Neuromedin U partially mediates leptin-induced hypothalamo-pituitary adrenal (HPA) stimulation and has a physiological role in the regulation of the HPA axis in the rat.


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**Abbreviations:** aCSF artificial cerebrospinal fluid; CNS central nervous system; CRH corticotrophin releasing hormone; HPA hypothalamo-pituitary-adrenal; ICV intracerebroventricular; i.p. intraperitoneal; NMU neuromedin U; NMU2R NMU 2 receptor; NPY neuropeptide Y; NIRS non-immune rabbit serum; PVN paraventricular nucleus; RPA ribonuclease protection assay.
Abstract

Intracerebroventricular (ICV) administration of the hypothalamic neuropeptide neuremedin U (NMU) or the adipostat hormone leptin increases plasma ACTH and corticosterone. The relationship between leptin and NMU in the regulation of the hypothalamo-pituitary adrenal (HPA) axis is currently unknown. In this study, leptin (1nM) significantly increased the release of CRH from ex-vivo hypothalamic explants by $207 \pm 8.4\% \ (p < 0.05 \ vs. \ basal)$, an effect blocked by the administration of anti-NMU immunoglobulin (IgG). ICV administration of leptin (10μg, 0.625nmol) increased plasma ACTH and corticosterone 20 minutes post injection [(plasma ACTH (pg/ml): vehicle 63 ± 20; leptin, 135 ± 36; p < 0.05) (plasma corticosterone (ng/ml): vehicle, 285 ± 39, leptin, 452 ± 44; p < 0.01)]. These effects were partially attenuated by the prior administration of anti-NMU IgG. Peripheral leptin also stimulated ACTH release, an effect attenuated by prior ICV administration of anti-NMU IgG. We examined the diurnal pattern of hypothalamic NMU mRNA expression and peptide content, plasma leptin and plasma corticosterone. The diurnal changes in hypothalamic NMU mRNA expression were positively correlated with hypothalamic NMU peptide content, plasma corticosterone and plasma leptin. ICV administration of anti-NMU IgG significantly attenuated the dark phase rise in corticosterone [corticosterone (ng/ml), vehicle, 493 ± 38; NMU IgG, 342 ± 47 (p < 0.05)]. These studies suggest that NMU may play a role in the regulation of the HPA axis and partially mediate leptin-induced HPA stimulation.
**Introduction**

Activity of the hypothalamo-pituitary adrenal (HPA) axis is altered by obesity and caloric deprivation. Leptin, the protein product of the *Lep* (obese) gene, is released into the circulation from adipose tissue and regulates food intake, energy expenditure and the HPA axis in part, via the hypothalamus (1-4). Leptin influences hypothalamic neurons via the long variant isoform of the leptin receptor, Ob-Rb (1;5;6).

The effects of leptin on the HPA axis are complex. The Ob-Rb is abundant on corticotropin-releasing hormone (CRH) neurons in the parvolesional region of the paraventricular nucleus (PVN) (7). It has been reported that administration of anti-CRH antibodies or the CRH receptor antagonist alpha-helical CRH$_{(9-41)}$ ($\alpha$-hCRH$_{(9-41)}$) inhibits the anorectic effects of leptin (8;9). These authors suggest that leptin partially regulates energy balance via hypothalamic CRH.

However, leptin has also been reported to have an inhibitory effect on the HPA axis. Leptin deficient (*ob/ob*) mice are hypercorticosteronemic (10). Administration of leptin to fasted rodents (11), *ob/ob* mice (11) or immobilization-stressed rodents (12) inhibits plasma ACTH and corticosterone levels. In addition, CRH mRNA expression in the PVN is increased in fasted rats, which have low circulating levels of leptin and *ob/ob* mice, in which leptin is absent (11;12). This apparent inverse relationship between plasma leptin and plasma corticosterone appears to support the hypothesis that leptin inhibits the HPA axis (11). However, recent publications suggest that leptin can stimulate the HPA axis under certain circumstances. Intracerebroventricular (ICV) administration of leptin has been shown to stimulate corticosterone secretion at the onset of the dark phase (13).
addition, a single ICV injection of leptin in the early light phase has shown to
dose-dependently increase plasma ACTH and corticosterone secretion (14). ICV injection of
leptin also increases the expression of CRH and its receptor, CRH-R\textsubscript{2} in the PVN (15).
Leptin may therefore have both inhibitory and stimulatory roles in the regulation of the
HPA axis. The mechanism by which leptin interacts with the HPA axis is currently
unknown.

Neuromedin U (NMU) is a gastrointestinal and central nervous system (CNS) peptide
which has been reported to reduce food intake (16;17), activate the sympathetic nervous
system (18) and increase energy expenditure (17;19). These effects are similar to those
observed following leptin administration (20-22). NMU also potently stimulates the HPA
axis and induces stress-related behaviors (16). Within the hypothalamus, NMU mRNA is
predominately expressed in the arcuate, suprachiasmatic and dorsomedial nuclei (17;23),
which also express the Ob-R\textsubscript{b} (24). NMU immunoreactive fibers project to the PVN (25).
ICV or intra-PVN administration of NMU to rats significantly increases plasma ACTH
and corticosterone (16;26). Incubation of \textit{ex-vivo} hypothalamic explants with NMU
significantly increases the release of CRH (16). The effects of NMU on the HPA axis are
thought to be mediated via the NMU 2 receptor (NMU2R) (16), which is expressed in the
ependymal layer of the third ventricle, the PVN (17;19;23;27;28) and the arcuate nucleus
(23).

NMU mRNA expression in the arcuate nucleus is reduced following a 48 hour fast, when
leptin concentrations are low, and reduced in \textit{ob/ob} mice, where leptin is absent (17).
Incubation of \textit{ex-vivo} hypothalamic explants with leptin increases the release of NMU in
vitro (16). However there is little other published data on the interaction between leptin and NMU systems with regards to regulation of the HPA axis.

Here we investigate the hypothesis that hypothalamic NMU in part mediates the leptin-induced activation of the HPA axis by using an anti-NMU immunoglobulin G (anti-NMU IgG) to block endogenous NMU signaling. We have also used anti-NMU IgG to investigate the role of endogenous hypothalamic NMU in the normal diurnal rise in plasma corticosterone.
METHODS AND MATERIALS

Animals

Male Wistar rats (specific pathogen free; Charles River, Margate, UK), weighing 200-250 g, were maintained in individual cages under controlled temperature (21-23 C) and light (12 h light/dark cycle, lights on at 0700 h) with *ad libitum* access to food (RM1 diet, SDS UK Ltd. Witham, UK), unless otherwise described. Food intake and body weight were measured daily throughout the study and all rats were handled daily to habituate and minimize any stress. All animal experimentation was conducted in accordance with accepted standards of humane animal care and carried out under the 1986 British Animals (Scientific Procedures) Act, License number 70/5516.

Materials

Recombinant murine leptin was a gift from M. Chiesi and N. Levens of Novartis (Basel, Switzerland). Reagents for the *ex-vivo* hypothalamic explant experiments were supplied by BDH (Poole, Dorset, UK). All peptides were reconstituted at the beginning of each study in vehicle (non-immune rabbit serum (NIRS)).

Antibody purification

The anti-NMU IgG was produced and purified using methods previously described (29;30).

Intracerebroventricular (ICV) cannulation and injection

Animal surgical procedures and handling were carried out as previously described (30). All compounds were injected using a 27 gauge stainless steel injector placed in and
projecting 1mm below the tip of the cannula. Cannula placement was confirmed by a positive dipsogenic response to angiotensin II (150ng). Only those animals showing a positive dipsogenic response were included in the data analysis (>98%). All animals were habituated to the injection process by a subsequent saline injection.

**Leptin and immunoblockade of leptin by anti-NMU IgG on hypothalamic CRH release**

A static incubation system was used as described previously (16). Briefly, *ad libitum* fed male Wistar rats were killed by decapitation and the whole brain immediately removed. The brain was mounted with the ventral surface uppermost and placed on a vibrating microtome (Microfield Scientific Limited, Dartmouth, UK). A 1.7mm slice was taken from the basal hypothalamus and blocked lateral to the Circle of Willis. The hypothalamic slices were incubated in individual chambers containing 1ml artificial cerebrospinal fluid (aCSF), (20mM NaHCO$_3$, 126mM NaCl, 0.09mM Na$_2$HPO$_4$, 6mM KCl, 1.4mM CaCl$_2$, 0.09mM MgSO$_4$, 5mM glucose, 0.18mg/ml ascorbic acid and 100µg/ml aprotinin (Bayer, Haywards Heath, UK)) equilibrated with 95% O$_2$ and 5% CO$_2$.

The tubes were placed on a platform in a water bath maintained at 37 C. After an initial 2 hour equilibration period, the hypothalami were incubated for 45 minutes in 600µl aCSF (basal period), before being challenged with leptin (1, 10 and 100nM), 1nM leptin combined with anti-NMU IgG (1:20), anti-NMU IgG alone (1:20) or NIRS (1:20) in 600µl aCSF for 45 minutes. The viability of the tissue was verified by 45 minutes of exposure to aCSF containing 56mM KCl. Isotonicity was maintained by substituting K$^+$ for Na$^+$. Hypothalamic explants that failed to show peptide release above that of basal in response to hyperkalaemic aCSF were excluded from the data analysis (<10%). The experiment
was repeated twice, with eight to twelve hypothalamic slices given each treatment. At the end of each period, aCSF was collected and stored at –20 C until measurement of CRH by RIA. To confirm whether the effect of anti-NMU IgG was specific for leptin, *ex vivo* hypothalamic explants were incubated in either 1000nM ghrelin combined with anti-NMU IgG or 1000nM ghrelin with NIRS. This dose of ghrelin has previously been shown to stimulate CRH release (31).

**Anti-NMU IgG immunoblockade on the effects of ICV leptin on the HPA axis**

*Ad libitum* fed male Wistar rats received a single ICV injection, between 0900 and 1000 h, of either vehicle, leptin (10µg, 0.625nmol) alone, anti-NMU IgG (5µl, concentration 150mg/ml) alone or leptin (0.625nmol) and anti-NMU IgG (5µl) together. The same dose of anti-NMU IgG has previously been shown to block the inhibitory actions on food intake of 1nmol NMU administered ICV (30). Morimoto *et al* reported that ICV administration of leptin significantly increased plasma ACTH and corticosterone 20 minutes post-injection (14). Rats were therefore killed by decapitation 20 minutes post-injection (*n* = 10-15 / group / time point) and trunk blood was collected into plastic lithium heparin tubes containing 0.6mg aprotinin and plastic tubes containing potassium EDTA (final concentration of 1.2-2 mg EDTA/ml blood). Plasma was separated by centrifugation, frozen and stored at –20 C for the measurement of ACTH and corticosterone by immunoradiometric assay (IRMA) and RIA respectively, according to the manufacturer’s protocol.
Anti-NMU IgG immunoblockade on the effects of peripheral leptin on the HPA axis

Ad libitum fed male Wistar rats received a single ICV injection, between 0900 and 1000 h, of either vehicle or anti-NMU IgG (1μl). Five minutes post injection, animals received an i.p. injection of either leptin (1.1mg/kg) or saline. Animals were killed by decapitation 20 minutes post ICV injection (n = 10/group) and trunk blood was collected into plastic lithium heparin tubes containing 0.6mg aprotinin and plastic tubes containing potassium EDTA (final concentration of 1.2-2mg EDTA/ml blood). Plasma was separated by centrifugation, frozen and stored at -20 C prior to the measurement of ACTH and corticosterone by IRMA and RIA respectively.

Diurnal changes in hypothalamic NMU mRNA expression and peptide content, plasma leptin and corticosterone levels.

Ad libitum fed male Wistar rats were decapitated at the following time points 0700 h (lights on), 1100 h, 1500 h, 1700 h, 1900 h, (lights off), 2100 h, 2300 h, 0300 h, and 0700 h) (n = 15 / per time point). At each time point hypothalami were dissected out and snap frozen in liquid nitrogen.

For quantification of hypothalamic NMU mRNA expression, hypothalamic mRNA was extracted (n = 10 / time point) using Tri-Reagent (Helena Biosciences, Sunderland, UK) according to the manufacturer’s protocol. Quantification of NMU mRNA expression was performed as previously described (32) using the Ambion RNase protection assay (RPA) III kit (Ambion Inc., TX) under conditions optimized within our laboratory. The NMU riboprobe corresponded to nucleotides 121 to 336 of the full length rat NMU sequence (Accession number NM_022239), a 215bp product. Briefly, 5μg RNA was hybridized
overnight at 42 C with 1.3 x 10^3 Bq ^32P[CTP] labeled riboprobe. Rat β-actin was used as an internal control (Ambion). Reaction mixtures were digested with RNase A/T1, the protected fragments precipitated and separated on a 4% polyacrylamide gel. The dried gel was exposed to a phosphorimager screen overnight and bands quantified by image densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

For quantification of hypothalamic NMU peptide content, peptide was extracted from hypothalami by boiling in 0.5M acetic acid (10ml/g hypothalamus) for 20 minutes (n = 5/time point). The hypothalamic extracts were cooled on ice and then stored at –20 C until determination of NMU peptide content by RIA. To normalize the data, the hypothalamic protein content was determined using the Coomassie Plus™ Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL).

Trunk blood was collected into plastic lithium heparin tubes containing 0.6mg aprotinin, separated by centrifugation, frozen and stored at -20 C until measurement of plasma leptin and corticosterone by RIA according to the manufacturer’s protocol.

**The effect of peripheral leptin on hypothalamic NMU mRNA and peptide expression**

*Ad libitum* fed male Wistar rats received a single i.p. injection, between 0900 and 1000 h of either saline or leptin (1.1mg/kg). This dose of leptin has previously been shown to activate hypothalamic neurons (33;34). Four hours post injection, animals were decapitated, brains removed and the hypothalamus dissected out and snap frozen in liquid nitrogen. Hypothalamic mRNA was extracted as previously described and hypothalamic NMU mRNA expression was quantified by RPA. To measure
hypothalamic NMU peptide content, peptide was extracted as described above and hypothalamic NMU content was quantified by RIA.

The effect of intracerebroventricular administration of anti-NMU IgG on the nocturnal rise of corticosterone

Hypothalamic NMU peptide content rose between 1900 and 2100 h (Figure 3A) therefore *ad libitum* fed male Wistar rats received a single ICV injection of either vehicle or anti-NMU IgG (5μl) just prior to the onset of the dark phase (1830 h) (n = 10-15 / group / time point) to block the rise in hypothalamic NMU peptide. Plasma corticosterone levels begin to rise from 1500 h (Figure 3B) and peak at 2100 h. In order to examine the blockade of endogenous NMU during the nocturnal rise in corticosterone, animals were decapitated at 1930 h. Trunk blood was collected into plastic lithium heparin tubes containing 0.6mg aprotinin. Plasma was separated by centrifugation, frozen and stored at –20 C until measurement of corticosterone by RIA according to manufacturer’s protocol.

Radioimmunoassays (RIA)

CRH-immunoreactivity (IR) and NMU-IR were measured using established assays (16). The intra- and inter-assay variations of both assays were less that 10%. Plasma corticosterone (MP Biomedicals, Hampshire, UK) and leptin (Linco Research Inc., St. Louis, MO) levels were measured using commercially available RIA kits. The intra- and inter-assay coefficients of variation for plasma corticosterone were less than 10%. Plasma ACTH (IDS Ltd, Tyne and Wear, UK) was measured by IRMA. The intra- and inter-assay coefficients of variation for ACTH were less than 4%.
**Statistical analysis**

Data are presented as mean ± sem. Data from *ex-vivo* hypothalamic explant release experiments were compared by paired *t*-test between the basal period and the test period. For the ICV studies, groups were compared by one-way ANOVA followed by a *post-hoc* Fisher’s least significant difference test (Systat, Evanson, IL). Correlation between hypothalamic NMU mRNA and peptide expression, plasma corticosterone and leptin levels with respect to time was determined by Spearman Rank Order Correlation. In all cases *p* < 0.05 was considered statistically significant.
**Results**

**Leptin and immunoblockade by anti-NMU IgG on hypothalamic CRH release**

Incubation of *ex-vivo* hypothalamic explants with 1, 10 and 100nM leptin significantly increased CRH immunoreactivity (IR) [CRH-IR (percentage of basal ± sem) basal, 100 ± 11; leptin (1nM) 219 ± 20 (p < 0.05 vs. basal); leptin (10nM) 270 ± 19 (p < 0.01 vs. basal); leptin (100nM) 207 ± 18 (p < 0.01 vs. basal) n = 8-12 / treatment] (Figure 1A). The increase in CRH release (Figure 1A) from hypothalamic explants following incubation of leptin (1nM) was attenuated by anti-NMU IgG (1:20) [CRH-IR (percentage of basal ± sem) basal, 100 ± 7.7; leptin (1nM), 207 ± 8.4 (p < 0.05 vs. basal); leptin (1nM)/anti-NMU IgG, 116 ± 23 (p = n.s. vs. basal; p < 0.05 vs. leptin); anti-NMU IgG 92 ± 6.9 (p = n.s. vs. basal, p < 0.05 vs. leptin), NIRS 109 ± 7.4 (p = n.s. vs. basal; p < 0.05 vs. leptin) n = 8-12 / treatment] (Figure 1B). The effect of leptin on CRH release was not affected by incubation with NIRS (CRH-IR (percentage of basal ± sem) basal, 100 ± 18.5; leptin (1nM)/NIRS 167.4 ± 8.5 (p < 0.05 vs. basal). To confirm the specificity of the effect for leptin, hypothalamic explants were incubated with 1000nM ghrelin and either NIRS or anti-NMU IgG. Ghrelin stimulated CRH release, an effect which remained in the presence of anti-NMU IgG (data not shown).

**Anti-NMU IgG immunoblockade on the effect of ICV leptin on the HPA axis**

ICV administration of leptin significantly increased plasma ACTH and corticosterone at 20 minutes post-injection when compared to the vehicle injected group [plasma ACTH (pg/ml): vehicle, 73 ± 25; leptin (0.625nmol), 135 ± 36; p < 0.05 vs. vehicle; plasma corticosterone (ng/ml): vehicle, 284 ± 39; leptin (0.625nmol), 452 ± 44; p < 0.01 vs. vehicle] n = 10-15 / group] (Figure 2A and B). The leptin-induced increase in plasma
ACTH and corticosterone was attenuated by the administration of anti-NMU IgG [plasma ACTH (pg/ml): leptin / anti-NMU IgG, 43 ± 11; p = n.s. vs. vehicle; p < 0.01 vs. leptin; plasma corticosterone (ng/ml): leptin / anti-NMU IgG, 319 ± 44; p = n.s. vs. vehicle, p < 0.01 vs. leptin] (Figure 2A and B). Anti-NMU IgG alone had no effect on plasma ACTH or corticosterone [plasma ACTH (pg/ml): anti-NMU IgG, 74 ± 27; plasma corticosterone (ng/ml): anti-NMU IgG, 312 ± 38] (Figure 2A and B).

**Anti-NMU IgG immunoblockade on the effect of peripheral leptin on the HPA axis.**

Intraperitoneal administration of leptin significantly increased plasma ACTH at 20 minutes post injection when compared to the vehicle injected control group. The leptin induced increase in plasma ACTH was attenuated by ICV administration of anti-NMU IgG. Anti-NMU IgG alone had no effect on plasma ACTH [plasma ACTH (pg/ml) saline / vehicle, 86.4 ± 12.2; leptin / vehicle, 135.6 ± 24.4; saline / anti-NMU IgG, 59.8 ± 7.0, leptin / anti-NMU IgG 66.8 ± 13.7 p<0.05 leptin / vehicle vs. saline / vehicle, saline / anti-NMU IgG and leptin / anti-NMU IgG, n = 10 / group] (Figure 2C). Intraperitoneal injection of leptin caused a non-significant increase in plasma corticosterone 20 minutes post injection when compared to the vehicle injected control group. There was a slight attenuation of this effect by ICV administration of anti-NMU IgG although again, this was not significant [plasma corticosterone (ng/ml) saline / vehicle, 279.3 ± 36.6; leptin / vehicle 387.6 ± 53.2; saline / anti-NMU IgG 242.4 ± 46.0; leptin / anti-NMU IgG 296.1 ± 45.2 n = 10 / group].

**Diurnal changes in hypothalamic NMU mRNA expression and peptide content, plasma corticosterone and plasma leptin.**

Hypothalamic NMU mRNA expression showed a diurnal rhythm with levels peaking at 2300 h, 4 hours after lights off (Figure 3A). Hypothalamic NMU peptide content
displayed a diurnal variation reaching a nadir at 1100 h, 4 hours after lights on. NMU peptide content subsequently increased throughout the day peaking at the onset of the dark phase (1900 h) (Figure 3B). Hypothalamic NMU mRNA expression was positively correlated with NMU peptide content ($r^2 = 0.514$, $p < 0.02$).

As expected, plasma corticosterone displayed a diurnal variation, with levels rising towards the end of the light phase (1700 h) and peaking at 2100 h (Figure 3A). A similar diurnal pattern existed between plasma corticosterone and hypothalamic NMU peptide. However, this correlation did not achieve statistical significance ($r^2 = 0.303$, $p = 0.062$).

Plasma leptin exhibited a diurnal pattern, reaching a nadir at 1100 h, 4 hours after lights on. Plasma leptin began to rise at the onset of the dark phase (1900 h) and peaked at 2100 h (Figure 3C). Plasma leptin levels were positively correlated with hypothalamic NMU mRNA expression ($r^2 = 0.321$, $p < 0.05$) and peptide content ($r^2 = 0.588$, $p < 0.01$) (see Supplemental figures for regression plots).

The effect of peripheral leptin on hypothalamic NMU mRNA and peptide expression.

A single i.p. injection of leptin had no effect on hypothalamic NMU mRNA expression (saline, $24.9 \pm 1.2$ arbitrary units; 1.1mg/kg leptin, $22.4 \pm 1.2$ n = 10) or NMU peptide content (saline, $5.6 \pm 0.5$ fmol/μg protein; 1.1mg/kg leptin, $4.9 \pm 0.4$ fmol/μg protein n = 10) 4 hours post injection.
ICV administration of anti-NMU IgG on the nocturnal rise of plasma corticosterone

Blocking endogenous hypothalamic NMU signaling with an ICV injection of anti-NMU IgG at the onset of the dark phase attenuated the nocturnal rise in plasma corticosterone at 1930 h [plasma corticosterone (ng/ml): vehicle, 493 ± 36; anti-NMU IgG, 342 ± 48 (p < 0.05 vs. vehicle) n = 10 -15 / group] (Figure 4).
**Discussion**

The evidence for the role of leptin in the regulation of the HPA axis is conflicting. Models of leptin deficiency suggest leptin inhibits the HPA axis (11;35) and leptin administration has been reported to suppress HPA axis activity (12;36). However, there is also evidence that leptin can stimulate the HPA axis (13-15). These current studies suggest that leptin stimulates the release of CRH from *ex-vivo* hypothalamic explants. This is in accord with studies showing that leptin increases CRH release, peptide concentration and mRNA expression in the PVN (14;37). In our current experiments, ICV administration of leptin increased plasma ACTH and corticosterone 20 minutes post injection, in agreement with published data (14). Leptin therefore appears to stimulate the HPA axis via the release of CRH under these specific conditions. A number of hypothalamic circuits are thought to regulate different aspects of “stress” and HPA axis activation. It is therefore quite possible that leptin can play both stimulatory and inhibitory roles in the regulation of the neuroendocrine response to stress. For example, both neuropeptide Y (NPY) and CRH neurons are leptin responsive, although leptin inhibits NPY release whilst stimulating CRH (1). Both of these neuropeptides stimulate the HPA axis but may be differentially regulated by different types of stress (13).

Hypothalamic release of NMU from *ex-vivo* hypothalamic explants is stimulated by leptin (16) and hypothalamic NMU mRNA expression is reduced in models of low or absent circulating leptin (17). NMU increases the release of CRH from *ex-vivo* hypothalamic explants (16) while intra-PVN administration of NMU increases plasma ACTH and corticosterone (16). The increase in plasma ACTH and corticosterone following ICV administration of NMU is attenuated in CRH-deficient mice and in rodents pre-treated with α-hCRH₉₋₄₁ (38;39). Though it is currently unknown if NMU2R is co-expressed
with CRH neurons, NMU2R is expressed in the PVN (17;27;40) and ICV administration of NMU increases \textit{c-fos}, a marker of neuronal activation, in CRH neurons in the PVN (41;42). The authors of these reports have suggested that the stimulatory effect of NMU on the HPA axis is mediated via CRH.

A number of leptin-responsive neuropeptides have been shown to regulate the HPA axis, including NPY (43). Our data suggest that the actions of leptin on the HPA axis may be partially mediated by the NMU/CRH pathway. Co-administration of anti-NMU IgG with leptin blocks leptin-induced CRH release from \textit{ex-vivo} hypothalamic explants. Furthermore, ICV administration of anti-NMU IgG partially attenuates the leptin-induced increase in plasma ACTH and corticosterone \textit{in vivo} following ICV administration. Leptin is produced by adipose tissue, circulates and enters the hypothalamus via the blood brain barrier. The effect of peripheral administration of leptin on the HPA axis is therefore perhaps more relevant than ICV administration. Peripheral administration of leptin caused a significant increase in plasma ACTH at 20 minutes post-injection. This effect is attenuated by ICV pre-treatment with anti-NMU IgG. These results suggest that NMU may mediate some of the effects of leptin on the HPA axis via CRH. The arcuate nucleus is incompletely isolated by the blood brain barrier and is thus in direct communication with peripheral signals from the blood and the CSF (44). It is therefore possible that arcuate NMU neurons respond to circulating leptin, signaling to CRH neurons in the PVN leading to activation of the HPA axis.

Our results suggest that both hypothalamic NMU peptide content and mRNA expression vary diurnally in rhythms that correlate with the diurnal variation in plasma leptin and
corticosterone. Furthermore, ICV administration of anti-NMU IgG partly attenuated the normal night-time rise in plasma corticosterone suggesting that NMU may play a role in regulating the diurnal changes in plasma corticosterone. Whilst indicating hypothalamic NMU mRNA expression and peptide content at specific time points, the diurnal patterns do not give an indication of the dynamics of peptide synthesis and release. For example, it is unclear whether high levels of hypothalamic NMU peptide content indicate increased peptide release or replenishment of neuronal peptide levels following release. The hypothalamus expresses low levels of NMU making it difficult to carry out a detailed dynamic investigation. In this current study, samples were only taken every 4 hours. More frequent sampling is required to determine the exact temporal relationship between hypothalamic NMU mRNA expression and peptide content, plasma corticosterone and plasma leptin. It is possible that NMU may not mediate the initial rise in corticosterone but may be involved in the maintenance of high corticosterone levels at the beginning of the dark phase.

The hypothalamic pathways that control the HPA axis are complex and are not fully characterized. The results presented in this manuscript suggest that leptin may mediate a stimulatory action on the HPA axis by altering hypothalamic NMU expression and release.
Figure 1: Leptin-induced CRH release is attenuated by administration of anti-NMU IgG. CRH release from *ex-vivo* hypothalamic explants (n = 8–12/treatment) after a 45 min basal incubation period followed by (A) 45 min exposure to 1, 10, 100 nM leptin in aCSF and (B) 45 min exposure to 1 nM leptin, leptin (1 nM) / anti-NMU IgG (1:20) in aCSF, anti-NMU IgG (1:20) in aCSF and non-immune rabbit serum (NIRS) (1:20) in aCSF. Data expressed as a percentage of basal ± sem. Significant values are indicated *p < 0.05 vs. basal; **p < 0.01 vs. basal; #p < 0.05 leptin / NMU IgG vs. NMU IgG and NIRS.

Figure 2: The stimulatory effects of leptin on the HPA axis are blocked by ICV administration of anti-NMU IgG. Plasma ACTH (A) and corticosterone (B) 20 minutes post injection of ICV vehicle, leptin (0.625nmol) or anti-NMU IgG (5µl) (n = 10-12 /group). Plasma ACTH (C) 20 minutes post ICV injection of either anti-NMU IgG (5µl) or vehicle and peripheral administration of either leptin (1.1mg/kg) or saline. Plasma ACTH: a, p < 0.05 vs. vehicle; b, p < 0.05 vs. leptin /anti- NMU IgG; c, p < 0.05 vs. anti-NMU IgG. Plasma corticosterone: a, p< 0.01 vs. vehicle, b, p < 0.05 vs. leptin / anti-NMU IgG; c, p < 0.05 vs. anti-NMU IgG.

Figure 3: Diurnal patterns of hypothalamic NMU peptide content, plasma corticosterone and plasma leptin. Diurnal profiles of (A) hypothalamic NMU mRNA expression, (B) hypothalamic NMU peptide content, (C) plasma corticosterone and (D) plasma leptin at nine time points 0700, 1100, 1500, 1700, 1900 (lights out), 2100, 2300, 0300, 0700 h (n = 10 / time point). Open bar indicates light phase and black bar indicates dark phase.
Figure 4: Immunoblockade of hypothalamic NMU attenuates the nocturnal rise in corticosterone. *Ad libitum* fed male Wistar rats received an injection of either anti-NMU IgG (5µl) or vehicle in the early dark phase. Plasma corticosterone was measured at 1930 h (n = 10-12). Significance values as indicated * p < 0.05 vs. vehicle
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Figure 1
Figure 2
Figure 3
Figure 4