Research report

The effect of morphine on MC4 and CRF receptor mRNAs in the rat amygdala and attenuation of tolerance after their blockade

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Abstract

The relationships between the CRF, which enhances the proopiomelanocortin (POMC) biosynthesis, and POMC-derived peptides (opioids and melanocortins) might be a new target for rational treatment of morphine tolerance. In the present study, we investigated the effect of acute and chronic morphine administration on the level of CRF1 and melanocortin 4 receptor (MC4-R) mRNAs in the rat amygdala by quantitative real-time PCR method. Moreover, we investigated the effect of antagonists of melanocortin and CRF receptors, SHU9119 and $\alpha$-helical CRF (\textit{\alpha}h-CRF), respectively, administered bilaterally into the central nucleus of the amygdala, on morphine tolerance using tail-flick and paw withdrawal tests. Our study demonstrated that acute morphine administration decreased the level of MC4-R mRNA in the rat amygdala. This decrease was attenuated following chronic morphine administration, and mRNA level of MC4 receptors was gradually increased and, on 9th day of morphine administration, i.e. in the period when morphine tolerance already developed, the level was significantly increased in comparison with control and with the effect after single morphine dose. In contrast, morphine did not affect the CRF receptor. In behavioral study, we demonstrated that SHU9119 and \textit{\alpha}h-CRF significantly increased the antinociceptive effect of morphine, when they were injected into the amygdala prior to morphine administration in tolerant rats. We have shown for the first time the contribution of amygdalar melanocortin receptors to morphine tolerance, and we conclude that the altered melanocortin receptor function may play an important role in the development of morphine-induced tolerance. CRF and melanocortin peptides can modulate the phenomena in the same direction, in opposition to opioids. Therefore, antagonists of melanocortin receptors may be regarded as possible therapeutic modulators of morphine tolerance.

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1. Introduction

The effectiveness of morphine in the treatment of chronic pain is limited due to the development of tolerance to the analgesic effect of this drug. Many studies have been undertaken to investigate the mechanisms underlying this phenomenon. A new target for investigations into the morphine tolerance and its rational treatment might be the relationships between the CRF, which induces the proopiomelanocortin (POMC) synthesis, and POMC-derived peptides, opioids and melanocortins [9,24]. CRF has been reported to contribute to some symptoms of morphine withdrawal and relapse to its dependence [12,17,20]. CRF and melanocortin peptides ($\alpha$-MSH/ACTH) share a number of common central effects. The effects of $\alpha$-MSH may be secondary, due to CRF effects on melanocortin peptide release. CRF receptor agonists are potent stimulators of the synthesis of anterior pituitary POMC-derived peptides, primarily $\beta$-endorphin and $\alpha$-MSH [9,24], and can act on human pituitary tissue in vitro to promote the release of POMC-related peptides, and similar effect was also observed in vivo [6]. CRF may influence melanocortin and
opioid release and in this way modulate central mechanisms of analgesia [16].

CRF-containing cell bodies and fibers have been localized to the central nucleus of the amygdala, while their projections stretch to the hypothalamus and brainstem nuclei. Areas of the brain previously thought to be unrelated to pain processing, such as the limbic system have been shown to play a major role in the experience of pain in animals and humans. CRF may act on a large number of brain structures involved in pain processing. In the rat, the amygdala is a forebrain structure known to be important for the mediation of fear-like and avoidance behavior [10]. It is also critical for the activation of endogenous antinociceptive systems during exposure to certain environmental stressors. The central nucleus of the amygdala sends monosynaptic projections to the population of opioid-sensitive cells in the ventrolateral periaqueductal gray, that are known to be involved in nociception [11]. The central nucleus of the amygdala contains a relatively high concentration of CRF and also considerable density of μ-opioid and melanocortin (MC) receptors [4,14,18,23].

The question arises if the melanocortins, which are known as highly active peptides involved in many functions in the brain, similarly to CRF, influence the effect of chronic morphine administration, since one of their suggested functions is the modulation of nociceptive transmission. Two receptor subtypes for melanocortins, MC3 and MC4 receptors, are expressed within the brain [21]. Highly selective MC4 receptor agonist, administered centrally to rats, increased Fos-like immunoreactivity in the paraventricular nucleus, central nucleus of the amygdala and nucleus of the solitary tract [5].

In the present study, we investigated whether acute and chronic morphine administration influences the level of mRNA coding for MC4 and CRF1 receptors in the amygdala using quantitative real time-PCR method, which could be of a great benefit to elucidation of regulatory mechanisms involved in the behavioral response to opioid treatment. In the behavioral study, we investigated if the blockade of melanocortin and CRF receptors within the central nucleus of the amygdala modifies the action of morphine on nociceptive transmission. We studied also possible influence of MC4 receptor antagonist SHU9119, in comparison with CRF receptor antagonist α-helical-CRF (α-h-CRF), administered bilaterally into the central nucleus of the amygdala, on morphine tolerance in rats.

2. Materials and methods

2.1. Animals and surgery

Male Wistar rats (200–350 g) were housed in single cages lined with sawdust, under standard 12/12-h light/dark cycle (lights on at 08:00 h) with food and water available ad libitum. The experiments had the approval of the Local Bioethical Committee and were carried out according to the NIH Guide for the Care and Use of Laboratory Animals.

The rats were divided into groups and two types of experiments were carried out. Four groups of the separately housed rats (n = 4 each) were appropriated for biochemical tests, i.e. real-time PCR evaluation of the MC4 and CRF receptor mRNA levels in the amygdala. Animals for behavioral evaluation were anaesthetized with pentobarbital and stereotaxically (David Kopf stereotaxic table) implanted with bilateral 23 gauge, 5 mm steel guide cannulas aimed 4 mm above the central nucleus of the amygdala. The injection cannula (connected by polyethylene tubing with Hamilton syringe) was inserted into the guide cannula immediately before the injection and the injection cannula advanced to protrude 4 mm beyond the guide cannula tip, thus reaching the central nucleus of amygdala. The volume of injection was 5 μl delivered at a constant rate over a period of 2 min, and the injection cannula was removed 20 s after the completion of this procedure. Stereotoxic coordinates were based on the atlas of Paxinos and Watson [22]. With the tooth bar positioned at −3.3 mm, the coordinates were: posterior from bregma 2.5 mm, lateral ±4.2 mm and ventral 5.1 mm from the point of entry at the skull surface. Cannulas were fastened to the skull with dental cement and sealed with a stylet wire. After the surgery, animals were allowed a minimum 1 week of recovery before the experiment.

2.2. Drug administration

SHU9119 was provided by Phoenix Pharmaceuticals, USA. Alpha-helical CRF (αh-CRF) was purchased from Sigma, Germany. SHU9119 and αh-CRF were dissolved in distilled water and were injected by hand-held Hamilton syringe connected by polyethylene tubing with injection cannula. Control animals were injected with distilled water and tested according to the same time schedule as described below for the experimental groups.

After the completion of the experiment, the injections of methylene blue were made and, after 1 h, the animals were sacrificed, their brains were dissected and fixed in formalin for verification of the cannula position. The localization of the cannula was marked on the brain scheme (Paxinos and Watson [22]). The results obtained with animals with incorrect cannula position were excluded from calculations and, therefore, a number of animals in the experiment on development of tolerance was 8–10 and, after intraamygdalar injection of SHU9119 or αh-CRF, 6–8 rats per group were tested.

Morphine was injected at a dose of 10 mg/kg (i.p.) twice daily every 12 h. Morphine antinociceptive effect was estimated by tail-flick and paw withdrawal tests 15 min after the morning morphine administration. The rats were
rendered tolerant to morphine after 8 days of morphine injections (see Fig. 1).

On day 9 of the experiment, the rats were challenged with the same dose of morphine but 15 min earlier, bilateral, intraamygdalar injections of SHU9119 (0.15–1.5 μg) or αh-CRF (0.5–1 μg) were made. Antinociceptive effect was measured by tail-flick and paw withdrawal tests 15 min after morphine administration.

2.3. Apparatus and testing procedures

The tail-flick test was carried out using Analgesia Meter (Ugo Basile). A rat was gently restrained by hand and radiant heat was directed into the animal’s tail; 9-s cutoff time was employed to prevent tissue damage. The development of morphine tolerance was determined by measuring the respective test latency after drug administration. The paw withdrawal test was carried out using Paw Withdrawal Apparatus (model 33, IITC, Landing, NJ). The rats were placed in a plastic cage with glass floor and were allowed to habituate for 5 min before the experiment. The light was focused through the glass floor to the midplantar surface of the hindlimb and latency of the foot withdrawal was measured. The cutoff value was set at 20 s.

2.4. mRNA extraction and real-time PCR

Brains for biochemical evaluation of MC4 and CRF1 receptors were collected from four groups: Group 1 consisted of control rats, which were injected with distilled water for 8 days (i.p.), rats in group 2 were injected with distilled water (twice daily, i.p.) and, on day 8 of the experiment, they received a single morphine injection (10 mg/kg i.p.) and were sacrificed 15 min later. Animals chronically injected with morphine (for 8 days, 10 mg/kg i.p., twice daily) were sacrificed either 3 (Group 3) or 24 h (Group 4) after the last morphine injection. Rats from the above groups (n=4 for each group) were killed, their brains were isolated and amygdala was dissected and subjected to further analysis. Total mRNA was extracted according to Chomczynski method [7].

The iCycler iQ Real Time PCR Detection System for quantitative real-time detection of PCR products was used. For quantification of RNA targets, QIAGEN QuantiTect SYBR Green RT-PCR Kits were used, containing SYBR Green I as a fluorescent dye. Specificity of RT-PCR product was validated by constructing a melting temperature curve and the result was further confirmed by agarose gel electrophoresis. Threshold value (at least 10 times the mean standard deviation of fluorescence in all wells over the baseline cycles) for each sample was set in the exponential phase of PCR. For analyzing the data, we used standard curve method (series of template dilutions) and changes in fluorescence of discrete samples were transformed into differences in respect to control samples (non-treated animals). As a result, data were presented as percent (%) of control (mean ± S.E.M.). We used β-actin as a housekeeping gene.

The following sets of primers were used: rat β-actin RT-PCR primer sets 5'-GTT GGA TAC AGG CCA GAC TTT GTT G (25-mer), 5'-GAG GGT AGG CTG GCC TAT AGG CT (23-mer); rat MC4-R: 5'-GTA ATT GCG CCC TTC ATG TT (20-mer), 5'-TCG GGC GTT CTT TTT ATC AT (20-mer); and rat CRF-R1: 5'-ATG AGG ATG CGG ACA ATG TT (20-mer), 5'-GTG GAT GTT CGT CTG CAT TG (20-mer). All primers were synthesized by Sigma-ARK, Darmstadt, Germany.

2.5. Data analysis

The results were compared using the one-way ANOVA followed by Bonferroni’s test. The data are presented as means ± S.E.M.; p<0.05 was considered to be statistically significant, F values resulting from statistical analysis of the test results are also given.

3. Results

3.1. The development of morphine tolerance

Chronic morphine administration twice a day (10 mg/kg i.p.) resulted in development of tolerance to its antinociceptive effect. On the first day of the experiment, significant morphine antinociceptive effect was observed in tail-flick
and paw withdrawal tests as shown in Fig. 1. The latencies of the response in both tests indicate high antinociceptive effect of morphine till day 5 of the experiment, when compared to control group. Tolerance to systemic administration of morphine developed over days 6 and 7, and on day 8, latency of response was close to control values in both behavioral tests.

3.2. The effects of acute or chronic morphine administration on MC4-R and CRF-R1 mRNA level in the rat amygdala

MC4 receptor mRNA level was significantly decreased to 36% after acute morphine treatment as shown in Fig. 2A.

In contrast, chronic morphine treatment resulted in a time-dependent gradual increase in MC4 receptor mRNA in the amygdala (Fig. 2A), when compared to acute drug administration (99% vs. acute morphine and 35% vs. control group). Nine days of drug treatment significantly increased levels of MC4 receptor mRNA in this brain region, when measured on days 8 (0.86 ± 0.05, p < 0.05) and 9

Table 1

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<th>Effect of corticotrophin and melanocortin receptor antagonists, αh-CRF and SHU9119 administered alone bilaterally into the amygdala of naïve rats measured by tail-flick (TF) and paw withdrawal (PWD) tests 15 min after their injection</th>
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<td>Control</td>
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<td>PWD test</td>
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<td>15 min: 5.8 ± 0.5</td>
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Data are expressed as means of the measured test latencies in seconds ± S.E.M. for groups of four animals each.

Fig. 2. Changes in the level of mRNAs coding for: (A) MC4-R, (B) CRF-R1 and (C) control gene, β-actin in the amygdala of the rats after acute (15 min after the administration) and chronic (3 and 24 h after the last dose) morphine treatment (10 mg/kg i.p., twice daily at 12-h intervals). Data are expressed as the percent of control mRNA level ± S.E.M. for four animals in each experimental group; *p < 0.01 vs. control group, †p < 0.05 and ##p < 0.001 vs. acute morphine treatment.

Fig. 3. The effect of bilateral, intraamygdalar injections of αh-CRF (0.5 and 1 μg) on morphine tolerance (10 mg/kg i.p.) as measured by tail-flick (A) and paw withdrawal (B) tests on day 9 of morphine administration. Data are expressed as the reaction latencies in seconds ± S.E.M. for groups of six to eight animals each; #p < 0.001 vs. control group and ##p < 0.001 vs. control group chronically injected with morphine (for 9 days, twice daily, 10 mg/kg i.p.).
(1.35 ± 0.17, p < 0.001) after chronic morphine treatment ($F_{3,12} = 15.55$), in comparison with acute morphine administration and also when compared with control. The up-regulation of MC4 receptor mRNA was statistically significant in comparison with acute administration in both cases, although the effect observed on day 9 was more pronounced.

CRF-R1 mRNA was regulated in a significant manner by neither acute nor chronic morphine administration (Fig. 2B). The observed effect did not reach statistical significance at any time point tested, although a decreasing tendency was observed after acute morphine administration when compared to control group.

The mRNA level of control gene, $\beta$-actin, remained unaffected (Fig. 2C) throughout the entire testing period.

### 3.3. Effect of bilateral intraamygdalar $\alpha$-CRF micro-injection on nociceptive threshold and morphine tolerance

CRF receptor antagonist, $\alpha$-CRF administered bilaterally at a dose of 0.5 μg into the central nucleus of the amygdala of naive animals, did not affect the nociceptive threshold (Table 1).

Bilateral intraamygdalar injection of $\alpha$-CRF (0.5 and 1 μg) 15 min before the morphine injection on day 9 reversed the morphine tolerance (Fig. 3). In the tail-flick test, the results were as follows, when the data were expressed as a percent of control response: 162% ± 2.5 ($p < 0.001$) and 170% ± 3.6 ($p < 0.001$) for $\alpha$-CRF at doses of 0.5 and 1 μg, respectively ($F_{4,33} = 25.6$). In the paw withdrawal test, the results were: 112% ± 0.1 (0.5 μg of $\alpha$-CRF) and 189% ± 2.9 (1 μg of $\alpha$-CRF). The effect was significant at both tested doses in the tail-flick test (Fig. 3A); however, in paw withdrawal test ($F_{4,33} = 14.5$), it was significant ($p < 0.001$) only for the $\alpha$-CRF dose of 1 μg (Fig. 3B).

### 3.4. Effect of bilateral intraamygdalar SHU9119 micro-injection on nociceptive threshold and morphine tolerance

Melanocortin receptor antagonist, SHU9119 (0.15 μg) administered bilaterally into the amygdala of naive animals did not cause any effect itself (Table 1).

In the rats tolerant to morphine, the blockade of brain melanocortin receptors by SHU9119 resulted in reversion of morphine tolerance. The effect was dose-dependent in the tail-flick test, in which intraamygdalar injection of SHU9119 (0.15 and 1.5 μg) significantly potentiated the antinociceptive effect of morphine (149% ± 2.5, $p < 0.001$ and 181% ± 3.6, $p < 0.001$, respectively; $F_{4,33} = 28$) in rats tolerant to its analgesic effects (Fig. 4A). The morphine antinociception estimated in the paw withdrawal test in rats pretreated with SHU9119 at the doses of 0.15 and 1.5 μg was enhanced (113% ± 0.4 and 151% ± 2.0, respectively). In the paw withdrawal test ($F_{4,33} = 9.8$), the behavioral results after SHU9119 reached significance ($p < 0.05$) only at its higher dose (Fig. 4B).

### 4. Discussion

The present results show for the first time that an antagonist of MC4 receptor, administered into the rat central nucleus of the amygdala, influences morphine tolerance. Moreover, the results of the current study demonstrated that the level of MC4 receptor mRNA, decreased after single morphine administration, was gradually increased parallel to the development of morphine tolerance. These results indicate that MC4 receptor in the amygdala may be involved in the development of some aspects of morphine tolerance; however, one should bear in mind that changes in mRNA level are not always followed by alterations in the protein level.

The amygdala belongs to the structures involved in major effects of opiates, like rewarding properties. Alvaro et al. [2] demonstrated that chronic morphine administration resulted in a decreased in MC4 receptor mRNA level in the striatum and periaqueductal gray, which was accom-
panied by a concomitant decrease in melanocortin receptor level measured by quantitative radioligand binding and autoradiography [2]. McNally and Akil [19] provide evidence for a role of the amygdalar CRF in opioid dependence. Furthermore, the authors conclude that the increased expression of the CRF gene in the amygdala is a consequence of down-regulation of opiate receptor signaling. In addition to opioid and CRF receptors, the melanocortin receptors are also expressed in the amygdala [21], however, still little is known about the role of amygdalar melanocortin system in the antinociceptive effects of morphine. A role of MC4 receptor in long-term opiate actions is further supported by behavioral studies demonstrating that melanocortins antagonize various functional effects of opiate treatment, e.g. they reduce opiate opiate actions is further supported by behavioral studies effects of morphine. A role of MC4 receptor in long-term amygdalar melanocortin system in the antinociceptive [21], however, still little is known about the role of melanocortin receptors are also expressed in the amygdala signaling. In addition to opioid and CRF receptors, the melanocortin receptors are also expressed in the amygdala [21], however, still little is known about the role of melanocortin system in the antinociceptive effects of morphine. A role of MC4 receptor in long-term opiate actions is further supported by behavioral studies demonstrating that melanocortins antagonize various functional effects of opiate treatment, e.g. they reduce opiate self-administration [28], decrease opiate physical dependence [8] or there is a correlation between the relative potency of various melanocortin peptides to block tolerance and their ability to activate the MC4 receptor in vitro [13,28]. Moreover, morphine has been shown to increase plasma α-MSH level [15,29], which may be also a consequence of morphine stimulatory effect on CRF secretion. Down-regulation of melanocortin receptors may result from higher release of α-MSH on the first days of morphine administration. Therefore, intraamygdalar administration of SHU9119, may result in attenuation of α-MSH action, which in consequence may restore morphine antinociception. Furthermore, the peripheral multiple i.p. administration of α-MSH, along with repeated morphine administration attenuated the development of tolerance in mice [27], which may suggest the involvement of repeated stress in the effect. Another possibility is that the effect of melanocortins on morphine tolerance could be different at peripheral and central level. The effect of SHU9119 may also be explained by influence on intracellular signal transduction mechanisms. In contrast to morphine, the agonists of MC4 receptors activate adenylyl cyclase (AC) and cAMP formation [1,3]. The antagonist of melanocortin receptor could potentiate the inhibitory effect of morphine on cAMP pattern by antagonizing the stimulatory effect of melanocortins.

The results obtained after intraamygdalar administration of αh-CRF are in line with other animal experimental studies. In our series of experiments, the acutely administered opioid did not modulate the CRF-R1 mRNA in the rat amygdala. In contrast, 24 h after the last morphine injection, at the time when we injected SHU9119 to the amygdala, the level of MC4 receptor mRNA was higher in comparison with single injection of morphine. This finding could correlate with our in vivo experiments, where SHU9119, by blocking the amygdalar MC4 receptors, was effective in restoring morphine analgesic effect when compared to control animals tolerant to morphine. Song and Takemori [25] reported that αh-CRF (9−41), a CRF receptor antagonist, increased the morphine antinociceptive ED50 estimated by tail-flick test, when it was administered intrathecally (i.t.) in tolerant mice. The activation of the CRF receptor is involved in morphine withdrawal signs and relapse to morphine dependence. The morphine withdrawal signs, including jumping, teeth chatter, writhing, shakes, lacrimation, piloerection, irritability and diarrhea, were attenuated by pretreatment with CRF receptor antagonist, αh-CRF (10 μg i.c.v.). Pretreatment with the CRF-R1 antagonist significantly attenuated several behavioral signs of naltrexone-induced morphine withdrawal [17]. In our study, the level of mRNA coding for CRF1 receptor was not changed upon morphine administration. This is in agreement with another study, namely, Zhou et al. [30] demonstrated that neither the activity of the HPA axis nor the β-endorphin and CRF systems in the brain are related by steady-state occupancy of opioid receptors with the long-acting opioid agonist [30]. Chronic methadone, a long-acting opioid agonist, did not alter CRF mRNA in the hypothalamus, POMC and CRF-R1 mRNA in the anterior lobe and neurointermediate/posterior lobe of the pituitary. No change was found in CRF mRNA levels in the frontal cortex, olfactory bulb and amygdala.

Previously, we have demonstrated that at the level of the spinal cord, melanocortin receptor ligands antagonize the action of opioid peptides [26]. The data obtained in the present studies, which were focused on the supraspinal levels, indicate that melanocortin peptides also at this level of CNS display their antagonistic potential toward opioids. The preliminary communication of Zhou et al. [31] indicates that after s.c. administration of another MC4 receptor antagonist, HS-131, several signs of naloxone-precipitated withdrawal were significantly attenuated and that the effect of the MC4 receptor antagonist was comparable to that observed for the neurokinin NK1 receptor antagonist, GR82334, previously shown to reduce the reaction to opioid withdrawal [31].

In conclusion, we hypothesize that gradual increase in MC4 receptor expression, which follows the chronic morphine administration, may be an important adaptation contributing to chronic opiate effects in the brain. Moreover, MC4 receptor function in the amygdala has been demonstrated in vivo, since intraamygdalar injection of SHU9119 reversed morphine tolerance, in a similar manner to αh-CRF, which also increased morphine efficacy in morphine tolerant rats. In conclusion, our data demonstrated for the first time the important role of MC4 receptors located in the amygdala, and confirmed the modulatory effect of melanocortin receptor antagonist on morphine tolerance. Our data suggested also that, at least partly, the effect of CRF, which releases α-MSH in several central nervous system regions, might act behaviorally in the same direction as melanocortins. Moreover, antagonists of melanocortin receptors may be regarded as possible therapeutically modulators of morphine tolerance. In summary, these findings indicate that morphine in animal model of tolerance modulates endogenous melanocortin pathways in the amygdala.
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References