Heliyon 6 (2020) e03947

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

# Cocaine has some effect on neuromedin U expressing neurons related to the brain reward system

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ARTICLE INFO

*Keywords:*
Neuroscience
Physiology
Endocrinology
Addiction medicine
Behavioral medicine
Biological psychiatry
Neuromedin U
Neuropeptide
Cocaine
c-Fos
Brain reward system
NAc
CPu
BLA
CPP test
Mouse

ABSTRACT

Neuromedin U (NMU) is a bioactive neuropeptide, highly distributed in the gastrointestinal tract and the central nervous system. NMU has various physiological functions related to feeding behavior, energy metabolism, stress responses, circadian rhythmicity and inflammation. Recently, several reports indicate that the central NMU system plays an important role in the reward systems in the brain. However, the underlying molecular mechanisms are not yet fully defined. In this study, we found that some of cocaine-induced c-Fos immunoreactive cells were co-localized with NMU in the nucleus accumbens (NAc), caudate putamen (CPu), and basolateral amygdala (BLA), which are key brain regions associated with the brain reward system, in wild type mice. Whereas, a treatment with cocaine did not influence the kinetics of NMU or NMU receptors mRNA expression in these brain regions, and NMU-knockout mice did not show any higher preference for cocaine compared with their control mice. These results indicate that cocaine has some effect on NMU expressing neurons related to the brain reward system, and this suggests NMU system may have a role on the brain reward systems activated by cocaine.

## 1. Introduction

Neuromedin U (NMU) is a bioactive neuropeptide, first isolated from porcine spinal cords [1]. NMU mediates a variety of physiological functions, including the regulation of feeding behavior, energy expenditure, circadian rhythm, stress response, and inflammation [2, 3, 4, 5, 6, 7]. Particularly, NMU has drawn attention as a potent anorexigenic peptide because intracerebroventricular administration of NMU significantly reduces food intake [7]. Transgenic mice overexpressing NMU are hypophagic and exhibit reduced body weight [8], and NMU gene knockout mice (NMU-KO) exhibit obesity with hyperphagia with "binge-like eating", reducing energy expenditure and locomotor activity [9]. Furthermore, a mutation in the human NMU gene has also been linked to obesity [10]. Based on these lines of evidence, NMU is suggested to be an endogenous anorexigenic peptide.

Recently, it has been reported that there is a close interaction between the regulation of feeding behavior and the brain reward systems [11]. In addition to metabolic systems, that maintain homeostasis, brain reward systems also play an important role in feeding behavior [11]. Especially, palatable food intake triggers neuroadaptive responses in the brain reward system similar to those seen in drug abuse with cocaine [11, 12].

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https://doi.org/10.1016/j.heliyon.2020.e03947
Received 14 November 2019; Received in revised form 20 December 2019; Accepted 5 May 2020

Several studies have shown a link between the NMU system and the brain reward system [13, 14, 15, 16, 17, 18, 19]. NMU is highly expressed in the area associated with the brain reward system, including the nucleus accumbens (NAc) [13, 14]. The receptor of NMU, especially neuromedin U receptor 2 (NMUR2), is also expressed in the NAc [13, 15]. Central administration of NMU decreases non-sensitized amphetamine-evoked activity, dopamine release in the NAc, expression of CPP [16] and attenuates alcohol-induced reward in rodents [17]. Moreover, a genome-wide allelic association study shows that a single nucleotide polymorphism in NMUR2 is associated with alcohol abuse in humans [18]. Furthermore, intra-NAc administration of NMU prevents non-sensitized cocaine evoked activity [15], selectively reduced palatable and rewarding peanut butter consumption [19]. These evidences support the role of the NMU system in reward processes. However, the detailed mechanisms are not yet fully defined.

Here, we have investigated the functional relevance of the NMU system in the brain reward system by using cocaine treatment. We found that some of cocaine-induced c-Fos-expressing neurons were co-localized with NMU immunoreactive neurons in the nucleus accumbens (NAc), caudate putamen (CPu), and basolateral amygdala (BLA), which are critical regions of the brain closely associated with the brain reward system. On the other hand, cocaine treatment did not affect either on NMU nor NMU receptors mRNA expression levels in these regions, and NMU knockout mice showed the same extent of cocaine preference compared with their control mice. Our data indicates that cocaine has some effect on NMU expressing neurons related to the brain reward system, and this suggests NMU system may have a role for the brain reward system activated by cocaine.

## 2. Materials and methods

### 2.1. Mice

C57BL/6J mice were initially obtained from KBT Oriental (Saga, Japan). Mice deficient in the gene encoding NMU (NMU-KO mice) have been developed as described previously [9], and backcrossed more than 15 times into C57BL/6J mice. The male C57BL/6J mice used for each experiment were 9–11 weeks of age at the onset of testing for immunohistochemical analysis and gene expression analysis. And the male NMU-KO mice and their control (WT) mice used in the CPP test were 20–24 weeks old. All mice were maintained on a 12 h:12 h light/dark cycle and fed *ad libitum* with standard diet (MFG Oriental Yeast, Tokyo, Japan). All animal experiments were performed according to the procedure approved by the Oita University Faculty of Medicine Committee on Animal Research.

### 2.2. Drugs

Cocaine was purchased from Takeda Pharmaceutical Company (Tokyo, Japan). Cocaine was dissolved in saline (0.9% NaCl) and injected intraperitoneally at 10 or 20 mg/kg in 200 μL saline.

### 2.3. Histology and immunostaining

C57BL/6J male mice were injected intraperitoneally with saline or cocaine (20 mg/kg). Ninety minutes after the injection, mice were anesthetized and perfused transcardially with 15 ml of 0.1 M phosphate buffered saline (PBS) (pH 7.4) and then with 20 ml of fixative containing 4% paraformaldehyde (PFA) in PBS. For c-Fos staining, brains were removed, post-fixed with 4% PFA at 4 °C, and kept for 24 h in PBS containing 20% sucrose. Frozen serial sections (40 μm thick) were prepared on a microtome (REM-710; Yamato Koki, Saitama, Japan), treated with 0.3% hydrogen peroxide for 1 h to inactivate endogenous peroxidases, and then incubated overnight at 4 °C with rabbit anti-c-Fos antibody (diluted 1:2000; EMD Millipore Corp., Darmstadt, Germany) in PBS. Sections were washed for 30 min with PBS, incubated for 2 h with a biotinylated secondary antibody, followed by incubation with peroxidase-labelled streptavidin (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). The staining reaction was performed for 3 min at room temperature using DAB-buffer tablets (EMD Millipore Corp.). Sections were then mounted on slides, dehydrated, and coverslipped with Entellan New mounting medium (EMD Millipore Corp.). Sections were viewed and photographed with a BZ-9000 microscope (Keyence, Osaka, Japan). c-Fos-positive cell number was quantified from the specific brain area of each image with ImageJ v1.51J8 software (http://rsb.info.nih.gov/ij). The specific brain areas were defined as follows: NAc, $1200 \times 900\ \mu m^2$; CPu, $200 \times 200\ \mu m^2$; BLA, $340 \times 500\ \mu m^2$ (Figure 1a). c-Fos immunoreactivity was identified as a blue-black oval-shaped nucleus distinguishable from the background.

For double immunofluorostaining of NMU and c-Fos immunoreactive cells, mice were perfused and fixed as described above and brains were sectioned at 40 μm thick ($n = 4$ per group). Sections were stained overnight at 4 °C with antiserum against NMU (1:1000, kindly provided by Prof. Miyazato at NCVC) [9] and goat anti-c-Fos antibody (1:1000, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) in PBS, washed three times for 15 min with PBS, and incubated for 2 h at room temperature with Alexa Fluor 488 donkey anti-rabbit IgG or Alexa Fluor 594 donkey anti-goat IgG antibody diluted 1:450 (Invitrogen, Waltham, MA, USA). After washing with PBS, sections were mounted on slides and coverslipped with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories). Sections were viewed and photographed with an LSM 710 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Regions were defined as mentioned above and the number of c-Fos-positive cells or NMU immunoreactive cells therein were counted. c-Fos and NMU images were separated and quantified individually using ImageJ software so that the percentage of c-Fos-positive and NMU-positive neurons could be calculated. Brain regions were identified using a mouse brain atlas [20].

### 2.4. Isolation of tissues

The brain was placed into a metal matrix (ASI Instruments, Warren, MI, USA) for dissection. The NAc, CPu, and BLA were dissected from a coronal slice dependent on the stereotaxic coordinates according to the mouse brain atlas [17]. The targeted brain regions were defined according to the mouse brain atlas as follows. NAc: 0.38–3.20 mm anterior to posterior from the bregma, −2.5 to 2.5 mm lateral from the midline, and 3.75–5.50 mm ventral from the skull surface at the bregma; CPu: 0.38–3.20 mm anterior to posterior from the bregma, 0.5–3.0 mm lateral from the midline, and 2.50–3.75 mm ventral from the skull surface at the bregma; BLA: −0.58 to −2.58 mm anterior to posterior from the bregma, 2.25–3.25 mm and −2.25 to −3.25 mm lateral from the midline, and 4.20–5.20 mm ventral from the skull surface at the bregma.

### 2.5. Quantitative real time PCR (qPCR)

Total RNA was prepared from the NAc, CPu, BLA, and hypothalamus (Hypo) by using an RNeasy mini kit (Qiagen) or TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed by using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo). Total RNA (250 ng) was subjected to quantitative real-time PCR performed with a SYBR Fast qPCR Kit (KAPA Biosystems, Wilmington, MA, USA) on a Light Cycler PCR platform (Roche Diagnostics, Basel, Switzerland). Primers used for qPCR are listed in Table 1. PCR was performed by using the following protocol: 95 °C for 3 min, followed by 45 cycles in total at 95 °C for 10 s and 60 °C for 30 s, then 72 °C for 1 s. Generation of specific PCR products was confirmed by melting curve analysis and DNA gel electrophoresis. Data were analyzed by using the ΔΔCt method, with normalization against *GAPDH* mRNA expression.

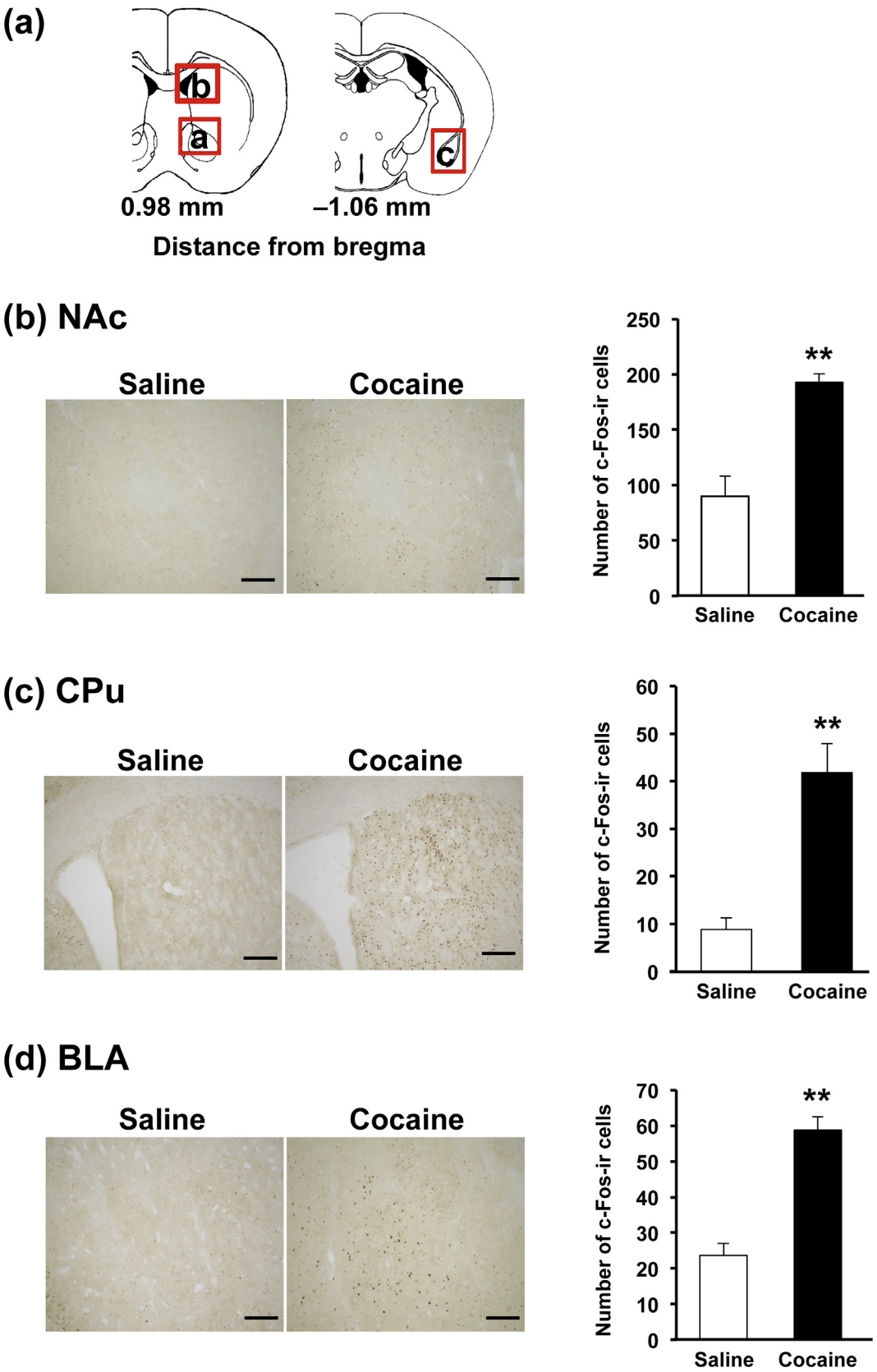


**Figure 1.** Cocaine-induced c-Fos expression in C57BL/6J mouse brain. Saline or cocaine was injected intraperitoneally to C57BL/6J mice and mice were sacrificed 90 min after injection ($n = 6$ per group). (A) Diagrams of brain sections indicating distance from bregma. Square regions labelled a–c show regions c-Fos immunoreactive cells were significantly increased in cocaine-injected groups compared with saline-injected groups. a: NAc, nucleus accumbens; b: CPu, caudate putamen; c: BLA, basolateral amygdala. (B) Representative images of cocaine-treatment-augmented c-Fos-expressing cells in NAc, CPu, and BLA. And the number of c-Fos immunoreactive (c-Fos-ir) cells was counted in the NAc, CPu, and BLA. All values are mean ± SEM. $^{**}P < 0.005$, for cocaine vs saline. Scale bars, 200 μm.

**Table 1.** Primers used in the present study.

| Genes (Genbank Accession number) | | Primers | Product length (bp) |
|---|---|---|---|
| NMU (NM_019515.1) | Forward | 5′-GTCCTCTGTTGTGCATCCGTT-3′ | 130 |
| | Reverse | 5′-GCGTGGCCTGAATAAAAAGTA-3′ | |
| NMUR1 (NM_010341.1) | Forward | 5′-CGTCATCCTGCGCAACAAG-3′ | 223 |
| | Reverse | 5′-CACACTCAGGGCTGTGACAT-3′ | |
| NMUR2 (NM_153079.4) | Forward | 5′-TGTCACCACGGTTAGCATTGA-3′ | 218 |
| | Reverse | 5′-GTTTGGTGACTGTGCAGGTG-3′ | |
| GAPDH (NM_008084.2) | Forward | 5′-CGTCCCGTAGACAAAATGGT-3′ | 177 |
| | Reverse | 5′-GAATTTGCCGTGAGTGGAGT-3′ | |

### 2.6. Conditioned place preference (CPP) test

Mice used in the CPP test were male NMU-KO mice (n = 17) and WT (n = 19) mice. The CPP test was conducted with slightly modified procedure as described in a previous report [21]. Briefly, the CPP test was conducted in a three-chamber apparatus (MED Associates, St. Albans, VT, USA) consisting of a small middle chamber that connected two large chambers that differed in floor and wall conditions [21]. On Day 0, mice were allowed to move freely between the three chambers for 20 min. On Days 1–3, mice were confined to one of the large chambers for 20 min immediately after they had received saline. Four hours later, they received 10 mg/kg cocaine and were confined to the other large chamber for 20 min. On Day 4, mice were placed in the middle chamber and allowed to move freely in the three chambers for 20 min. Actual time spent in the cocaine-paired compartment of WT and NMU-KO mice at pre-test and post-test was determined. And CPP score was calculated as a ratio as follows: [time spent in cocaine injection compartment at test day (sec)] – [time spent in cocaine injection compartment at pre-test (sec)]/total time (sec). In addition, we have also examined locomotor activity for 30 min after cocaine treatment. The activity level was recorded by a video camera, and the traveled distance was analyzed using the SMART software system (Panlab, Barcelona, Spain).

### 2.7. Statistics

All values are given as means ± SEM. Comparisons between groups were made by Student's *t*-test using PRISM. $P < 0.05$ was defined as statistically significant.

## 3. Results

### 3.1. Effects of cocaine on c-Fos protein expression levels in C57BL/6J mouse brain

First, we examined cocaine-induced c-Fos expression in the central nervous system of C57BL/6J mice. We found c-Fos-positive neurons in several brain regions such as the ventral orbital cortex (VO), the medial part of the anterior olfactory nucleus (AOM), the posterior part of the anterior olfactory nucleus (AOP), the caudate putamen (CPu), the nucleus accumbens (NAc), the lateral septal nucleus (LSI), the CA3 field of the hippocampus (CA3), the basolateral amygdala (BLA), and the periaqueductal gray (PAG). To quantify the c-Fos expression levels in those brain areas with or without cocaine injection, we analyzed the number of c-Fos-expressing cells by using ImageJ software. The numbers of c-Fos immunoreactive cells in each region (mean ± SEM) were shown in Table 2. Effects of acute administration of cocaine on c-Fos-positive cells in the brain were most notably seen in the NAc, CPu, and BLA (Figure 1), which are key brain regions of the brain reward system. Thus, we focused on these three brain regions.

### 3.2. Co-localization of c-Fos and NMU immunoreactive cells with cocaine treatment

To determine the relationship between the NMU system and the brain reward system activated by cocaine, we examined whether NMU-expressing neurons were activated by cocaine treatment. We performed double immunohistochemical staining and examined co-localization of NMU and c-Fos expression in the NAc, CPu, and BLA. Cocaine-induced c-Fos positive cells were detected in some of NMU immunoreactive cells in the NAc, CPu, and BLA (ratio of c-Fos positive cells in NMU immunoreactive cells in the NAc: 16.25% ± 9.87, CPu: 29.17% ± 2.41, BLA: 50.00% ± 14.83) (Figure 2).

### 3.3. Kinetics of NMU, NMUR1, and NMUR2 mRNA expression levels with cocaine treatment in the NAc, CPu, and BLA

Next, we examined whether cocaine treatment had any direct effect on the mRNA kinetics of NMU-related molecules such as NMU, NMUR1, and NMUR2 in the NAc, CPu, and BLA. Cocaine treatment induced no significant change in NMU, NMUR1, or NMUR2 mRNA expression levels in those brain areas (Figure 3).

**Table 2.** The numbers of c-Fos immunoreactive cells by treatment of saline or cocaine in brain regions of C57BL/6J mice.

| Brain regions | Saline | Cocaine | P value |
|---|---|---|---|
| VO | 107.00 ± 14.57 | 215.40 ± 37.91 | 0.028 |
| AOM | 78.80 ± 1.62 | 113.80 ± 10.82 | 0.012 |
| AOP | 98.40 ± 27.13 | 192. 00 ± 29.28 | 0.047 |
| NAc | 90.00 ± 18.08 | 193.00 ± 7.46 | 0.0009* |
| CPu | 8.83 ± 2.46 | 41.80 ± 6.13 | 0.0005* |
| LSI | 7.33 ± 0.02 | 22.40 ± 5.63 | 0.021 |
| CA3 | 15.33 ± 2.19 | 26.40 ± 3.84 | 0.023 |
| BLA | 23.67 ± 3.37 | 58.80 ± 3.75 | 0.00007* |
| PAG | 51.83 ± 8.88 | 90.00 ± 14.79 | 0.046 |

The data were shown mean ± SEM. *$P < 0.001$, for cocaine vs saline.

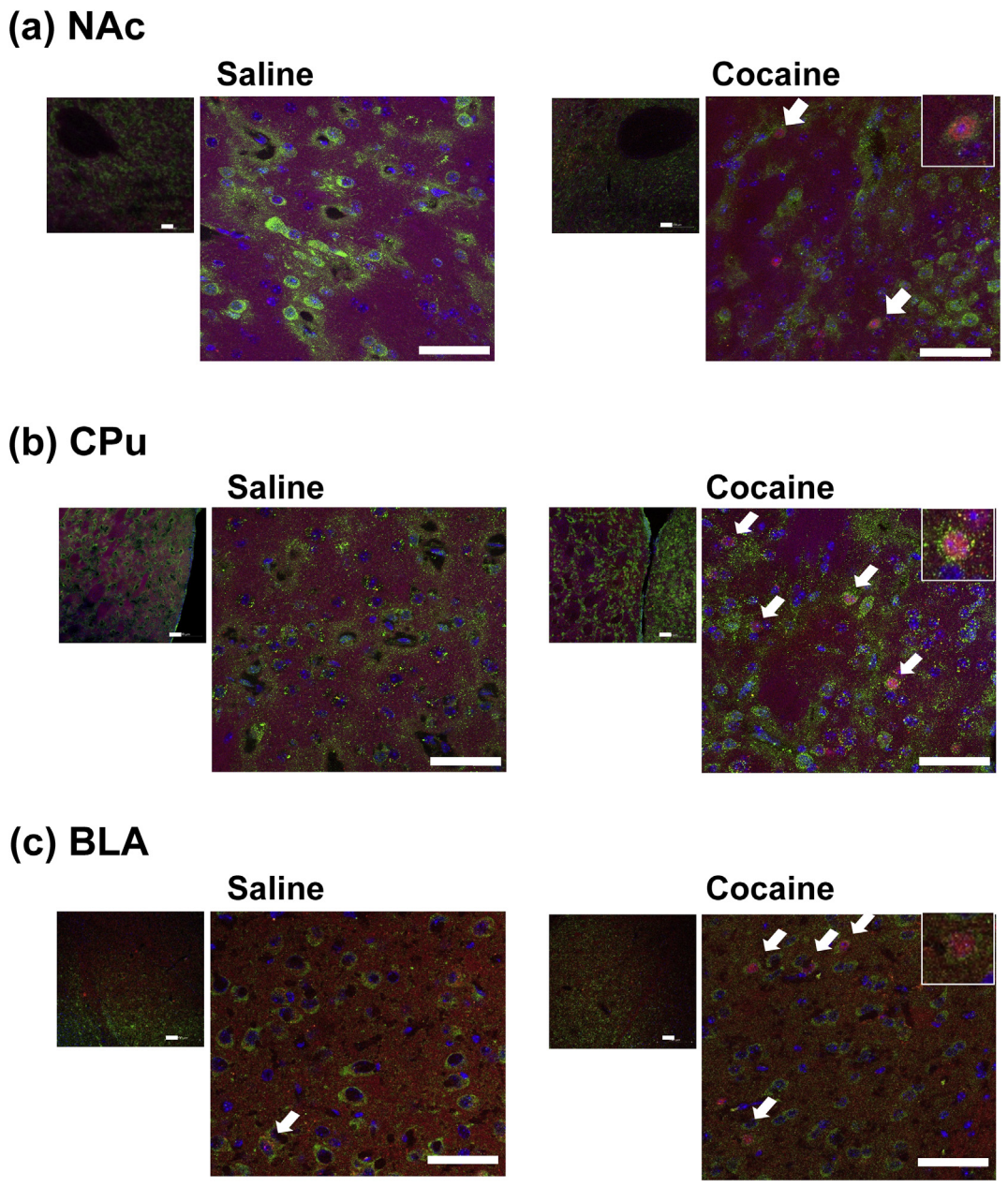


**Figure 2.** NMU is expressed in some of cocaine-induced c-Fos immunoreactive cells in the NAc, CPu, and BLA. Representative images of immunofluorescence staining for c-Fos (red), NMU (green), and DAPI (blue). Following cocaine treatment, some c-Fos immunoreactive cells (red) are co-localized (white arrows) with NMU (green) in the NAc, CPu, and BLA. Scale bars, 50 μm.

### *3.4. CPP test for cocaine in NMU-KO mice*

Furthermore, to determine whether NMU plays a crucial role in cocaine preference *in vivo*, we performed the CPP test for cocaine in NMU-KO mice and WT mice. We have examined body weights and food intake of WT or NMU-KO mice. As reported before [9], NMU-KO mice showed obese phenotype (WT: 29.75 ± 0.36 g; NMU-KO: 36.10 ± 0.80 g; $P < 0.001$), and food intake was also increased in NMU-KO mice compared with WT mice (WT: 4.14 ± 0.28 g; NMU-KO: 5.29 ± 0.26 g; P = 0.016). In the CPP experiment, actual time spent in the cocaine-paired compartment at pre-test and post-test was no change between WT mice and NMU-KO mice (Figure 4a). The CPP score was not significantly different between NMU-KO and WT mice conditioned with cocaine treatment (Figure 4b). Furthermore, the locomotor activity level defined by distance of moving in 30 min after cocaine treatment was not different between WT and NMU-KO mice (WT: 61.07 ± 9.71 m/30min; NMU-KO: 66.64 ± 8.44 m/30min; P = 0.674).

## 4. Discussion

In this study, we found that cocaine-induced c-Fos expression in the brain was co-localized with some of NMU-expressing cells in the critical regions of the brain reward system such as the NAc, CPu, and BLA. This finding implies that NMU itself may have some physiological function concerning the brain reward system activated by cocaine treatment. On the other hand, we also showed that cocaine treatment did not considerably affect mRNA expression levels of NMU-related molecules in these brain regions, and did not significantly affect the cocaine preference in NMU-KO mice. Based on these data, NMU seems to have a partial role on the brain reward system activated by cocaine treatment.

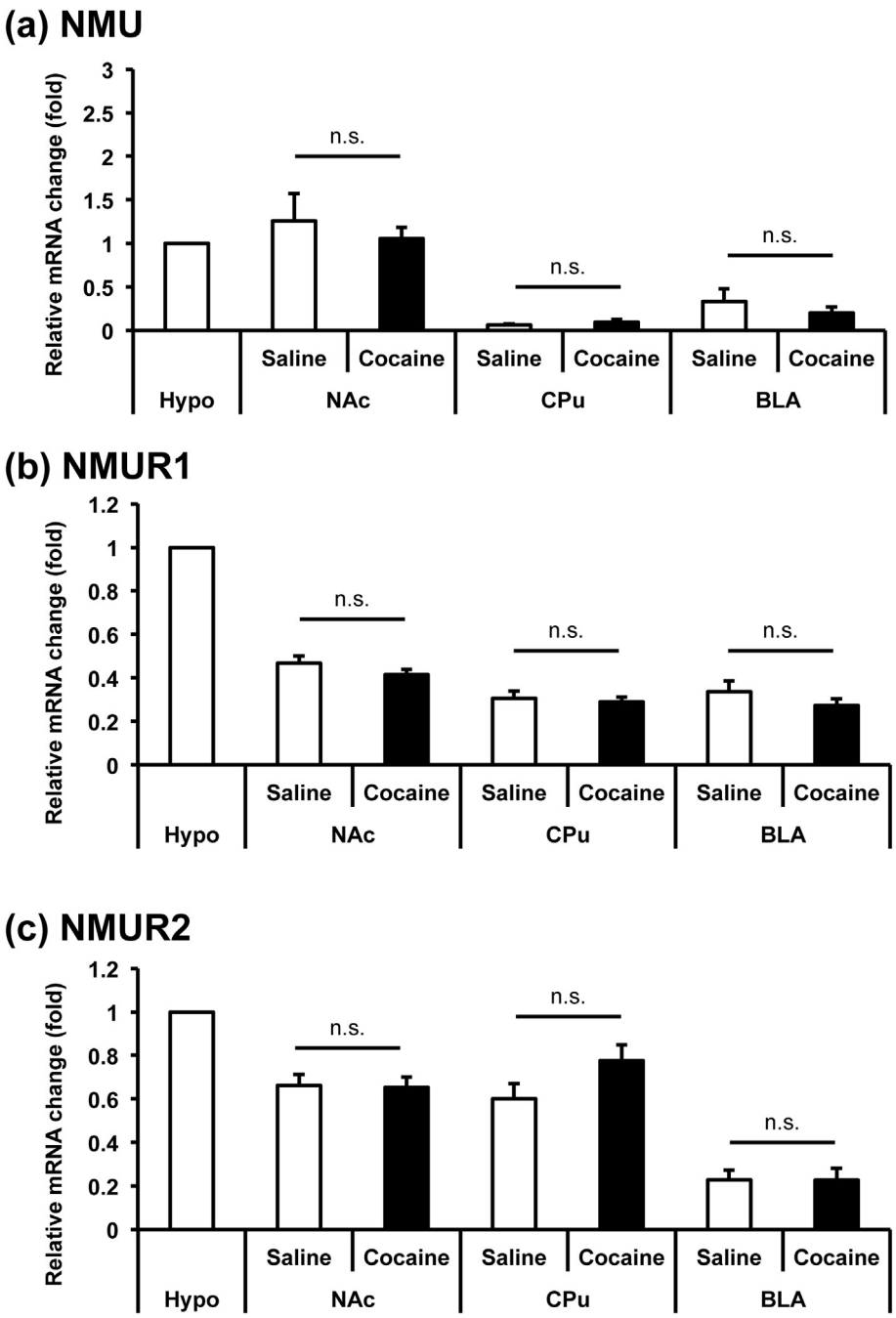


**Figure 3.** Kinetics of NMU, NMUR1, and NMUR2 mRNA expression with cocaine treatment in the NAc, CPu, and BLA. mRNA expression levels of (A) NMU, (B) NMUR1, and (C) NMUR2 relative to GAPDH mRNA expression were determined by quantitative PCR. For all parameters, there was no difference between the cocaine-injected group and saline-injected group. $n$ = 9–11 per group of NAc and CPu samples, $n$ = 4–5 per group of BLA samples. All values are mean ± SEM; n.s., not significant for cocaine vs saline. Abbreviations: Hypo, hypothalamus.

NMU is a neuropeptide that is highly conserved in mammals and is widely expressed both in the central nervous system and peripheral tissues [22, 23]. NMU has a variety of physiological functions including energy metabolism, circadian rhythm, stress, inflammation, and feeding behavior [2, 6, 7, 9]. In our previous study, we have reported that NMU-KO mice showed an obese phenotype with "binge-like eating" behavior, which is closely associated with the brain reward systems [9, 11, 12]. Hedonic eating and drug abuse share common functional mechanisms mediated by the brain reward system via the mesolimbic dopamine system [11]. Over the last decade, some of the appetite regulatory peptides, such as ghrelin, glucagon-like peptide-1 (GLP1), neuropeptide Y (NPY), agouti-related peptide (AgRP), pro-opiomelanocortin (POMC), and cocaine-amphetamine regulated transcript (CART), were reported to have a pivotal role in the process of drug reinforcement or brain reward system [13, 24, 25]. NMU and NMUR2, one of the NMU receptors mainly expressed in the central nervous system, have recently been also shown to regulate the reinforcement value of amphetamine-evoked locomotion [16], alcohol [17] and preference for obesogenic, palatable food [19, 26].

In our results, some of NMU immunoreactive cells were co-localized with cocaine-induced c-Fos-expressing neurons. As previously reported, both NMU and NMUR2 are expressed in the NAc [13, 22], and our data demonstrated that cocaine treatment activated some NMU neurons in the NAc. On the other hand, unexpectedly, cocaine treatment had no discernible effect on the kinetics of NMU, NMUR1, and NMUR2 mRNA in the specific brain regions related to the reward system, including the NAc. This difference might be a result of the regimen used on this study. It was also reported that there was no difference in NMU or NMUR2 expression levels in the NAc of alcohol-consuming rats [17]. And a different pattern of c-Fos activation might be obtained in mice consuming

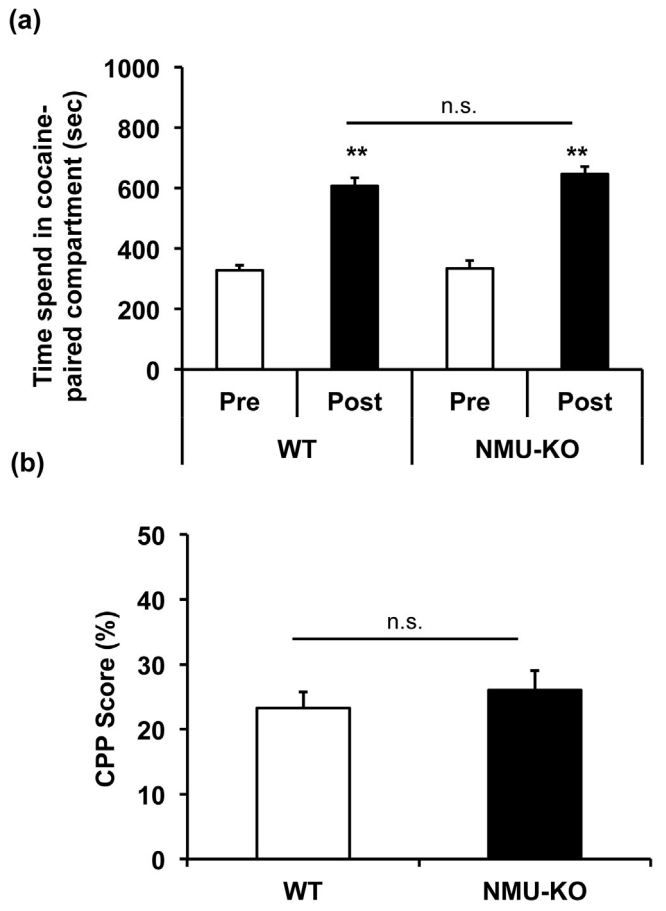


**Figure 4.** Cocaine-induced CPP score in NMU-KO mice and WT mice. Evaluation of abuse potential of cocaine in WT and NMU-KO mice, assessed by CPP test. CPP score for cocaine treatment was not significantly different between NMU-KO and WT mice. (a) Actual time spent in the cocaine-paired compartment of WT and NMU-KO mice at pre-test and post-test. (b) CPP score is determined as a ratio of [time spent in cocaine injection compartment at test day (sec)] – [time spent in cocaine injection compartment at pre-test (sec)]/total time (sec). $n = 19$ in WT, $n = 17$ in NMU-KO mice. All values are mean ± SEM; n.s., $^{**}P < 0.005$, for post vs pre in each mice group, n.s., not significant for WT mice vs NMU-KO mice.

alcohol or cocaine for a prolonged period. Previous studies have shown that repeated cocaine exposure alters the expression of genes and proteins in specific brain regions associated with addiction [27], and causes dysregulation of expression of NMUR2 [28]. Since the influence of chronic cocaine treatment on the NMU system was not performed in our model, it will be worth examining the expression levels of NMU and NMU receptors with repeated cocaine treatment.

In this study, we demonstrated that NMU-KO mice did not show any higher preference for cocaine compared with their control mice. Cocaine-evoked molecular changes in brain regions linked to addiction, such as the NAc, may be associated with behavioral changes associated with repeated exposure to cocaine. Other studies in mice demonstrate that repeated NMU administration in the regimen of cocaine sensitization decreases cocaine-evoked hyperactivity, and NMUR2 knockdown in the pre synapses of the NAc potentiates cocaine sensitization [15]. From this point of view, our results might be coming from the difference on the regimen of cocaine treatment or the difference on sensitization to cocaine in each conditioned mouse. As NMU-KO mice are constitutively deleted of NMU gene; it raises the possibility that some other pathways are compensatory and mask the behavioral phenotype. Furthermore, we need to consider the effect of Neuromedin S (NMS), which is another ligand for NMUR1 and NMUR2 [29]. NMS also has many physiological functions in the central nervous system and is involved in such processes as circadian rhythm and feeding behavior [29, 30]. However, there is no report on the functional relevance between the NMS system and the brain reward system. Although this is the first study using NMU-KO mice to examine the relationship between the NMU system and the brain reward system activated by cocaine treatment, it would be also interesting to investigate the differences in the effects of cocaine between NMS null, NMUR1 null, or NMUR2 null mice and their control mice. Regarding the link between NMU system and the brain reward system to the clinical target for drug abuse, NMUR2 agonist which was reported to be a candidate of anti-obesity drug, might suppress cocaine evoked behavior [13]. Further studies are needed to elucidate the detailed mechanism in this area.

In conclusion, although cocaine treatment did not considerably influence NMU or NMU receptors gene expression, and NMU-KO mice did not show any difference in their preference for cocaine compared with their control mice, this study indicated that cocaine has some effect on NMU-expressing neurons in the NAc, CPu, and BLA. Based on these results, NMU may have an effect on the brain reward system activated by acute cocaine treatment.

## Declarations

### *Author contribution statement*

Madoka Anan, Ryoko Higa, Kenshiro Shikano, Masahito Shide and Akinobu Soda: Performed the experiments; Analyzed and interpreted the data.

Magdeline E. Carrasco Apolinario: Performed the experiments; Wrote the paper.

Kenji Mori, Mikiya Miyazato and Kenji Kangawa: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Toshitaka Shin and Hiromitsu Mimata: Contributed reagents, materials, analysis tools or data.

Takatoshi Hikida: Conceived and designed the experiments; Analyzed and interpreted the data.

Toshikatsu Hanada: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Kazuwa Nakao: Analyzed and interpreted the data; Wrote the paper.

Reiko Hanada: Conceived and designed the experiments; Wrote the paper.

### *Funding statement*

This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JP18K08522 to R. Hanada, T. Hanada and T. Hikida; JP16H06568 and JP18H02542 to T.Hikida) and by grants from the Smoking Research Foundation, and The Naito Foundation (to R. Hanada). This work was performed, in part, under the Collaborative Research Program of Institute for Protein Research, Osaka University, ICR-18-03.

### *Competing interest statement*

The authors declare no conflict of interest.

### *Additional information*

No additional information is available for this paper.

## Acknowledgements

We thank Ryohei Umeda, Natsuki Matsuno, and Hitoshi Teranishi, Nakatsu Daiichi Hospital and Yasuko Ito of Furugo Clinic, for their helpful advice and suggestions.

## References


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

[2] R. Hanada, M. Nakazato, N. Murakami, S. Sakihara, H. Yoshimatsu, K. Toshinai, T. Hanada, T. Suda, K. Kangawa, S. Matsukura, T. Sakata, A role for neuromedin U in stress response, Biochem. Biophys. Res. Commun. 289 (2001) 225–228.

[3] T. Hanada, Y. Date, T. Shimbara, S. Sakihara, N. Murakami, Y. Hayashi, Y. Kanai, T. Suda, K. Kangawa, M. Nakazato, Central actions of neuromedin U via corticotropin-releasing hormone, Biochem. Biophys. Res. Commun. 311 (2003) 954–958.

[4] V.G. Martinez, L. O'Driscoll, Neuromedin U: a multifunctional neuropeptide with pleiotropic roles, Clin. Chem. 61 (2015) 471–482.

[5] M. Moriyama, A. Matsukawa, S. Kudoh, T. Takahashi, T. Sato, T. Kano, A. Yoshimura, M. Kojima, The neuropeptide neuromedin U promotes IL-6 production from macrophages and endotoxin shock, Biochem. Biophys. Res. Commun. 341 (2006) 1149–1154.

[6] K. Nakahara, R. Hanada, N. Murakami, H. Teranishi, H. Ohgusu, N. Fukushima, M. Moriyama, T. Ida, K. Kangawa, M. Kojima, The gut-brain peptide neuromedin U is involved in the mammalian circadian oscillator system, Biochem. Biophys. Res. Commun. 318 (2004) 156–161.

[7] M. Nakazato, R. Hanada, N. Murakami, Y. Date, M.S. Mondal, M. Kojima, H. Yoshimatsu, K. Kangawa, S. Matsukura, Central effects of neuromedin U in the regulation of energy homeostasis, Biochem. Biophys. Res. Commun. 277 (2000) 191–194.

[8] T.J. Kowalski, B.D. Spar, L. Markowitz, M. Maguire, A. Golovko, S. Yang, C. Farley, J.A. Cook, G. Tetzloff, L. Hoos, R.A. Del Vecchio, T.M. Kazdoba, M.F. McCool, J.J. Hwa, L.A. Hyde, H. Davis, G. Vassileva, J.A. Hedrick, E.L. Gustafson, Transgenic overexpression of neuromedin U promotes leanness and hypophagia in mice, J. Endocrinol. 185 (2005) 151–164.

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

[10] I. Hainerova, S.S. Torekov, J. Ek, M. Finkova, K. Borch-Johnsen, T. Jorgensen, O.D. Madsen, J. Lebl, T. Hansen, O. Pedersen, Association between neuromedin U gene variants and overweight and obesity, J. Clin. Endocrinol. Metab. 91 (2006) 5057–5063.

[11] P.J. Kenny, Reward mechanisms in obesity: new insights and future directions, Neuron 69 (2011) 664–679.

[12] M. Lutter, E.J. Nestler, Homeostatic and hedonic signals interact in the regulation of food intake, J. Nutr. 139 (2009) 629–632.

[13] E. Jerlhag, Gut-brain axis and addictive disorders: a review with focus on alcohol and drugs of abuse, Pharmacol. Ther. 196 (2019) 1–14.

[14] Y.C. Lee, J.A. Ball, D. Reece, S.R. Bloom, Neuromedin N: presence and chromatographic characterization in the rat, FEBS Lett. 220 (1987) 243–246.

[15] J.M. Kasper, D.L. McCue, A.J. Milton, A. Szwed, C.M. Sampson, M. Huang, S. Carlton, H.Y. Meltzer, K.A. Cunningham, J.D. Hommel, Gamma-aminobutyric acidergic projections from the dorsal raphe to the nucleus accumbens are regulated by neuromedin U, Biol. Psychiatr. 80 (2016) 878–887.

[16] D. Vallof, J. Vestlund, J.A. Engel, E. Jerlhag, The anorexigenic peptide neuromedin U (NMU) attenuates amphetamine-induced locomotor stimulation, accumbal dopamine release and expression of conditioned place preference in mice, PLoS One 11 (2016) e0154477.

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

[18] G.J. Lydall, N.J. Bass, A. McQuillin, J. Lawrence, A. Anjorin, R. Kandaswamy, A. Pereira, I. Guerrini, D. Curtis, A.E. Vine, P. Sklar, S.M. Purcell, H.M. Gurling, Confirmation of prior evidence of genetic susceptibility to alcoholism in a genome-wide association study of comorbid alcoholism and bipolar disorder, Psychiatr. Genet. 21 (2011) 294–306.

[19] D. Vallof, A.L. Kalafateli, E. Jerlhag, Brain region-specific neuromedin U signalling regulates alcohol-related behaviours and food intake in rodents, Addiction Biol. (2019) e12764.

[20] K.B. Franklin, G. Paxinos, The Mouse Brain in Stereotaxic Coordinates, third ed., Academic Press, San Diego, CA, 2008.

[21] T. Hikida, S. Kaneko, T. Isobe, Y. Kitabatake, D. Watanabe, I. Pastan, S. Nakanishi, Increased sensitivity to cocaine by cholinergic cell ablation in nucleus accumbens, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 13351–13354.

[22] A.D. Howard, R. Wang, S.S. Pong, T.N. Mellin, A. Strack, X.M. Guan, Z. Zeng, D.L. Williams Jr., S.D. Feighner, C.N. Nunes, B. Murphy, J.N. Stair, H. Yu, Q. Jiang, M.K. Clements, C.P. Tan, K.K. McKee, D.L. Hreniuk, T.P. McDonald, K.R. Lynch, J.F. Evans, C.P. Austin, C.T. Caskey, L.H. Van der Ploeg, Q. Liu, Identification of receptors for neuromedin U and its role in feeding, Nature 406 (2000) 70–74.

[23] M. Kojima, R. Haruno, M. Nakazato, Y. Date, N. Murakami, R. Hanada, H. Matsuo, K. Kangawa, Purification and identification of neuromedin U as an endogenous ligand for an orphan receptor GPR66 (FM3), Biochem. Biophys. Res. Commun. 276 (2000) 435–438.

[24] H.R. Berthoud, H. Munzberg, C.D. Morrison, Blaming the brain for obesity: integration of hedonic and homeostatic mechanisms, Gastroenterology 152 (2017) 1728–1738.

[25] M.C. Gumbs, J.K. van den Heuvel, S.E. la Fleur, The effect of obesogenic diets on brain Neuropeptide Y, Physiol. Behav. 162 (2016) 161–173.

[26] C.R. Benzon, S.B. Johnson, D.L. McCue, D. Li, T.A. Green, J.D. Hommel, Neuromedin U receptor 2 knockdown in the paraventricular nucleus modifies behavioral responses to obesogenic high-fat food and leads to increased body weight, Neuroscience 258 (2014) 270–279.

[27] R.C. Pierce, B. Fant, S.E. Swinford-Jackson, E.A. Heller, W.H. Berrettini, M.E. Wimmer, Environmental, genetic and epigenetic contributions to cocaine addiction, Neuropsychopharmacology 43 (2018) 1471–1480.

[28] J.M. Kasper, A.E. Smith, J.D. Hommel, Cocaine-evoked locomotor activity negatively correlates with the expression of neuromedin U receptor 2 in the nucleus accumbens, Front. Behav. Neurosci. 12 (2018) 271.

[29] K. Mori, M. Miyazato, T. Ida, N. Murakami, R. Serino, Y. Ueta, M. Kojima, K. Kangawa, Identification of neuromedin S and its possible role in the mammalian circadian oscillator system, EMBO J. 24 (2005) 325–335.

[30] K. Mori, M. Miyazato, K. Kangawa, S. Neuromedin, Discovery and functions, Results Probl. Cell Differ. 46 (2008) 201–212.