cys<sup>0</sup>)-rat NMU (1–23) or (Cys<sup>0</sup>)-rat NMS (1–20), was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (Pierce, Rockford, IL, USA). New Zealand White rabbits were immunized by s.c. injection of these conjugates emulsified with Freund's complete adjuvant. Antibody specificity and titer were confirmed by RIA (Hosoda *et al.* 2000). Anti-rat NMU antibody did not crossreact with rat NMS, and anti-rat NMS antibody did not crossreact with NMU. Neutralizing activity was verified by calcium mobilization assay using Chinese hamster ovary cells stably expressing NMU or NMS receptor (Mori *et al.* 2005).

### Feeding experiments

A stainless steel cannula was implanted into one of the lateral cerebral ventricles by a method that has been described previously (Ida *et al.* 2005). Each rat was sham injected with saline before the study and weighed and handled daily. Only animals demonstrating progressive weight gain following surgery were used for subsequent experiments. First, to compare the potencies of the acute suppressive effects of NMU and NMS on feeding, sham was injected separately at a dose of 0.1, 0.5, or 1.0 nmol/10  $\mu$ l saline into the lateral cerebral ventricle of free-moving rats ( $n=6$ /group), with saline only as a control, via a 27-gauge cannula connected to a 50  $\mu$ l Hamilton syringe at 1845 h. The 3-h food intake during the first quarter of the dark period (1900–2200 h) was then examined. Although we were unsure whether these doses were physiological, we did not use a dose exceeding 1.0 nmol in this study, as we had found beforehand that this produced abnormal behavior. Secondly, to determine whether NMU and NMS acted downstream of the leptin signaling pathway, rats were pretreated with i.c.v. injection of anti-NMU IgG (6.5 nmol) or anti-NMS IgG (5.5 nmol), with almost equivalent neutralizing activity, or with normal rabbit IgG (6.5 nmol) as a control, 30 min before leptin treatment. Leptin (i.c.v.: 3.5 or 10  $\mu$ g/rat, i.p.: 1.0 or 3.0 mg/kg body weight) was administered i.c.v. or i.p. at 1845 h, and the 12-h food intake was then measured. Thirdly, to further confirm the effect of anti-NMU IgG pretreatment on the leptin-induced decrease of food intake, 13 nmol anti-NMU IgG or 13 nmol normal rabbit IgG were injected i.c.v. 30 min before leptin treatment. For this experiment, leptin was administered i.p. (3 mg/kg body weight) or i.c.v. (10  $\mu$ g/rat).

### Quantification of mRNAs

Each of six rats was killed by decapitation 2 h after i.c.v. injection of saline, 0.5 nmol NMU, or 0.5 nmol NMS. All experiments were performed twice using new rats ( $n=6$ /group). The Arc and PVN were collected by punch-out from frozen brain slices using a method described previously (Mori *et al.* 2005). Levels of expression of mRNAs for agouti-related peptide (AGRP) and POMC in the Arc and CRH in the PVN were measured by real-time quantitative PCR, as described previously (Nakahara *et al.* 2004a,b). AGRP mRNA was evaluated as one of the control for no influence by i.c.v. injection of NMU and NMS. Total RNA was extracted from each tissue by using an RNeasy Micro kit (Qiagen) and synthesized into first-strand cDNA by using an iScript cDNA Synthesis kit (Bio-Rad Laboratories). A single tissue sample was sufficient for measuring the mRNA levels. An aliquot of first-strand cDNA (40–100 ng tissue equivalent) was quantified on an iCycler (Bio-Rad Laboratories) by using iQ SYBR Green Supermix (Bio-Rad Laboratories) with primers to amplify glyceraldehyde 3-phosphate dehydrogenase (GAPDH), AGRP, POMC, and CRH specifically. The primer sets used for rat AGRP, POMC, and CRH, and GAPDH were as follows: AGRP: sense, 5'-TCTGAAGAAGACAGCAGCAGACCGA-3', antisense, 5'-AGCGACGCGGAGAA CGAGACT-3'; POMC: sense, 5'-GACCTCACCACGGAA AGCAACCTG-3', antisense, 5'-ACTTCCGGGGATTTTCAGTCAAGGG-3'; CRH: sense, 5'- ATCTCACCTTCCAC CTTCTG-3', antisense, 5'-GTGTGCTAAATGCAG AATCG-3', and GAPDH: sense, 5'-CGGCAAGTTCAACGGCACA-3', antisense, 5'-AGACGCCAGTAGACTCCA CGACA-3'.

To examine whether i.p. administration of leptin affected the expression of NMS mRNA, hypothalamic levels of NMS mRNA were quantified 2 and 12 h after i.p. injection of leptin (3 mg/kg body weight) or saline, using real-time quantitative PCR by the method described above with some modifications. Each of six rats was killed by decapitation 2 and 12 h after leptin or saline treatment. Total RNA was extracted and synthesized into first-strand cDNA using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). A single tissue sample was sufficient for measuring the level of mRNA. An aliquot of first-strand cDNA was quantified on a 7300 Real-time PCR System (Applied Biosystems) using TaqMan Gene Expression Master Mix (Applied Biosystems) with primers to amplify  $\beta$ -actin and NMS specifically. For these two genes, probe/primer kits were purchased from Applied Biosystems (TaqMan Gene Expression Assay ID: Rn00667869\_m1, GenBank NM: NM031144 for  $\beta$ -actin and Assay ID: Rn02349491, GenBank NM: NM\_001012233 for NMS).

### Measurement of $\alpha$ -MSH secretion in Arc tissue cultures

Adult male Wistar rats were killed by decapitation at 1845 h. Each brain was quickly removed and cut with a Vibratome

(Leica Microsystems GmbH, Wetzlar, Germany: VT 1000S) into 750  $\mu$ m thick coronal slices including both sides of the Arcs. Using a Pasteur pipette with an inner diameter of 1.2 mm, the Arcs were dissected out from each brain slice under microscopic observation. Three Arc explants/well were incubated at 37 °C for 30 min under 95% air and 5% CO<sub>2</sub> in each of 5 wells/group in 96-well plates with 200  $\mu$ l oxygen-saturated DMEM (Gibco, Invitrogen) containing 10 mM HEPES, 2% FCS, and penicillin (100 U/ml)/streptomycin (100  $\mu$ g/ml). Then the medium was changed to fresh medium with or without NMU or NMS at either 0.5 or 1.0 nmol/ml. After 1 h of incubation with slight shaking at 37 °C, all the culture medium was collected and centrifuged at 5000 g for 10 min at 4 °C. The supernatant was used to measure  $\alpha$ -MSH with an ELISA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA).

### In vitro recording of neuronal firing activity

*In vitro* multiple unit activity was recorded from the Arc and PVN in hypothalamic slice cultures plated on a microelectrode array dish (MEAD: Multi Channel Systems MCS GmbH, Reutlingen, Germany). A MEAD biosensor has a square recording area with sides 1000  $\mu$ m long. Within this area, 60 electrodes are aligned in an 8×8 layout grid; the electrode diameter is 10  $\mu$ m, and the inter-electrode distance is 100  $\mu$ m. Each Arc and PVN slice was carefully cut with a respective thickness of 700 and 500  $\mu$ m with a Vibratome slicer under cooling, and the upper surface of the slice was placed downward on the MEAD biosensor. The slice position was adjusted to ensure that the Arc and PVN were located over the electrode array and held lightly from above by mesh. DMEM containing 10 mM HEPES was then put carefully into the dish, and the biosensor was connected to a computer via an input amplifier and the MEAD connector housed on a custom-built microscope stage. The incubator consisted of a 16.5×16.5 cm steel base and an aluminum block through which the temperature was controlled (TC02, Multi Channel System MCS GmbH). Records were taken corresponding to the site of each microelectrode. The neuronal firing activity frequencies were calculated at 30-s intervals and displayed on the computer screen using software developed by Multi Channel Systems MCS GmbH. After stable multiple unit activity recordings had been obtained, either NMU or NMS was added to the culture medium at a final concentration of 0.5 nmol/ml, and the recording was continued. After 20 min of incubation, the medium was changed to fresh medium without NMU or NMS to confirm the recovery of multiple unit activity.

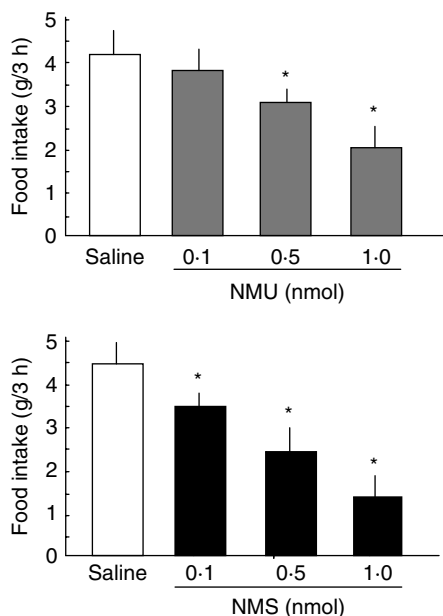
### Statistical analysis

The data (means  $\pm$  S.E.M.) were analyzed statistically by ANOVA with the *post hoc* Fisher's test at a significance level of  $P<0.05$ . In addition, the data (food intake, NMS mRNA,  $\alpha$ -MSH secretion, and multiple unit activity) were evaluated using two-way ANOVA (NMU and NMS).

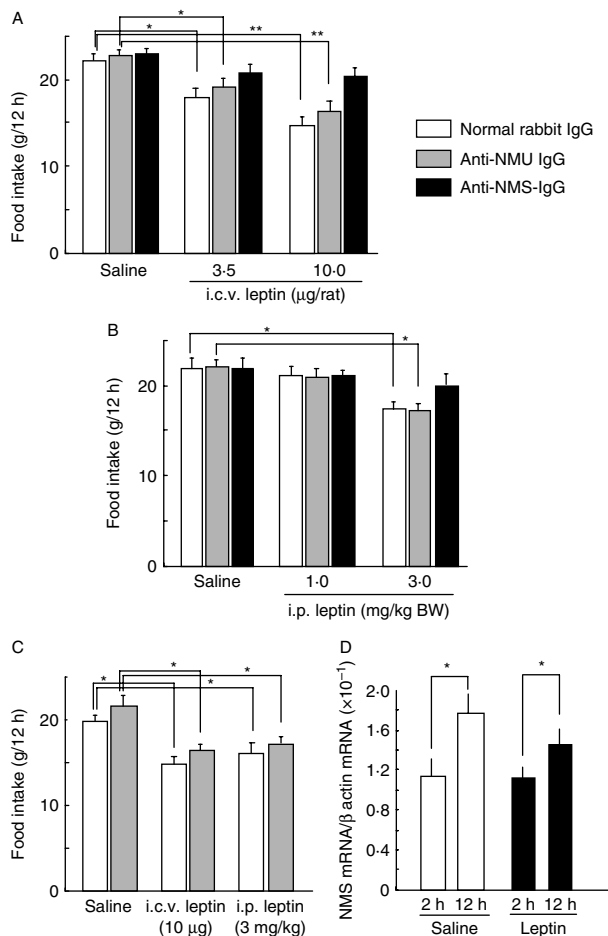
## Results

I.c.v. injection of either NMU or NMS decreased 3-h food intake during the first quarter of the dark period in a dose-dependent manner (Fig. 1). Although NMS treatment elicited suppressive effects with smaller doses than NMU, there was no significant difference between NMS and NMU ( $F(1,30)=3.375$ ,  $P=0.07$ ).

There were no significant differences in 12-h food intake between saline-treated rats preinjected i.c.v. with normal rabbit IgG, anti-NMU IgG, or anti-NMS IgG (Fig. 2A and B). I.c.v. injection of leptin caused a significant and dose-dependent decrease of food intake in rats that had been pretreated with normal rabbit IgG or anti-NMU IgG, but not with anti-NMS IgG. No significant difference was observed in these decreases between rats pretreated with normal rabbit IgG and those pretreated with anti-NMU IgG (Fig. 2A). I.p. injection of leptin at 3 mg/kg, but not at 1 mg/kg, also caused a significant decrease of food intake in rats pretreated with normal rabbit IgG or anti-NMU IgG, but not in rats pretreated with anti-NMS IgG (Fig. 2B). No significant difference in these decreases was observed between rats pretreated with normal rabbit IgG and those pretreated with anti-NMU IgG (Fig. 2B). As anti-NMU IgG did not suppress the leptin-induced decrease of food intake, we confirmed this using a larger dose of anti-NMU IgG. As shown in Fig. 2C,



**Figure 1** Comparison of food intake in rats after i.c.v. injection of neuromedin U (NMU) and neuromedin S (NMS). Each bar and vertical line represents the mean  $\pm$  S.E.M. ( $n=6$ ). Food intake of free-feeding rats was examined for 3 h during the first quarter of a dark period (from 1900 to 2200 h) in NMU- or NMS-treated rats. Various doses of NMU or NMS were injected at 1845 h. Asterisks indicate significant differences ( $P<0.05$ ) between reagent-treated and saline-treated groups.



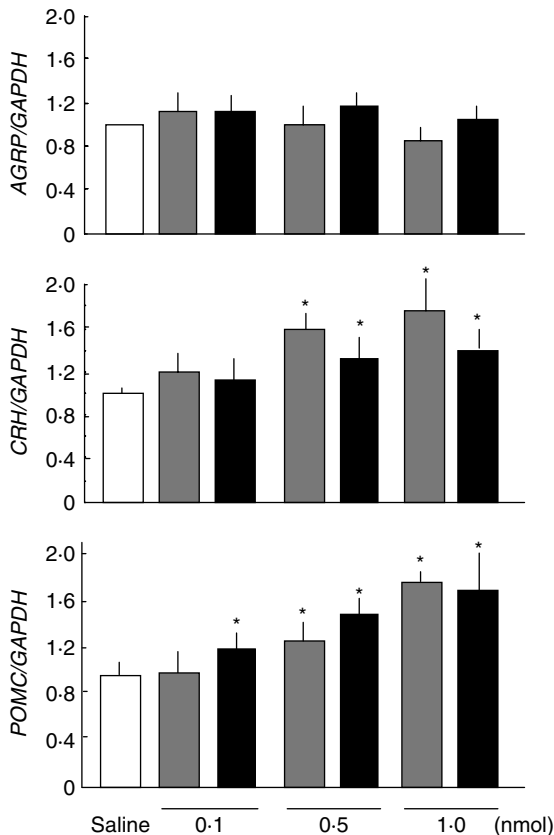
**Figure 2** (A and B) Effect of pretreatment with normal rabbit IgG (6.5 nmol), anti-NMU IgG (6.5 nmol) and anti-NMS IgG (5.5 nmol) on leptin-induced suppression of food intake. Specific antibody for NMU or NMS, or normal rabbit IgG as a control, was injected i.c.v. 30 min before administration of leptin. Leptin or saline was administered i.c.v. (A) or i.p. (B) just before the dark period, and the 12-h food intake was then measured. (C) Effect of i.c.v. pretreatment with normal rabbit IgG (13 nmol: white bar) and anti-NMU IgG (13 nmol: gray bar) on the leptin-induced suppression of food intake. Each bar and vertical line represents the mean  $\pm$  S.E.M. ( $n=6$ /group). Asterisks indicate significant differences ( $*P<0.05$ ,  $**P<0.01$ ). (D) Effect of i.p. administration of leptin on the expression of hypothalamic NMS mRNA. Leptin (3 mg/kg body weight) was administered i.p. just before the dark period. Hypothalamic NMS mRNA levels were measured 2 and 12 h after leptin treatment ( $n=6$ /group). Asterisks indicate significant differences ( $*P<0.05$ ).

although i.c.v. pretreatment with a large dose of anti-NMU IgG in saline-treated rats resulted in a tendency for an increase of food intake during the dark period in comparison with pretreatment using the same dose of normal rabbit IgG, the difference between them was not significant. Pretreatment with anti-NMU IgG did not suppress the leptin-induced decrease in food intake because no significant difference was observed in these decreases between rats pretreated with normal rabbit IgG and those pretreated with anti-NMU IgG (Fig. 2C).

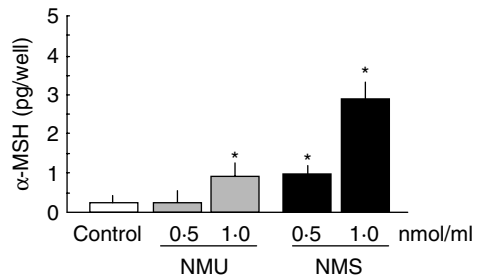
The expression of *NMS* mRNA was higher at 12 h than at 2 h in both saline- and leptin-treated mice ( $F(1,20)=15.72$ ,  $P=0.0008$ ). On the other hand, there was no significant difference in *NMS* mRNA levels between leptin and saline treatment ( $F(1,20)=1.208$ ,  $P=0.2848$ ) (Fig. 2D).

Quantitative PCR analysis revealed that the expression of mRNAs for *CRH* and *POMC* was increased by i.c.v. injection of NMU or NMS (Fig. 3). There was no significant change in *AGRP* mRNA levels. Expression of *POMC* mRNA was significantly increased by NMU at 0.5 and 1.0 nmol and by NMS at a level as low as 0.1 nmol. *CRH* mRNA expression tended to be increased more by NMU than by NMS, but this difference was not significant.

When Arc explants were incubated with medium containing NMU and NMS, secretion of  $\alpha$ -MSH was significantly increased by NMU at 1.0 nmol/ml or NMS at 0.5 or 1.0 nmol/ml, but not by NMU at 0.5 nmol/ml (Fig. 4). There was significant difference between NMS and NMU ( $F(1,20)=9.587$ ,  $P=0.0057$ ).



**Figure 3** Effects of i.c.v. injection of saline (white bar), NMU (gray bar), and NMS (black bar) on *CRH* mRNA expression in the paraventricular nucleus (PVN) and on *AGRP* and *POMC* mRNA expression in the arcuate nucleus (Arc). Samples were collected 2 h after i.c.v. injection. Each bar and vertical line represents the mean  $\pm$  S.E.M. ( $n=16$ ). Asterisks indicate significant differences ( $P<0.05$  versus saline-treated group).

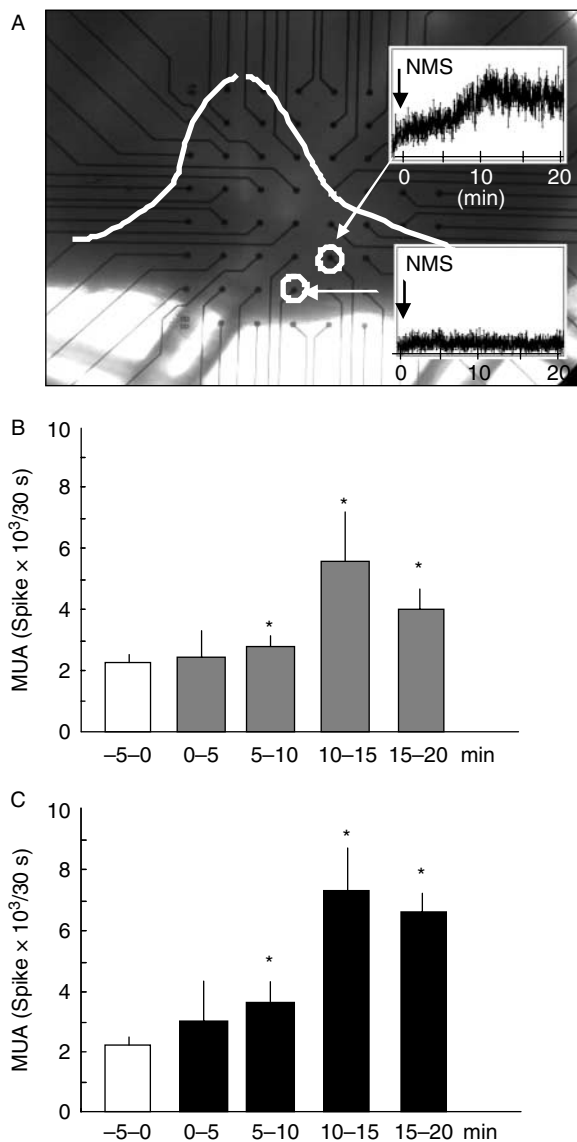


**Figure 4** Effects of NMU and NMS on  $\alpha$ -MSH secretion from cultured Arc explants. Each bar and vertical line represents the mean  $\pm$  S.E.M. Asterisks indicate significant differences ( $P<0.05$  versus control group).

*In vitro* electrophysiological analysis using MEAD showed that NMU and NMS increased the number of spikes in both Arc (Fig. 5) and PVN (Fig. 6) slices. In each experiment, the effects of NMU and NMS on neuronal activity were evaluated by calculation of the average number of spikes at 5-min intervals from each electrode because all NMU- or NMS-responsive neurons do not respond with a similar temporal profile, and by excluding the data from the electrode showing no change in firing rate within 20 min after NMU or NMS treatment. When the average number of spikes per 30 s was calculated at 5-min intervals from each electrode, the first significant increase in neuronal activity in the Arc slice was observed during 5–10 min after NMU or NMS treatment, and the highest activity with both the treatments was observed between 10 and 15 min (Fig. 5). With both the treatments, the increase in the number of spikes was greatest near the outside of the Arc. There was no change in neuronal activity near the central area (data not shown). On the other hand, the first significant increase of neuronal activity in the PVN slice was observed at 0–5 min after NMU or NMS treatment, and the highest activity was observed at 5–10 min after NMU treatment and at both 0–5 and 15–20 min after NMS treatment (Fig. 6). As shown in Figs 5 and 6, there was no significant difference between NMU and NMS (Fig. 5,  $F(1,24)=3.236$ ,  $P=0.08$ ; Fig. 6,  $F(1,24)=0.01$ ,  $P=0.94$ ).

## Discussion

We previously reported that i.c.v. injection of NMS decreased 12-h food intake during the dark period in rats, and that this anorexigenic effect was more potent and persistent than that observed with the same dose of NMU (Ida *et al.* 2005). In this study, i.c.v. injection of NMU or NMS decreased 3-h food intake during the first quarter of the dark period. This acute inhibitory effect of NMU and NMS was dose dependent and was more potent with NMS than with NMU because a smaller dose of NMS significantly suppressed food intake. These results, together with our previous observations (Ida *et al.* 2005), indicate that NMS-induced suppression of food intake is more potent both acutely and sustainably than



**Figure 5** Effects of NMU and NMS on *in vitro* neuronal activity (multiple unit activity; MUA) in cultured Arc slices plated on a microelectrode array dish. (A) The photo represents the physical relationship between the electrodes on the dish and the hypothalamic slice (represented by hind gray shadow) including Arc (broad outline shown by the white curve). The insets are two samples of MUA recording for 20 min after NMS treatment. Each record corresponds to the sites of microelectrode shown in the white circles. The black arrow in each recording indicates the point of NMS treatment. Upper recording indicates an increase in firing rate, and lower one shows no change in firing rate. (B and C) Effects of NMU (gray bars) and NMS (black bars) on the total average number of spikes recorded at 5-min intervals in four individual experiments. In each experiment, the average spike number for each 5-min interval was first calculated, with the inclusion of all data that showed an increase in firing rate after NMU or NMS treatment. Then the total average spike number was obtained for the four experiments. Each bar and vertical line represents the mean  $\pm$  S.E.M. ( $n=4$ ). Asterisks indicate significant differences ( $P<0.05$  versus pretreatment).

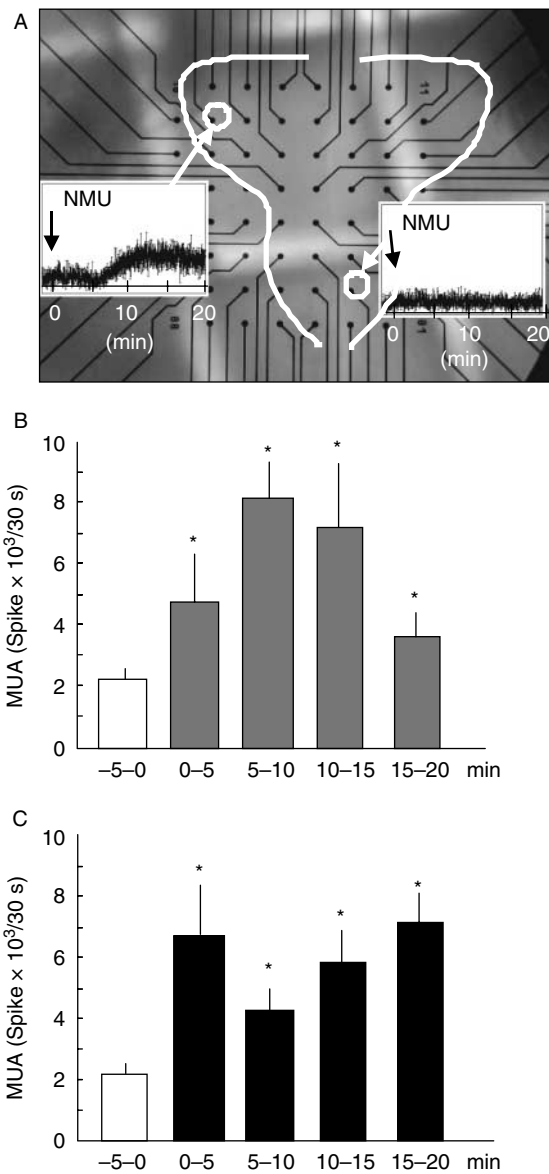
NMU. As NMS and NMU activate recombinant NMUR1 and NMUR2 expressed in Chinese hamster ovary cells with almost the same affinity (Mori *et al.* 2005), the difference in their potencies of food intake suppression is not due to a difference in their affinities for NMUR1 and NMUR2.

We previously reported that the anorexigenic effect of NMU is independent of leptin in NMU KO mice because NMU reduces food intake in leptin-deficient mice (ob/ob mice) and leptin reduces intake in NMU KO mice (Hanada *et al.* 2004). However, leptin stimulates the secretion of NMU in hypothalamic explants *in vitro* (Wren *et al.* 2002). In addition, leptin-induced reduction of food intake is partly attenuated by i.c.v. injection of anti-NMU IgG (Jethwa *et al.* 2005). These latter results suggest that NMU may act partly downstream from the action of leptin. However, the anti-NMU IgG used in the previous reports (Wren *et al.* 2002, Jethwa *et al.* 2005) recognized the C-terminal regions of NMU, suggesting that this antibody binds to not only NMU but also NMS. If so, NMS also may act partly downstream from the action of leptin. To examine this possibility, we produced antibodies specific to NMU and NMS recognizing their N-terminal regions with little homology. The decrease of food intake induced by i.c.v. and i.p. treatment with leptin was blocked by pretreatment with the antibody specific to NMS but not by the antibody specific to NMU. This lack of effect in the latter case did not seem to be due simply to the dosage of anti-NMU because a large dose of anti-NMU IgG also failed to block it. These results suggest that NMS, but not NMU, acts downstream of the leptin signaling pathway.

When the hypothalamic expression of NMS mRNA was evaluated by real-time PCR, basal levels were significantly higher at 12 h (0700 h) than at 2 h (2100 h) after saline treatment, suggesting a circadian rhythm of NMS mRNA levels (Mori *et al.* 2005). On the other hand, there was no significant difference in NMS mRNA levels between leptin and saline treatment. Therefore, leptin might stimulate NMS release, but not synthesis. The source of the NMS released upon leptin stimulation was unclear. Granted that leptin stimulates the release of NMS from the SCN, there is no evidence for any leptin receptor in the SCN. Further studies will be required to elucidate the relationship between leptin and NMS or NMU.

The target of NMU and NMS for suppression of feeding seems to be the Arc and/or PVN. Radiolabeled NMU has been shown to bind to the Arc and PVN *in vitro* by receptor autoradiography (Mangold *et al.* 2008), and expression of *NMUR2* mRNA has been confirmed in the PVN (Sakamoto *et al.* 2007). Therefore, it has been assumed that cFos expression in both nuclei after i.c.v. injection of NMU and NMS may be due to direct action on them (Ida *et al.* 2005).

Although the mechanism of NMU- and NMS-induced suppression of food intake is known to involve the hypothalamic anorexigenic peptides POMC in the Arc or CRH in the PVN, or both, several incongruous problems with regard to this suppression of food intake remain to be solved. Our quantitative analysis of mRNA expression and



**Figure 6** Effect of NMU and NMS on *in vitro* neuronal activity (multiple unit activity; MUA) in cultured PVN slices plated on a microelectrode array dish. (A) The photo represents the physical relationship between the electrodes on the dish and the hypothalamic slice (represented by hind gray shadow) including PVN (broad outline shown by white curve) slice. The insets are two samples of MUA recording for 20 min after NMU treatment. Other details are the same as in Fig. 5. (B and C) Effects of NMU (gray bars) and NMS (black bars) on the total average number of spikes recorded at 5-min intervals in four individual experiments. Total average spike numbers were obtained as described in the caption to Fig. 5. Each bar and vertical line represents the mean  $\pm$  S.E.M. ( $n=4$ ). Asterisks indicate significant differences ( $P<0.05$  versus pretreatment).

our *in vitro* electrophysiological analysis in the Arc and PVN demonstrated that both NMU and NMS increased *POMC* mRNA expression in the Arc and *CRH* mRNA expression in the PVN and increased the neuronal firing rates in both

nuclei. Although it is not clear whether the increase in firing rate in the Arc and PVN in response to NMU and NMS resulted in the increase in mRNA expression in these nuclei, it is likely that a direct action of NMU and NMS on neuronal firing rate in the Arc and PVN and an increase in *POMC* and *CRH* mRNAs are involved in NMU- and NMS-induced suppression of food intake. In these analyses, however, we observed subtle differences in the effects of NMU and NMS. Expression of *POMC* mRNA was stimulated more by NMS than by NMU, and expression of *CRH* mRNA was stimulated more (although not significantly) by NMU than by NMS. Although the time-dependent pattern of the increase in neuronal activity caused by NMU in the PVN was similar to that in the Arc, the time-course patterns of the NMS-induced changes in neuronal activity differed between the Arc and the PVN.

Hanada *et al.* (2004) reported previously that i.c.v. injection of NMU in rats did not affect the expression of *POMC* mRNA in the Arc but augmented *CRH* mRNA expression in the PVN. The reasons for the differences in the observed effects of NMU on *POMC* mRNA expression between this study and the previous one are unknown. One possible explanation may be that the *in situ* hybridization method used in the previous study (Hanada *et al.* 2004) might have been incapable of detecting the small change in the level of *POMC* mRNA after NMU injection. Although the expression of *POMC* mRNA was increased by NMU at 0.5 and 1.0 (but not 0.1) nmol, NMS increased it at 0.1 nmol. In addition to these differences in *POMC* mRNA expression,  $\alpha$ -MSH secretion from Arc explants in culture was significantly increased by NMU at 1.0 (but not 0.5) nmol, whereas NMS increased it at both 0.5 and 1.0 nmol. These results indicate that the more potent suppression of food intake by NMS than by NMU may be due to more potent stimulation of  $\alpha$ -MSH synthesis and release in the Arc because *CRH* mRNA expression was not greater with NMS than with NMU. In this study, we do not know which is the primary instrument of NMU-induced suppression of food intake between  $\alpha$ -MSH and *CRH*. It has been shown that food intake in *CRH* KO mice is not reduced after NMU injection (Hanada *et al.* 2003). In contrast, Thompson *et al.* (2004) have demonstrated that chronic administration of NMU into the PVN stimulates the hypothalamus-pituitary-adrenal axis but does not influence food intake or body weight.

Our *in vitro* electrophysiological analysis indicates that NMU and NMS directly stimulate the neuronal firing in the Arc and PVN. In the Arc, a significant increase in firing rate in response to both NMU and NMS started after 5 min of treatment. In the PVN, on the other hand, it was observed within 5 min after treatment. In addition to *CRH* neurons, the PVN includes vasopressin and oxytocin neurons. Both NMU and NMS stimulate the secretion of these hormones through direct action on NMUR2 in vasopressin and oxytocin neurons (Sakamoto *et al.* 2007, 2008). In this case, too, the action of NMS is more potent than that of NMU (Sakamoto *et al.* 2007, 2008). Therefore, the differences in the

time course or strength of neuronal firing in response to NMU and NMS in the Arc and PVN might arise from differences in the populations of neurons responding to NMU and NMS in these nuclei.

In conclusion, we have demonstrated that NMU and NMS increase the expression of *POMC* and *CRH* mRNA in the Arc and PVN respectively and increase the neuronal firing rates in these nuclei with different potencies. We also showed that NMS, but not NMU, may act downstream of the leptin signaling pathway. These results may partly explain the difference in potency of feeding suppression between NMU and NMS *in vivo*, and suggest that NMS and NMU may share an anorexigenic action that is dependent on physiological conditions.

However, a paradox in the NMU and NMS observations remains to be explained: i.e., why the receptors for NMS and NMU are the same but the downstream mechanisms of feeding regulation by NMS and NMU are different? In addition, it is also unclear why NMU *KO* mice develop obesity (Hanada *et al.* 2004), whereas NMUR2 *KO* mice are lean and smaller than normal (Zeng *et al.* 2006, Novak 2009, Peier *et al.* 2009). Of course, as mentioned above, NMS may act on an undefined receptor other than NMU1R and NMU2R. Alternatively, NMU may act on an undefined receptor other than these two. Several studies have demonstrated, on the other hand, that the mRNAs encoding NMU, NMS, NMU1R, and NMU2R each have an intrinsic rhythmic expression in the SCN or hypothalamus with a different circadian pattern (Graham *et al.* 2003, Nakahara *et al.* 2004a,b, Mori *et al.* 2005, Jethwa *et al.* 2006). As the SCN sends neural projections into the PVN and Arc (Vrang *et al.* 1995, Saeb-Parsy *et al.* 2000), these different rhythmic expressions may be related to the different effects of NMS and NMU.

Recently, Egecioglu *et al.* (2009) demonstrated that long-term central NMU treatment reduced body weight, food intake, and adiposity in diet-induced obese mice, but not in lean mice fed a standard diet, and that female (but not male) NMUR2 *KO* mice fed a high-fat diet were protected from central NMU-induced body weight loss in comparison with wild-type littermates. In addition, Bechtold *et al.* (2009) have demonstrated that proNMU(104–136) is a novel modulator of energy balance since central administration of proNMU(104–136) caused a significant but transient (~4 h) increase in feeding, although both food intake and body weight decreased over the following 24 h. Further studies will be required to elucidate the mechanism responsible for regulation of feeding and energy metabolism by NMU, NMS, and proNMU, including their receptors.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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