THE BEHAVIORAL EFFECTS OF OBESTATIN AND PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE ON ANALGESIC TOLERANCE TO MORPHINE AND MORPHINE WITHDRAWAL IN MICE

Summary of Ph.D. Thesis

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2014

List of publications related to the Thesis

I. **Nándor Lipták**, Roberta Dochnal, Anikó Babits, Krisztina Csabafi, Júlia Szakács, Gábor Tóth, Gyula Szabó. The effect of pituitary adenylate cyclase-activating polypeptide on elevated plus maze behavior and hypothermia induced by morphine withdrawal. Neuropeptides, 2012, 46, 11–17. Impact factor: 2.067

II. **Nándor Lipták**, Roberta Dochnal, Krisztina Csabafi, Júlia Szakács, Gyula Szabó. Obestatin prevents analgesic tolerance to morphine and reverses the effects of mild morphine withdrawal in mice. Regulatory Peptides, 2013, 186, 77-82. Impact factor: 2.05

1. Introduction

Morphine was originally isolated from opium by German pharmacist Friedrich Wilhelm Adam Sertürner. Sertürner was the first who used the name "morphine" in 1817 in one of his publication. This new opium compound took its name from the Greek god of dreams Morpheus due to its sedative effect. In clinical medicine, morphine is still the best analgesic drug and using for alleviation of acute and chronic pain (e.g. postoperative pain, please see reviews: *Busch, 1987; Blaudszun et al., 2012*). Unfortunately, morphine has a lot of negative side effects especially in chronic treatments, for example tolerance to morphine-induced analgesia, sedation, reduced euphoria, reduced libido, loss of appetite, etc. After morphine withdrawal, these aversive effects become more expressed, for example hyperalgesia, anxiety, depression, etc. In spite of intense research there are still unsolved problems in the clinical pratice (e.g. analgesic tolerance to morphine, undesired side effects after morphine withdrawal).

Morphine exerts its effect on specific G-protein coupled opioid receptors: κ opioid receptors, KOR; μ opioid receptors, MOR; δ opioid receptors, DOR (*Pert and Snyder, 1973*) and orphan receptor like receptor-1, ORL-1 (*Mollereaua et al., 1994*). These receptors have numerous subtypes, for more details please see review: *Snyder S. H. and Pasternak G. W., 2003*. All of opioid receptors are present in the central nervous system. MORs, DORs and KORs mediate analgesia, but all of these receptors have other physiological effects. MORs mediates respiratory depression, sedation, reward/euphoria, nausea, urinary retention and constipation. KORs have dysphoric, aversive, sedative and diuretic effects. DORs have effect on reward/euphoria, respiratory depression and constipation.

In this Ph.D. thesis work the behavioral effects of obestatin and PACAP on analgesic actions of morphine and morphine withdrawal were examined in mice.

2. Aims

The aims of the current work:

- to examine the effects of PACAP on naloxone precipitated morphine withdrawal using EPM and withdrawal jumping test.
- to analyze the effects of obestatin on naloxone precipitated morphine withdrawal using EPM and open-field test.

- to investigate the actions of obestatin on morphine-induced acute tolerance and analgesic tolerance to morphine using the tail-flick test.

3. Materials and methods

3.1 Animals

Male CFLP white mice (30 ± 5 g of weight) of an outbred strain (Domaszék, Hungary) were used. They were kept under a standard light–dark cycle (lights on between 07.00 and 19.00 h) with food and water available ad libitum. The animals were kept and treated according to the rules of the Ethical Committee for the Protection of Animals in Research (Faculty of Medicine, University of Szeged, Hungary).

3.2. Surgery

For intracerebroventricular (i.c.v.) cannulation, the mice were anesthetized with intraperitoneal (i.p.) injection of sodium pentobarbital (Nembutal, Phylaxia-Sanofi, Budapest, Hungary; 50 mg/ kg or Euthasol^{R}, Produlab Pharma B.V. Raamsdonksveer, The Netherlands; 60 mg/kg), and a polyethylene cannula was inserted into the right lateral cerebral ventricle and cemented to the skull with cyanoacrylatecontaining instant glue. The experiments were started 4 days afteri.c.v. cannulation. Upon conclusion of the experiments, methylene blue were injected into the cerebral ventricle of the decapitated animals and the position of the cannula was inspected visually. Mice with improper cannula placement were excluded from the statistical analysis.

3.3. Drugs

For i.c.v. treatments, PACAP-38 (syntethized by Gábor Tóth), obestatin 1-23 (Anaspec, Inc., USA) and [D-Lys3]-GHRP-6, (Sigma Aldrich, USA) were dissolved in artificial cerebrospinal fluid (aCSF) and injected in a volume of 2 µl. For testing the morphine effects, subcutaneous (s.c.) morphine-HCl (Sigma-Aldrich) and naloxone-HCl (Sigma-Aldrich) injections were used.

3.4. Assesment of naloxone-precipitated withdrawal jumping in mice treated graded doses of morphine

Precipitated withdrawal jumping latency was measured in mice treated with morphine in the

presence and absence of PACAP after naloxone (1 mg/kg, s.c.) administration. Immediately after naloxone or saline injection, mice were placed on a circular platform. The precipitated abstinence syndrome was measured by scoring the latency to the apperance of stereotyped jumping from a circular platform 35 cm indiameter and 70 cm high (*Azarov et al., 1992*) . A cut off time of 15 min was used. The rectal body temperatures and body weights of all animals were also measured 15, 30, 60 min after naloxone injection, and changes in both parameters were calculated.

3.5. Elevated plus maze (EPM)

The elevated plus maze (EPM) is an accepted model for examining anxiety-like behavior in mice (*Lister, 1987*). Conditions that decrease time spent in the open arms are associated with anxiety-like behavior, whereas increased time spent in the open arms is associated with an anxiolytic effect. The EPM apparatus (Columbus Instruments, Columbus, Ohio, USA)consists of four arms (87-mm wide, 155-mm long) elevated 63.8 cm above the ground, with two arms enclosed by 16.3-cm-high opaque walls and illuminated with 60 W light situated 1 m above the maze. The combination of height, luminosity and open space is assumed to induce anxiety-like behavior in the animal. Behavioral testing was conducted between 11.00 and 13.00 h. Mice were carried to the experimental room in their home cages and habituated to the laboratory for at least 30 min before testing. Only one EPM apparatus per testing room was present. The apparatus was thoroughly cleaned between mice. Mice were placed in the center of the maze facing toward an enclosed arm and there behavioral activity were recorded for 10 min (*Schulteis et al., 1998*). The following behavioral parameters were monitored: the time spent in open arms and the entries into open arms compared to the total time (%OAT) and entries (%OAE) and the total activity which was defined as the total number of crosses between any two arms.

3.6. Open field (OF)

Obestatin effects on mild morphine withdrawal were also tested by the Conducta System (Experimetria Ltd., Budapest, Hungary). The apparatus consists of five black-painted testing boxes (40cm×50cm×50cm each) set in an isolated room; the movements of mice were detected by high-density arrays of infrared diodes. One animal was placed in one box, the apparatus is able to test 5 mice at the same time and there is no connection between them. The floor of the box was washed with ethanol (96%), water and dried prior to the next animal testing. On test day, mice were transported to the testing room and the percentage of time spent in the center and ambulation

distances in the center were recorded individually for each animal and separately for each box.

3.7. Tail-flick

Obestatin effect on morphine-evoked analgesic response tested by the tail-flick system (IITC Life Science, California, USA) described by *D'Amour and Smith, 1941*. All experiments were started with an initial tail-flick latency measurement, pain sensitivity was measured 15, 30, 60 min after peptide challenge in acute dose-response experiments and 60, 90, 120 min after morphine treatment in acute morphine experiment (day 1). In tolerance studies, pain sensitivity was measured 60 min after morphine injection. For tail-flick measurement, animals were habituated to the experimental room at least 30 min prior to testing. During the measurement, they were loosely restrained and the tail was positioned so that the light beam focused on the tail approximately 1–2 cm from the base. Tail stimulation was delivered at different sites in consecutive measures to prevent tissue damage. The analgesic effect was expressed according to this equation:

analgesic effect (%) = $(TFn - TF_0)/ (TFmax - TF_0) \times 100$,

where TF_0 is the tail-flick latency in the preliminary test mentioned above or (in tolerance studies) before morphine injection. TFn is the value of a repeated corresponding measurement n (15, 30, 60 or 60, 90, 120 min) after obestatin or/and morphine injection, and TFmax indicates the cutoff (20 s).

3.8. Statistical analysis

Statistical analysis of the elevated plus maze, open-field and jump test data was made by one-way analysis of variance (ANOVA) followed by Sidak or Tukey post-hoc test. Tail-flick experiments were analyzed using two-way repeated measures ANOVA, where drug effect (between subjects), time effect (within subjects) and their interactions were analyzed. In presence of interactions between drug and time, drug differences depend on time and vice versa, so in case of significant interaction drug effects were tested on each time point and time differences were tested in each group by Sidak post-hoc test. A probability value, $P < 0.05$ was considered statistically significant.

4. Major results

4.1. Influence of PACAP on naloxone-precipitated morphine withdrawal symptoms

Fifteen minutes after naloxone treatment PACAP blunted hypothermia induced by morphine withdrawal [$F(3,37) = 32.97$, $P < 0.034$]. However, 30 and 60 min after withdrawal PACAP had no significant effect on body temperature.

4.2. The effect of naloxone on EPM behaviors in mice treated with obestatin

Obestatin alone had no effect on the EPM behavior compared to control mice. Obestatin treated mice undergoing withdrawal showed decreased tendency in both parameters compared to the morphine withdrawal mice that did not receive obestatin, but the differences were not significant (%OAT: [$F_{(4,38)} = 7.11$, *P < 0.086*]; %OAE: [$F_{(4,38)} = 7,11$, *P < 0.227*].

4.3. The effect of naloxone and obestatin on OF behavior in mice treated with morphine

Obestatin alone had no significant effect on both parameters compared to control mice. Obestatin significantly decreased the percentage of time spent in the center in mice undergoing naloxone-precipitated mild morphine withdrawal [$F_{(4,51)}=10.998$, *P<0,045*]. Obestatin had no significant effect on the percentage of ambulation distance in center in mice treated with morphine and naloxone [$F_{(4,51)}=13,149$, *P<0,998*]. Naloxone precipitated mild morphine withdrawal caused significant increase in both parameters compared control mice and mice treated with morphine (the percentage of time spent in the center: [$F_{(4,51)=}10.998$, *P<0,001*]; the percentage of ambulation distance in the center: [$F(4,51)=13,149$, $P<0,005$]).

4.4. The effect of obestatin on analgesic effect induced by acute morphine treatment (1st day)

Mice treated with morphine showed significant higher pain sensitivity 90 and 120 min after morphine injection compared to first measurement (60 min) of the same group [$F_{(3,28)}= 12.482$, *P<0,001*] and significant lower pain-related behavior compared control in all time of measurements. Obestatin maintained the analgesic effect of morphine 90 and 120 min after morphine injection in mice treated with morphine receiving obestatin compared to mice treated with morphine (90 min: [$F_{(3,28)}= 6.285$, *P<0,01*]; 120 min: [$F_{(3,28)}= 6.285$, *P<0,001*]. Drug - time interactions (drug-time [$F_{(3,28)}=7.198$, *P<0,001*]; time [$F_{(3,28)}=7.912$, *P<0,003*]; drug [$F_{(3,28)}=45.175$, *P<0,003*]) were significant.

4.5. The effect of obestatin on analgesic tolerance to morphine

Morphine tolerant mice showed significant higher pain sensitivity on the 3rd and 5th day of experiments compared to the 1st day of the same group [$F_{(3,28)}$= 67.693, *P<0,001*] and significant lower pain-related behavior compared control on the 1st and 3rd day, but not on the 5th day. Morphine tolerant mice receiving obestatin displayed significant higher pain sensitivity on the 5th day compared the 1st day of the same group [$F_{(3,28)}$= 8.693, *P<0,001*]. Obestatin diminished the analgesic tolerance to morphine on the 5th day in morphine tolerant mice receiving obestatin compared with morphine tolerant mice [$F_{(3,28)}$= 8.693, *P<0,001*]. Drug - time interactions (drug-time [$F_{(3,28)}$=15.813, *P<0,001*]*;* time [$F_{(3,28)}$=25.473, *P<0,003*]*;* drug [$F_{(3,28)}$=62.100, *P<0,003*]) were significant**.**

4. Discussion

In this Ph.D. thesis work the behavioral effects of obestatin and PACAP on analgesic actions of morphine and morphine withdrawal were examined in mice. Up to this point, the behavioral effects on PACAP and obestatin on morphine-induced behavioral changes has been a poorly examined research field and the present data may provide a new orientation for neuropeptides research.

PACAP had no significant effect on the EPM behavior compared to morphine withdrawal mice. Moreover, mice chronically treated with PACAP and morphine jumped off the platform earlier than mice treated with morphine after withdrawal. Thus, PACAP enhanced this aversive effect of opioid withdrawal. Although, we found that naloxone induced hypothermia in mice treated with morphine was decreased by PACAP.

Obestatin displayed an inhibitory effect on %OAT in EPM and the time spent and ambulation distance in the center of the OF undergoing withdrawal. Although, our result was not significant in the EPM tests ($P < 0.086$), it followed the same tendency that we have recorded in the open field test after naloxone treatment. In tail-flick we also recorded that obestatin significantly prolonged the analgesic effect of acute morphine 90 and 120 min after morphine treatment and prevented the analgesic tolerance to morphine in the fifth day of chronic morphine treatment.

5. Summary

In summary, the effects of two neuropeptides, PACAP and obestatin were examined on morphine-induced behavioral changes in mice.

PACAP had no effect on EPM during naloxone-precipitated mild morphine withdrawal and shortened withdrawal jump latency induced by naloxone in mice treated with morphine. However, PACAP blunted the hypothermia induced by morphine withdrawal, but this positive effect of PACAP presented only 15 minutes after withdrawal.

Obestatin reversed the effects of mild morphine withdrawal on EPM on OF in mice. Interestingly, obestatin enhanced the analgesic effect of acute morphine and prevented the analgesic tolerance to morphine in tail-flick test in spinal and supraspinal level, suggesting the role of GHSR-1a receptor in this action of obestatin.

The underlying mechanisms of these effects of obestatin remained unclear; probably obestatin exerts its behavioral effects via ERK 1/2 activation and/or via GHSR-1a receptor.

6. Acknowledgements

Firstly, I am grateful to **Professor Gyula Szabó** for providing me the opportunity for research at the Department of Pathophysiology, Faculty of Medicine, University of Szeged.

I am especially thankful to my colleagues, **Roberta Dochnal, Anikó Babits, Krisztina Csabafi and Júlia Szakács** for their help in research.

I would like to say thank to Professor Gábor Tóth for his kindly donation of PACAP-38.

I wish to appreciatively acknowledge the technical assistance of **Gusztáv Kiss, Ágnes Pál and Ildikó Sípos**.

Last but not the least; I would like to thank to my family for their constant support.

This Ph.D. thesis work was supported by ETT-Grant (355-08/2009); TÁMOP 4.2.1./B-09/KONV-2010-0005 and TÁMOP-4.2.2/B-10/1-2010-0012.

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