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Chronic administration of NMU into the paraventricular nucleus stimulates the HPA axis but does not influence food intake or body weight

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Abstract

Hypothalamic neuromedin U (NMU) appears to have a role in the regulation of appetite and the hypothalamo–pituitary–adrenal (HPA) axis. Acute administration of NMU into the paraventricular nuclei (iPVN) increases plasma adrenocorticotrophic hormone and corticosterone, and inhibits food intake in fasted rats. No studies have as yet investigated the chronic effects of centrally administered NMU. We investigated the effect of twice-daily iPVN injections of 0.3 nmol NMU for 7 days on food intake, body weight, the HPA axis, and behavior in freely fed rats. Chronic iPVN NMU was not associated with a decrease in food intake or body weight. Chronic iPVN NMU produced a typical behavioral response on day 1 and day 4 of the study, and resulted in the elevation of plasma corticosterone present 18 h after the final injection. These results suggest NMU may have a role in the regulation of the HPA axis and behavior.

Keywords: Neuromedin U; Chronic administration; HPA axis; Stress; Food intake

Neuromedin U (NMU) is a 23-amino acid peptide originally isolated from porcine spinal cord and named because of its strong contractile properties in the uterus [1]. NMU mRNA and peptide are found in the brain, pituitary and thyroid glands, and the gastrointestinal and urogenital tracts [2–10]. This wide distribution suggests smooth muscle contraction is not the only role of NMU. Although several biological roles, including control of blood pressure, ion transport, adrenocortical function, and cardiovascular function, have been investigated [11–14], the precise physiological role of NMU is still unknown.

There is evidence that hypothalamic NMU is involved in the regulation of appetite, the hypothalamo–pituitary–adrenal (HPA) axis, and behavior. Within the hypothalamus, NMU mRNA is concentrated in the arcuate nucleus (Arc) [5,10,15], with various hypothalamic nuclei, in particular the paraventricular nucleus (PVN), receiving dense innervation from NMU-immunoreactive fibers [5,15–17]. The NMU receptor predominantly expressed in central nervous system tissue, NMU2R, is found in the PVN and the wall of the 3rd ventricle [15]. Intracerebroventricular (ICV) administration of NMU causes marked induction of Fos-like immunoreactivity in the PVN, which is significantly reduced by the pre-treatment with anti-NMU IgG [18]. Therefore, NMU may be synthesized

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in the Arc and released in the PVN to act via the NMU2R.

Acute ICV and intra-PVN (iPVN) administration of NMU suppresses food intake [19] and stimulates the HPA axis [18,19]. These effects may be mediated by corticotrophin releasing hormone (CRH). NMU stimulates the release of CRH from hypothalamic explants in vitro [19] and increases c-fos expression in the CRH-containing parvocellular regions of the PVN [18]. ICV and iPVN injection of NMU induces a behavioral response [19–21] consisting of a marked increase in grooming and locomotion and a decrease in sleeping which is comparable to that induced by CRH [22–24], and raises plasma adrenocorticotrophic hormone (ACTH) and corticosterone [18,19]. The NMU induced behavior response is blocked by the administration of the CRH antagonist, α-hCRH, or anti-CRH IgG, and the NMU induced increase in locomotor activity in wild-type mice is absent in CRH knockout (KO) mice [20]. In addition, central administration of CRH decreases food intake in rats [23–26] and the NMU induced decrease in dark phase feeding is abolished in CRH KO mice [27]. NMU may therefore influence the HPA axis and appetite via effects on CRH.

Hypothalamic NMU appears to play a role in the regulation of appetite, behavior, and the HPA axis. However, there are no published reports on the effects of chronic NMU administration. The current study aimed to investigate the effects of chronic (7 days) iPVN administration of NMU on food intake, body weight, behavior, and the HPA axis in rats.

Research design and methods

Materials. Full length neuromedin U (NMU-23) was purchased from IAF Biochem, Quebec, Canada. NMU was freshly prepared for each injection period from freeze-dried aliquots. Male Wistar rats (Specific pathogen free, Charles River, UK) weighing 250–300 g were maintained in individual cages under controlled temperature (21–23 °C) and light conditions (12:12 h light:dark cycle, lights on 07:00 h, lights off 19:00 h), with ad libitum access to food (RM1 diet; SDS, Witham, UK) and water. Animal procedures undertaken were approved by the British Home Office Animals Scientific Procedures Act 1986.

Intraparaventricular cannulation and injection. Cannulation was carried out as previously described [28]. Briefly, animals were anesthetized and implanted with unilateral 26-gauge stainless steel guide cannula projecting immediately above the paraventricular nucleus using a Kopf stereotactic frame (David Kopf, Tujunga, CA) and coordinates were calculated using the rat brain atlas of Paxinos and Watson [29]. Three stainless steel screws were inserted into the cranium, and the cannula was fixed to these with dental cement. After surgery, a wire stylet was inserted into the guide cannulas to prevent blockage. The animals were allowed 1 week to recover from surgery. They were then accustomed to handling on a daily basis, and their body weight and food intake were monitored. After the 7 day recovery period, rats received injections of 1 μl saline 0.9% on days −4 and −1 to habituate them to the injection procedure. Injections of a substance into discrete hypothalamic nuclei result in less non-specific side effects than administration into the 3rd ventricle (ICV).

All compounds were dissolved in 0.9% saline and administered in a volume of 1 μl by a stainless steel injector projecting 0.5 mm into the PVN (iPVN) over 1 min. Only those animals with correct placement of cannulas were included in the data analysis. Correct cannula placement was confirmed histologically at the end of the study period [19]. Briefly, after decapitation, animals were injected with 1 μl India ink, and their brains were rapidly removed and snap-frozen in liquid nitrogen using paraformaldehyde as a cryopreservative. A freezing sled microtome (Shandon Southern Products, Cheshire, UK) was used to take 20 μm coronal sections. Cannula placement was assessed by an observer blinded to the intended cannula placement and was considered acceptable if the cannula tract was seen in the location of the PVN. Animals with cannula placement more than 0.5 mm away from the coordinates used to identify the PVN or with India ink detectable in the cerebral ventricular system were excluded from all data analysis (<20%). There was no visual evidence of damage to neuronal tissue in the area surrounding the cannula tract.

Study design. On day 0 rats were separated into two experimental groups (11–15 per group): group 1 (saline control) received twice-daily iPVN injections of saline daily for 7 days (days 1–7); group 2 (NMU) received twice-daily injections of 0.3 nmol NMU-23 for 7 days (days 1–7).

These twice-daily iPVN injections were administered during the early light phase (09:00 h) and during the late light phase (16:00 h). Animals received their first injection (saline or 0.3 nmol NMU-23) on day 1 at 16:00 h. The final iPVN injection of saline or NMU-23 was at 16:00 h on day 7. The dose of 0.3 nmol NMU-23 was based on a dose previously shown to inhibit food intake when administered iPVN [19].

Food weight (g) was measured before each injection and 1 h post-injection, thus allowing the calculation of 1 h, overnight (16:00–09:00 h), daytime (09:00–16:00 h), and 24 h food intake. Body weight (g) was recorded daily from 1 day before the start of the experiment (day 0).

Behavioral studies. Behavior was observed twice during the study: once following the first injection, (16:00 h on day 1) and again following the 16:00 h injection on day 4. Rats (n = 5–7 from each treatment group) were observed continuously for 1 h post-injection by observers blinded to the experimental treatment. Behavior was classified, as previously described, into seven different categories: feeding, drinking, grooming, rearing (defined as stationary with front paws elevated), head down (defined as stationary with all four paws on the cage bottom), locomotion (defined as moving around the cage, with all four paws moving), or sleeping [19]. Each rat was observed for 15 s every 5 min during the test session. This 15 s period was further divided into three 5 s periods, and the behavior of the rat was scored in each section of the time period. Each rat had a total of 36 behaviors recorded per hour.

Effects of chronic iPVN NMU on circulating pituitary hormones. The final injection was given at 16:00 h on day 7. All rats were killed by decapitation during the early light phase on day 8. This time point was chosen in order to observe only the chronic effects of NMU on the hormones measured, rather than any acute effects of the final injection. Trunk blood was collected into plastic tubes containing potassium EDTA (final concentration of 2 mg EDTA/ml blood) (Sarstedt, Leicester, UK) for ACTH analysis and into lithium heparin tubes containing 0.6 mg aprotinin (Bayer, Haywards Heath, UK) for corticosterone, prolactin (Prl), thyroid stimulating hormone (TSH), luteinizing hormone (LH), and follicular stimulating hormone (FSH) analysis. Plasma was separated by centrifugation, frozen on dry ice, and stored at −70 °C until assayed.

Effects of chronic iPVN NMU on tissue weights. Epididymal fat pads (chosen as a representative fat pad), interscapular brown adipose tissue (BAT), and adrenal glands were removed and weighed.

Radioimmunoassay. Plasma concentrations of Prl, TSH, LH, and FSH were measured by radioimmunoassay (RIA) using reagents and methods provided by the National Institute of Diabetes and Digestive and Kidney Diseases and the National Hormone and Pituitary

Program (Dr. A. Parlow, Harbor University of CA, Los Angeles Medical Center) as previously described [30]. Plasma corticosterone was measured using a commercial RIA kit (ICN Biomedicals, Costa Mesa, CA) and plasma ACTH was measured using a solid phase immunoradiometric assay (Euro-Diagnostica, Arnhem, The Netherlands) according to manufacturer's instructions.

Statistics. Data are presented as means ± SEM. One-way ANOVA with post hoc least significance Tukey's test (Systat) was used for comparison of the effects on food intake and changes in body weight between rats receiving NMU or saline. Unpaired *t* test was used for comparison of the HPA axis, pituitary hormones, and tissue weights between rats receiving NMU or saline. As behavioral observation data were not normally distributed, Kruskal–Wallis test was used for comparisons between treatment groups. In all cases, $P < 0.05$ was considered to be statistically significant.

Results

Investigation of the effect of chronic iPVN administration of NMU on food intake

In freely fed rats, there was no significant difference in food intake between NMU (0.3 nmol) and saline-treated rats in the first hour following any iPVN injection, or at any further time point investigated (09:00–16:00 h, 16:00–09:00 h, and 24 h time period). In addition, cumulative food intake over the study period was not significantly different between the experimental groups (Fig. 1).

Investigation of the effect of chronic iPVN administration of NMU on body weight

Before the onset of the study, there was no significant difference in body weight between the two experimental groups (day 0 body weight: 259.4 ± 6.1 [saline] vs. 261.8 ± 4.4 g [NMU]; NS). Twice-daily administration of NMU or saline for 7 days produced no significant difference in body weight between the two groups (day 1: 259.9 ± 6.7 [saline] vs. 264.2 ± 4.2 g [NMU], NS; day 8: 254.6 ± 8.2 [saline] vs. 262.6 ± 5.4 g [NMU], NS). (Fig. 2). The saline- and NMU-treated groups both maintained their body weight during the entire study period. Other investigators [31–37] have previously observed a maintenance of body weight in saline-injected control groups during chronic hypothalamic administration studies.

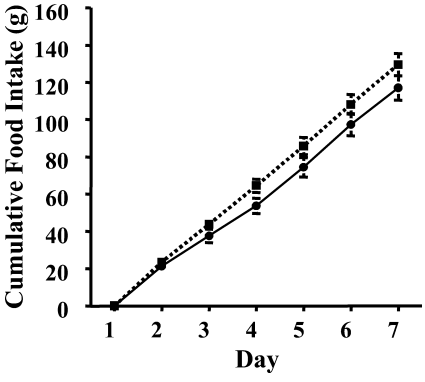

Fig. 1. Effects of twice daily iPVN administration of saline or NMU-23 (0.3 nmol) on cumulative food intake (mean food intake (g) ± SEM) throughout the study (days 1–7). Animals received their first injection on day 1 with twice daily injections thereafter (days 2–7). Solid line (——) saline. Dashed line (-----) NMU. There was no significant difference between the saline control and NMU-treated group on food intake throughout the treatment period.

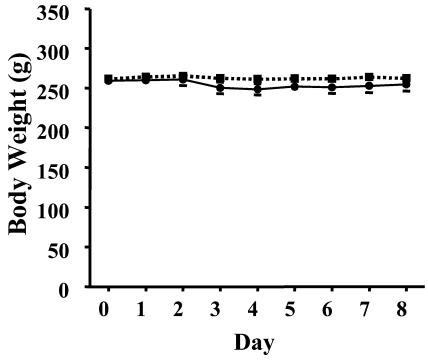

Fig. 2. Effects of twice daily iPVN administration of saline or NMU-23 (0.3 nmol) on daily body weight (mean bodyweight (g) ± SEM). Solid line (——) saline. Dashed line (-----) NMU. There was no significant difference between the saline control and NMU-treated group on body weight throughout the treatment period.

Table 1
The effect of chronic iPVN NMU on tissue weights

Tissue	Tissue weight (g/100 g body weight)	
	Saline-treated	NMU-treated
WAT	1.04 ± 0.07	1.12 ± 0.06
BAT	0.12 ± 0.01	0.12 ± 0.01
Adrenals	0.025 ± 0.004	0.021 ± 0.003

Saline or NMU-23 (0.3 nmol) was administered iPVN twice daily for 7 days. On day 8 animals were sacrificed and epididymal WAT, BAT, and adrenals were removed and weighed. Data represent means ± SEM; $n = 10–14$ rats per group.

There was no significant difference in white adipose tissue (WAT), BAT or adrenal gland weight between NMU-treated and saline-treated animals, either expressed as an absolute value or relative to body weight (Table 1).

Investigation of the effect of chronic iPVN administration of NMU on the HPA axis

Chronic administration of NMU produced an elevation of plasma corticosterone of 305.4 ± 23.8% compared to saline-treated, at 18 h following the final 16:00 h injection (29.7 ± 10.4 [saline] vs. 90.7 ± 21.6 ng/ml [NMU], $n = 10–14$/group; $P = 0.02$) (Fig. 3A). Plasma ACTH in animals receiving twice daily iPVN

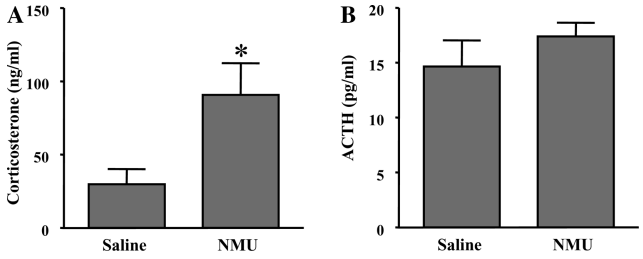

Fig. 3. Plasma concentration of corticosterone (A) and ACTH (B) at 18 h after iPVN administration of saline or NMU-23 (0.3 nmol) for 7 days; $n = 10–14$/group. Data expressed as mean plasma hormone levels ± SEM. $^*P < 0.05$ (vs. saline).

NMU injections did not differ from levels in saline-treated animals (116.3 ± 5.7%) at 18 h following the final 16:00 h injection (14.9 ± 1.9 [saline] vs. 17.3 ± 1.0 pg/ml [NMU], $n = 10–14$/group; NS) (Fig. 3B).

Chronic NMU treatment did not alter plasma pituitary hormones—Prl, TSH, LH or FSH—measured 18 h after the final injection (data not shown).

Investigation of the effect of chronic iPVN administration of NMU on behavior

NMU produced typical behavioral changes [19,20] which were quantified during both investigation periods (day 1 and day 4). The number of observations of each defined behavior was calculated and expressed as a percentage of the total number of observations in the hour after iPVN injection of NMU or saline. On day 1, the behavior of the animals was observed following the first study injection (Fig. 4A). After iPVN administration of 0.3 nmol NMU, there was a significant marked increase in grooming behavior compared with saline-treated animals, this being the most prevalent NMU-induced behaviour [median percentage of observations (interquartile range), saline, 8.3% (8.3–11.1%); NMU, 44.4% (29.2–61.1%), $n = 5–7$/group, $P = 0.012$]. In contrast, sleeping was significantly reduced in the NMU-treated group [saline 41.7% (16.7–58.3%); NMU, 0% (0–2.8%), $n = 5–7$/group, $P = 0.009$]. During the 1 h behavior observation period on day 4 (Fig. 4B) there was again a marked significant increase in grooming [saline, 16.7% (11.1–27.8%); NMU, 50% (43.1–56.9%), $n = 5–7$/group, $P = 0.006$] and significant decrease in sleeping [saline, 36.1% (33.3–44.4%); NMU, 1.4% (0–19.4%), $n = 5–7$/group, $P = 0.016$]. Interestingly, there was a significant decrease in drinking in the NMU-treated group on day 4 [saline, 5.6% (5.6–8.3%); NMU, 0% (0–2.1%), $n = 5–7$/group, $P = 0.006$]. However, this effect was not evident on day 1 [saline, 0% (0–2.8%); NMU, 5.6% (4.2–8.3%), $n = 5–7$/group, NS]. All behaviors observed were readily assigned to the predefined treatment categories. No adverse behaviors were observed in either treatment group.

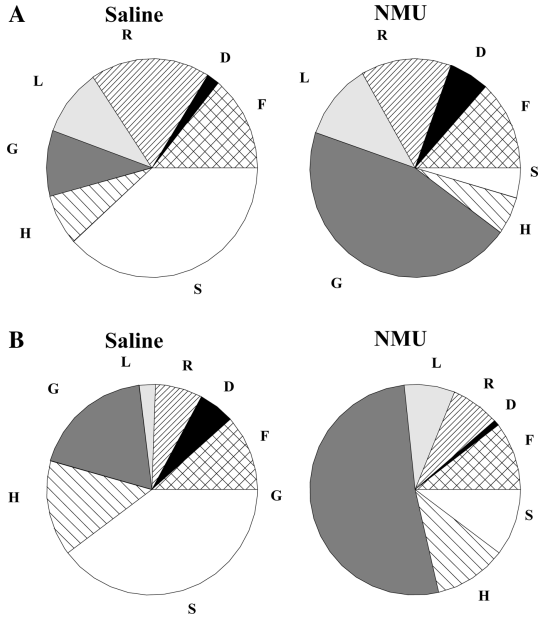

Fig. 4. Proportion of observations for each of the pre-defined behaviors in the hour after iPVN injection of saline or NMU-23 (0.3 nmol), on (A) day 1 and (B) day 4. ($n = 5–7$/group). G, grooming; D, drinking (rat drinking from water bottle); F, feeding (rat eating chow); R, rearing (stationary with front paws lifted off cage floor); S, sleeping; H, head down (stationary with all four paws on cage floor); and L, locomotor (moving about the cage).

Discussion

Several studies have explored the acute effects of ICV and iPVN administered NMU on feeding, behavior, and the HPA axis in rats. A number of hypothalamic peptides synthesized in the Arc are involved in both the regulation of food intake and the HPA axis, including cocaine and amphetamine regulated transcript (CART) [30], neuropeptide Y [38], and agouti-related protein [39]. In the present study, we investigated the effect of chronic iPVN administration of NMU on food intake, body weight, the HPA axis, and behavior in rats. All previous published studies have examined the effects of a single central NMU injection.

We have demonstrated up-regulation of the HPA axis following chronic 7-day administration of NMU into the PVN at 18 h post the final injection. It has previously been shown that a single iPVN injection of NMU leads to an acute prominent rise in plasma ACTH and corticosterone [18,19,40]. Wren et al. [19] observed an approximately 3-fold increase in plasma ACTH following acute NMU (0.3 nmol) administration compared to saline at 20 min post-injection and an approximately 1.8-fold increase in plasma corticosterone at 20 min post-injection. Following the HPA axis activation caused by acute ICV administration of NMU, ACTH, and corticosterone return to baseline after 60 and 180 mins, respectively [18]. In the present study, chronic

NMU resulted in an elevation of corticosterone which was still raised 3-fold compared to saline-treated animals 18 h after the last study injection. It is well recognized that following acute activation of the HPA axis there is a rapid increase in plasma corticosterone levels, which in turn negatively regulates the release of ACTH. However, in models of chronic stress, basal corticosterone levels are often elevated without any significant change in baseline ACTH concentrations [41–43]. This may be due to a dissociation between ACTH and corticosterone in such studies and might explain why ACTH was not significantly altered in our study. That ACTH is unchanged when it might be expected to be suppressed suggests that ACTH release is the cause of the elevated corticosterone. It would be interesting to examine ACTH and corticosterone levels at different time points following chronic administration of NMU. Additionally, data on the diurnal levels of corticosterone in the NMU-treated animals would help one to identify whether there is a change in the circadian pattern of corticosterone release in these animals. Although it was not possible in the present study because brains were sectioned to verify cannula placement, it would also be interesting to measure hypothalamic CRH in chronic NMU-treated animals.

Intra-PVN administration of NMU resulted in a significant increase in grooming and a decrease in time spent sleeping in the hour following each behavioral study. This typical behavioral response has been shown in acute studies and seems likely to be mediated by CRH [19,20]. The CRH circuit which regulates this specific behavioral response is thought to be independent of CRH regulation of the HPA axis [44]. Informal observation of the animals after each NMU injection suggested that the animals continued to display typical grooming and arousal responses post-injection throughout the study, though these effects were not formally assessed. Our results suggest that escape from the behavioral effects of iPVN NMU does not occur.

While certain forms of chronic stress increase adrenal gland size [45,46], other models of chronic stress do not affect adrenal gland mass [45]. In the present study, no difference was found in adrenal gland weight between the saline and NMU-treated groups. It is possible a longer NMU treatment period might result in a change in adrenal gland weight.

Twice daily administration of 0.3 nmol NMU (09:00 and 16:00 h) to freely fed rats had no statistically significant effect on food intake compared to saline injected controls at any of the time periods measured. This dose has previously been shown to inhibit food intake in fasted rats 1 h following iPVN administration [19]. Others have shown that NMU suppresses dark-phase food intake and fasting-induced feeding in rats when administered ICV [15,16,19,47–50]. Although other centrally active anorectic peptides, including α-melanocyte-stimulating hormone (α-MSH) [51] and CART [52], suppress feeding in both freely fed and fasted rats, timing of injection and satiety state of the animals appear to be crucial to the anorectic effect of NMU. In the present study, NMU administered iPVN to freely fed rats in the early (09:00 h) or late (16:00 h) light phase (i.e., satiated states) had no effect on food intake at 1 h. Similarly, Ivanov et al. [48], showed that although ICV administration of NMU (4 nmol) to fasted rats reduced food intake, the same dose had no effect in freely fed rats. Therefore, NMU may participate in the physiological regulation of feeding only under specific circumstances. Nevertheless, it has been shown that the expression of arcuate NMU is reduced following fasting [15], and is hence more highly expressed in freely fed animals. It is therefore possible that exogenous NMU administered into the PVN of freely fed, satiated rats may not be able to suppress food intake through the NMU pathway, since endogenous levels would be high and this system may already be activated. It would be interesting to perform further studies investigating the effect of chronic NMU administration during the dark phase. Both the saline and NMU-treated groups did not increase, but rather maintained, their body weights throughout the study period. This is not unusual in chronic hypothalamic studies and may be attributed to the mild stress of the experiment [31–37]. However, it is possible that had the rats maintained their normal growth rates, differences in body weight between the groups may have been observed.

In conclusion, NMU appears to be involved in several hypothalamic circuits including those controlling feeding and the neuroendocrine stress axis. Our data provide further evidence that NMU has a role in the regulation of stress and the HPA axis.

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