Vrije Universiteit Brussel

# Effects of neuromedin U-8 on stress responsiveness and hypothalamus-pituitary-adrenal axis activity in male C57BL/6J mice. IF(2019) 3,684

De Prins, An; Allaoui, Wissal; Medrano Moya, Mireia; Van Eeckhaut, Ann; Ballet, Steven; Smolders, Ilse; De Bundel, Dimitri

*Published in:*
Hormones and Behavior

*DOI:*
10.1016/j.yhbeh.2019.104666

*Publication date:*
2020

*License:*
CC BY

*Document Version:*
Accepted author manuscript

Link to publication

*Citation for published version (APA):*
De Prins, A., Allaoui, W., Medrano Moya, M., Van Eeckhaut, A., Ballet, S., Smolders, I., & De Bundel, D. (2020). Effects of neuromedin U-8 on stress responsiveness and hypothalamus-pituitary-adrenal axis activity in male C57BL/6J mice. IF(2019) 3,684. *Hormones and Behavior*, *121*, [104666]. https://doi.org/10.1016/j.yhbeh.2019.104666

**Copyright**
No part of this publication may be reproduced or transmitted in any form, without the prior written permission of the author(s) or other rights holders to whom publication rights have been transferred, unless permitted by a license attached to the publication (a Creative Commons license or other), or unless exceptions to copyright law apply.

**Take down policy**
If you believe that this document infringes your copyright or other rights, please contact openaccess@vub.be, with details of the nature of the infringement. We will investigate the claim and if justified, we will take the appropriate steps.

Download date: 18. Apr. 2024

# Effects of neuromedin U-8 on stress responsiveness and hypothalamus-pituitary-adrenal axis activity in male C57BL/6J mice

De Prins An [a,*], Allaoui Wissal [a,*], Medrano Moya Mireia [a], Van Eeckhaut Ann [a], Ballet Steven [b], Smolders Ilse [a], De Bundel Dimitri [a,#]

* *Shared first authors*

[a] Department of Pharmaceutical Chemistry, Drug Analysis and Drug Information, Center for Neurosciences (C4N), Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium

[b] Research Group of Organic Chemistry, Departments of Chemistry and Bioengineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium

[#] **Corresponding author:**
Prof. Dr. Dimitri De Bundel
Department of Pharmaceutical Chemistry, Drug Analysis and Drug Information, Research Group Experimental Pharmacology, Center for Neurosciences (C4N), Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, 1090 Brussels, Belgium
Phone number: +3224774113
E-mail address: dimitri.de.bundel@vub.be

**Double names:**
An De Prins: first name “An”, last name “De Prins”
Ann Van Eeckhaut: first name “Ann”, last name “Van Eeckhaut”
Dimitri De Bundel: first name “Dimitri”, last name “De Bundel”
Mireia Medrano Moya: first name “Mireia”, last name “Medrano Moya”

**Declarations of interest:** none

**Abstract**

Neuromedin U (NMU) is a highly conserved neuropeptide that has been implicated in the stress response. To better understand how it influences various aspects of the stress response, we studied the effects of intracerebroventricular NMU-8 administration on stress-related behavior and activity of the hypothalamus-pituitary-adrenal (HPA) axis in male C57BL/6J mice. We investigated these NMU-8 effects when mice remained in their home cage and when they were challenged by exposure to forced swim stress. NMU-8 administration resulted in increased grooming behavior in mice that remained in their home cage and in a significant increase in c-Fos immunoreactivity in the paraventricular hypothalamus (PVH) and arcuate nucleus (ARC). Surprisingly, NMU-8 administration significantly decreased plasma corticosterone concentrations. Furthermore, NMU-8 administration increased immobility in the forced swim test in both naïve mice and mice that were previously exposed to swim stress. The effect of NMU-8 on c-Fos immunoreactivity in the PVH was dependent on previous exposure to swim stress given that we observed no significant changes in mice exposed for the first time to swim stress. In contrast, in the ARC we observed a significant increase in c-Fos immunoreactivity regardless of previous stress exposure. Interestingly, NMU-8 administration also significantly decreased plasma corticosterone concentrations in mice that were exposed to single forced swim stress, while this effect was no longer observed when mice were exposed to forced swim stress for a second time. Taken together, our data indicate that NMU-8 regulates stress responsiveness and suggests that its effects depend on previous stress exposure.

**Keywords**

Neuromedin U (NMU); stress-related behavior; c-Fos immunoreactivity; paraventricular nucleus (PVH); arcuate nucleus (ARC); forced swim test; hypothalamus-pituitary-adrenal axis.

**Abbreviations**

ACTH, adrenocorticotropic hormone; ARC, arcuate nucleus; CRH, corticotrophin-releasing hormone; HPA, hypothalamic-pituitary-adrenal; i.c.v., intracerebroventricular; i.p., intraperitoneal; NMU, neuromedin U; NMUR, neuromedin U receptor; POMC, pro-opiomelanocortin; PVH, paraventricular nucleus of the hypothalamus; TBS, tris-buffered saline.

## 1. Introduction

Neuromedin U (NMU) is a member of the neuromedin family that was originally isolated from porcine spinal cord (Minamino et al., 1985). NMU is coded by the *Nmu* gene and shows a remarkable amino acid sequence homology across animals, suggesting a strong evolutionary pressure to maintain its structure and function (Brighton et al., 2004). In mammals, NMU was found to occur in two major molecular forms: an extended 25 (NMU-25) or 23 (NMU-23) amino acid peptide and a truncated version of 9 (NMU-9) or 8 (NMU-8) amino acid C-terminal fragment (Brighton et al., 2004; Mitchell et al., 2009). The C-terminal amidated heptapeptide is entirely conserved in mammals and the C-terminal amidated octapeptide NMU-8 is the shorted peptide fragment that exerts the same biological effects as its longer endogenous isoforms, NMU-25 in humans or NMU-23 in rodents (Brighton et al., 2004). NMU-like immunoreactivity has been detected in neurons of the brain, spinal cord and mesenteric plexus (Brighton et al., 2004). NMU isoforms activate two G protein-coupled receptors known as NMUR1 receptors, which are mainly expressed in the periphery, and NMUR2 receptors, which are predominantly expressed in spinal cord and brain (Gartlon et al., 2004; Howard et al., 2000). Interestingly, different distribution patterns of NMUR2 mRNA have been observed in different species. Indeed, whereas NMUR2 mRNA was mainly detected in the paraventricular nucleus of the hypothalamus (PVH) in the rat brain (Graham et al., 2003; Howard et al., 2000), it was found to be abundantly expressed in the arcuate nucleus (ARC) and around the ventromedial hypothalamus in the mouse brain (Graham et al., 2003).

Current literature indicates a role for NMU in the control of smooth muscle contraction, blood pressure, nociception, inflammation, food intake and regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Brighton et al., 2004; Mitchell et al., 2009). The HPA axis is a major neuroendocrine system that regulates the stress response (Levy and Tasker, 2012). Activation of the HPA axis leads to the release of corticotropin releasing hormone (CRH) by the PVH, adrenocorticotropic hormone (ACTH) by the pituitary and corticosterone (cortisol in humans) by the adrenal cortex. Corticosterone further mediates the stress response by initiating metabolic and behavioral coping mechanisms and by exerting negative feedback on the HPA axis (Holsboer and Ising, 2010). The ARC was recently shown to also play a role in the negative feedback for corticosterone secretion (Leon-Mercado et al., 2017). The ARC is located at the base of the median eminence, and the blood-brain barrier is more permissive in the ventromedial part of the ARC. As a result, the ARC has been proposed as a key region for direct input from both systemic and pituitary blood. Moreover, the ARC projects to the PVH and has been suggested to thus contribute to HPA axis activity (Leon-Mercado et al., 2017; Palkovits, 2008).

Previous studies have shown that exogenous NMU administration elevates stress biomarkers and induces stress-related behavior in rodents. A single intracerebroventricular (i.c.v.) administration of NMU-23 to rats was reported to induce stress-related behavior such as face washing and grooming

(Gartlon et al., 2004; Hanada et al., 2001; Wren et al., 2002). These effects were at least partially mediated by CRH (Hanada et al., 2001; Wren et al., 2002). It was further demonstrated that i.c.v. administration of NMU-23 increased plasma levels of several endocrine hormones including arginine vasopressin, oxytocin, ACTH and corticosterone in rats (Ozaki et al., 2002; Wren et al., 2002). c-Fos gene expression levels in, amongst other brain regions, the PVH and ARC, were observed in rats following i.c.v. administration of NMU-23 (Niimi et al., 2001; Ozaki et al., 2002; Yokota et al., 2004). Direct administration of NMU-23 into the PVH or ARC gave similar results, namely a remarkable grooming behavior in rats, together with increased locomotor activity and reduced feeding episodes (Novak et al., 2006; Wren et al., 2002). Additionally, activation of the HPA axis was reported after microinjection of NMU-23 in the PVH with increased plasma concentrations of ACTH and corticosterone in rats (Wren et al., 2002). Repeated NMU-23 administration (twice per day for 7 days) resulted in elevated concentrations of plasma corticosterone, but not ACTH, and increased grooming behavior in rats (Thompson et al., 2004). Interestingly, NMU knockout mice exhibit a diminished behavioral response to stressful situations, such as substantial room temperature increases or immobilization stress (Nakahara et al., 2004). These stressors also result in a significant increase in plasma corticosterone levels in wildtype but not in NMU knockout mice, suggesting that NMU may be a key stress hormone (Nakahara et al., 2004). These effects of central administration of NMU-23 were attributed to activation of hypothalamic NMUR2 receptors (Graham et al., 2003; Hanada et al., 2001; Howard et al., 2000; Peier et al., 2009; Zeng et al., 2006). However, while the aforementioned studies consistently found that central administration of NMU peptides results in elevated c-Fos expression in the PVH and ARC, several studies were unable to confirm the effects on circulating corticosterone. Indeed, acute i.c.v. administration of NMU-23 was also shown to have no significant effect in plasma corticosterone concentrations in rats (Gartlon et al., 2004) and in mice (Vallof et al., 2017). Moreover, another study found no effect on plasma corticosterone following i.c.v. delivery of NMU-23 for 14 days in mice (Peier et al., 2011). Similarly, there have been contrasting findings regarding the effect of central administration of NMU on stress-related behaviors. A recent study found that NMU-23 may improve stress-coping in mice subjected to the forced swim test (Tanaka and Telegdy, 2014). This suggests that the effect of administration of NMU isoforms may depend on previous exposure to stress and the activity of the HPA axis.

In the present study, we aimed to further investigate these described discrepancies in the effects of central administration of NMU and performed a systematic set of experiments on the role of NMU-8 in stress coping at the behavioral, cellular and endocrinal level in the commonly used C57BL/6J mouse strain. We studied the effects of i.c.v. administration of NMU-8, the smallest active NMU fragment acting as a high-affinity agonist on NMUR1 and NMUR2, on stress-related behavior and activity in the home cage and when mice were challenged by exposure to forced swim stress. In all conditions, immunoreactivity of c-Fos, a marker of neuronal activation, was assessed in the PVH and ARC. Finally,

plasma corticosterone concentrations were measured to assess the putative downstream effects of NMU-8 on HPA axis activity.

## 2. Material and methods

### 2.1 Peptides

NMU-8 (H-Tyr-Phe-Leu-Phe-Arg-Pro-Arg-Asn-$NH_2$) was manually synthesized by conventional 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid phase peptide synthesis on Rink Amide AM resin (0.45 – 0.60 mmol $g^{-1}$, ChemImpex, USA) as described by De Prins *et al.* (De Prins et al., 2018a). The structure of the pure peptide was confirmed by high-resolution mass spectrometry on a Waters Micromass Q-Tof micro spectrometer with electrospray ionization. The purity of NMU-8 was more than 95 % according to high-performance liquid chromatography analysis. Mouse NMU-23 (H-Phe-Lys-Ala-Glu-Tyr-Gln-Ser-Pro-Ser-Val-Gly-Gln-Ser-Lys-Gly-Tyr-Phe-Leu-Phe-Arg-Pro-Arg-Asn-$NH_2$) was purchased from Phoenix Pharmaceuticals (USA), with a purity ≥95 % guaranteed by the manufacturer.

### 2.2 Animals

Adult (7 weeks) male C57Bl/6J mice were obtained from Janvier (France) and habituated to the animal facility for one week minimum prior to experiments. Mice were housed in groups of 4-6 (1290 eurostandard type III cages, Tecniplast, Italy) upon arrival and single-housed (1264C eurostandard type II cages, Tecniplast, Italy) at the start of the experiments in a temperature (18-24°C) and humidity (30-70%) controlled environment with a 12/12 h light/dark cycle. Mice had free access to food pellets (A03, SAFE, France) and water. Cages were minimally enriched with wooden gnawing blocks and nesting material. Mice did not receive a shelter following surgery to prevent damage to the implanted cannula. All procedures were approved by the Ethical Committee for Animal Experiments of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel and were carried out in accordance with the European Community Council Directives (2010/63/EU).

### 2.3 Stereotactic surgery

Mice were anesthetized with 4 % isoflurane (1000 mg/g, Iso-Vet®, Dechra Veterinary Products, The Netherlands) in an induction chamber. Anesthesia was maintained during the entire duration of the surgery with 2-3 % isoflurane delivered via a facemask. Ketoprofen (5 mg/kg, Ketofen®, 10 mg/mL, Merial, France) was administered subcutaneously to prevent post-operative pain and inflammation. Artificial tears (Tears Naturale, Alcon, United Kingdom) were applied to the eyes to prevent dehydration. A 3 mm guide cannula (26 GA, Plastics One, Roanoke, USA) was implanted stereotactically in the left ventricle of the mice using the following coordinates relative to bregma; +1.00 mm medial-lateral, -0.34 mm anterior-posterior and -2.20 mm ventral-dorsal. At the end of the surgical procedure, mice received 1 mL saline (0.9 % NaCl, Baxter, Belgium) intraperitoneally (i.p.) and were placed on a heating pad until awake. After surgery, mice were single-housed to prevent damage to the cannula and could recover for at least one week. During the recovery period, mice were habituated to the experimental procedures. A dummy (Plastics One, Roanoke, USA) was placed in the cannula to

prevent clogging. The position of the implanted guide was verified *post mortem* and compared to the anatomical Mouse Brain Atlas (Paxinos and Franklin, 2004). Correct implantation of the cannula was verified before unblinding. We observed off-target cannulation in 12 out of 146 mice. These mice were excluded from our analysis.

**2.4 Behavioral assessment**

All mice were acclimatized to the testing room and handled for 5 minutes per day starting three days prior to the behavioral assessments. Experiments were performed in the light phase of the light/dark cycle between 9:00 AM and 2:00 PM. The experimenters were blinded to treatment for the whole duration of the study. I.c.v. injections of vehicle (0.9% sterile saline), NMU-8 (0.5 nmol or 5 nmol dissolved in sterile saline) or NMU-23 (5 nmol dissolved in sterile saline) were carried out via the implanted guide cannula using a 33 GA injection needle (extending 1 mm beyond the tip of the guide cannula, Plastics One, Roanoke, USA), connected to a micro-injection pump (CMA 400 Syringe Pump, CMA/Microdialysis, Sweden) at a flow rate of 0.5 µL/min during 2 min. Following i.c.v. administration, the injection needle was left in place for 1 min to avoid reflux of the injected liquid.

*2.4.1 Home cage behavior*

Mice were randomly divided into three different groups and received an i.c.v. administration of saline or NMU-8 (0.5 or 5 nmol). Immediately after drug administration, mice were returned to their home cage and video monitored for 20 min. Behavioral analysis was performed with EthoVision xT11.5 software (Noldus, The Netherlands) and animals were scored for the following behaviors: grooming (body and face), digging and explorative behavior (distance moved).

*2.4.2 Forced swim test*

Passive stress-coping behavior was assessed by subjecting a separate cohort of mice to a single forced swim test, 15 min after i.c.v. administration of saline or NMU-8 (5 nmol). Mice were placed in a glass tank cylinder (30 cm diameter) filled with 30 cm of water at 25 ± 1 °C and video monitored during 5 min. The experiment was performed at a light intensity of 400 lux (Bentea et al., 2015). Immobility, swimming and climbing behavior were assessed during the experiment. Immobility was defined as the absence of movement in at least three paws. When mice used one paw occasionally to keep their head above the water surface, this was still considered as immobility. Climbing was defined as the use of two paws or more with vertical movement along the wall of the water tank. Swimming was defined as the use of two paws or more with horizontal movement in the tank. The predominant behavior present in ≥ 3 sec in each 5 sec epoch was scored offline by an observer blinded to treatment for the complete duration of the trial, resulting in 60 counts per mouse.

*2.4.3 Modified forced swim test*

To assess NMU-8 effects on stress responsiveness in a condition where stress was already present, another separate cohort of mice was subjected to a modified version of the mouse forced swim test. The modified version corresponds to the original forced swim test for rats, described by Porsolt *et al.* (Porsolt et al., 1977). Briefly, mice were exposed to a 15-min forced swim session on day one, 24 hours prior to subjection to a 5-min forced swim test. On the second day, NMU-8 (5 nmol) or saline was administered i.c.v. fifteen min before the 5-min forced swim test. All experimental and scoring conditions were identical to those described in 2.4.2.

**2.5 Immunohistochemistry**

To evaluate c-Fos immunoreactivity mice were intracardially perfused 90 min after drug treatment in all experimental conditions. Briefly, mice were deeply anesthetized with sodium pentobarbital i.p. (Doléthal®, 200 mg/mL, Vétoquinol, France) and perfused with phosphate buffered saline (PBS, Sigma-Aldrich, Germany) followed by 4 % paraformaldehyde (Sigma-Aldrich, Germany) for 5 min at a rate of 10 mL/min. After perfusion, brains were dissected and postfixed overnight in 4 % paraformaldehyde in PBS. 40-µm coronal sections were cut using a vibratome (Leica VT1000S, Leica Biosystems, Germany) and stored at -20 °C in a Tris-buffered saline (TBS) solution (50 mM Tris, pH 7.6, Sigma-Aldrich, Germany) containing 30 % glycerol (Millipore, Merck, Germany) and 30 % ethylene glycol (VWR International, USA).

Free-floating sections were rinsed three times for 10 min with Tris-buffered saline (TBS) and TBS containing 3% bovine serum albumin (Sigma-Aldrich, Germany) and 0.3% Triton-X (Sigma-Aldrich, Germany) for 1 h at room temperature under gentle agitation. Next, sections were incubated overnight in primary rabbit or goat anti-c-Fos antibody in blocking buffer (1:500; #2250, Cell Signaling, USA or sc-52, Santa Cruz Biotechnology, USA) at 4 °C. Co-labeling with guinea pig anti-CRH antibody (1:5000; T-5007, Peninsula Laboratories International, Inc., USA) or rabbit anti-proopiomelanocortin (POMC, 1:400; H-029-30, Phoenix Europe GmbH, Germany) was used to delineate PVH or ARC, respectively. The next day, sections were rinsed three times with TBS containing 0.1% Triton-X and incubated with secondary antibodies for 45 min at room temperature and protected from light. Secondary antibodies used were Cy™2-labeled donkey anti-guinea pig (1:200; #706-225-148, Jackson ImmunoResearch Laboratories, USA), Cy™3-labeled goat anti-rabbit (1:500; #111-165-003, Jackson ImmunoResearch Laboratories, USA), Cy™3-labeled donkey anti-goat (1:400; #705-165-147, Jackson ImmunoResearch Laboratories, USA) and Cy™5-labeled donkey anti-rabbit (1:400; #711-175-152, Jackson ImmunoResearch Laboratories, USA). Immunoreactivity to c-Fos was visualized with a confocal laser scan microscope (Zeiss, Axio Observer with LSM 710-6NLO configuration, Zeiss International, Germany) and c-Fos positive cells were manually quantified using the digital imaging system ImageJ (National Institutes of Health, USA). Essentially, for a given experiment the image with

the highest signal for c-Fos was used to define a fixed threshold to improve the signal-to-noise for quantification of the number of c-Fos positive profiles in all the obtained images. Next, the PVH or ARC (median eminence not included) was contoured and the area was measured on all images based on the labelling for CRH or POMC. Only images where the PVH and ARC were clearly visible and on which the profile corresponded to the representative images were used for further analysis. Finally, circular c-Fos positive profiles were analyzed blinded to treatment and expressed as number of c-Fos cells per square micrometer ($\mu m^2$).

### 2.6 Plasma corticosterone measurements

Plasma corticosterone concentrations were measured 10 min following i.c.v. administration of saline, NMU-8 (5 nmol) or NMU-23 (5 nmol) when mice remained in the home cage. When mice were subjected to swim stress after administration of saline or NMU-8 (5 nmol), blood was collected 10 min following the 5-min forced swim test. Mice were anesthetized with sodium pentobarbital i.p. and blood was collected from the heart by cardiac puncture and stored in $K_3$EDTA-coated tubes (Vacutest Kima S.R.L, Italy). To obtain plasma, blood samples were centrifuged for 15 min at 2500 g at 4 °C. The supernatant was collected and stored at -20 °C until analysis. Corticosterone plasma concentrations were measured using an enzyme-linked immunosorbent assay kit (ab108821, Abcam, UK) according to the manufacturer's recommended protocol (sensitivity: 0.28 ng/mL, intra-assay coefficient of variance: 5.3%, inter-assay coefficient of variance: 10.6% as indicated by the manufacturer).

### 2.7 Data analysis and statistical evaluation

Graphical representations and statistical analyses were performed using GraphPad Prism 6.01 software (GraphPad Software, Inc., USA). Data are expressed as dot blots with designation of median values. For comparison of two groups, Mann-Whitney U test was performed. For comparison of multiple groups, Kruskal-Wallis test followed by Dunn's *post hoc* test was employed. When more than one variable was evaluated, two-way ANOVA followed by Dunnett's *post hoc* test was used. The α value was set at 0.05 for each statistical test. Effect size estimates were determined using Cohen's d for Mann-Whitney U tests and eta-squared ($\eta^2$) for all ANOVA statistics reported within the text.

## 3. Results

### 3.1 Behavioral effects of central administration of NMU-8

Home cage behavior was assessed over a 20-min interval after i.c.v. administration of saline or NMU-8 (0.5 and 5 nmol). We found a significant increase in grooming behavior following i.c.v. administration of 5 nmol NMU-8 ($p=0.012$, $U=44$, Cohen's $d=1.06$; Figure 1A), while no significant effects were observed on total digging activity ($p>0.05$, $U=84.5$, Cohen's $d=0.24$; Figure 1B) and overall locomotor activity as measured by total distance moved ($p>0.05$, $U=73$, Cohen's $d=0.45$; Figure 1C). Further analysis of grooming behavior over 5-min time bins showed both a time [2-way ANOVA, time factor: $F(3,102) = 3.56$, $p=0.017$, $\eta^2=0.12$] and treatment effect [2-way ANOVA, treatment factor: $F(2,34) = 5.55$, $p=0.0082$, $\eta^2=0.18$]. Dunnett's multiple comparisons test revealed no significant effects on all measures for the lowest dose of NMU-8 (Dunnett's *post hoc* test, $p>0.05$, Figure S1) but a significant increase of grooming behavior after injection of 5 nmol NMU-8 at 5-10 min (saline vs 5 nmol NMU-8, Dunnett's *post hoc* test, $p= 0.033$), 10-15 min (saline vs 5 nmol NMU-8, Dunnett's *post hoc* test, $p= 0.012$) and 15-20 min (saline vs 5 nmol NMU-8, Dunnett's *post hoc* test, $p= 0.0011$) (Figure S1).

To assess whether NMU-8 increased stress vulnerability in the forced swim test, mice received an i.c.v. administration of saline or NMU-8 (5 nmol) followed 15 min later by a single 5-min forced swim test. We found that NMU-8 significantly increased immobility in the forced swim test compared to controls ($p=0.0065$, $U=16.5$, Cohen's $d=1.56$; Figure 1D), while swimming ($p>0.05$, $U=56.5$, Cohen's $d=0.11$; Figure 1E) and climbing ($p>0.05$, $U=36$, Cohen's $d=0.73$; Figure 1F) behavior was not significantly affected. We next evaluated whether NMU-8 would affect passive stress-coping behavior differently when mice were also previously exposed to swim stress. Here, mice were subjected to a 15-min forced swim session on the first day, and on the following day they received an i.c.v. administration of saline or NMU-8 (5 nmol) followed 15 min later by a 5-min forced swim test. Under these experimental conditions, NMU-8 also significantly increased immobility in the forced swim test compared to controls ($p=0.034$, $U=19$, Cohen's $d=1.12$; Figure 1G) while swimming ($p>0.05$, $U=36.5$, Cohen's $d=0.32$; Figure 1H) and climbing ($p>0.05$, $U=21.5$, Cohen's $d=0.98$; Figure 1I) behavior were not significantly altered.

### 3.2 Effects of NMU-8 on c-Fos immunoreactivity

The PVH was delineated using an antibody against CRH (Figure 2A). We found that i.c.v. administration of NMU-8 (5 nmol) significantly increased c-Fos immunoreactivity in mice that remained in the home cage (c-Fos: $p=0.017$, $U=2$, Cohen's $d=0.51$, Figure 2B; PVH area: $p>0.05$, $U=12$, Cohen's $d=0.42$). However, NMU-8 (5 nmol) did not significantly increase c-Fos expression in the PVH when mice were subjected to a single 5-min forced swim test (c-Fos: $p>0.05$, $U=10$, Cohen's $d=0.27$, Figure 2C; PVH area: $p>0.05$, $U=3$, Cohen's $d=1.51$). Here, it can be noted that following a single 5-min forced swim test, baseline c-Fos immunoreactivity in the PVH was high compared to the mice that

remained in the home cage (p=0.0043, U=0, Cohen's d=2.93). When mice were pre-exposed to a 15-min forced swim stress session on the first day, NMU-8 (5 nmol) significantly increased c-Fos expression in the PVH (c-Fos: p=0.032, U=1, Cohen's d=2.17, Figure 2D; PVH area: p>0.05, U=0.84, Cohen's d= -0.14) when administered 15 min prior to a 5-min forced swim test on the second day.

The ARC was delineated using an antibody against POMC (Figure 2E). We observed that i.c.v. administration of NMU-8 (5 nmol) significantly increased c-Fos immunoreactivity in mice that remained in the home cage (c-Fos: p= 0.0095, U=0, Cohen's d=2.75, Figure 2F; ARC area: p>0.05, U=10, Cohen's d= -0.09). Moreover, we found that NMU-8 significantly increased c-Fos expression in POMC cells (p=0.0095, U=0, Cohen's d=2.75; Figure S2), but the total c-Fos expression in the ARC was not restricted to POMC cells. Following administration of NMU-8 (5 nmol) only 15.7±2.5% of c-Fos expressing cells also expressed POMC. When NMU-8 (5 nmol) was administered 15 min before a single 5-min forced swim test, we also observed a significant increase in c-Fos immunoreactive cells compared to control mice (c-Fos: p=0.019, U=1, Cohen's d=2.21, Figure 2G; ARC area: p>0.05, U=5, Cohen's d= 0.97). Furthermore, when mice were pre-exposed to a 15-min forced swim stress on the first day, NMU-8 (5 nmol) significantly increased c-Fos expression in the ARC when administered 15 min before the 5-min forced swim test on the second day of the experiment (c-Fos: p=0.016, U=0, Cohen's d=2.83, Figure 2H; ARC area: p>0.05, U=4, Cohen's d= -0.96).

### 3.3 Effects of NMU-8 on the plasma corticosterone concentration

NMU-8 (5 nmol) administration resulted in a significant decrease of the corticosterone concentration in plasma 10 min post-injection compared to controls (p=0.019, U=14, Cohen's d=1.32; Figure 3A) in mice that remained in the home cage. When NMU-8 (5 nmol) was administered 15 min before a 5-min forced swim test, it resulted in significantly decreased plasma corticosterone concentrations 10 min after the test compared to saline controls (p=0.0082, U=4.5, Cohen's d=1.87; Figure 3B). In mice that were subjected to a 15-min forced swim on the first day, administration of NMU-8 (5 nmol) 15 min before the 5-min forced swim test on the second day did not result in a significant decrease in the plasma corticosterone concentration compared to controls (p>0.05, U=12, Cohen's d=0.066; Figure 3C). In mice that underwent a single forced swim test, the concentration of corticosterone in plasma was significantly higher compared to mice that remained in the home cage (Kruskal-Wallis; home cage versus single forced swim test: p<0.0001, home cage versus modified forced swim test: p>0.05, H=17.45, $\eta^2$=0.86).

### 3.4 Effects of NMU-23 on the plasma corticosterone concentration

To investigate whether the observed effects on plasma corticosterone were dependent on the NMU isoform we used in our experiments, we also investigated the effect of central administration of NMU-23. We observed that i.c.v. NMU-23 (5 nmol) administration resulted in a significant decrease of the corticosterone concentration in plasma 10 min post-injection compared to saline controls ($p=0.033$, $U=6$, Cohen's $d=1.48$; Figure 4) in mice that remained in the home cage.

## 4. Discussion

In the present study we refined existing knowledge on the effects of central NMU-8 administration on the stress response. We confirmed previous observations that central administration of NMU-8 stress-related behavior and c-Fos expression in the PVH and ARC of C57BL6/J mice. However, in contrast to other studies we observed that central administration of NMU-8 evoked a significant decrease in plasma corticosterone. We propose that the effects of NMU-8 may depend on previous stress exposure.

### 4.1 NMU-8 effects in home cage conditions

Our study showed that i.c.v. administration of NMU-8 in a dose of 5 nmol increased grooming behavior in mice that remained in the home cage. This finding is in line with previous studies, reporting increased grooming behavior in rats following central NMU-23 administration (Gartlon et al., 2004; Hanada et al., 2001; Wren et al., 2002). This effect of NMU-23 was attributed to activation of the HPA axis, sinceit resulted in activation of CRH cells in the PVH and treatment with CRH antagonists blocked the stress response (Gartlon et al., 2004; Hanada et al., 2001; Wren et al., 2002). While these studies also reported a NMU-23-induced increase in overall locomotor activity in rats (Gartlon et al., 2004; Hanada et al., 2001), we could not observe this effect in C57BL/6J mice. Moreover, another study showed decreased locomotor activity in NMU knockout mice compared to wildtype littermates (Hanada et al., 2004). These inconsistencies may be due to the different time span for observation. Indeed, while we observed locomotor activity over a time span of 20 min, other studies explored activity for 120 min up to 24 hours (Gartlon et al., 2004; Hanada et al., 2004).

At the cellular level, we found that the NMU-8-induced grooming behavior was accompanied by increased c-Fos expression in the PVH and ARC. Our findings are consistent with previous observations of increased c-Fos immunoreactivity in both the PVH and ARC of mice or rats treated with NMU (Ivanov et al., 2002; Nagai et al., 2018; Nakahara et al., 2004; Niimi et al., 2001). In the PVH, the increase in c-Fos expression was observed in CRH-containing cells and in the ARC it was observed in POMC cells. However, increased c-Fos expression was not restricted to these cell types or brain regions. The CRH-containing cells have been described to release CRH upon their activation (McEwen, 2007; Zhang et al., 2017). We therefore expected that the increased expression of c-Fos in the hypothalamus following administration of NMU-8 would be associated with an increase in the plasma concentration of corticosterone. Surprisingly, we found that NMU-8 decreases the plasma concentration of corticosterone in C57Bl/6J mice as quickly as 10 min following its central administration. When administered centrally, NMU-23 administration (0.3-1 nmol i.c.v.) was previously shown to increase plasma corticosterone in male Wistar rats (Ozaki et al., 2002; Wren et al., 2002). In line with this observation, repeated administration of NMU-23 (0.3 nmol) in the PVH of male Wistar rats for a duration of 7 days resulted in a significant increase in plasma corticosterone when measured one day after the final injection (Thompson et al., 2004). Moreover, lowered plasma corticosterone

concentrations were also reported in mice lacking the functional NMU peptide compared to their wildtype littermates in naïve conditions (Hanada et al., 2004). In contrast, another study found no significant effect of central administration of NMU-23 on plasma corticosterone in male Sprague-Dawley rats (Gartlon et al., 2004). Similarly, central administration of NMU-23 (1 µg i.c.v.) had no significant effect on plasma corticosterone in male NMRI mice (Vallof et al., 2017). Moreover, central delivery of NMU-23 for 14 days did not affect plasma corticosterone in male C57BL6/J mice (Peier et al., 2011). The notion that we used NMU-8 cannot explain differences with literature given that it is also an agonist of NMUR1/2 and when we administered NMU-23 (5 nmol i.c.v.) we similarly observed a significant decrease in plasma corticosterone. We note however that the highest dose of NMU-8 used in our study is slightly higher compared to those used in previous literature. Given that cerebrospinal fluid is rapidly cleared from the brain through meningeal lymphatic vessels (Ahn et al., 2019), we cannot exclude that NMU-8 would reach the periphery after i.c.v. administration. This may be relevant given that NMU-8 was initially reported to exert biphasic effects on corticosterone. Administration of a single systemic dose of NMU-8 (6 µg/100g sc) was reported to induce a transient increase in serum corticosterone in adult female Wistar rats (Malendowicz et al., 1993). However, 6-day regimen of systemic NMU-8 administration significantly increased serum corticosterone in a low dose (1.5 µg/100g s.c.) whereas a high dose (6 µg/100g s.c.) did not affect basal serum corticosterone in female Wistar rats (Malendowicz et al., 1994). Interestingly, in the same study, the highest dose of NMU-8 suppressed the ACTH-induced increase in serum corticosterone (Malendowicz et al., 1994). Based on these findings, the authors concluded higher doses of NMU-8 may exert a direct inhibitory effect on adrenal function (Malendowicz et al., 1994). We acknowledge that these pioneering studies were carried out in female rats and that the lack of inclusion of female mice is a weakness of our study.

### 4.2 NMU-8 effects in a stressful context

Given its previously described effects on HPA axis activity, we hypothesized that NMU would aggravate stress-related behaviors, such as passive stress-coping in the forced swim test. We indeed observed that central NMU-8 administration increased immobility in a 5-min forced swim test. However, this finding contrasted with a previous study in which i.c.v. administration of NMU-23 resulted in a decreased immobility while increasing climbing and swimming time in a modified version of the forced swim test, ascribing antidepressant-like effects to NMU-23 (Tanaka and Telegdy, 2014). In this study, mice were exposed to a 15-min forced swim session on day one, followed by NMU-23 treatment and a 5-min forced swim test on day two (Tanaka and Telegdy, 2014). Therefore, we carried out an experiment where we administered NMU-8 under these experimental conditions. Interestingly, we again found increased immobility in mice treated with NMU-8. One potential explanation of the observed discrepancy is the difference in used mouse strains. While our experiments were performed with mice of the widely used inbred strain C57BL/6J, the reported study used the outbred CFLP mouse strain. Indeed, mouse strain is an important factor in both baseline performances and pharmacological

responses to antidepressants in the forced swim test (Lucki et al., 2001). Likewise, it is possible that NMU acts differently in these mouse strains. It can also be noted that we used the truncated peptide NMU-8 in contrast to the longer isoform NMU-23 in the previously reported study. However, NMU-8 has been shown to act as a full agonist on NMUR1 and NMUR2 without loss in potency (Brighton et al., 2004; De Prins et al., 2018a; De Prins et al., 2018b). This makes it unlikely that the observed differences are due to differences in the used peptide forms. Moreover, we showed that NMU-23 has similar effects as NMU-8 on plasma corticosterone. Interestingly, it has been suggested that NMU peptides may also act through other, yet undiscovered receptors (Martinez and O'Driscoll, 2015). In this context, we cannot fully exclude that the use of a different isoform did not contribute to the behavioral outcomes in the forced swim test, given the assumption that a different isoform could also exert effects independently of NMUR2. However, it is worth mentioning that stress-coping behavior has been previously investigated in NMUR2 knockout mice, using the mouse tail suspension test (Zeng et al., 2006). Similar to the forced swim test, the mouse tail suspension test induces an inescapable and stressful state (Cryan et al., 2005). Interestingly, the mentioned study did not report significant differences between NMUR2 knockout mice and their wildtype littermates in the mouse tail suspension test (Zeng et al., 2006). This may suggest that the loss of NMUR2 function does not critically affect passive stress-coping behavior in the mouse tail suspension test.

We found that c-Fos expression was high in the PVH after a single 5-min forced swim test. This corresponds to previous literature findings demonstrating that swim-stress increases c-Fos positive cells in the PVH, a stress-sensitive region and key modulator of HPA axis (Duncan et al., 1993; Stone et al., 2007). However, we found that central administration of NMU-8 did not further elevate c-Fos expression in the PVH under these experimental conditions while it did increase c-Fos immunoreactivity in the ARC. Interestingly, when mice were previously exposed to swim stress, c-Fos immunoreactivity in the PVH following a 5-min forced swim test was less pronounced. Under these experimental conditions, NMU-8 increased neuronal activity in both the PVH and ARC. Overall, it appears that central administration of NMU-8 increases c-Fos expression in both the PVH and ARC, but that these effects depend on prior stress exposure.

Corticosterone levels in plasma typically increase upon stress exposure. Interestingly, a previous study demonstrated that short-term immobilization stress increased plasma corticosterone in wildtype mice while no effect was seen in NMU knockout mice (Nakahara et al., 2004). However, we found that NMU-8 decreased plasma corticosterone levels in naïve mice as well as in mice subjected to a single 5-min forced swim test. Intriguingly, the corticosterone-reducing effect of NMU-8 was not observed when mice were pre-exposed to a 15-min forced swim session one day before the 5-min forced swim test. Importantly, the increase in baseline plasma corticosterone levels compared to mice that remained in the home cage was also less pronounced when mice were subjected to a second forced swim test. Our data

indicate that the effects of NMU may depend on previous stress exposure but the mechanism through which central administration of NMU-8 decreases plasma corticosterone remains unknown.

### 4.3 Conclusions

Altogether, our findings contribute to the better understanding of the effects of NMU on stress-responsiveness. We conclude that NMU-8 increases c-Fos activity in the PVH and ARC and stress-related behaviors in C57BL/6J mice, while it surprisingly decreases corticosterone plasma concentrations. Importantly, we found that the observed effects of NMU-8 were dependent on previous stress exposure. We hypothesize NMU might be an interesting target to further explore novel treatments for stress-related disorders. However, the inconsistencies found with literature at both the behavioral and endocrinal level emphasize the need for further investigation of the mechanisms through which NMU affects physiological functions.

## Figure legends

**Figure 1. Effects of central NMU-8 administration on stress-related behavior in C57BL/6J mice.** Intracerebroventricular (i.c.v.) administration of saline or NMU-8 (5 nmol) was carried out at the onset of a 20 min observation period and the total time spent grooming, digging and the total distance moved were scored by an observer blinded to treatment while mice remained in the home cage (A-C). In a separate series of experiments, we investigated the effect of i.c.v. administration of saline or NMU-8 (5 nmol) on immobility, swimming and climbing behavior in a 5 min forced swim test in naïve mice (D-F) or in mice exposed to a 15 min forced swim session one day before the 5 min forced swim test session (G-I). NMU-8 was administered 15 min before the forced swim test. Data are presented as a dot blot for individual values with designation of the median and n = 9-14 per group. * $P<0.05$, ** $P<0.01$ versus saline controls analyzed by Mann-Whitney U test.

**Figure 2. Effects of central NMU-8 administration on expression of the immediate early gene c-Fos in the paraventricular hypothalamus (PVH) and in the arcuate nucleus (ARC) of C57BL/6J mice.** Representative images of c-Fos positive cells in the PVH, co-labeled with an antibody against CRH (A) and ARC, co-labeled with an antibody against POMC (E). Quantification by an observer blinded to treatment shows that intracerebroventricular (i.c.v.) administration of NMU-8 (5 nmol) differentially affected the amount of c-Fos immunoreactive cells/$\mu m^2$ in both the ARC and PVH of mice that remained in their home cage (B,F), mice subjected to a single forced swim test (C,G) or mice subjected to a 15 min forced swim session one day before the forced swim test (D,H). Data are presented as a dot blot for individual values with designation of the median and n = 4-6 per group. * $P<0.05$, ** $P<0.01$ versus saline controls analyzed by Mann-Whitney U test. *3V, third ventricle; CRH, corticotropic releasing hormone; DMH, dorsomedial hypothalamus; LHA, lateral hypothalamus; ME, median eminence; POMC, pro-opiomelanocortin.*

**Figure 3. Effects of central NMU-8 administration on the plasma concentration of corticosterone in C57BL/6J mice.** Plasma corticosterone was measured 10 min after intracerebroventricular (i.c.v.) administration of NMU-8 in mice remaining in the home cage (A), 10 min after a 5 min forced swim test in naïve mice (B) or 10 min after a 5 min forced swim test in mice that were subjected to a 15 min forced swim session one day earlier (C). NMU-8 was administered 15 min before the forced swim test. Data are presented as a dot blot for individual values with designation of the median and n = 5-9 per group. * P<0.05, ** P <0.01 versus saline controls analyzed by Mann-Whitney U test.

**Figure 4. Effects of central NMU-23 administration on the plasma concentration of corticosterone in C57BL/6J mice.** Plasma corticosterone was measured 10 min after intracerebroventricular administration of NMU-23 (5 nmol) in mice remaining in the home cage. NMU-23 significantly decreased plasma corticosterone concentrations compared to saline treated mice. Data are presented as a dot blot for individual values with designation of the median and n=6-7 per group. *P<0.05 versus saline controls analyzed by Mann-Whitney U test.

## Figures

**FIGURE 1**

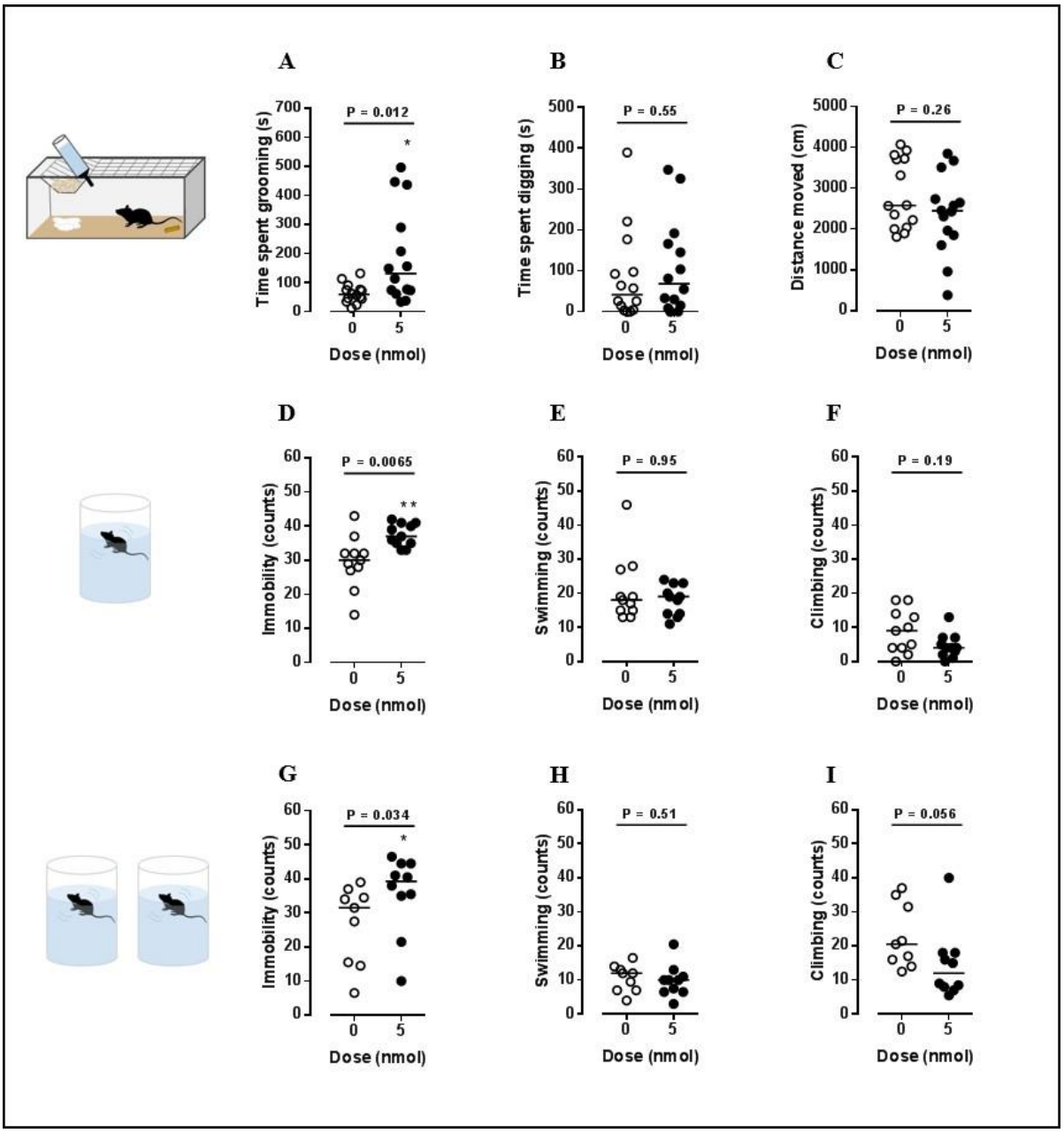


**FIGURE 2**

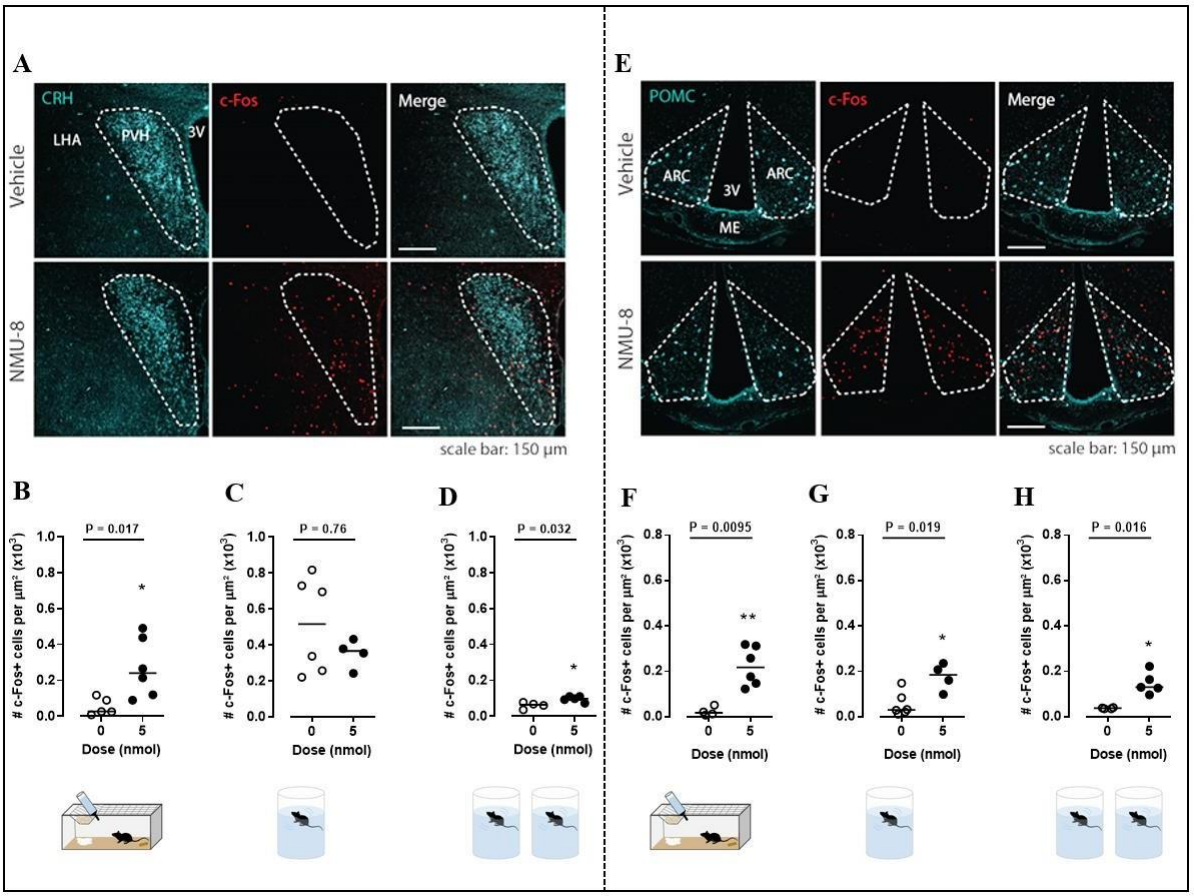

A
CRH
c-Fos
Merge
LHA
PVH
3V
Vehicle
NMU-8
scale bar: 150 µm
E
POMC
c-Fos
Merge
ARC
3V
ARC
ME
Vehicle
NMU-8
scale bar: 150 µm
B
C
D
F
G
H
# c-Fos+ cells per µm² (x10³)
P = 0.017
P = 0.76
P = 0.032
P = 0.0095
P = 0.019
P = 0.016
Dose (nmol)
0
5

**FIGURE 3**

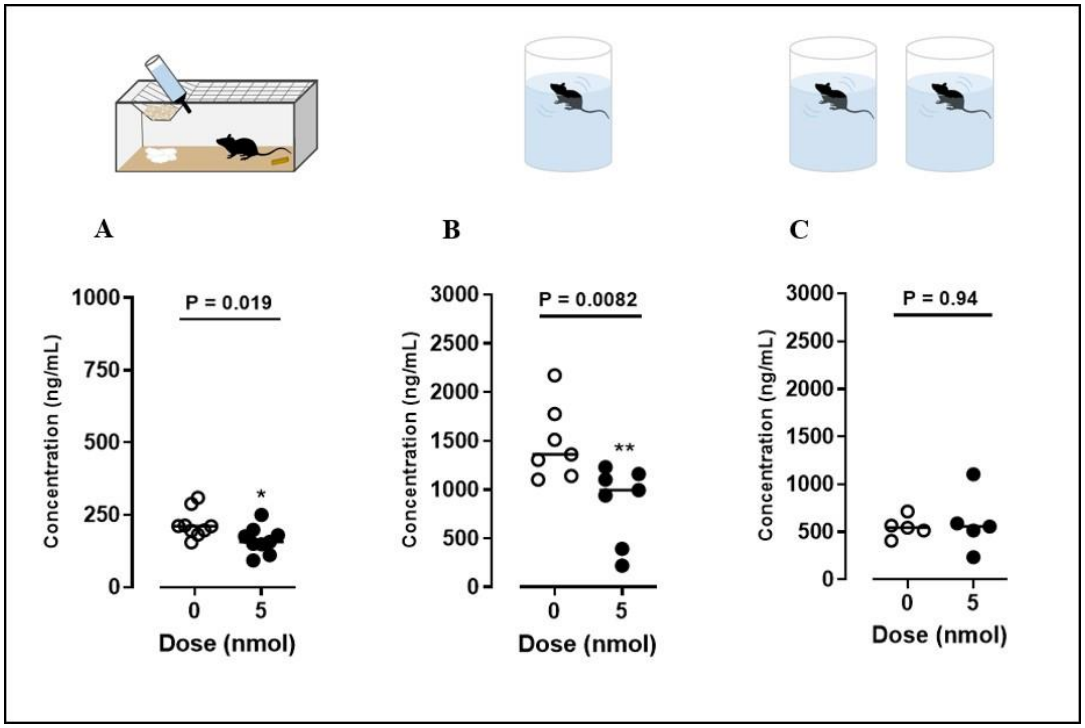
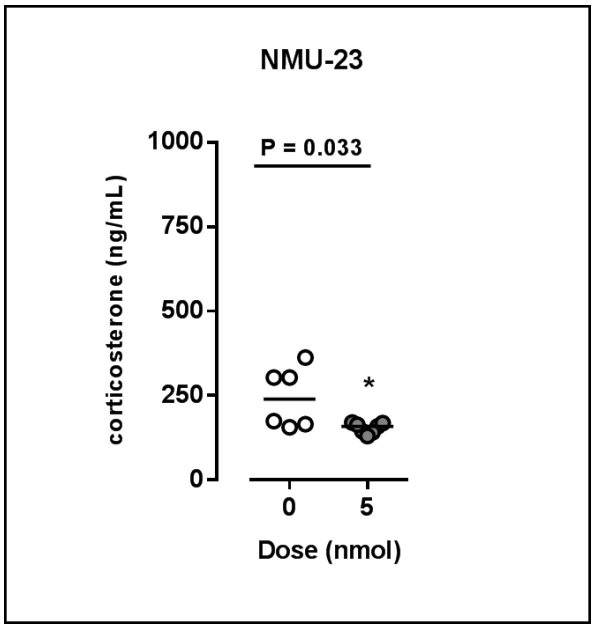

A
B
C
Concentration (ng/mL)
1000
750
500
250
0
P = 0.019
*
0
5
Dose (nmol)
3000
2500
2000
1500
1000
500
0
P = 0.0082
**
P = 0.94

**FIGURE 4**


NMU-23
P = 0.033
corticosterone (ng/mL)
1000
750
500
250
0
*
0
5
Dose (nmol)

## Funding

ADP was supported by the Flanders Innovation & Entrepreneurship Agency (VLAIO). This work was supported by the Queen Elisabeth Medical Foundation (G.S.K.E – 2017-2019), the Fund for Scientific Research Flanders (FWO grant G.0287.16) and the Strategic Research Program of the Vrije Universiteit Brussel (SRP49).

## Author contributions

A.D.P., D.D.B., I.S. and W.A. designed the experiments. A.D.P, W.A and M.M.M. performed the experiments. D.D.B. and I.S. supervised the research study. A.D.P., W.A., D.D.B. and I.S. wrote the manuscript. A.V.E., M.M.M. and S.B. revised the manuscript. All authors provided critical feedback and helped shape the research and manuscript.

## References


Ahn, J.H., Cho, H., Kim, J.H., Kim, S.H., Ham, J.S., Park, I., Suh, S.H., Hong, S.P., Song, J.H., Hong, Y.K., Jeong, Y., Park, S.H., Koh, G.Y., 2019. Meningeal lymphatic vessels at the skull base drain cerebrospinal fluid. Nature 572, 62-66.

Bentea, E., Demuyser, T., Van Liefferinge, J., Albertini, G., Deneyer, L., Nys, J., Merckx, E., Michotte, Y., Sato, H., Arckens, L., Massie, A., Smolders, I., 2015. Absence of system xc- in mice decreases anxiety and depressive-like behavior without affecting sensorimotor function or spatial vision. Prog Neuropsychopharmacol Biol Psychiatry 59, 49-58.

Brighton, P.J., Szekeres, P.G., Willars, G.B., 2004. Neuromedin U and its receptors: structure, function, and physiological roles. Pharmacological reviews 56, 231-248.

Cryan, J.F., Mombereau, C., Vassout, A., 2005. The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. Neurosci Biobehav Rev 29, 571-625.

De Prins, A., Martin, C., Van Wanseele, Y., Skov, L.J., Tomboly, C., Tourwe, D., Caveliers, V., Van Eeckhaut, A., Holst, B., Rosenkilde, M.M., Smolders, I., Ballet, S., 2018a. Development of potent and proteolytically stable human neuromedin U receptor agonists. Eur J Med Chem 144, 887-897.

De Prins, A., Martin, C., Van Wanseele, Y., Tömböly, C., Tourwé, D., Caveliers, V., Holst, B., Van Eeckhaut, A., Rosenkilde, M.M., Smolders, I., Ballet, S., 2018b. Synthesis and in Vitro Evaluation of Stabilized and Selective Neuromedin U-1 Receptor Agonists. ACS Med Chem Lett 9, 496-501.

Duncan, G.E., Johnson, K.B., Breese, G.R., 1993. Topographic patterns of brain activity in response to swim stress: assessment by 2-deoxyglucose uptake and expression of Fos-like immunoreactivity. J Neurosci 13, 3932-3943.

Gartlon, J., Szekeres, P., Pullen, M., Sarau, H.M., Aiyar, N., Shabon, U., Michalovich, D., Steplewski, K., Ellis, C., Elshourbagy, N., Duxon, M., Ashmeade, T.E., Harrison, D.C., Murdock, P., Wilson, S., Ennaceur, A., Atkins, A., Heidbreder, C., Hagan, J.J., Hunter, A.J., Jones, D.N., 2004. Localisation of NMU1R and NMU2R in human and rat central nervous system and effects of neuromedin-U following central administration in rats. Psychopharmacology 177, 1-14.

Graham, E.S., Turnbull, Y., Fotheringham, P., Nilaweera, K., Mercer, J.G., Morgan, P.J., Barrett, P., 2003. Neuromedin U and Neuromedin U receptor-2 expression in the mouse and rat hypothalamus: effects of nutritional status. Journal of neurochemistry 87, 1165-1173.

Hanada, R., Nakazato, M., Murakami, N., Sakihara, S., Yoshimatsu, H., Toshinai, K., Hanada, T., Suda, T., Kangawa, K., Matsukura, S., Sakata, T., 2001. A role for neuromedin U in stress response. Biochemical and biophysical research communications 289, 225-228.

Hanada, R., Teranishi, H., Pearson, J.T., Kurokawa, M., Hosoda, H., Fukushima, N., Fukue, Y., Serino, R., Fujihara, H., Ueta, Y., Ikawa, M., Okabe, M., Murakami, N., Shirai, M., Yoshimatsu, H., Kangawa, K., Kojima, M., 2004. Neuromedin U has a novel anorexigenic effect independent of the leptin signaling pathway. Nature medicine 10, 1067-1073.

Holsboer, F., Ising, M., 2010. Stress hormone regulation: biological role and translation into therapy. Annual review of psychology 61, 81-109, C101-111.

Howard, A.D., Wang, R., Pong, S.S., Mellin, T.N., Strack, A., Guan, X.M., Zeng, Z., Williams, D.L., Jr., Feighner, S.D., Nunes, C.N., Murphy, B., Stair, J.N., Yu, H., Jiang, Q., Clements, M.K., Tan, C.P., McKee, K.K., Hreniuk, D.L., McDonald, T.P., Lynch, K.R., Evans, J.F., Austin, C.P., Caskey, C.T.,

Van der Ploeg, L.H., Liu, Q., 2000. Identification of receptors for neuromedin U and its role in feeding. Nature 406, 70-74.

Ivanov, T.R., Lawrence, C.B., Stanley, P.J., Luckman, S.M., 2002. Evaluation of neuromedin U actions in energy homeostasis and pituitary function. Endocrinology 143, 3813-3821.

Leon-Mercado, L., Herrera Moro Chao, D., Basualdo, M.D., Kawata, M., Escobar, C., Buijs, R.M., 2017. The Arcuate Nucleus: A Site of Fast Negative Feedback for Corticosterone Secretion in Male Rats. eNeuro 4.

Levy, B.H., Tasker, J.G., 2012. Synaptic regulation of the hypothalamic–pituitary–adrenal axis and its modulation by glucocorticoids and stress. Front Cell Neurosci 6.

Lucki, I., Dalvi, A., Mayorga, A.J., 2001. Sensitivity to the effects of pharmacologically selective antidepressants in different strains of mice. Psychopharmacology (Berl) 155, 315-322.

Malendowicz, L.K., Nussdorfer, G.G., Markowska, A., Tortorella, C., Nowak, M., Warchol, J.B., 1994. Effects of neuromedin U (NMU)-8 on the rat hypothalamo-pituitary-adrenal axis. Evidence of a direct effect of NMU-8 on the adrenal gland. Neuropeptides 26, 47-53.

Malendowicz, L.K., Nussdorfer, G.G., Nowak, K.W., Mazzocchi, G., 1993. Effects of neuromedin U-8 on the rat pituitary-adrenocortical axis. In Vivo 7, 419-422.

Martinez, V.G., O'Driscoll, L., 2015. Neuromedin U: a multifunctional neuropeptide with pleiotropic roles. Clin Chem 61, 471-482.

McEwen, B.S., 2007. Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol Rev 87, 873-904.

Minamino, N., Sudoh, T., Kangawa, K., Matsuo, H., 1985. Neuromedins: novel smooth-muscle stimulating peptides identified in porcine spinal cord. Peptides 6 Suppl 3, 245-248.

Mitchell, J.D., Maguire, J.J., Davenport, A.P., 2009. Emerging pharmacology and physiology of neuromedin U and the structurally related peptide neuromedin S. British journal of pharmacology 158, 87-103.

Nagai, H., Kaisho, T., Yokoyama, K., Asakawa, T., Fujita, H., Matsumiya, K., Noguchi, J., Tsuchimori, K., Nishizawa, N., Kanematsu-Yamaki, Y., Dote, K., Inooka, H., Sakamoto, J.I., Ohtaki, T., Asami, T., Takekawa, S., 2018. Differential effects of selective agonists of neuromedin U1 and U2 receptors in obese and diabetic mice. Br J Pharmacol 175, 359-373.

Nakahara, K., Kojima, M., Hanada, R., Egi, Y., Ida, T., Miyazato, M., Kangawa, K., Murakami, N., 2004. Neuromedin U is involved in nociceptive reflexes and adaptation to environmental stimuli in mice. Biochem Biophys Res Commun 323, 615-620.

Niimi, M., Murao, K., Taminato, T., 2001. Central administration of neuromedin U activates neurons in ventrobasal hypothalamus and brainstem. Endocrine 16, 201-206.

Novak, C.M., Zhang, M., Levine, J.A., 2006. Neuromedin U in the paraventricular and arcuate hypothalamic nuclei increases non-exercise activity thermogenesis. Journal of neuroendocrinology 18, 594-601.

Ozaki, Y., Onaka, T., Nakazato, M., Saito, J., Kanemoto, K., Matsumoto, T., Ueta, Y., 2002. Centrally administered neuromedin U activates neurosecretion and induction of c-fos messenger ribonucleic acid in the paraventricular and supraoptic nuclei of rat. Endocrinology 143, 4320-4329.

Palkovits, M., 2008. Stress-induced activation of neurons in the ventromedial arcuate nucleus: a blood-brain-CSF interface of the hypothalamus. Ann N Y Acad Sci 1148, 57-63.

Paxinos, G., Franklin, K.B., 2004. The Mouse Brain in Stereotaxic Coordinates. Compact second edition. Elsevier Academic Press, San Diego.

Peier, A., Kosinski, J., Cox-York, K., Qian, Y., Desai, K., Feng, Y., Trivedi, P., Hastings, N., Marsh, D.J., 2009. The antiobesity effects of centrally administered neuromedin U and neuromedin S are mediated predominantly by the neuromedin U receptor 2 (NMUR2). Endocrinology 150, 3101-3109.

Peier, A.M., Desai, K., Hubert, J., Du, X., Yang, L., Qian, Y., Kosinski, J.R., Metzger, J.M., Pocai, A., Nawrocki, A.R., Langdon, R.B., Marsh, D.J., 2011. Effects of peripherally administered neuromedin U on energy and glucose homeostasis. Endocrinology 152, 2644-2654.

Porsolt, R.D., Le Pichon, M., Jalfre, M., 1977. Depression: a new animal model sensitive to antidepressant treatments. Nature 266, 730-732.

Stone, E.A., Lehmann, M.L., Lin, Y., Quartermain, D., 2007. Reduced evoked fos expression in activity-related brain regions in animal models of behavioral depression. Prog Neuropsychopharmacol Biol Psychiatry 31, 1196-1207.

Tanaka, M., Telegdy, G., 2014. Neurotransmissions of antidepressant-like effects of neuromedin U-23 in mice. Behav Brain Res 259, 196-199.

Thompson, E.L., Murphy, K.G., Todd, J.F., Martin, N.M., Small, C.J., Ghatei, M.A., Bloom, S.R., 2004. Chronic administration of NMU into the paraventricular nucleus stimulates the HPA axis but does not influence food intake or body weight. Biochemical and biophysical research communications 323, 65-71.

Vallof, D., Ulenius, L., Egecioglu, E., Engel, J.A., Jerlhag, E., 2017. Central administration of the anorexigenic peptide neuromedin U decreases alcohol intake and attenuates alcohol-induced reward in rodents. Addict Biol 22, 640-651.

Wren, A.M., Small, C.J., Abbott, C.R., Jethwa, P.H., Kennedy, A.R., Murphy, K.G., Stanley, S.A., Zollner, A.N., Ghatei, M.A., Bloom, S.R., 2002. Hypothalamic actions of neuromedin U. Endocrinology 143, 4227-4234.

Yokota, M., Ozaki, Y., Sakamoto, F., Yamada, S., Saito, J., Fujihara, H., Ueta, Y., 2004. Fos expression in CRF-containing neurons in the rat paraventricular nucleus after central administration of neuromedin U. Stress 7, 109-112.

Zeng, H., Gragerov, A., Hohmann, J.G., Pavlova, M.N., Schimpf, B.A., Xu, H., Wu, L.J., Toyoda, H., Zhao, M.G., Rohde, A.D., Gragerova, G., Onrust, R., Bergmann, J.E., Zhuo, M., Gaitanaris, G.A., 2006. Neuromedin U receptor 2-deficient mice display differential responses in sensory perception, stress, and feeding. Mol Cell Biol 26, 9352-9363.

Zhang, R., Asai, M., Mahoney, C.E., Joachim, M., Shen, Y., Gunner, G., Majzoub, J.A., 2017. Loss of hypothalamic corticotropin-releasing hormone markedly reduces anxiety behaviors in mice. Mol Psychiatry 22, 733-744.

## Supplemental figures

**FIGURE S1**

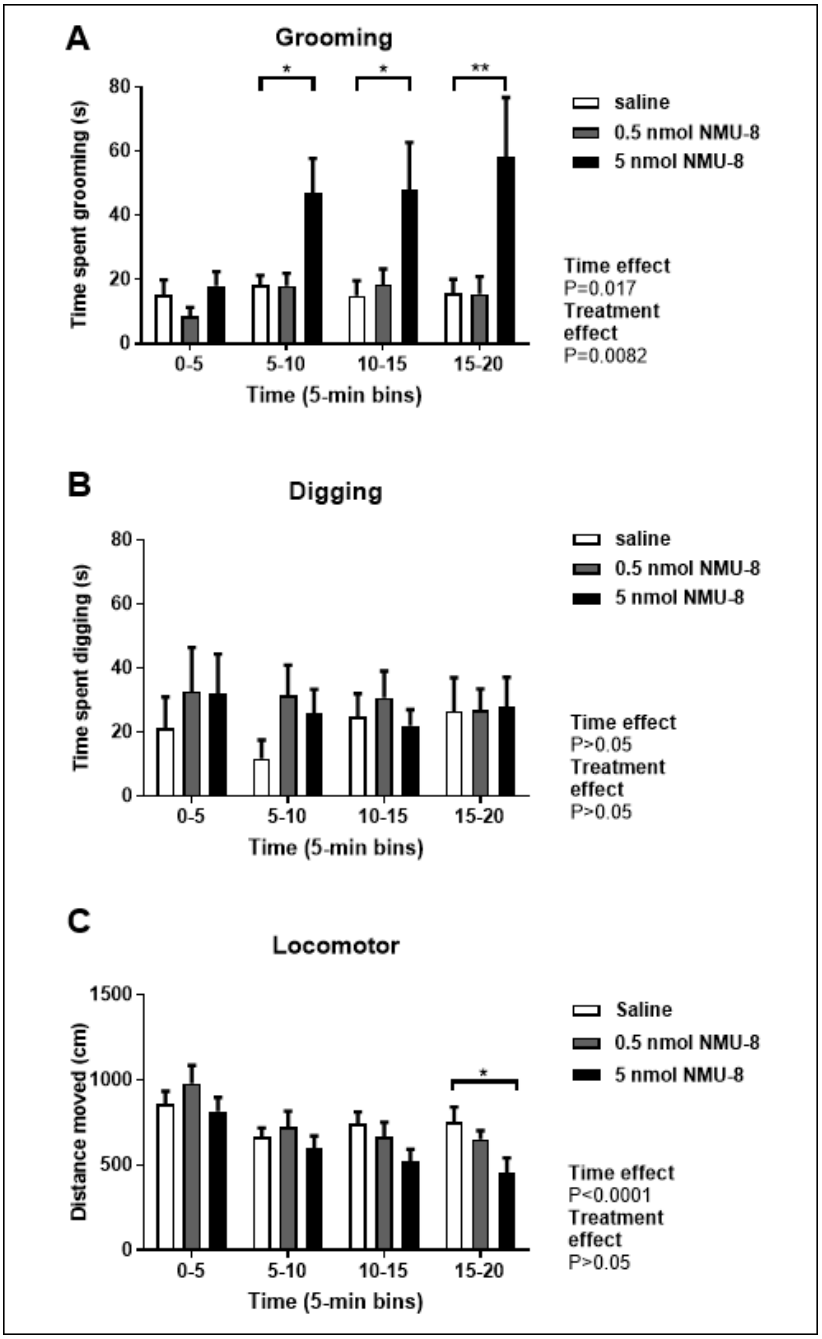



**Figure S1. Effects of central NMU-8 administration on home cage behavior.** Time spent grooming (A), digging (B) and total distance moved (C) in the home cage after intracerebroventricular administration of saline or NMU-8 (0.5 or 5 nmol), divided in 5-min time bins. A significant increase in grooming behavior after injection of 5 nmol NMU-8 is observed at 5-10 min, 10-15 min and 15-20 min compared to saline controls (A). No significant effects of NMU-8 on time spent digging were observed (B). However, at 15-20 min, 5 nmol NMU-8 decreased locomotor activity as measured by the

distance moved (C). Data are presented as bars with mean±SEM and n=11-14 per group. *P<0.05, **p<0.01 versus saline controls, analyzed using two-way ANOVA followed by Dunnett's multiple comparisons test.

**FIGURE S2**

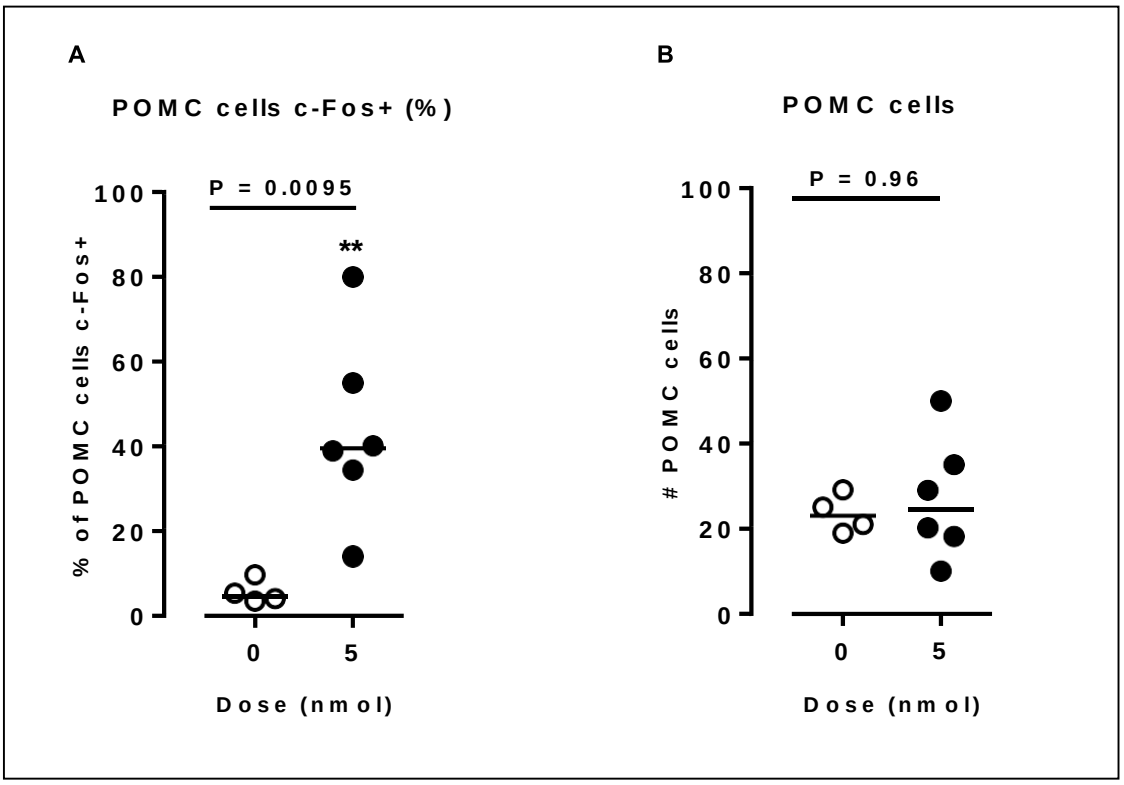



**Figure S2. Effects of central NMU-8 administration on POMC cells in the arcuate nucleus.** Intracerebroventricular administration of NMU-8 (5 nmol) significantly increased the percentage of POMC cells expressing c-Fos (A), while the number of POMC expressing cells remained unchanged (B) in mice that remained in the home cage. Data are presented as a dot blot for individual values with designation of the median and n=4-6 per group. **P<0.01 versus saline controls analyzed by Mann-Whitney U test.