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ORIGINAL ARTICLE

Mu Opioid Agonistic Effect on Neuropeptide Gene Expression Levels Involved in Hypothalamic Feeding Regulation

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Abstract

Background: The regulation of food intake in the hypothalamus is one of most complicated through the integration of various neuroendocrine mechanisms. In this region, orexigenic and anorexigenic peptides play a role by responding to different stimuli. Additionally, central opioidergic systems are involved in the regulation of feeding behavior. Several neuropeptides expressed in the hypothalamus also contribute to the regulation of food intake. The aim of this study was to investigate the effects of mu opioidergic agonist/antagonist molecules on both orexigenic and anorexigenic peptides gene expression levels in the hypothalamus.

Methods: In our study, 48 male Wistar Albino rats were divided into 4 groups as control, morphine, naloxone and morphine+naloxone. The control group received subcutaneous SF solution for 5 days; morphine group received morphine at a dose of 10 mg/kg/day for 5 days; naloxone group SF was administered for 5 days and naloxone at a dose of 3 mg/kg 1.5 hours after the last injection: morphine+naloxone group received naloxone 1.5 hours after 5 days of morphine injection. Hypothalamus tissues were isolated from brains at the end of experimental period. Anorexigenic and orexigenic peptide expression levels were analysed by RT-PCR method. Differences between groups were statistically analyzed using one-way factorial ANOVA and Tukey post-hoc test.

Results: Morphine administration results in a decrease in the expression levels of OX2R and LepR genes, but did not change ORXA, OX1R, AgRP, NPY, POMC gene expression. Naloxone administration increased AgRP and NPY expression while decreasing OX2R, LepR and APLNR gene expression levels.

Conclusions: Our findings suggest that morphine may affect the gene expression of molecules related to regulation of nutrition and metabolism in the hypothalamus. Further studies are needed to clarify the possible mechanistic effects of mu opiodergic activity on the central control of feeding in morphine dependence manner.

Key words: AgRP, apelin, hypothalamus, neuropeptides, NPY, morphine dependence, POMC, rat

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INTRODUCTION

Opioid peptides are primarily used in pain management (1). The hypothalamus and several other brain regions contain opioid receptors (2). Three major types of opioid receptors have been identified in the central nervous system such as mu, delta and kappa (3). Morphine is a mu opioid receptor (MOR) agonist widely used for preventing pain sensation in myocardial infaction and cancer issues (4). It is pointed that consecutive administration of morphine may cause dependence and tolerance development (5). Naloxone is often used for threating morphine addiction as an opioid receptor antagonist. Even a single dose of naloxone exposure is sufficient to show withdrawal symptoms. Because of this feature, it is frequently used in experimental morphine studies to detect withdrawal symptoms and addiction (6).

Central opioid systems are also involved in the regulation of feeding behaviour (7). Injection of morphine and various morphine derivatives into various hypothalamic regions increases food intake (8). It has been reported that morphine can increase food intake when injected systemically or into brain regions such as nucleus accumbens (NAc), amygdala, ventral tegmental area (VTA), hypothalamus (4, 9).

The hypothalamus controls food intake and energy balance. It has over 40 regions and nuclei related to many neuroendocrinological regulation processes (10). Morphine dose-dependently increased food intake when injected into the paraventricular nucleus (PVN) and perifornical hypothalamus, whereas injection of naloxone into the PVN decreased food intake (11). In addition, pretreatment with naloxone was found to prevent morphine-induced feeding behaviour (12). Injections of morphine and other selective μ-opioid agonists to lateral septum area have also been reported to trigger feeding (13). Hypothalamic neuropeptides and their receptors play essential roles in the regulation of feeding behavior (14). Agouti-related protein (AgRP) (15), neuropeptide Y (NPY) (16), pro-opiomelanocortin (POMC) (17), orexin A (ORXA), orexin receptor type 1 (OX1R), orexin receptor type 2 (OX2R) (18), leptin receptor (LepR) (19), apelin and apelin receptors (APLNR) (20) plays roles by inducing or inhibiting food intake in hypothalamic region. The regulation of the motivational aspect of feeding by opioid systems in the mesolimbic region is important in food intake (21). Recent studies have focused on neuropeptides found specifically in the arcuate nucleus, PVN and lateral hypothalamus. It is thought that direct or indirect effects of these neuropeptides may be effective in the relationship between opiate and food intake (14). It is important to understand the relationship of these neuropeptides in the hypothalamus with morphine dependence and changes in feeding behaviour. Therefore, the aim of this study was to examine the possible changes in hypothalamic neuropeptides and receptor expression levels in morphine dependence manner. In addition, comparative analyses of changes in these neuropeptide expressions will provide a holistic perspective in understanding of neuroendocrinological regulation of feeding in hypothalamus under opioidergic influence.

MATERIALS AND METHODS

Test Animals

Forty-eight 10-week-old male Wistar Albino rats weighing 300-350 g were used in this study. The animals were placed in groups of four in standard plastic cages (temperature 22 ± 2°C) with food and water on a 12-hour light/dark cycle. They were fed standard rat chow and tap water. The animals were randomly divided into four groups as control group (C), morphine addiction group (M), naloxone group (N) and morphine addiction+naloxone group (M+N). In order to create a dependency model, morphine (10 mg/kg) was administered intraperitoneally to the morphine groups every morning between 09:00 and 10:00 for 5 days. Groups C and N were administered saline for 5 days. Naloxone (3 mg/kg) was injected into naloxone groups 1.5 hours after the last injection, and saline was injected into other groups. All experimental procedures in this study were approved by Necmettin Erbakan University Experimental Medicine Application and Research Centre Animal Experiments Local Ethics Committee (Ethics Number: 041-2023). The experimental studies were carried out in this centre. Animal rights are protected under the 'Guide for the Care and Use of Laboratory Animals'.

Experimental protocol

Total RNA isolation

Brain tissues of rats from all groups were rapidly removed. Hypothalamus brain regions were isolated. They

were immediately frozen in liquid nitrogen and stored at -80°C for gene expression analysis. The TRIzol method was used to isolate total RNA. Spectrophotometric analysis and agarose gel electrophoresis were used to determine the density and quality of the total RNA samples. To eliminate possible cDNA contamination, DNAse-I (Thermo Scientific; EN0521) digestion was performed according to the manufacturer's directions. Bio-Rad iScriptTM cDNA Synthesis Kit (#170- 8891, USA) was used to synthesise cDNA from total RNA samples.

Primer design and Quantitative real time-PCR analysis Primers for AgRP, NPY, POMC, ORXA, OX1R, OX2R, LepR, apelin and ALPNR and the reference genes (PGK1, RPL13A and GAPDH) were generated using the IDT PrimerQuest (https://eu.idtdna.com/site) program. Quantitative expression analysis of target and reference genes was performed using a real time PCR device (Bio-Rad CFX Connect Real Time PCR System). Briefly, polymerase chain reaction was performed by 10 μl of 2X SyberGreen master mix, 5 pMol each primer and 2 μl cDNA in 20 μl dH2O total volume. The temperature profile of the reaction was set as +95 ˚C 10 min denaturation than 40 cycles of 95˚C 30 sec, 60˚C 30 sec and 72˚C 30 sec.

Statistical analysis

For the OX1R parameter, 95% confidence level (α=0.05) and 91% statistical power were targeted, effect size was accepted as 0.64 and total standard deviation was accepted as 0.3. Based on these data, the minimum group size was calculated as n=12 (22). For gene expression analysis, Ct values of all genes were normalized to the Ct values of PGK1, RPL13A and GAPDH reference genes and ΔCt values were calculated. Gene expression differences groups were expressed as mean ± SEM values and analyzed by one-way factorial ANOVA with Tukey post-hoc test. *p<0.05 is considered significant.

RESULTS

AgRP gene expression levels were significantly higher in the M+N group compared to the M group (p<0.05, Figure 1A). NPY gene expression levels were significantly higher in the N and M+N groups compared to the K group (p<0.001, Figure 1B). NPY gene expression was also significantly higher in the N group compared to the M group (p<0.01). However, there was no significant difference in POMK gene expression levels between the groups (Figure 1C).

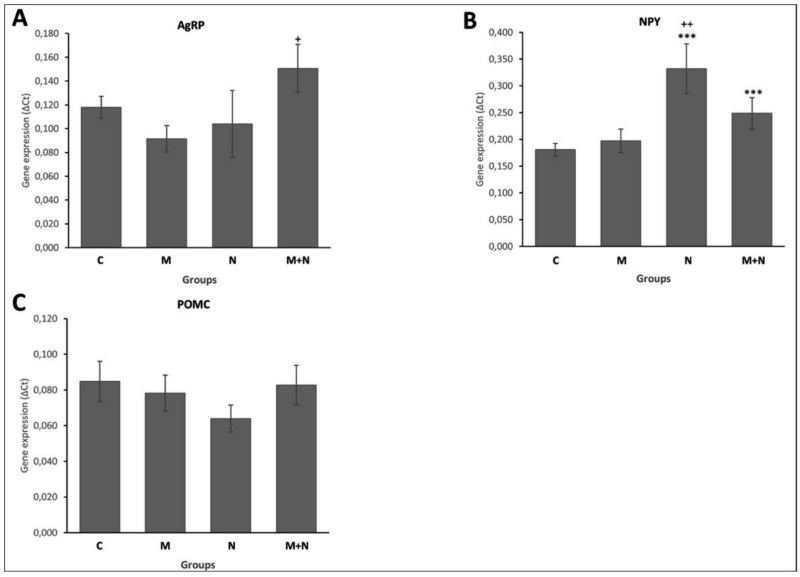

Figure 1: Changes in hypothalamic expression of AgRP, NPY and POMC

Bar graphs showing gene expression levels of AgRP (A), NPY (B) and POMC (C). All data +p<0.05 is considered significant according to the morphine group. were expressed as mean ± SEM values and analysed by one-way factorial ANOVA with Tukey post-hoc test. ***p<0.001 is considered significant according to the control group; ++p<0.01, +p<0.05 is considered significant according to the morphine group.

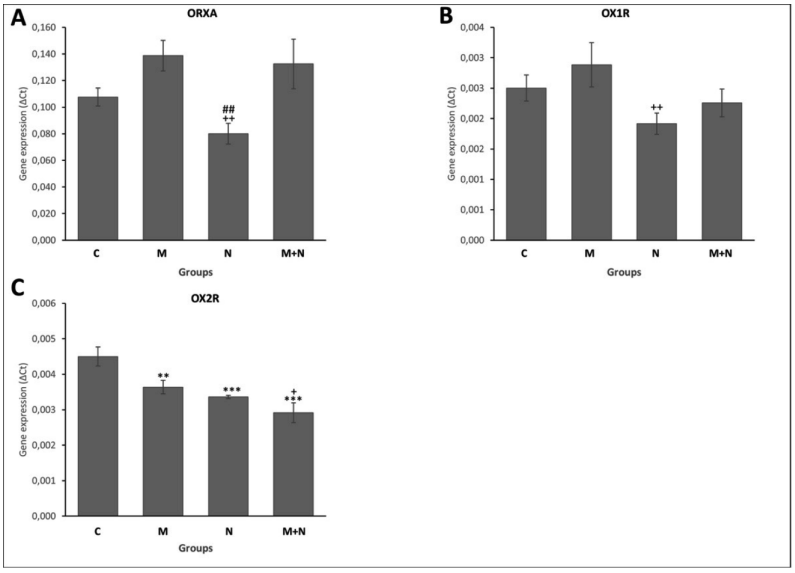

Figure 2: Changes in hypothalamic expression of ORXA, OX1R and OX2R.

Bar graphs showing gene expression levels of ORXA (A), OX1R (B) and OX2R (C). All data were expressed as mean ± SEM values and analyzed by one-way factorial ANOVA with Tukey post-hoc test. ***p<0.001, **p<0.01 is considered significant according to the control group; ++p<0.01, +p<0.05 is considered significant according to the morphine group; ##p<0.01 is considered significant according to the M+N group.

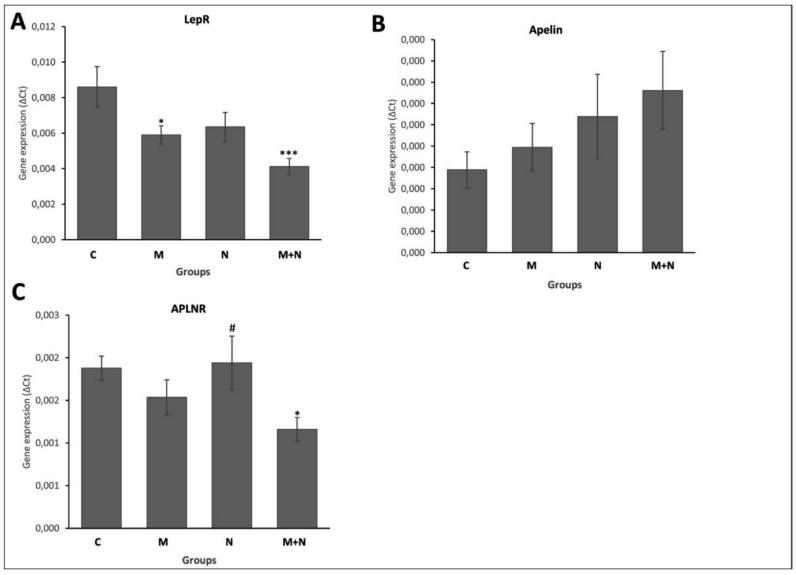

Figure 3: Changes in hypothalamic expression of LepR, Apelin and APLNR.

Bar graphs showing gene expression levels of LepR (A); apelin (B) and APLNR (C). All data were expressed as mean ± SEM values and analyzed by one-way factorial ANOVA with Tukey post-hoc test. *** p<0.001, *p<0.05 is considered significant according to the control group; #p<0.05 is considered significant according to the M+N group.

ORXA gene expression was significantly decreased in the N group (Figure 4.18.) compared to the M ($p<0.01$) and M+N group ($p<0.01$) (Figure 2A). OX1R gene expression was significantly decreased in the N group compared to the M group ($p<0.01$) (Figure 2B). OX2R gene expression levels were significantly decreased in the M ($p<0.01$), N ($p<0.001$) and M+N ($p<0.001$) groups compared to the control group. Also, OX2R gene expression was significantly decreased in the M+N group compared to the M group ($p<0.05$) (Figure 2C).

LepR expression was significantly decreased in the M ($p<0.05$) and the M+N ($p<0.001$) group compared to control (Figure 3A). There was no significant difference in apelin gene expression level between the groups (Figure 3B). APLNR gene expression was decreased in the M+N group compared to the control ($p<0.05$), whereas it was significantly increased in the N group compared to the M+N group ($p<0.05$) (Figure 3C).

DISCUSSION

In our study, we examined the possible changes in the levels of some hypothalamic neuropeptides that are involved in feeding as a result of morphine dependence. In the morphine group the level of OX2R and LepR were lower than in the control group. In the withdrawal group, NPY expression were higher than in the control group, while OX2R, LepR and APLNR levels were lower. In the naloxone-only group, NPY level were increased, whereas OX2R level were decreased in comparison to the control group. Since these hypothalamic neuropeptides are involved in many food intake signals, we may consider that opioids play a larger role in appetite regulation than previously recognized (23). A wide range of hypothalamic neuropeptide levels were analysed in our study. Many mechanisms arising from peripheral organs such as adipose tissue, gastrointestinal tract and skeletal muscles and many factors in central nervous system regulate food intake. The hypothalamus is the main regulatory centre of these mechanisms. Many studies have questioned the effects of opioids on food intake and hyperphagia (24, 25). The fact that biochemical measurements investigated in human and animal models show opioidergic signalling disorders in obese individuals increases the importance of this issue (26, 27).

Orexigenic AgRP-NPY and anorexigenic POMC hypothalamic neurons have been reported to be critical regulators of feeding and foraging behaviour (28). AgRP and NPY co-expressed at highest levels in the arcuate nucleus (29, 30). In our study, we found that hypothalamic AgRP expression was higher in the withdrawal group than in the morphine group. Opioids have been reported to inhibit AgRP-expressing neurons directly and indirectly via the MOR. This suggests that it is mediated by activating opioid receptors in non-AGRP neurons (23). In our study, NPY expression was decreased in the control and morphine groups compared to the naloxone group. This may suggest that NPY neurons have little effect on the hyperphagia effect of opioid addiction. Our results and recent studies contradict the view that the ability of NPY to potently stimulate food intake depends in part on the functioning of mu- and kappa-opioid receptors (31). Regional variation may account for this discrepancy. An autoregulatory feedback mechanism in POMC expression mediated by beta-endorphin acting through mu-opioid receptors in POMC neurons was also revealed (32). In our study there was no difference between POMC expressions. Given the specific regions of the hypothalamus, it seems likely that there are differences between groups. Orexin has been reported to reduce the electrical activity of POMC cells mainly by regulating synaptic inputs (33). Orexinergic neurons are found mainly in the lateral hypothalamus. They increase food intake by projecting to many regions (18). Orexinergic neurons located in the hypothalamus evoke their effects via two metabotropic receptors: OX1R and OX2R (34). In and around ARCPOMC cells there is strong immunoreactivity for orexin receptors (35). ORXA is a potent agonist of OX1R and OX2R (36). μ-, δ- and κ-opioid receptors are thought to modulate orexin neurons in the hypothalamus (14). In our study, the increasing trend in ORXA expression in morphine groups may support this viewpoint. In addition, according to our results, we can say that this effect of orexin on opiate exposure is mediated by OX2R rather than OX1R.

Leptin and apelin are adipokines that regulate food intake and are expressed in the hypothalamus. They exert their effects by binding to their receptors (LepR and APLNR, respectively) (37, 38). Leptin is responsible for satiety signalling. Leptin increases the firing rate of the POMC neurons and suppresses the frequency of the action potentials of the AgRP and NPY neurons (39). In our study, LepR expression was decreased in morphine groups. This may indicate the importance of hypothalamic LepRs in the hyperphagic effect of morphine. In

our previous study, we found that opioid exposure did not change APLNR expression in the hippocampus (40). In the present study, we did not find any difference in hypothalamic APLNR in morphine group, consistent with our previous study. However, APLNR was increased in the naloxone group. Moreover, this increase was suppressed by morphine in withdrawal group. This may indicate that hypothalamic apelin receptors are more likely to be activated by apelinergic signals from different regions during opiate exposure. Furthermore, this suppression increases the importance of the apelinergic system in opiate addiction, given their hyperphagic effects.

Opiates may inhibit or activate hypothalamic neuropeptides and receptors involved in regulation of feeding. Further studies with region- and neuron-specific transgenic animal models are necessary for a better understanding of these effects.

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Abbreviations list

APLNR: apelin receptors
LepR: leptin receptor
MOR: mu opioid receptor
Nac: nucleus accumbens
ORXA: orexin A
OX1R: orexin receptor type 1
OX2R: orexin receptor type 2
POMC: pro-opiomelanocortin
PVN: paraventricular nucleus
VTA: ventral tegmental area

Ethics approval and consent to participate

This project has been approved by Necmettin Erbakan University Experiments Animals Local Ethics Committee (Project Number: 2023-041 Date: 15.09.2023)

Consent for publication

There is no data on any individual in our study.

Availability of data and materials

The data that support the finding of this study are available from corresponding author upon reasonable request.

Competing interests

The authors declare no competing interest.

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Authors' contributions

Conceptualization and methodology: F.B.K.C., Z.E.T. and S.K. Data curation and project administration: F.B.K.C., Z.E.T., A.S., K.Z.C.S., and S.K. Investigation and data analysis: Z.E.T., A.S., K.Z.C.S., C.E.G. and E.K. Manuscript writing—original draft: F.B.K.C., C.E.G., E.K., Y.A.C., and S.K. Manuscript editing and manuscript review: F.B.K.C., E.K., Y.A.C. and S.K.

This manuscript has been read and approved by all the authors and that each author believes that the manuscript represents honest work.

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