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

Ph.D. Thesis

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List of abbrevations

5-HT: 5-hydroxytryptamine (serotonin)

aCSF: artificial cerebrospinal fluid

BNST: bed nucleus of stria terminalis

cAMP: cyclic adenosine monophosphate

CNS: central nervous system

CREB: cAMP-responsive element binding protein

DORs: δ opioid receptors

Elk1: Ets-like transcription factor 1

EPM: elevated plus maze

ERK 1/2: extracellular signal-regulated kinase1/2

Ets: E26-AMV virus oncogene cellular homolog, a transcription factor

GHSR-1a: growth hormone secretagogue receptor type 1a

H3: histone 3

KORs: κ opioid receptors

MORs: μ opioid receptors

mRNA: messenger ribonucleic acid

MSK1: Mitogen- and stress-activated kinase 1

NMDA: N-Methyl-D-aspartate

OF: open field

ORL-1: orphan receptor like receptor-1

PACAP: pituitary adenylate cyclase-activating polypeptide

PENK: proenkephalin

PDYN: prodynorphin

Pol: RNA polymerase II transcription complex

PPE: preproenkephalin

SP: substance P

VIP: vasoactive intestinal polypeptide

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: 2.056

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I. Krisztina Csabafi, Miklós Jászberényi, Zsolt Bagosi, **Nándor Lipták**, Gyula Telegdy. Effects of kisspeptin-13 on the hypothalamic-pituitary-adrenal axis, thermoregulation, anxiety and locomotor activity in rats. *Behavioural Brain Research* 241 (2013) 56– 61. Impact factor: 3.327

List of conference abstracts related to the Thesis

I. **Nándor Lipták**, Roberta Dochnal, Anikó Babits, Krisztina Csabafi, Gábor Tóth, Gyula Szabó. Morphine withdrawal anxiety influenced by pituitary adenylate cyclase-activating polypeptide (PACAP) in mice. *Frontiers in Neuroscience* (2011) doi: 10.3389/conf.fnins.2011.84.00172.

II. **Nándor Lipták**, Anikó Babits, Krisztina Csabafi, Júlia Szakács, Gábor Tóth, Gyula Szabó. The role of pituitary adenylate cyclase-activating polypeptide in morphine withdrawal

induced anxiety and locomotor activity in mice. *Acta Physiologica* 202 (2011) Supplement: 684.

III. **Nándor Lipták**, Roberta Dochnal, Krisztina Csabafi, Júlia Szakács, Gyula Szabó, The effects of obestatin on morphine withdrawal-induced behavioral changes in mice. *Clinical Neuroscience* 65 (2012) 42.

IV. **Nándor Lipták**, Roberta Dochnal, Krisztina Csabafi, Júlia Szakács, Gyula Szabó. The effect of obestatin on behavioral responses induced by morphine withdrawal in mice. *Conference abstract of PhD Scientific Meeting. School of PhD Studies, Semmelweis University* (2012).

V. **Nándor Lipták**, Júlia Szakács, Krisztina Csabafi, Gyula Szabó. Obestatin enhances the acute analgesic effect of morphine and prevents analgesic tolerance in mice. *14th Conference of the Hungarian Neuroscience Society* (2013).

List of conference abstracts not related to the Thesis

I. Anikó Babits, Krisztina Csabafi, **Nándor Lipták**, Júlia Szakács, Gyula Szabó. The effect of Antalarmin on cocaine-induced locomotion in mice. *Frontiers in Neuroscience* (2011) doi: 10.3389/conf.fnins.2011.84.00085.

II. Krisztina Csabafi, Miklós Jászberényi, Zsolt Bagosi, **Nándor Lipták**, Géza Tóth, Mária Wollemann, Gyula Telegdy. The effect of TYR-D-ALA-GLY-PHE-D-NLE-ARG-PHE, a synthetic opiate on the hormonal and behavioral stress response. *Acta Physiologica* 202 (2011) Supplement: 684.

III. Júlia Szakács, Roberta Dochnal, Anikó Babits, **Nándor Lipták**, Krisztina Csabafi, Gyula Szabó. Role of obestatin in morphine-induced behavioural responses. *Acta Physiologica* 202 (2011) Supplement: 684.

IV. Krisztina Csabafi, Zsolt Bagosi, **Nándor Lipták**, Júlia Szakács, Gyula Telegdy. The effect of kisspeptin on the hormonal and behavioural aspects of the stress response. *Clinical Neuroscience* 65 (2012) 13.

V. Krisztina Csabafi, Zsolt Bagosi, **Nándor Lipták**, Gyula Telegdy. A kisspeptin szerepe a hypothalamusz-hipofízis-mellékvesekéreg rendszer és a viselkedés szabályozásában. *Conference of the Hungarian Physiological Society* (2012).

VI. Júlia Szakács, Krisztina Csabafi, **Nándor Lipták**, Roberta Dochnal, Gyula Szabó. Az obestatin viselkedésre kifejtett hatásai egérben. *Conference of the Hungarian Physiological Society* (2012).

VII. Krisztina Csabafi, Miklós Jászberényi, Zsolt Bagosi, **Nándor Lipták**, Gyula Telegdy. Effects of neuropeptide SF on corticosterone release, locomotor activity and body temperature. *14th Conference of the Hungarian Neuroscience Society* (2013).

VIII. Júlia Szakács, Krisztina Csabafi, **Nándor Lipták**, Gyula Szabó. Behavioural effects of cocaine and the ghrelin-associated peptide obestatin in mice. *14th Conference of the Hungarian Neuroscience Society* (2013).

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1 Introduction

1.1 Short historical and clinical overview about morphine

Morphine was originally isolated from opium by German pharmacist Friedrich Wilhelm Adam Sertürner. In 1806, he published a scientific paper about the results of his experiments in which he claimed the isolation of "*principium somniferum*," the narcotic principle of opium. Opium, is the dried latex purified from *Papaver somniferum*, contains two main groups of alkaloids: phenanthrenes and isoquinolines. Phenanthrenes such as morphine (approximately 10-15 % of opium), codeine, and thebaine have effects in the central nervous system (CNS); on the contrarily, isoquinolines (e.g. papaverine and noscapine) have no significant actions in the CNS. Morphine was firstly distributed by its inventor in 1817, thus, morphine-extract is used by humans almost in the past two centuries. 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. Morphine was widely used after the perfection of the hypodermic needle in 1857. The next big breakthrough in morphine research was the discovery of heroin (diacetylmorphine), in 1874, by Alder Wright. Heroin was given its name due to its "heroic fight" against pain and morphine addiction. Heroin was believed to be an effective analgesic opiate without provoking addiction and also was marketed as a cough suppressant by Bayer in 1898. A few years later, heroin turned out to cause more severe addiction than morphine, so the production and distribution of heroin was prohibited in the great majority of the countries. For more historical details, please see the excellent review of *Schiff P. L. Jr., 2002.*

Currently, there are at least 11 licensed, medical prescription-required morphine-containing pharmaceutical products available in Hungary *(Online Drug Databese, National Institute of Pharmacy, 2014)*. In clinical medicine, morphine is still considered to be the best analgesic drug used 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 unwanted side effects especially in chronic treatments, for example tolerance to morphine-induced analgesia, sedation, reduced euphoria, reduced libido, loss of appetite, etc. After naloxone-precipitated morphine withdrawal, these aversive effects become more expressed, for example hyperalgesia, anxiety, depression, etc. Naloxone is one of the most widely used

withdrawal-provoking drug in the clinical practice and there are 9 licensed naloxone-containing pharmaceutical products available in Hungary, all of these drugs are medical prescription-required *(Online Drug Databese, National Institute of Pharmacy, 2014)*. These symptoms after withdrawal are called *morphine withdrawal syndrome*. Due to extended human consumption, a lot of studies examined the aversive impacts of morphine treatment and morphine withdrawal, both in humans and rodents. 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).

1.2 Opioid receptors, opiate peptides, and their role in regulation of pain and anxiety in the CNS

1.2.1 Opioid receptor distribution and endogenous opiate peptides

Opiates are the ligands which bind to their specific receptors, the opioid receptors. This nomenclature was used throughout the Ph.D. thesis. Opiates can be exogenous (e.g. morphine, codeine, thebaine etc.) or endogenous (see this section). Morphine exerts its effect on specific G-protein coupled opioid receptors: κ opioid receptors (KORs), μ opioid receptors (MORs), δ opioid receptors (DORs) (Pert et al., 1973; *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*. Naturally, these receptors have endogenous ligands, called endogenous opiate peptides. The firstly discovered and purified opiate peptide was β-endorphin sourced from human brain (*Li et al., 1976*); and the last identified peptides were endomorphin-1 and endomorphin-2 from the bovine brain (*Zadina et al., 1997*) and human cortex (*Hackler et al., 1997*). Up to this point, there are 22 different identified endogenous opiate peptides (please see review: *Fichna et al., 2007*).

All of the opioid receptors are present in the CNS. The following parts of the introduction section are focusing on studies about experiments using rodent animal models. *KORs, the specific receptors of dynorphins,* have been detected in the ventral tegmental area, substantia nigra, nucleus accumbens, caudate putamen, various hypothalamus, and the amygdala of the rat and mouse brain (please see review *Bruijnzeel, A. W., 2009*); and in various regions in the mouse and rat spinal cord. KOR messenger ribonucleic acids (mRNAs)

are highly expressed in the locus coeruleus of the mouse brain, whereas only weakly expressed in this region of the rat brain. *MORs, the specific receptors of endomorphins, casomorphins, etc*., were detected in the cerebral cortex at a low level (only in layer VI), in the amygdala, in the thalamic nuclei, in the medial raphe nucleus. Intense expression of MOR mRNAs were detected in the rat and mouse spinal cord lumbar dorsal horn, and moderate expression in the lumbar ventral horn. *DORs, the specific receptors of enkephalins and deltorphins*, were observed in the cerebral cortex (layers II, III, V and VI), little or no detectable expressions in globus-, ventral pallidum and in the thalamic nuclei in the mouse brain. High DOR mRNAs expression were detected in the mouse entopeduncular nucleus, and moderate DOR mRNAs present were observed throughout the laminae I-VI of the mouse spinal dorsal horn (please see review: *Minami and Satoh, 1995*). KORs, MORs and DORs are co-expressed in many regions of the CNS of rodents.

ORL-1, the specific receptor of nociceptin/orphanin FQ, is highly expressed in the cortex (layers II to VI), the anterior olfactory nuclei, the dentate gyrus of the hippocampus, the amygdala, the bed nucleus of the stria terminalis, the substantia nigra, the ventral tegmental area in the mouse and rat brain. In the rat spinal cord this receptor is also expressed: in the cell bodies of rat dorsal root ganglia, in the ventral horn (laminae VIII-IX), in the interneurons of the dorsal horn (see review *Mollereau et al, 2000*). In the mouse spinal cord, ORL-1 is identified in the superficial layers of the dorsal horn and around the central canal (*Narita et al., 1999*).

1.2.2 The effects of opiates on pain

Endogenous opiate peptides are natural analgesic “drugs” of the central and peripheral nervous system. All type of opioid receptor mediates analgesia. Morphine can bind to MORs, DORs and KORs, but morphine exerts its highest analgesic effect through the activation of the MORs (*Matthes et al., 1996*). Endogenous and exogenous opiates can inhibit pain both in spinal and supraspinal level.

At spinal level, opiates have presynaptic inhibitory effect on excitatory transmission in superficial dorsal horn in rodents. Opiates inhibit the release of pronociceptive neurotransmitters in the spinal cord via prevention of Ca^{2+} influx. The most important

consequence of this prevention is the suppression of substance P (SP) release from primary sensory neurons, because there is no nociceptive information transmission without SP release in the spinal cord. At the postsynaptic membrane, opioids evoke hyperpolarization by opening K^+ channels, thereby preventing excitation or propagation of action potentials in second order projection neurons. In addition, opioids inhibit sensory neuron-specific tetrodotoxin-resistant Na^+ channels and excitatory postsynaptic currents evoked by glutamate receptors, for example N-Methyl-D-aspartate (NMDA) receptors in the spinal cord (please see review: *Stein, 2013*).

Endomorphin 2 can act on MORs directly to induce antinociception in the spinal cord, or (at supraspinal level) enhance the release of Met-enkephalin, dynorphin-A, etc via descending inhibitory pathways. Noradrenaline and 5-hydroxytryptamine (5-HT) are the most important neurotransmitters which are involved in descending inhibition. Two important areas of the brainstem are involved in reducing pain: the periaquaductal grey and the nucleus raphe magnus (see review*: Steeds, 2013)*. Endomorphin 1 also affects directly on MORs, or enhances the release of noradrenalin and serotonin. Thus, endogoneous opiates have very complex effects on physiological antinociception system (please see review: F*ichna et al., 2007*).

There are several behavioral methods for analyzing analgesic actions of morphine in rodents. For example, tail-flick, hot-plate, etc. for measuring thermal-; Randall Selitto test, rodent pincher test, etc. for measuring mechanical-; and formalin test, capsaicin test for measuring chemical nociceptive agents induced behavior.

Morphine has analgesic effect both in mice and rats. There are lots of studies which demonstrated the development of analgesic tolerance to morphine in mice (*Ninan and Kulkarni*, 2000; *Mamiya et al., 2001*; *Contet et al., 2008*; etc.) and rats (e.g. *Advokat et al., 1987; Rawls et al., 2010; Ranjbar-Slamloo et al., 2012*) using behavioral methods. For more experimental details, see section **1.2.3.**

1.2.3 The role of neuropeptides in morphine-induced behavioral changes

In the past four decades, the role of endogenous neuropeptides in morphine-induced behavioral changes was examined by our Department. These investigations focused on the effects of the natriuretic peptide family and (vasoactive intestinal polypeptide) VIP family on

morphine-evoked behavioral responses, especially on morphine-induced analgesia and morphine withdrawal. Brain natriuretic peptide and C-type natriuretic peptide are the members of the natriuretic peptide family. C-type natriuretic peptide depressed the acute analgesic effect of morphine, blocked the development of acute tolerance to morphine (*Babarczy et al., 1995*). Brain natriuretic peptide also decreased the acute analgesic effect of morphine (*Babarczy et al., 1996*).

VIP decreased the analgesic effect of single injection of morphine and the development of chronic tolerance to morphine (*Mácsai et al., 1998*). PACAP blocked the nalgesic effect of acute morphine and enhanced the analgesic tolerance to morphine (*Mácsai et al., 2002*). Moreover, PACAP significantly enhanced the naloxone precipitated withdrawal jumping.

1.2.4 The effects of opiates on anxiety

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. The regulation of anxiety is a complex system in rodents. The areas of the limbic system (included the hippocampus, amygdala, anterior thalamic nucleus, septum, prefrontal cortex and the fornix) have a key role in the modulation of anxiety. Principally, the GABA-A, 5-HT, cholecystokinin, nor-adrenalin, neuropeptide Y neurotransmitter systems are responsible for regulation of anxiety and stress-response.

There are numerous behavioral tests for measuring anxiety-like behavior in rodents. These tests may divide into five types:

- exploratory behavior tests (e.g. elevated plus maze (EPM), open-field (OF), light-dark box, etc.);
- social behavior tests;
- reflexive fear tests (e.g. acoustic startle test, conditioned freezing test, etc.);
- conflict tests (e.g. Vogel conflict test, four-plate test, etc.);

- defensive behavior tests (e.g. shock-probe burying test, marble burying test, etc.).

For more details, see review (*Rotzinger et al., 2010*).

Endomorphin 1 decreases the normal preference for the closed arms on EPM in mice (*Asakawa et al., 1998*); this is the only one report measuring the effect of endomorphins on anxiety in rodents. The role of KORs/dynorphin system in anxiety is not clearly understood; contradictory studies were published in this issue (please see review: *Schwarzer, 2009*). Activation of DORs reduces anxiety-like behavior in the central amygdala in rats (*Randall-Thompson et al., 2010*).

Morphine has anxiolytic action in rats injected peripherally (*Koks et al., 1999*) and centrally (*Motta et al., 1995*). Naloxone-precipitated morphine withdrawal has a clear anxiogenic effect in rats *(Schulteis et al., 1998; Zhang and Schulteis, 2008*). Repeated subcutaneous morphine administration in increasing doses also evokes anxiety-like behavior in mice (*Hodgson et al., 2008*). Furthermore, both naloxone-precipitated (*Hodgson et al., 2008*) and spontaneous morphine withdrawal (*Buckman et al., 2009*) caused increased elevated plus maze open-arm time in mice. This elevated plus maze behavior was blocked by SL327 (a selective ERK 1/2 inhibitor) after its injection into the amygdala (*Hofford et al., 2009*) indicating the role of ERK 1/2 in this unexpected behavior of mice.

1.2.5 Intracellular changes evoked by acute and chronic morphine treatment in the CNS in rodents

All type of opioid receptors couple to G-proteins and subsequently inhibit adenylate cyclase in the presence of an agonist. Thus, *acute morphine* administration reduces intracellular cAMP level (*Sharma et al., 1975*); decrease the conductance of voltage-gated Ca^{2+} channels and open rectifying K^{+} channels. Furthermore, acute morphine treatment causes a rapid and transient increase extracellular signal-regulated kinase (ERK) phosphorylation in the nucleus accumbens (NAcc), central amygdala, prefrontal cortex, lateral bed nucleus of stria terminalis (BNST) in mice (*Valjent et al., 2004*). Acute single morphine injection does not change the proenkephalin (PENK) and prodynorphin (PDYN) mRNA level in the nucleus

accumbens or striatum (*Turchan et al., 1997*) in mice. On the other hand, acute intermittent morphine treatment increases the preprodynorphin and KOR mRNA levels, but does not alter preproenkephalin (PPE) mRNA level in the rat cortex and cerebellum (*Wang et al., 1999*).

One of the first consequences of chronic morphine treatment is the opioid receptor endocytosis (please see review of *von Zastrow et al., 2003*) and desensitization of opioid receptors. Desensitization is a progressive loss of receptor function under continued exposure to opiate agonists *(Murányi et al., 2013)*. Opioid receptors desensitizatied by the G-protein coupled receptor kinases and second messenger regulated protein kinases (*Polakiewicz et al., 1998*). Desensitization and endocytosis may play an important role in the early stages of analgesic tolerance to morphine and morphine dependence. In contrast to acute treatment, chronic exposure to morphine causes a gradual loss in the opioid-mediated adenylate cyclase inhibition and a marked increase in adenylate cyclase activity (*Nevo et al., 1998*). This phenomenon called adenylate cyclase super activation and is supposed to participate in the development of morphine tolerance and dependence but the underlying mechanism of this super activation has not been clarified yet. This increased cAMP level (which was increased directly via opioid receptors or indirectly via D1 dopamine receptors) induces a marked elevation in ERK 1/2 phosphorylation during morphine dependence. After naloxone precipitated withdrawal, this elevation becomes more expressed both in mice and rats (*Li et al., 2010*). ERK2 can enter into the nucleus and regulates transcription of mRNAs of cFos, dynorphin, etc. via phosphorylation of mitogen- and stress-activated kinase 1 (see **Figure 1**). Repeated morphine administration reduces the PENK mRNA level in the nucleus accumbens and in the striatum, but elevates the PDYN mRNA level in both those structures in mice (*Turchan et al., 1997*). Withdrawal from chronic morphine treatment also causes increased PPE mRNA level in rat periaqueductal gray (*Fukunaga et al., 1998*). These elevations may be regulated via increased ERK 1/2 phosphorylation (*Ji et al., 2002*).

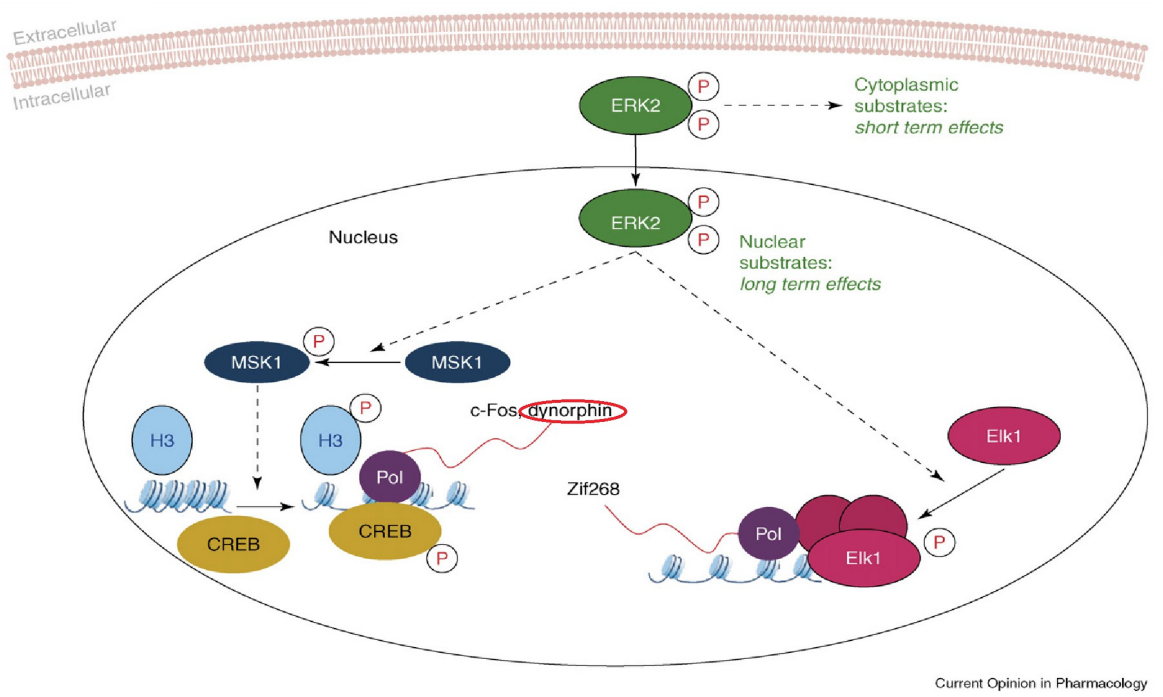


Figure 1: The Regulation of transcription by ERK2 in the mice striatum. Figure adapted from: *Girault et al., 2007*.

ERK 1/2 is activated by almost all of drug of abuse (e. g. cocaine, nicotine, morphine, tetrahydrocannabinol), therefore ERK 1/2 phosphorylation might be the common "step" in the development of drug-induced plasticity and addiction (for more details please see review: *Girault et al., 2007*).

1.2.6 PACAP

PACAP was originally isolated from ovine hypothalamus by its potent activity in stimulating cAMP production in rat anterior pituitary cells (*Arimura, 1992*). PACAP is a neuropeptide and member of a VIP superfamily that includes numerous peptides, e.g. VIP, secretin, helodermin, glucagon, galanin, etc. (*Christophe, 1993*; *Gourlet et al., 1998*). PACAP has two amidated forms: PACAP-38 and PACAP-27 (*Miyata et al., 1990*). The presence of PACAP was detected in hypothalamus, medial and ventral areas of the diencephalon, central thalamic nuclei, amygdala, BNST, septum, hippocampus, cingulate and entorhinal cortex, substantia nigra, nucleus accumbens, globus pallidus and sacral spinal cord (*Dietl et al., 1990; Joo et al., 2004*). Two receptor classes were described for PACAP in mammalian

tissues: type I and type II. Type II was further divided into three subclasses: PAC1, VPAC1 and VPAC2. PAC1 receptors are more selective for PACAP than VIP, but VPAC1 and VPAC2 receptors show similar affinity for PACAP-27, PACAP-38 and VIP (*Cauvin et al., 1990; Gourlet et al., 1996*). Type I receptors stimulate both adenylate cyclase and phospholipase C, thus being coupled to dual transduction pathways, involving interactions with G proteins of both Gs- and Gq-type. PACAP-38 and PACAP-27 were also effective in increasing cAMP release, cellular cAMP content, and total cAMP production in a dose-dependent in rat neuroepithelial cells (*Zhou et al., 2001*). Behavioral studies examined the PACAP effect on motor stimulation and conditioned place preference (CPP) induced by morphine (*Marquez et al., 2009*); analgesic tolerance to morphine *(Mácsai et al., 2002*); mechanical hyperalgesia and thermal allodynia (*Sándor et al., 2010*). Earlier experiments done by our research group demonstrated that PACAP diminished the antinociceptive effect of acute morphine and enhanced the analgesic tolerance to morphine pellet (*Mácsai et al., 2002*). Low doses of PACAP (0.03 and 0.3 μg), which had no effect on basal motor activity, enhanced morphine-induced (5 mg/kg, s.c.) motor stimulation and PACAP-deficient mice exhibited reduced morphine-induced motor stimulation (*Marquez et al., 2009*). Morphine-induced CPP following a single alternate-day saline/morphine (10 mg/kg, s.c.) conditioning was blunted in PACAP-deficient mice compared to their wild type littermates (*Marquez et al., 2009*).

1.2.7 Ghrelin and obestatin

Obestatin and ghrelin belong to the same neuropeptide family. Ghrelin, a 28-amino acid peptide, was isolated from rat stomach and identified as an endogenous ligand for (growth hormone secretagogue receptor type 1a) GHSR-1a (*Kojima et al., 1999*). Ghrelin and GHSR-1a mRNA was detected in peripheral organs (e.g. stomach, lung, heart, kidneys, etc.), and in the central nervous system. GHSR-1a was identified in rodent hypothalamus, pons, medulla oblongata, cortex, midbrain and spinal cord *(Zigman et al., 2006; Vergano et al., 2008*). Ghrelin immunoreactive neurons in the hypothalamus densely innervate proopiomelanocortin neurons (*Cowley et al., 2003*); suggesting that ghrelin can regulate the release of β-endorphin and the analgesic effect of endogenous opiates. Interestingly, peptides encoded by the ghrelin gene (acylated ghrelin, unacylated ghrelin and obestatin) can activate extracellular signal-related kinase 1/2 human pancreatic islet endothelial cells (*Favaro et al., 2012*). Furthermore,

[D-Lys3]-GHRP-6 blocked the obestatin-induced cell survival in human pancreatic islet cells (*Granata et al., 2008*); hence GHSR-1a may participate in the physiological effects of obestatin. Ghrelin can prevent mechanical hyperalgesia (*Garcia et al., 2008*) and inflammatory pain (*Sibilia et al., 2006*). In addition, a very recent study has claimed that ghrelin can induce a dose- and time-dependent analgesic effect in the acute heat-induced pain in mice (*Wei et al., 2013*). This effect of ghrelin was significantly antagonized by [D-Lys3]-GHRP-6, a specific GHS-R1a antagonist, and by naloxone, indicating the involvement of endogenous opioid system in the analgesic effect of ghrelin (*Wei et al., 2013*). The role of ghrelin in reward (please see reviews in this issue: (*Dickson el at., 2011; Skibicka and Dickson, 2011*) and in anxiety (*Andrews et al., 2011*) are well-examined research areas, but the role of obestatin in these research fields has not been clarified yet.

Obestatin, a ghrelin-associated peptide derived from the preproghrelin was discovered by *Zhang et al., 2005*. Obestatin was purified from the rat stomach and was initially reported to reduce food intake, gastric emptying and intestinal motility (*Zhang et al., 2005; Bresciani et al., 2006)*. It was also characterized as an activator of the orphan G protein-coupled GPR39 receptor and was found to be the main ligand for it. The highest levels of GPR39 mRNA were detected by *in situ* hybridization in the amygdala, the hippocampus, and the auditory cortex, while lower levels were found in several other brain regions but surprisingly no expression of GPR39 was found in the hypothalamus in mice (*Jackson et al., 2006*). GPR39 receptor has two splice variants, GPR39-1a and GPR39-1b. GPR39-1a is expressed selectively in the gastrointestinal tract, whereas GPR39-1b has a wider expression pattern, including nuclei in the central nervous system, for example the amygdala, and hippocampus (*Egerod et al., 2007*). Later studies reported that GPR39 may not have obestatin as a main ligand (*Chartrel et al., 2007; Holst et al., 2007; Lauwers et al., 2007*). After these findings, Zhang et al. confirmed that their original result was unreproducible (*Zhang et., 2007*) and subsequent results suggested that glucagon-like peptide-1 receptor (GLP-1R) is the receptor of obestatin (*Granata et al., 2008; Favaro et al., 2012*). Moreover, a few *in vitro* studies claimed that obestatin stimulated ERK1/2 phosphorylation, in rat tumor somatotroph cells (*Pazos et al., 2009*); in human pancreatic islet cells (*Favaro et al., 2012*; *Granata et al., 2008*) and in human retinal pigment epithelial cells (*Camiña et al., 2007*). In the past few years metabolic and body weight-regulating effect of obestatin has been investigated in detail, however, there are only a few reports, which examined the role of obestatin in exploratory behavior and its analgesic effect. A previous study on the EPM indicated that obestatin induced elevation of

the %OAT and %OAE in rat (*Carlini et al., 2007*). These data were later confirmed by *Ishitobi et al., 2012*: intracerebroventricular administration of antisense DNA for GPR39-1b caused anxiolytic-like effect in rats in two different behavioral tests. The same research group discovered that ghrelin decreased the %OAT in the EPM and increased the ambulation time in the OF test in rats and neonatal chicks (*Carlini et al., 2002; Carvajal et al., 2009*).

1.3 Aims

The aims of the current works were the following points:

- 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.

2 Materials and methods

2.1 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).

2.2 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 after i.c.v. cannulation. Upon conclusion of the experiments, methylene blue was 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.

2.3 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 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.

2.4 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.

2.4.1 Influence of PACAP on naloxone-precipitated morphine withdrawal symptoms

We used twice daily injections (09.00 and 19.00 h.) of graded doses of morphine as follows: day 1: 20 mg/kg, day 2: 40 mg/kg, day 3: 60 mg/kg, day 4: 80 mg/kg or saline (*Contet et al., 2008*). Mice were also treated once a day (09.30 h) with either PACAP (500 ng/2 µl) or aCSF i.c.v. On the test day (day 5) animals received in the morning a single morphine injection (100 mg/kg s.c., 9.00 h) or saline (s.c.). Ninety minutes later aCSF or PACAP was given (10.30 h, i.c.v.). Withdrawal signs were evoked by naloxone administration (1 mg/kg, s.c.) 2 h after the final morphine treatment. Precipitated morphine withdrawal syndrome was induced by 1 mg/kg naloxone-HCl as described by *Azarov et al., 1992.* For specific groups and treatments see **section 3.1.**

2.5 Elevated plus maze

The 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 homecages 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 was 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.

2.5.1 The effects of PACAP on EPM behavior

Mice were treated once a day (09.30 h) with either PACAP (500 ng or 1 μg i.c.v. or aCSF for 3 consecutive days. On the test day (day 4) PACAP or aCSF was administered 30 min before EPM assessment. The parameters were monitored as mentioned above.

2.5.2 The effect of naloxone on EPM behavior in mice treated with morphine

We used twice daily injections (09.00 and 17.00 h.) of graded doses of morphine as follows: day 1: 10 mg/kg, day 2: 20 mg/kg, day 3: 40 mg/kg or saline (*Hodgson et al., 2008*). On the test day (day 4) animals received a single dose of morphine (20 mg/kg, s.c.) or saline (s.c.) in the morning at 09.00 h. Naloxone treatment in a dose of 0.1 mg/kg, or 0.2 mg/kg, s.c. Preceded behavioral assessment by 5 min. The behavioral changes were measured 2 h after the final morphine treatment with EPM test (*Higgins and Sellers, 1994; Schulteis et al., 1994*). For treatment schedule, see **section 3.1.**

2.5.3 The effect of naloxone and PACAP on EPM behavior in mice treated with morphine

Morphine administration regimen was the same as in **2.5.2.** In addition, mice were treated once a day with either PACAP (500 ng/2 μl) or aCSF (i.c.v.) at 09.30 h. On the test day (day 4) animals received the final morphine dose (20 mg/kg) s.c.), or saline (s.c.). 9.00 h and either aCSF or PACAP (i.c.v.) was given at 10.30 h. Naloxone treatment and behavioral assessment were conducted in the same way as outlined in the **2.5.2** with one exception that only the higher dose of naloxone was used (0.2 mg/kg, s.c.). For treatment schedule, see **section 3.3.**

2.5.4 The effect of naloxone and obestatin on EPM behavior in mice treated with morphine

Morphine (08.00 and 16.00 h) and naloxone administration regimen was the same as in **2.5.3.** In addition, mice were treated once a day with either obestatin (1.5 μg/2 μl) or aCSF (i.c.v.) at 08.15 h. On the test day (day 4) animals received a single dose of morphine (20 mg/kg, s.c.) or saline (s.c.) at 08.00 h and either aCSF or obestatin (i.c.v.) was given at 09.45 h. For specific groups and treatments see **section 3.5.**

2.6 Open field

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.

2.6.1 The effect of graded doses of acute obestatin on OF behavior in mice

Obestatin was administrated i.c.v. at graded doses: 0.5 - 2 μg. Mice were tested 15 min after the obestatin treatment for 10 minutes.

2.6.2 The effect of naloxone on OF behaviors in mice treated with obestatin

We used twice daily injections of saline. Mice were also treated once a day with either obestatin (1.5 μg/2 μl, i.c.v., respectively) or aCSF (i.c.v.) at 08.15 h. On the test day (day 4) animals received saline (s.c.) at 08.00 h and either aCSF or obestatin (i.c.v.) was given at 09.45 h. Naloxone treatment in a dose of 0.2 mg/kg, s.c. preceded behavioral assessment by 5 min. The behavioral changes were measured 2 h after the final saline treatment in the OF.

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

Treatment protocol was the same as in **2.5.4.** For specific groups and treatments see **section 3.5.**

2.7 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).

2.7.1 The analgesic effect of graded doses of acute obestatin

Obestatin was administrated i.c.v. at graded doses: 0.5 - 2 μg. Mice were tested 15, 30 and 60 min after the obestatin treatment.

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

Mice were treated 10 mg/kg morphine (s.c.) or saline (s.c.) at 09.00 h, an hour before to first tail-flick measurement. Obestatin or aCSF were injected i.c.v. at 09.45 h. The analgesic response was measured 60, 90 and 120 min after the morphine injection. For treatment schedule see **section 3.9.**

2.7.3 The effect of obestatin on analgesic tolerance to morphine

To develop morphine tolerance, mice received either morphine (10 mg/kg, s.c.) or saline twice daily for four days, at 09.00 and 16.00 *(Mamiya et al, 2011)*. Mice also treated with either obestatin or aCSF once a day at 09.45 h. On the fifth day, morphine was administrated only in the morning at 09.00 h. Obestatin treatment was the same as previous days. Analgesic effect was measured on the 1st, 3rd and 5th days in the morning at 10.00 h. For treatment schedule see **section 3.10.**

2.8 Statistical analysis

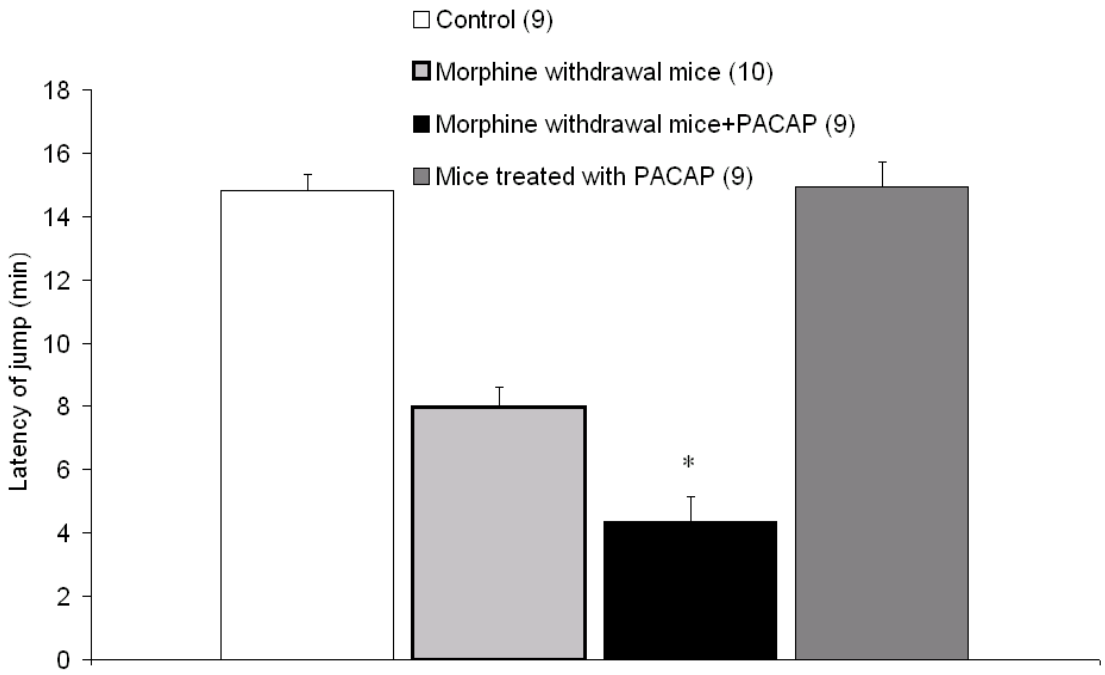
Statistical analysis of the elevated plus maze, open-field and jump test data was made by one-way analysis of variance (ANOVA) followed by Sidak or Tukey post-hoc test. Tail-flick experiments were analyzed using two-way repeated measures ANOVA, where drug effect (between subjects), time effect (within 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. The numbers in parentheses indicate the number of mice per group.

3 Results

3.1 Influence of PACAP on naloxone-precipitated morphine withdrawal symptoms

Repeated treatment of PACAP shortened jump latency in mice treated with morphine and challenged with naloxone [$F_{(3,37)} = 23{,}73$, $P < 0{,}023$] (**Fig. 2**). (Tukey post-hoc)

Figure 2. The effect of PACAP on naloxone-precipitated withdrawal jumping in mice



treated with graded doses of morphine. Bars represent the latency of jump, vertical lines on the top of the bars denote S.E.M., *: $p < 0.05$ vs. morphine withdrawal mice.

Mice received the following treatments:

- Control mice: Twice daily injections of saline (Sal), s.c. (09.00 and 19.00 h.) and once daily injection aCSF, i.c.v. (09.30.) for 4 consecutive days. On the test day, mice given: Sal, s.c. (09.00 h), aCSF, i.c.v. (10.30 h) and Sal, s.c. (11.00 h).

- Morphine withdrawal mice: Twice daily injections of graded doses of morphine (Mor), s.c. (09.00 and 19.00 h.) and once daily injection aCSF (09.30.), i.c.v., s.c. for 4 consecutive days. On the test day, mice given: Mor, s.c. (09.00 h), aCSF. i.c.v. (10.30 h.), and Sal, s.c., (11.00 h).

- Morphine withdrawal mice + PACAP: Twice daily injections of graded doses of Mor, s.c. (09.00 and 19.00 h.) and once daily injection PACAP

(09.30.), i.c.v., s.c. for 4 consecutive days. On the test day, mice given: Mor, s.c. (09.00 h), PACAP (i.c.v.), and naloxone (Nal) s.c., (11.00 h).

- Mice treated with PACAP: Twice daily injections of Sal, s.c. (09.00 and 19.00 h) and once daily injection PACAP, i.c.v. (09.30.) for 4 consecutive days. On the test day, mice given: Sal, s.c. (09.00 h), PACAP, i.c.v. (10.30 h) and Sal, s.c. (11.00 h).

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 (**Fig. 3**). (Tukey post-hoc)

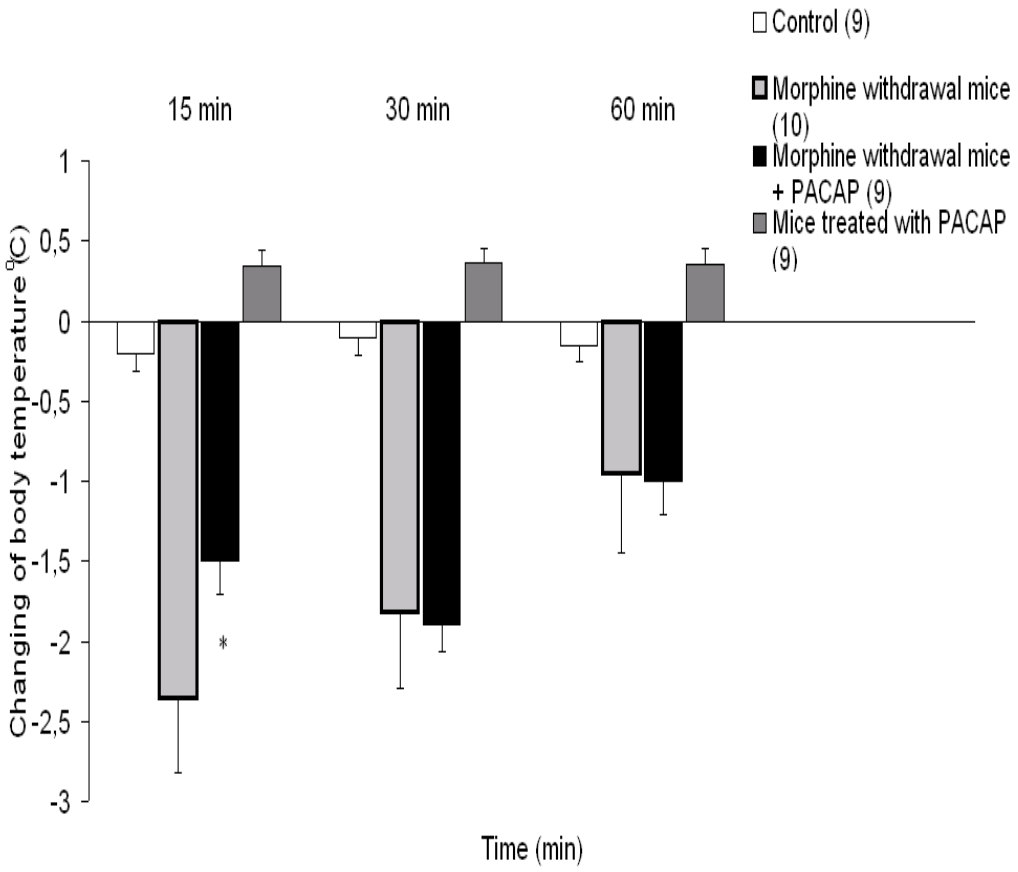


Figure 3. The effect of PACAP on hypothermia induced by naloxone in mice treated with morphine. Bars represent the decreasing of body temperature, vertical lines on the top of the bars denote S.E.M., *: $p < 0.05$ vs. mice given morphine and naloxone. Treatment schedule was the same as was described under **Figure 2.**

PACAP treatment alone did not influence the body weight of mice upon withdrawal (data not shown).

3.2 The effects of PACAP on EPM behavior

Both doses of PACAP increased the %OAT, but no significant difference was observed. There was a slight increase upon the higher dose of PACAP compared to control mice [$F_{(2,27)} = 3.63$, $P < 0,0614$]. PACAP had no effect on total activity (data not shown).

3.3 The effect of naloxone on EPM behavior in mice treated with morphine

Naloxone (0.2 mg/kg, s.c.) administration in mice treated with morphine significantly increased the %OAT compared to the control mice and mice treated with morphine [$F_{(3,42)} = 3,97$, $P < 0,0146$]. Naloxone also significantly enhanced the %OAE compared to the control mice and mice receiving 0.1 mg/kg naloxone [$F_{(3,42)} = 4,18$, $P < 0,0117$]. Naloxone (0.1 mg/kg, s.c.) had no significant effect on withdrawal behavior compared to the mice treated with morphine (**Fig. 4**). (Tukey post-hoc)

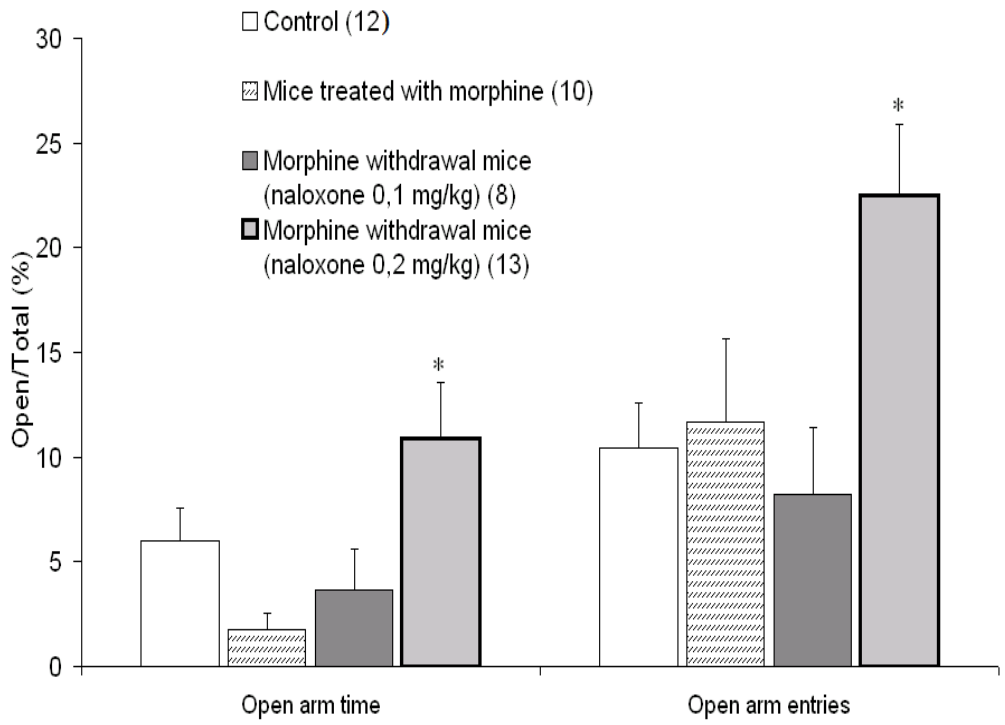


Figure 4. The effect of naloxone on EPM behavior in mice treated with morphine. Bars represent the open-arm time/total time rate and the number of open arm entries/total entries rate, vertical lines on the top of the bars denote S.E.M., *: $p < 0.05$ vs. control mice and mice treated with morphine.

Mice received the following treatments:

- Control mice: Twice daily injections of Sal, s.c. (09.00 and 17.00 h.) for 3 consecutive days. On the test day, mice given: Sal, s.c. (09.00 h) and Sal, s.c. (10.55 h).
- Mice treated with morphine: Twice daily injections of graded doses of Mor, s.c. (09.00 and 17.00 h.) for 3 consecutive days. On the test day, mice given: Mor, s.c. (09.00 h) and Sal, s.c., (10.55 h).
- Morphine withdrawal mice (Nal 0,1 mg/kg): Twice daily injections of graded doses of Mor, s.c. (09.00 and 17.00 h.) for 3 consecutive days. On the test day, mice given: Mor, s.c. (09.00 h) and Nal, s.c., (10.55 h).

- Morphine withdrawal mice (Nal 0,2 mg/kg): Twice daily injections of graded doses of Mor, s.c. (09.00 and 17.00 h.) for 3 consecutive days. On the test day, mice given: Mor, s.c. (09.00 h) and Nal, s.c., (10.55 h).

3.4 The effect of naloxone and PACAP on EPM behavior in mice treated with morphine

In combination with PACAP (for treatment details see Table 3), naloxone administration in mice treated with morphine again significantly increased %OAT compared to the control mice and mice treated with morphine [$F_{(3,35)}$ = 5,32, P < 0,0037]. However, PACAP had no significant effect on EPM compared to morphine withdrawal group (**Figure 6**). (Tukey post-hoc)

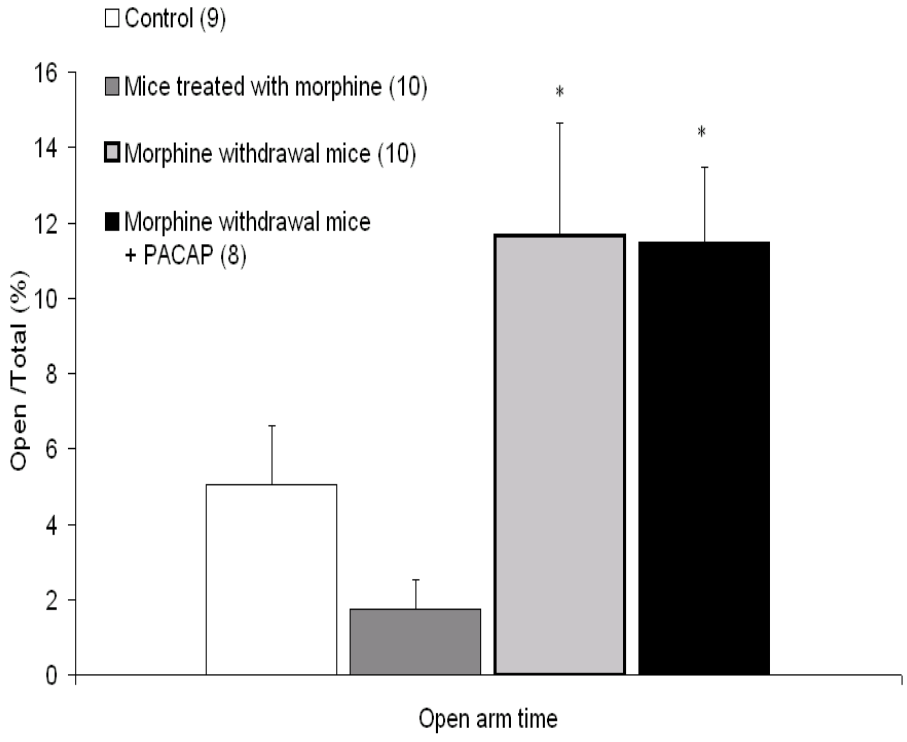


Figure 5. The effect of naloxone and PACAP on EPM behavior in mice treated with morphine. Bars represent the open-arm time/total time rate, vertical lines on the top of the bars denote S.E.M., *: p < 0.05 vs. control mice and mice treated with morphine.

Mice received the following treatments:

- Control mice: Twice daily injections of Sal, s.c. (09.00 and 17.00 h.) and once daily injection aCSF, i.c.v. (09.30.) for 3 consecutive days. On the test day, mice given: Sal, s.c. (09.00 h), aCSF, i.c.v. (10.30 h) and Sal, s.c. (10.55 h).

- Mice treated with morphine: Twice daily injections of graded doses of Mor, s.c. (09.00 and 17.00 h.) and once daily injection aCSF (09.30.), i.c.v. for 3 consecutive days. On the test day, mice given: Mor, s.c. (09.00 h), aCSF. i.c.v. (10.30 h.), and Sal, s.c., (10.55 h).

- Morphine withdrawal mice: Twice daily injections of graded doses of Mor, s.c. (09.00 and 17.00 h.) and once daily injection aCSF (09.30.), i.c.v. for 3 consecutive days. On the test day, mice given: Mor, s.c. (09.00 h), aCSF, i.c.v. (10.30 h.), and Sal, s.c., (10.55. h).

- Morphine withdrawal mice + PACAP: Twice daily injections of graded doses of Mor, s.c. (09.00 and 19.00 h.) and once daily injection PACAP (09.30.), i.c.v. for 3 consecutive days. On the test day, mice given: Mor, s.c. (09.00 h), PACAP, i.c.v. (10.30 h.), and Sal, s.c., (10.55. h).

Total motor activity was reduced in mice treated with morphine, a response that was not altered in mice treated with naloxone. However, PACAP had no significant effect on total activity compared to morphine withdrawal group (**Figure 6**). (Tukey post-hoc)

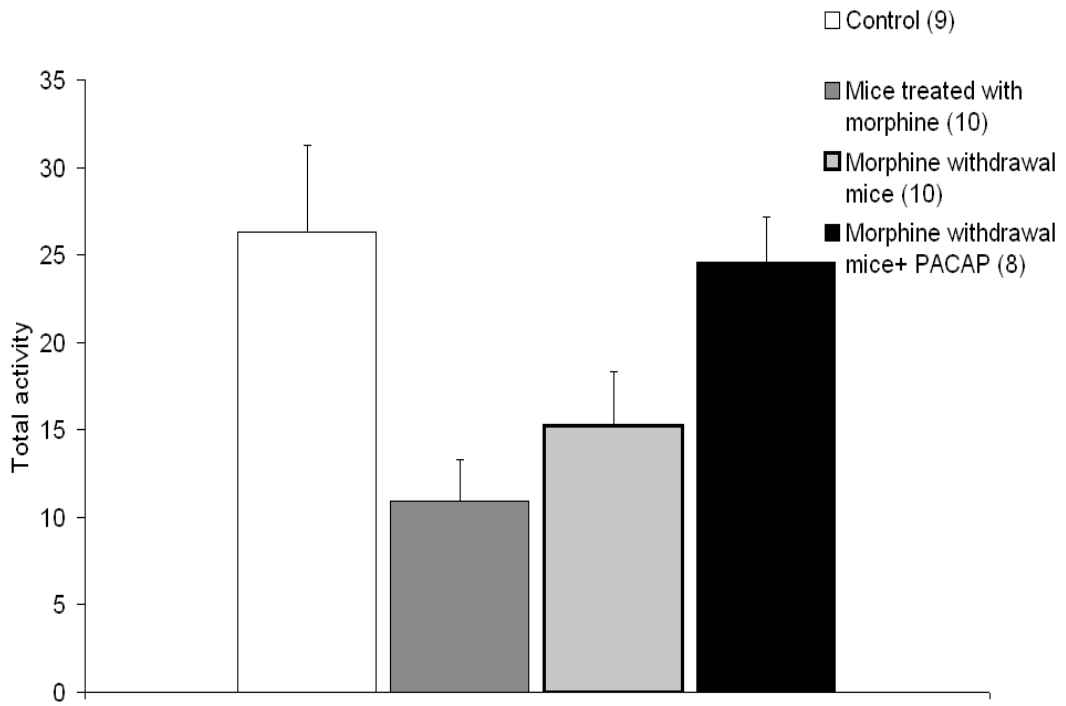


Figure 6. Bars represent the total activity; vertical lines on the top of the bars denote S.E.M. Treatment schedule was the same as was described under **Figure 5.**

3.5 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 (**Figure 7A, B)** 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, $P < 0,227$]. (Sidak post-hoc)

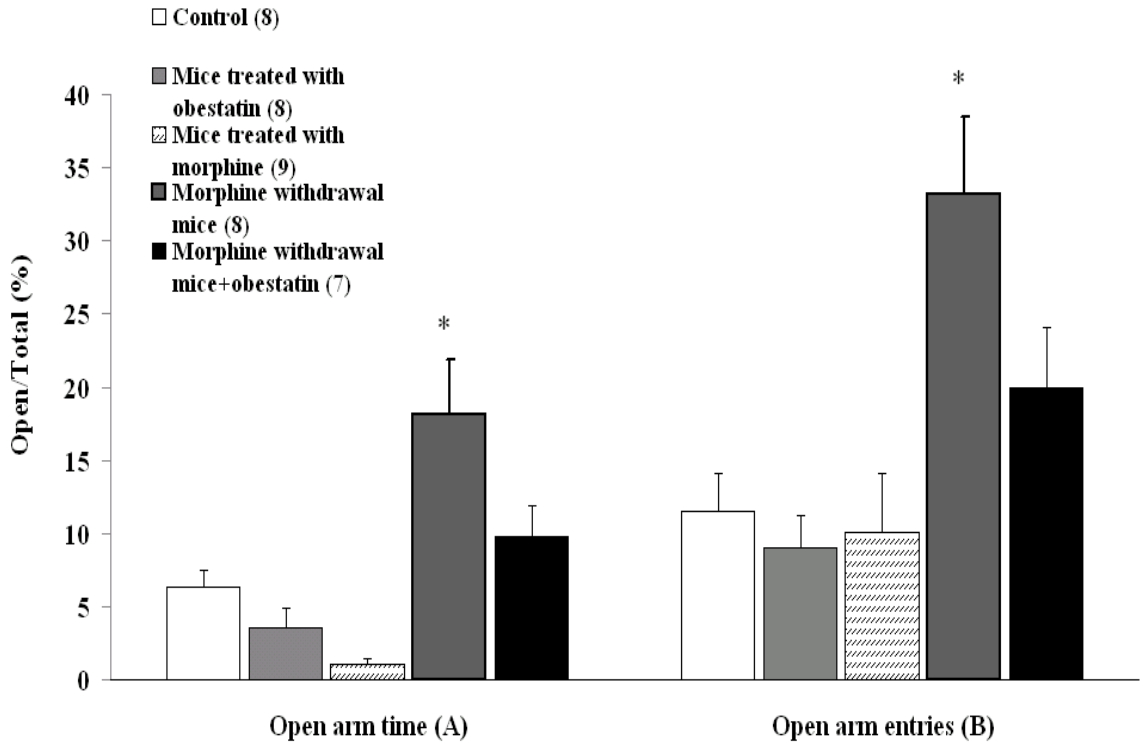


Figure 7. Bars represent the %OAT (**Fig. 7A**) and the %OAE (**Fig. 7B**), vertical lines on the top of the bars denote S.E. M. A *: $p<0.05$ vs. control mice, mice treated with morphine and mice treated with obestatin. B *: $p<0.05$ vs. control mice, mice treated with morphine and mice treated with obestatin. Naloxone caused a significant increase in both parameters in morphine treated mice compared with control mice and mice treated with morphine [$F_{(4,38)} = 11{,}01$, $P < 0{,}002$].

Mice received the following treatment:

- Control mice: Twice daily injections of Sal, s.c. (08.00 and 16.00 h.) and once daily injection aCSF, i.c.v. (08.15 h) for 3 consecutive days. On the test day, mice given: Sal, s.c. (08.00 h), aCSF, i.c.v. (09.45 h) and Sal, s.c. (9.55 h).

- Mice treated with obestatin: Twice daily injections of Sal, s.c. (08.00 and 16.00 h.) and once daily injection obestatin, i.c.v. (08.15 h) for 3 consecutive days. On the test day, mice given: Sal, s.c. (08.00 h), obestatin, i.c.v. (09.45 h) and Sal, s.c. (9.55 h).

- Mice treated with morphine: Twice daily injections of graded doses of Mor, s.c. (08.00 and 16.00 h) and once daily injection aCSF (08.15 h), i.c.v. for 3 consecutive days. On the test day, mice given: Mor, s.c. (08.00 h), aCSF. i.c.v. (09.45 h and Sal, s.c., (9.55 h).

- Morphine withdrawal mice: Twice daily injections of graded doses of Mor, s.c. (08.00 and 16.00 h.) and once daily injection aCSF (08.15 h), i.c.v. for 3 consecutive days. On the test day, mice given: Mor, s.c. (08.00 h), aCSF, i.c.v. (09.45. h.), and Nal, s.c., (9.55. h).

- Morphine withdrawal mice + obestatin: Twice daily injections of graded doses of Mor, s.c. (08.00 and 16.00 h) and once daily injection obestatin (08.15 h), i.c.v. for 3 consecutive days. On the test day, mice given: Mor, s.c. (08.00 h), obestatin, i.c.v. (09.45. h), and Nal, s.c., (9.55. h).

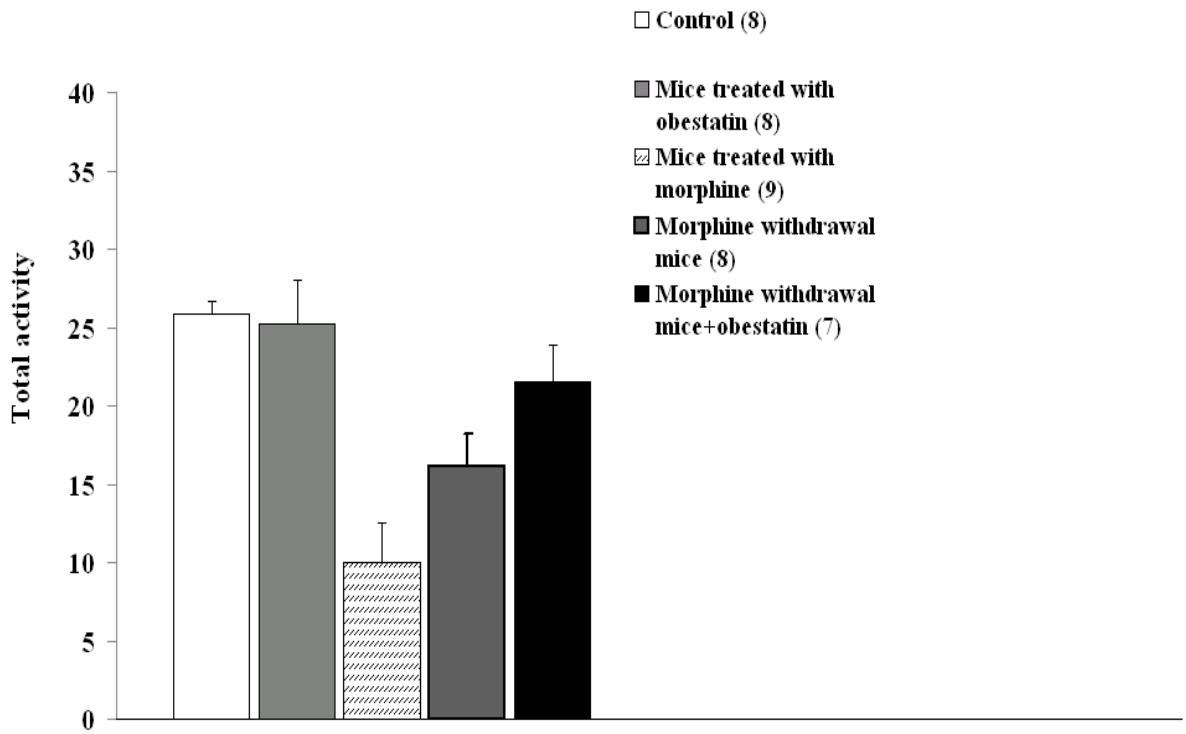


Figure 8. The effect of naloxone and obestatin on total activity in mice treated with morphine. Morphine withdrawal mice receiving obestatin did not show significant changes in

total activity compared to morphine withdrawal mice [$F_{(4,38)}$ =9,243, *P < 0,682*].Bars represent the total activity; vertical lines on the top of the bars denote S.E. M. Treatment schedule was the same as was described under **Figure 7.**

3.6 The effect of graded doses of acute obestatin on OF behavior in mice

The 1.5 µg/2µl dose of obestatin had a moderate decreasing effect on the percentage of time spent in the center compared to control mice so this dose of obestatin was selected for the following experiments (data not shown).

3.7 The effect of naloxone on OF behaviors in mice treated with obestatin

Naloxone alone had no effect on the percentage of time spent in the center and ambulation distance in the center. Mice treated with naloxone and obestatin did not show any changes in these parameters (data not shown).

3.8 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*] **(Figure 9B)**. 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*] **(Figure 9A)**. 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]). (Sidak post-hoc)

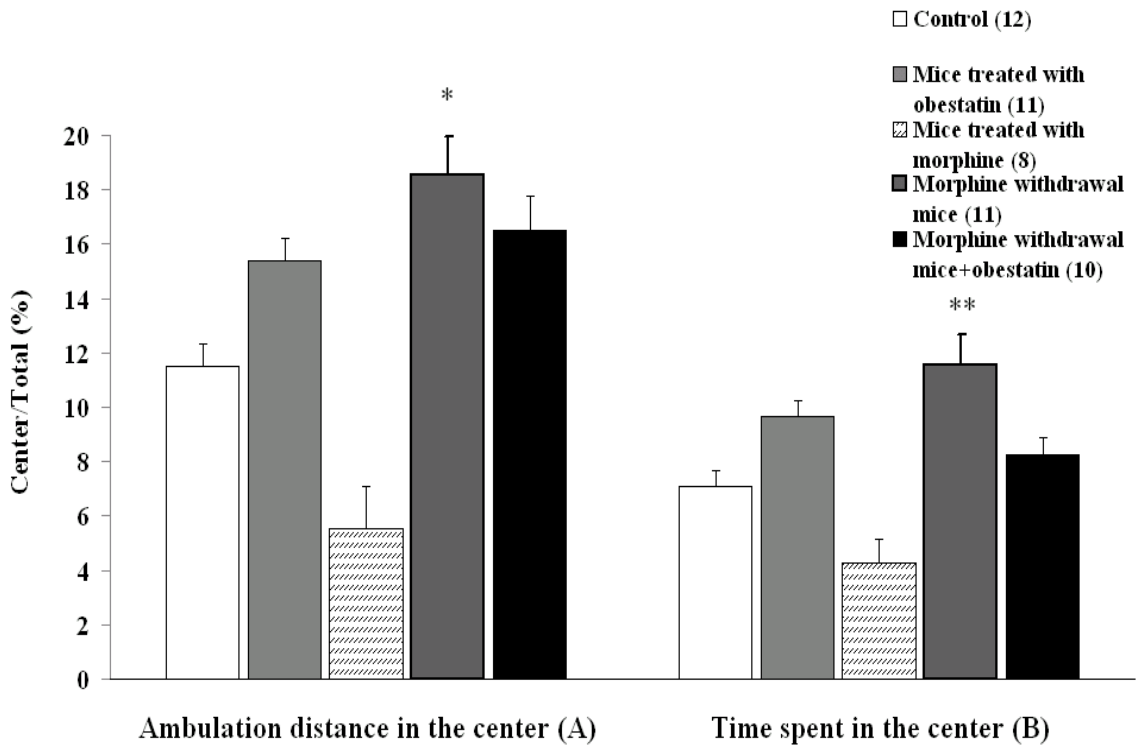


Figure 9. Bars represent the percentage of ambulation distance in the center (**Figure 9A**) and the percentage of time spent in the center (**Figure 9B**) vertical lines on the top of the bars denote S.E. M. **Figure 9A** *: $p<0.05$ vs. control mice and mice treated with morphine. **Figure 9B** **: $p<0.05$ vs. control mice, mice treated with morphine and morphine withdrawal mice receiving obestatin. Treatment schedule was the same as was described under **Figure 7.**

3.9 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 **(Figure 10).** (Sidak post-hoc)

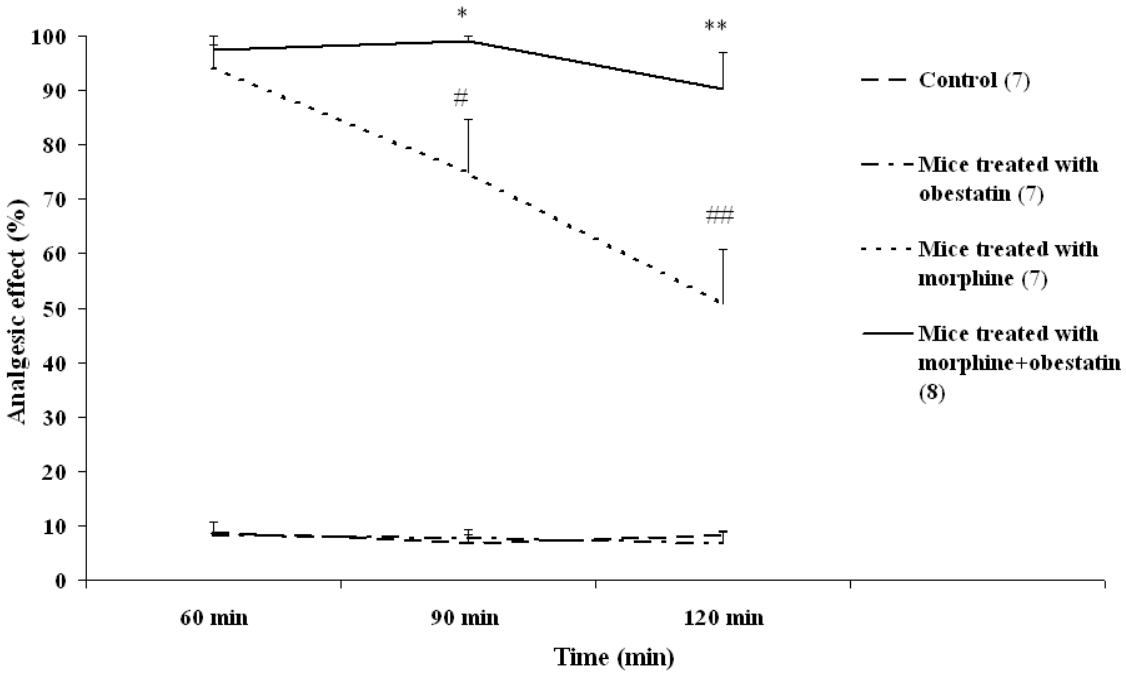


Figure 10. Curves represent the percentage of analgesic effect; vertical lines on the curves denote S.E. M. *: p <0.05 vs. mice treated with morphine and control mice at 90th min; **: p <0.05 vs. mice treated with morphine and control mice at 120th min; #: p <0.05 vs. mice treated with morphine at 60th min; ##: p<0.05 vs. mice treated with morphine at 60th min and 90th min. Mice received the following treatment:

- Control mice: Sal, s.c. (09.00 h.) and aCSF, i.c.v. (09.45 h).
- Mice treated with obestatin: Sal, s.c. (09.00 h.) and obestatin, i.c.v. (09.45 h).
- Mice treated with morphine: Mor, s.c. (09.00 h) and injection aCSF i.c.v. (09.45 h).
- Mice treated with morphine + obestatin: Mor, s.c. (09.00 h.) and obestatin, i.c.v. (09.45 h).

3.10 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 **(Figure 11).** (Sidak post-hoc)

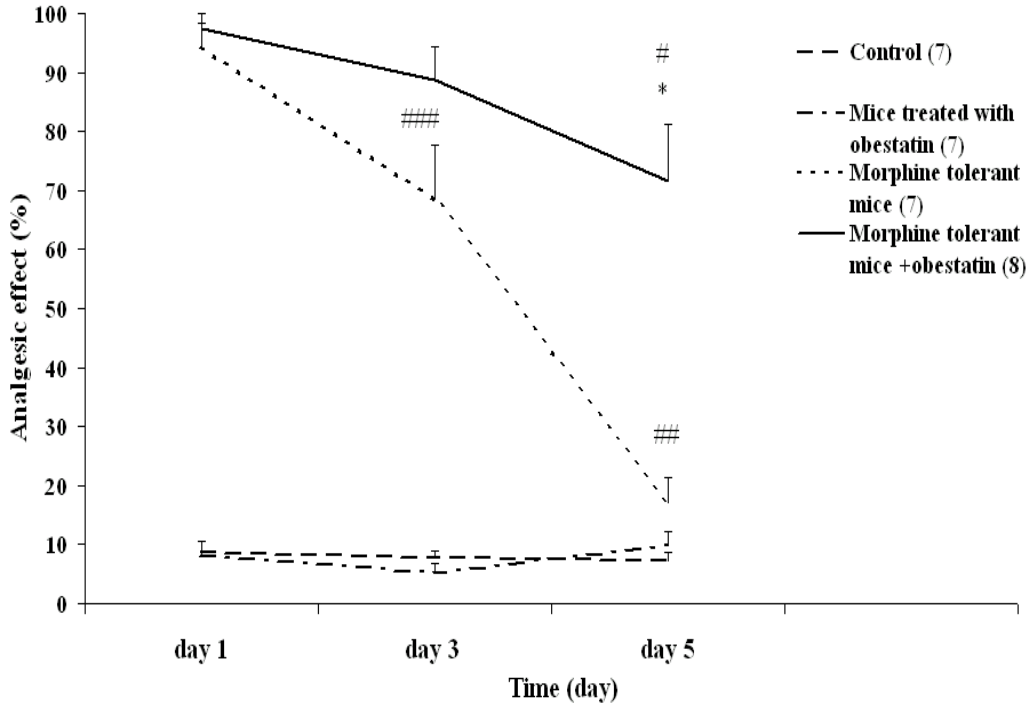


Figure 11. Curves represent the percentage of analgesic effect; vertical lines on the curves denote S.E. M., *: p <0.05 vs. morphine tolerant mice and control mice on the 5th day; #: p <0.05 vs. morphine tolerant mice receiving obestatin on the 1st day; ##: p<0.05 vs.

morphine tolerant mice on the 3rd and 1st day; ###: $p<0.05$ vs. morphine tolerant mice on 1st day.

Mice received the following treatment:

- Control mice: Twice daily injections of Sal, s.c. (09.00 and 16.00 h.) and once daily injection aCSF, i.c.v. (09.45 h) for 4 consecutive days. On the test day, mice given: Sal, s.c. (09.00 h) and aCSF, i.c.v. (09.45 h).
- Mice treated with obestatin: Twice daily injections of Sal, s.c. (09.00 and 16.00 h.) and once daily injection obestatin, i.c.v. (09.45 h) for 4 consecutive days. On the test day, mice given: Sal, s.c. (09.00 h), and obestatin, i.c.v. (09.45 h).
- Morphine tolerant mice: Twice daily injections of Mor, s.c. (09.00 and 16.00 h) and once daily injection aCSF (09.45 h), i.c.v. for 4 consecutive days. On the test day, mice given: Mor, s.c. (08.00 h), aCSF. i.c.v. (09.45 h and Sal, s.c., (9.55 h).
- Morphine tolerant mice + obestatin: Twice daily injections of Mor, s.c. (09.00 and 16.00 h.) and once daily injection obestatin (09.45 h), i.c.v. for 3 consecutive days. On the test day, mice given: Mor, s.c. (08.00 h), and obestatin, i.c.v. (09.45. h.).

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.

4.1 The effects of PACAP and obestatin on morphine withdrawal

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 might provide a new orientation for neuropeptides research.

Chronic morphine withdrawal is known to be anxiogenic in humans and, using the EPM, was proved to be anxiogenic in rats as well (*Schulteis et al., 1998; Zhang and Schulteis, 2008*). In contrast to rats, both naloxone-precipitated *(Hodgson et al., 2008*), and spontaneous opioid withdrawal (*Buckman et al., 2009*) exhibited reduced anxiety-like behaviors on EPM in mice. This increased open arm time may be regarded as escape or defensive behavior or decrease feeling of fear (*Hodgson et al., 2008*). Moreover, ERK 1/2 inhibitors can block the increased open arm-time after morphine withdrawal on EPM *(Hofford et al., 2009*).In contrast to EPM results, naloxone-precipitated morphine withdrawal caused a rise in plasma and brain corticosterone levels (*Hodgson et al., 2008*). It is known that morphine treatment alone and naloxone precipitated morphine withdrawal increase the ERK 1/2 phosphyration in mice *(Li et al., 2010; Park et al., 2012*). In addition, according to in vitro studies, obestatin can stimulate ERK1/2 phosphorylation (see references in section 1.2.6.).

Morphine indirectly decreases the intracellular level of cAMP due to inhibition of the enzyme adenylate cyclase (*Beitner et al., 1989; Kuriyama et al., 1978*), but PACAP increases the activity of adenylate cyclase, thus increasing the intracellular level of cAMP (*Absood et al., 1992*). In contrast to the acute morphine treatment, withdrawal from chronic morphine significantly upregulates the mRNA level of adenylate cyclase in the nucleus raphe magnus, which causes an increase in cAMP level and hyperalgesia *(Bie et al., 2005)*. PACAP alone caused hyperthermia in a dose dependent manner in rat *(Pataki et al., 2000)* with 1000 ng induced the largest elevation in body temperature. The hyperthermic effect of PACAP may be mediated via a cyclooxygenase-involved pathway, and it suggests that PACAP may play an important role in thermoregulation (*Pataki et al., 2003)*. Morphine was able to induce either

hyperthermia (through DOR and KOR opioid receptors) or hypothermia (through l-opioid receptors) depending on the dose administrated, but naloxone- precipitated morphine withdrawal evoked hypothermia *(Wang et al., 2008)*. In our study, we found that naloxone induced hypothermia in mice treated with morphine was decreased by PACAP (**Figure 3**).

Naloxone (0.2 mg/kg, s.c.) administration in mice treated with morphine significantly increased the %OAT compared to the control mice and the mice treated with morphine. Moreover, naloxone significantly increased the %OAE compared with control mice and mice received 0.1 mg/kg naloxone (**Figure 4**). We also examined the effect of chronic PACAP administration on naloxone induced morphine withdrawal behavior using EPM. In other experiments, acute administration of PACAP at dose dependent manner influenced morphine-induced locomotion: at lower doses (0.03 and 0.3 μg) increased, at higher dose inhibited (1 μg) morphine-induced locomotion *(Marquez et al., 2009)*. According to another study, single i.c.v. doses of 500 ng and 1 μg PACAP had locomotor stimulating effect in open field test, 30 min post-treatment in rat (*Adamik and Telegdy, 2004*). Therefore, in our experiments 30 min after last PACAP injection (500 ng) the EPM test was started. After naloxone-precipitated withdrawal, PACAP had no significant effect on the total motor activity compared to morphine withdrawal mice (**Figure 6**). A recent study suggested that chronic stress (footshock, forced swim, oscillation stress, etc.) increases PACAP mRNA expression in the bed nucleus of the stria terminalis and PACAP is anxiogenic (*Hammack et al., 2009*). We could not confirm these results; PACAP alone increased the open arm time/ total time rate and had no effect on total activity. Chronic PACAP treatment did not influence significantly the open arm time/ total time rate in mice undergoing naloxone-precipitated morphine withdrawal. These results allude to anxiolytic effect of PACAP, but differences were not significant compared to aCSF treated control mice ($P < 0.0614$). Jump latency is an accepted method to report the somatic signs of withdrawal (*Babarczy et al., 1996; Miller et al., 1983*). 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 **(Figure 2)**. A previous study carried out in our laboratory showed a similar result using morphine pellet implantation (*Mácsai et al., 2002*). Although withdrawal jumping is a somatic withdrawal symptom in mice and rats, perhaps this is a similar escape behavior, which we experienced on EPM tests. PACAP can inhibit spinal dynorphin release; and PACAP6-38, a specific PAC1 receptor inhibitor, reverses this effect in rat (*Liu et al., 2011*).

PACAP may exert its aversive effects on morphine-induced behavioral changes via mediation of dynorphin release.

To our knowledge, there are no *in vivo* studies which examined the effects of obestatin on morphine withdrawal in rodents. After chronic morphine treatment naloxone significantly increased the %OAT and %OAE compared to control mice and mice treated with morphine in the EPM (**Figure 7**). Our result supports the previous findings described by (*Hodgson et al., 2008*). The same experimental protocol was used in the OF test. Mice undergoing mild withdrawal spent significantly more time and travelled significantly more distance in the center of the open field compared to the control mice and mice treated with morphine (**Figure 9)**. To our knowledge this is the first study which has confirmed this effect of naloxone-precipitated mild morphine withdrawal in the open field test in mice. In line with literature, naloxone alone did not alter the behavior of mice in our experiments (*Hodgson et al., 2008; Ribeiro et al., 1998)*. We injected obestatin obestatin 15 minutes prior to test in all experiment due to our dose-response data and rapid degredation of obestatin *(Pan et al., 2006*). Obestatin showed maximal levels of ERK1/2 phosphorylation after 15 min of obestatin treatment in vitro (*Pazos et al., 2009*). In accordance with literature (*Carlini et al., 2007*), obestatin alone had no effect on total activity compared control mice (**Figure 8**). Morphine withdrawal mice receiving obestatin also showed no significant changes in total activity compared to morphine withdrawal mice (**Figure 8**). Chronic administration of obestatin alone had no significant effect on EPM (**Figure 7**) and OF parameters (**Figure 9**), so we cannot support the results of mentioned study in which obestatin caused a significant increase in the time spent in open arm in rats *(Carlini et al., 2007).* This contradiction alludes to the species-dependent impact of obestatin, although the amino acid-sequence of rat and mouse obestatin is completely same. Obestatin displayed an inhibitory effect on %OAT in EPM **(Figure 7)** and the time spent and ambulation distance in the center of the OF undergoing withdrawal **(Figure 9)**. 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.

4.2 The effects of obestatin on the acute and chronic analgesic actions of morphine

A previous study done by our research group demonstrated that PACAP diminished the acute analgesic effect of morphine and enhanced the analgesic tolerance to morphine *(Mácsai et al. 2002).* Due to these results, in this Ph.D. thesis work the effects of PACAP on analgesic actions of morphine were not examined.

The reduction of analgesic effect of the single injection of morphine **(Figure 10)** and the analgesic tolerance to morphine **(Figure 11)** were confirmed using tail-flick assay. In our experiments, obestatin alone had no significant analgesic effect. In tail-flick we also recorded that obestatin significantly prolonged the analgesic effect of acute morphine 90 and 120 min after morphine treatment **(Figure 10)** and prevented the analgesic tolerance to morphine in the fifth day of chronic morphine treatment **(Figure 11)**.

A recently published study claimed that i.c.v. injection of ghrelin (0.1, 1, 10 and 100 nmol/L) produced inhibition of systemic morphine (6 mg/kg, i.p.) analgesia in the tail withdrawal test in mice. Furthermore, [D-Lys3]-GHRP-6 injected 5 min before morphine failed to block the analgesic effect of morphine. In addition, this action of ghrelin was not blocked by pretreatment of i.c.v. injection of [D-Lys3]-GHRP-6. These results suggest that the anti-opioid effect of ghrelin do not interact with GHS-R1a (*Zeng et al., 2013*). Taken together, ghrelin and obestatin might exert their effects on morphine-induced analgesia in different pathways.

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. 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.

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7 References

Absood, A., Chen, D., Hakanson, R., Neuropeptides of the vasoactive intestinal peptide/helodermin/pituitary adenylate cyclase activating peptide family elevate plasma cAMP in mice: comparison with a range of other regulatory peptides, Regulatory Peptides, 1992, 40, 311–322.

Adamik, A., Telegdy, G., Involvement of different receptors in pituitary adenylate cyclase activating polypeptide induced open field activity in rats. Neuropeptides, 2004, 38, 16–20.

Advokat C., Burton P., Tyler C.B., Investigation of tolerance to chronic intrathecal morphine infusion in the rat. Physiology and Behavior, 1987, 39, 161-168.

Andrews Z.B., The extra-hypothalamic actions of ghrelin on neuronal function. Trends in Neuroscience, 2011, 34, 31–40.

Arimura A., Pituitary adenylate cyclase activating polypeptide (PACAP): discovery and current status of research. Regulatory Peptides, 1992, 37, 287–303.

Asakawa A., Inui A., Momose K., Ueno N., Fujino M.A., Kasuga M., Endomorphins have orexigenic and anxiolytic activities in mice. Neuroreport, 1998, 10, 2265-2267.

Azarov A.V., Szabó G., Telegdy G., Effects of atrial natriuretic peptide on acute and chronic effects of morphine. Pharmacology Biochemistry and Behavior, 1992, 43, 193–197.

Babarczy E., Vizi Z., Tóth G., Telegdy G., C-type natriuretic peptide can modify the acute and chronic effects of morphine. Neuropeptides, 1995, 29, 145-149.

Babarczy E., Vizi Z., Szabó G., Telegdy G., Effects of brain natriuretic peptide on effects of morphine in mice. Neuropeptides, 1996, 30, 438–442.

Beitner, D.B., Duman, R.S., Nestler, E.J., A novel action of morphine in the rat locus coeruleus: persistent decrease in adenylate cyclase. Molecular Pharmacology, 1989, 35, 559–564.

Bie B., Peng Y., Zhang Y., Pan Z.Z., CAMP-mediated mechanisms for pain sensitization during opioid withdrawal. Journal of Neuroscience, 2005, 25, 3824–3832.

Blaudszun G., Lysakowski C., Elia N., Tramer M.R., Effect of perioperative systemic alpha-2 agonists on postoperative morphine consumption and pain intensity: systematic review and meta-analysis of randomized controlled trials. Anesthesiology 2012, 116, 1312–22.

Bresciani E., Rapetti D., Dona F., Bulgarelli I., Tamiazzo L., Locatelli V., Torsello A., Obestatin inhibits feeding but does not modulate GH and corticosterone secretion in the rat. Journal of Endocrinological Investigation, 2006, 29, 16-18.

Bruijnzeel A.W., kappa-Opioid receptor signaling and brain reward function. Brain Research Rewiews, 2009, 62, 127–146.

Buckman S.G., Hodgson S.R., Hofford R.S., Eitan S., Increased elevated plus maze open-arm time in mice during spontaneous morphine withdrawal. Behavioral Brain Research, 2008, 197, 454–456.

Busch E. H.,Stedman P. M., Epidural Morphine for Postoperative Pain on Medical surgical Wards-A Clinical Review. Anesthesiology, 67, 1987, 101-103.

Camiña J.P., Campos J.F., Caminos J.E., Dieguez C., Casanueva F.F., Obestatin-mediated proliferation of human retinal pigment epithelial cells: Regulatory mechanisms. Journal of Cellular Physiology, 2007, 211, 1-9.

Carlini V.P., Monzon M.E., Varas M.M., Cragnolini A.B., Schioth H.B., Scimonelli T.N., de Barioglio S.R., Ghrelin increases anxiety-like behavior and memory retention in rats, Biochemical and Biophysical Research Communications, 2002, 299, 739-743.

Carlini V.P., Schioth H.B., de Barioglio S.R., Obestatin improves memory performance and causes anxiolytic effects in rats. Biochemical and Biophysical Research Communications, 2007, 352, 907-912.

Carvajal P., Carlini V.P., Schioth H.B., de Barioglio S.R., Salvatierra N.A., Central ghrelin increases anxiety in the open field test and impairs retention memory in a passive avoidance task in neonatal chicks. Neurobiology of Learning and Memory, 2009, 91, 402-407.

Cauvin A., Buscail L., Gourlet P., De N.P., Gossen D., Arimura A., Miyata A., Coy D.H., Robberecht P., Christophe J., The novel VIP-like hypothalamic polypeptide PACAP interacts with high affinity receptors in the human neuroblastoma cell line NB-OK. Peptides, 1990, 11, 773–777.

Chartrel N., Alvear-Perez R., Leprince J., Iturrioz X., Reaux-Le G.A., Audinot V., Chomarat P., Coge F., Nosjean O., Rodriguez M., Galizzi J.P., Boutin J.A., Vaudry H., Llorens-Cortes C., Comment on "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake". Science, 2007, 315, 766.

Christophe J., Type I receptors for PACAP (a neuropeptide even more important than VIP?). Biochimica et Biophysica Acta, 1993, 1154, 183–199.

Contet C., Filliol D., Matifas A., Kieffer B.L., Morphine-induced analgesic tolerance, locomotor sensitization and physical dependence do not require modification of mu opioid receptor, cdk5 and adenylate cyclase activity. Neuropharmacology 2008, 54, 475–486.

Cowley M.A., Smith R.G., Diano S., Tschöp M., Pronchuk N., Grove K.L., Strasburger C.J., Bidlingmaier M., Esterman M., Heiman M.L., Garcia-Segura L.M., Nillni E.A., Mendez P., Low M.J., Sotonyi P., Friedman J.M., Liu H., Pinto S., Colmers W.F., Cone R.D., Horvath T.L., The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron, 2003, 37, 649–661.

D'Amour F.E., Smith D.L. (1941), A method for determining loss of pain sensation, Journal of Pharmacology and Experimental Therapeutics, 1941, 72, 74–79.

Dennis S.G., Melzack R., Gutman S., Boucher F., Pain modulation by adrenergic agents and morphine as measured by three pain tests. Life Sciences, 1980, 26, 1247-1259.

Dickson S.L., Egecioglu E., Landgren S., Skibicka K.P., Engel J.A., Jerlhag, E., The role of the central ghrelin system in reward from food and chemical drugs. Molecular and Cellular Endocrinology, 2011, 340, 80-87.

Dietl M.M., Hof P.R., Martin J.L., Magistretti P.J., Palacios J.M., Autoradiographic analysis of the distribution of vasoactive intestinal peptide binding sites in the vertebrate central nervous system: a phylogenetic study. Brain Research 1990, 520, 14–26.

Egerod K.L., Holst B., Petersen P.S., Hansen J.B., Mulder J., Hokfelt T., Schwartz T.W., GPR39 splice variants versus antisense gene LYPD1: expression and regulation in gastrointestinal tract, endocrine pancreas, liver, and white adipose tissue. Journal of Molecular Endocrinology, 21, 2007, 1685-1698.

Favaro E., Granata R., Miceli I., Baragli A., Settanni F., Perin P.C., Ghigo E., Camussi G., Zanone M.M., The ghrelin gene products and exendin-4 promote survival of human pancreatic islet endothelial cells in hyperglycaemic conditions, through phosphoinositide 3-kinase/Akt, extracellular signal-related kinase (ERK)1/2 and cAMP/protein kinase A (PKA) signalling pathways. Diabetologia, 2012, 55, 1058–1070.

Fichna J., Janecka A., Costentin J., Do Rego J.C., The endomorphin system and its evolving neurophysiological role. Pharmacological Reviews, 2007, 59, 88-123.

Fukunaga Y., Nishida S., Inoue N., Miyamoto M., Kishioka S., Yamamoto H., Time course of morphine withdrawal and preproenkephalin gene expression in the periaqueductal gray of rats. Molecular Brain Research, 1998, 55, 221-231.

Garcia J.M., Cata J.P., Dougherty P.M., Smith R.G., Ghrelin prevents cisplatin induced mechanical hyperalgesia and cachexia. Endocrinology, 2008, 149, 455–460.

Girault J.A., Valjent E., Caboche J., Hervé D., ERK2: a logical AND gate critical for drug-induced plasticity? Current Opinion in Pharmacology, 2007, 7, 77-85.

Gourlet P., Vandermeers A., Vandermeers-Piret M.C., De N.P., Waelbroeck M., Robberecht, P., Effect of introduction of an arginine16 in VIP, PACAP and secretin on ligand affinity for the receptors. Biochimica et Biophysica Acta, 1996, 1314, 267–273.

Gourlet P., Vandermeers, A., Van R.J., De N.P., Cnudde J., Waelbroeck M., Analogues of VIP, helodermin, and PACAP discriminate between rat and human VIP1 and VIP2 receptors. Annals of the New York Academy of Sciences, 1998, 865, 247–252.

Granata R., Settanni F., Gallo D., Trovato L., Biancone L., Cantaluppi V., Nano R., Annunziata M., Campiglia P., Arnoletti E., Ghe C., Volante M., Papotti M., Muccioli G., Ghigo E., Obestatin promotes survival of pancreatic β cells and human islets and induces

expression of genes involved in the regulation of β cell mass and function. Diabetes, 2008, 57, 967–979.

Hackler L., Zadina J.E, Ge L.J., Kastin A.J. Isolation of relatively large amounts of endomorphin-1 and endomorphin-2 from human brain cortex. Peptides, 1997, 18, 1635-1639.

Hammack S.E., Cheung J., Rhodes K.M., Schutz K.C., Falls W.A., Braas K.M., May V., Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP) and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of the stria terminalis (BNST): roles for PACAP in anxiety-like behavior, Psychoneuroendocrinology, 2009, 34, 833–843.

Higgins G.A., Sellers E.M., Antagonist-precipitated opioid withdrawal in rats: evidence for dissociations between physical and motivational signs. Pharmacology Biochemistry and Behavior, 1994, 48, 1–8.

Hodgson S.R., Hofford R.S., Norris C.J., Eitan S., Increased elevated plus maze open-arm time in mice during naloxone-precipitated morphine withdrawal. Behavioural Pharmacology, 2008, 19, 805-811.

Hofford R.S., Hodgson S.R., Roberts K.W., Bryant C.D., Evans C.J., Eitan S., Extracellular signal-regulated kinase activation in the amygdala mediates elevated plus maze behavior during opioid withdrawal. Behavioural Pharmacology 2009, 20, 576-583.

Hofford R.S., Hodgson S.R., Roberts K.W., Bryant C.D., Evans C.J., Eitan S., Extracellular signal-regulated kinase activation in the amygdala mediates elevated plus maze behavior during opioid withdrawal. Behavioural Pharmacology, 2009, 20, 576-583.

Holst B., Egerod K.L., Schild E., Vickers S.P., Cheetham S., Gerlach L.O., Storjohann L., Stidsen C.E., Jones R., Beck-Sickinger A.G., Schwartz T.W., GPR39 signaling is stimulated by zinc ions but not by obestatin. Endocrinology, 2007, 148, 13-20.

Ishitobi Y., Akiyoshi J., Honda S., Ninomiya T., Kanehisa M., Tanaka Y., Tsuru J., Isogawa K., Kitamura H., Fujikura Y., Administration of antisense DNA for GPR39-1b causes anxiolytic-like responses and appetite loss in rats. Neuroscience Resesearch, 2012, 72, 257-262.

Jackson V.R., Nothacker H.P., Civelli O., GPR39 receptor expression in the mouse brain. Neuroreport, 2006, 17, 813-816.

Ji R.R., Befort K., Brenner G.J., Woolf C.J., ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity. The Journal of Neurosicence, 2002, 22, 478-485.

Joo K.M., Chung Y.H., Kim M.K., Nam R.H., Lee B.L., Lee K.H., Cha C.I., Distribution of vasoactive intestinal peptide and pituitary adenylate cyclase- activating polypeptide receptors (VPAC1, VPAC2, and PAC1 receptor) in the rat brain. Journal of Comparative Neurology, 2004, 476, 388–413.

Kojima M., Hosoda H., Date Y., Nakazato M., Matuso H., Kangawa K., Ghrelin is a growth hormone- releasing acylated peptide from stomach. Nature, 1999, 402, 656-660.

Koks S., Soosaar A., Voikar V., Bourin M., Vasar E., BOC-CCK-4, CCK(B)receptor agonist, antagonizes anxiolytic-like action of morphine in elevated plus-maze. Neuropeptides, 1999, 33, 63-69.

Kuriyama, K., Nakagawa, K., Naito, K., Muramatsu, M., Morphine-induced changes in cyclic AMP metabolism and protein kinase activity in the brain. Japanese Journal of Pharmacology, 1978, 28, 73–84.

Lauwers E., Landuyt B., Arckens L., Schoofs L., Luyten W., Obestatin does not activate orphan G protein-coupled receptor GPR39. Biochemical and Biophysical Research Communications, 2006, 351, 21-25.

Li C.H., Chung D., Doneen B.A., Isolation, characterization and opiate activity of β-endorphin from human pituitary glands. Biochemical and Biophysical Research Communications, 1976, 72, 1542–1547.

Li T., Hou Y., Cao W., Yan C.X., Chen T., Li S.B., Naloxone-precipitated withdrawal enhances ERK phosphorylation in prefrontal association cortex and accumbens nucleus of morphine-dependent mice. Neuroscience Letters 2010, 468, 348-352.

Li T., Houc Y., Caoc W., Yana C., Chena T., Sheng-bing., Naloxone precipitated withdrawal enhances ERK phosphorylation in prefrontal association cortex and accumbens nucleus of morphine-dependent mice, Neuroscience Letters, 2010, 468, 348–352.

Lister, R.G., The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology, 1987, 92, 180–185.

Liu N.J., Schnell S.A., Schulz S., Wessendorf M.W., Gintzler A.R., Regulation of spinal dynorphin 1-17 release by endogenous pituitary adenylyl cyclase-activating polypeptide in the male rat: relevance of excitation via disinhibition. The Journal of Pharmacology and Experimental Therapeutics, 2011, 336, 328-335.

Mácsai M., Szabó G., Telegdy G., Vasoactive intestinal polypeptide induces analgesia and impairs the antinociceptive effect of morphine in mice. Neuropeptides, 1996, 32, 557-562.

Mácsai M., Pataki I., Tóth G., Szabó G., The effects of pituitary adenylate cyclase-activating polypeptide on acute and chronic morphine actions in mice. Regulatory Peptides, 2002, 109, 57–62.

Mamiya T., Noda Y., Ren X., Nagai T., Takeshima H., Ukai M., Nabeshima T., Morphine tolerance and dependence in the nociceptin receptor knockout mice. Journal of Neural Transmission, 2001, 108, 1349-1361.

Marquez P., Bebawy D., Lelievre V., Coute A.C., Evans C.J., Waschek J.A., Lutfy K., The role of endogenous PACAP in motor stimulation and conditioned place preference induced by morphine in mice. Psychopharmacology, 2009, 204, 457–463.

Matthes H.W., Maldonado R., Simonin F., Valverde O., Slowe S., Kitchen I., Befort K., Dierich A., Le Meur M., Dollé P., Tzavara E., Hanoune J., Roques B.P., Kieffer B.L., Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. Nature, 1996, 383, 819-823.

Miller J.M., Poulsen-Burke J., Cochin J., Latency to first naloxone-induced jump as a measure of precipitated abstinence intensity in morphine-dependent mice and the interaction of set on this test, Life Sciences. 1983, 32, 2205–2213.

Minami M., Satoh M., Molecular biology of the opioid receptors: structures, functions and distributions. Neuroscience Research, 1995, 23, 121- 145.

Miyata A., Jiang L., Dahl R.D., Kitada C., Kubo K., Fujino M., Minamino N., Arimura A., Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary

adenylate cyclase activating polypeptide with 38 residues (PACAP38). Biochemical and Biophysical Research Communications, 199, 170, 643–648.

Mollereau C., Mouledous L., Tissue distribution of the opioid receptor-like (ORL1) receptor. Peptides, 2000, 21, 907–917.

Mollereau C., Parmentier M., Mailleux P., Butour J.L., Moisand C., Chalon P., Caput D., Vassart G., Meunier J.C., ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. FEBS Letters, 1994, 341, 33-38.

Motta V., Penha K., Brandao M.L., Effects of microinjections of mu and kappa receptor agonists into the dorsal periaqueductal gray of rats submitted to the plus maze test. Psychopharmacology, 1995, 120, 470–474.

Murányi M., Resat C., Kékesi O., Birkás E.,1 Fábián G.,1 Bozó B., Zentai A., Tóth G., Kicsi E.G., Mácsai M., Dochnal R., Szabó G., Szücs M., Ligand-specific regulation of the endogenous mu-opioid receptor by chronic treatment with mu-opioid peptide agonists. BioMed Research International, 2013, http://dx.doi.org/10.1155/2013/501086

Narita M., Mizoguchi H., Oji D.E., Narita M., Dun N.J., Hwang B.H., Nagase H., Tseng L.F., Identification of the G-protein-coupled ORL1 receptor in the mouse spinal cord by [35S]-GTPgammaS binding and immunohistochemistry. British Journal of Pharmacology, 1999, 128, 1300-1306.

Nevo I., Avidor-Reiss T., Levy R., Bayewitch M., Heldman E., Vogel Z., Regulation of adenylyl cyclase isozymes on acute and chronic activation of inhibitory receptors. Molecular Pharmacology, 1998, 54, 419–426.

Ninan I., Kulkarni S.K., Ketanserin reverses dizocilpine-supression of morphine dependence but not tolerance in mice. Brain Research, 2000, 876, 215-219.

Online Drug Databese, National Institute of Pharmacy, http://www.ogyi.hu/drug_database/, 13.05.2014.

Pan W., Tu H., Kastin A.J., Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin, Peptides. 2006, 27, 911-916.

Park S.H., Sim Y.B., Kang Y.J., Kim C.H., Kwon M..S, Suh H.W., The differential profiles of withdrawal symptoms induced by morphine and beta-endorphin administered intracerebroventricularly in mice. Neuroscience, 2012, 218, 216–325.

Pataki I., Adamik A., Jászberényi M., Mácsai M., Telegdy G., Pituitary adenylate cyclase-activating polypeptide induces hyperthermia in the rat. Neuropharmacology, 2000, 39, 1303–1308.

Pataki I., Adamik A., Jászberényi M., Mácsai M., Telegdy G., Involvement of transmitters in pituitary adenylate cyclase-activating polypeptide-induced hyperthermia. Regulatory Peptides, 2003, 115, 187–193.

Pazos Y., Alvarez C.J., Camina J.P., Al-Massadi O., Seoane L.M., Casanueva F.F., Role of obestatin on growth hormone secretion. Biochemical and Biophysical Research Communications, 2009, 390, 1377–1381.

Pazos Y., Alvarez C.J., Camina J.P., Al-Massadi O., Seoane L.M., Casanueva F.F., Role of obestatin on growth hormone secretion. Biochemical Biophysical Research Communication, 2009, 390, 1377–1381.

Pert C. B., Snyder S. H., Opiate receptor: demonstration in nervous tissue. Science, 1973, 179, 1011-1014.

Polakiewicz R.D., Schiefer S.M., Dorner L.F, Kansra V., Comb M.J., A Mitogen-activated Protein Kinase Pathway Is Required for μ-Opioid Receptor Desensitization. The Journal of Biological Chemistry, 1998, 273, 12402-12406.

Randall-Thompson J.F., Pescatore K.A., Unterwald E.M., A role for delta opioid receptors in the central nucleus of the amygdala in anxiety-like behaviors. Psychopharmacology, 2010, 212, 585-595.

Ranjbar-Slamloo Y., Azizi H., Fathollahi Y., Semnanian S., Orexin receptor type-1 antagonist SB-334867 inhibits the development of morphine analgesic tolerance in rats. Peptides, 2012, 35, 56-59.

Rawls S.M., Zielinski M., Patel H., Sacavage S., Baron D.A., Patel D., Beta-lactam antibiotic reduces morphine analgesic tolerance in rats through GLT-1 transporter activation. Durg and Alcohol Dependence, 2010, 107, 261-263.

Ribeiro S.J, De Lima T.C., Naloxone-induced changes in tachykinin NK3 receptor modu.lation of experimental anxiety in mice. Neuroscience Letters, 1998, 258, 155-158.

Rotzinger S., Lovejoy D.A., Tan L.A., Behavioral effects of neuropeptides in rodent models of depression and anxiety. Peptides, 2010, 4, 736-756.

Sándor K., Kormos V., Botz B., Imreh A., Bölcskei K., Gaszner B., Markovics A., Szolcsányi J., Shintani N., Hashimoto H., Baba A., Reglodi D., Helyes Z., Impaired nocifensive behaviours and mechanical hyperalgesia, but enhanced thermal allodynia in pituitary adenylate cyclase-activating polypeptide deficient mice. Neuropeptides, 2010, 44, 363–371.

Schiff P. L. Jr., Opium and its alkaloids. Am. J. Pharm. Educ, 66, 2002, 186-194.

Schulteis G., Markou A., Gold L.H., Stinus L., Koob G.F., Relative sensitivity to naloxone of multiple indices of opiate withdrawal: a quantitative dose- response analysis. Journal of Pharmacology and Experimental Therapeutics, 1994, 271, 1391–1398.

Schulteis G., Yackey M., Risbrough V., Koob G.F., Anxiogenic-like effects of spontaneous and naloxone-precipitated opiate withdrawal in the elevated plus-maze. Pharmacology Biochemistry and Behavior, 1998, 60, 727–731.

Schwarzer C., 30 years of dynorphins--new insights on their functions in neuropsychiatric diseases. Pharmacology & Therapeutics, 2009, 123, 353-370.

Sharma S.K, Nirenberg M., Klee W.A., Morphine receptors as regulators of adenylate cyclase activity. Proceedings of the National Academy of Sciences USA, 1975, 72, 590-594.

Sibilia V., Lattuada N., Rapetti D., Pagani F., Vincenza D., Bulgarelli I., Locatelli V., Guidobono F., Netti C., Ghrelin inhibits inflammatory pain in rats: involvement of the opioid system. Neuropharmacology, 2006, 51, 497–505.

Skibicka K.P., Dickson S.L., Ghrelin and food reward: The story of potential underlying substrates. Peptides, 2011, 32, 2265–2273.

Snyder S.H., Pasternak G.W., Historical review: Opioid receptors. Trends Pharmacol Sci, 2003, 24, 198-205.

Steeds C. E, The anatomy and physiology of pain. Surgery, 2013, 31, 49-53.

Stein C., Opioids, sensory system and chronic pain. European Journal of Pharmacology, 2013, doi: 10.1016/j.ejphar.2013.01.076.

Turchan J., Lasoń W., Budziszewska B., Przewłocka B., Effects of single and repeated morphine administration on the prodynorphin, proenkephalin and dopamine D2 receptor gene expression in the mouse brain. Neuropeptides, 1997, 31, 24-28.

Valjent E., Pagès C., Hervé D., Girault J.A., Caboche J., Addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in mouse brain. European Journal of Neuroscience, 2004, 19, 1826-1836.

Vergnano A.M., Ferrini F., Salio C., Lossi L., Baratta M., Merighi A., 2008. The gas trointestinal hormone ghrelin modulates inhibitory neurotransmission in deep laminae of mouse spinal cord dorsal horn. Endocrinology, 2008, 149, 306–312.

von Zastrow M., Svingos A., Haberstock-Debic H., Evans C., Regulated endocytosis of opioid receptors: cellular mechanisms and proposed roles in physiological adaptation to opiate drugs. Current Opinion in Neurobiology, 2003, 13, 348-353.

Wang X.M., Zhou Y., Spangler R., Ho A., Han J.S., Kreek M.J., Acute intermittent morphine increases preprodynorphin and kappa opioid receptor mRNA levels in the rat brain. Molecular Brain Research, 1999, 66, 184-187.

Wang Y.Q., Guo J., Wang S.B., Fang Q., He F., Wang R., Neuropeptide FF receptors antagonist, RF9, attenuates opioid-evoked hypothermia in mice. Peptides, 2008, 29, 1183–1190.

Wei J., Zhib X., Wang X., Zenga P., Zoua T., Yanga B., Wanga J., In vivo characterization of the effects of ghrelin on the modulation of acute pain at the supraspinal level in mice. Peptides, 2013, 43, 76-82.

Zadina J.E, Hackler L., Ge L.J., Kastin A.J., A potent and selective endogenous agonist for the μ-opiate receptor. Nature, 1997, 386, 499 – 502.

Zeng P., Chen J.X., Yang B., Zhi X., Guo F.X., Sun M.L., Wang J.L., Wei J., Attenuation of systemic morphine-induced analgesia by central administration of ghrelin and related peptides in mice. Peptides, 2013, 50, 42-49.

Zhang J.V., Klein C., Ren P.G., Kass S., Donck L.V., Moechars D., Hsueh A.J.W., Response to comment on "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake". Science, 2007, 315, 766.

Zhang J.V., Ren P.G., Avsian-Kretchmer O., Luo C.W., Rauch R., Klein C., Hsueh A.J.W., Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science, 2005, 310, 996-999.

Zhang Z., Schulteis G., Withdrawal from acute morphine dependence is accompanied by increased anxiety-like behavior in the elevated plus maze. Pharmacology Biochemistry and Behavior, 2008, 89, 392-403.

Zhou C.J., Yada T., Kohno D., Kikuyama S., Suzuki R., Mizushima H., Shioda S., PACAP activates PKA, PKC and Ca(2+) signaling cascades in rat neuroepithelial cells. Peptides, 2001, 22, 1111–1117.

Zigman J.M., Jones J.E., Lee C.E., Saper, C.B., Elmquist J.K., 2006. Expression of ghrelin receptor mRNA in the rat and mouse brain. Journal of Comparative Neurology, 2006, 548, 528–548.