

Gene Expression Profile of Calcium/Calmodulin-Dependent Protein Kinase II α in Rat's Hippocampus during Morphine Withdrawal

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Introduction: Calcium/calmodulin-dependent protein kinase II (CaMKII) which is highly expressed in the hippocampus is known to play a pivotal role in reward-related memories and morphine dependence.

Methods: In the present study, repeated morphine injections once daily for 7 days was done to induce morphine tolerance in male Wistar rats, after which gene expression profile of α -isoform of CaMKII (CaMKII α) in the hippocampus was evaluated upon discontinuation of morphine injection over 21 days of morphine withdrawal. Control groups received saline for 7 consecutive days. For gene expression study, rats' brains were removed and the hippocampus was dissected in separate groups on days 1, 3, 7, 14, and 21 since discontinuation of morphine injection. A semi-quantitative RT-PCR method was used to evaluate the gene expression profile.

Results: Tolerance to morphine was verified by a significant decrease in morphine analgesia in a hotplate test on day 8 (one day after the final repeated morphine injections). Results showed that gene expression of CaMKII α mRNA level on day 1, 3, 7, 14 and 21 of morphine withdrawal was significantly altered as compared to the saline control group. Post hoc Tukey's test revealed a significantly enhanced CaMKII α gene expression on day 14.

Discussion: It can be concluded that CaMKII α gene expression during repeated injections of morphine is increased and this increase continues up to 14 days of withdrawal then settles at a new set point. Therefore, the strong morphine reward-related memory in morphine abstinent animals may, at least partly be attributed to, the up-regulation of CaMKII α in the hippocampus over 14 days of morphine withdrawal.

1. Introduction

Morphine has analgesic and rewarding effects, which is mediated through μ -opioid receptors (Benyamin et al., 2008; Biala & Weglinska, 2006; He, Kim & Whistler, 2009; Walwyn, Miotto & Evans, 2010). Meanwhile, repeated administration of morphine results in the development of tolerance and dependence (Benyamin et al., 2008; Silverman, 2009). The chronic use of morphine gives rise to long-lasting changes in the brain which underlie the behavioral ab-

normalities associated with morphine dependence and withdrawal syndrome (Nestler, 2002; 2004b). This form of long-term plasticity in the brain requires relative stable changes in gene expression, which may alter neurotransmission and the structure of target neurons (McClung & Nestler, 2008; Robinson & Kolb, 2004). Recent studies have shown that the altered gene expression due to morphine exposure is responsible for behavioral changes in drug addicts (Prenus, Luscar, Zhu, Badisa & Goodman, 2012; Zhu, Badisa, Palm & Goodman, 2012). It has been shown that expression of many receptors and second messengers are affected by chron-

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ic morphine treatment. Among them Ca²⁺/calmodulin-dependent protein kinase 2 (CaMKII) is of great interest (Chen et al., 2008; Colbran, 2004; Nestler, 2004b; a). However, the underlying molecular and cellular mechanisms in morphine addiction and withdrawal syndrome are yet to be fully understood.

CaMKII is a serine/threonine protein kinase with its α and β isoforms highly expressed in the central nervous system and enriched at synaptic structures especially in the hippocampus (Chen et al., 2008; Martin & Arthur, 2012; Wayman, Lee, Tokumitsu, Silva & Soderling, 2008). CaMKII regulates number of important neuronal functions especially the neurotransmitter synthesis and release, modulation of ion channel activity, synaptic plasticity, learning, memory, and gene expression (Hudmon & Schulman, 2002). The α -isoform of CaMKII (CaMKII α) plays a pivotal role in learning and memory [for review see (Wayman, Lee, Tokumitsu, Silva & Soderling, 2008)]. It has been reported that transgenic mice lacking the CaMKII α are defective in long-term potentiation (LTP) and spatial learning (Yamauchi, 2005).

In a sense, opioid tolerance and addiction is a mode of neuronal plasticity similar to learning and memory (Nestler, 2004b). Evidence has shown that CaMKII inhibitors suppress the development and formation of tolerance to morphine when selectively administered to rats' brain (Fan, Wang, Qiu, Ma & Pei, 1999). Furthermore, inhibition of CaMKII α in the locus coeruleus significantly attenuates some signs of morphine withdrawal (Navidhamidi et al., 2012). It has also been demonstrated that CaMKII activity in the NAc shell is essential for relapse in morphine-seeking behaviors (Liu, Liu, Zhang & Yu, 2012; Liu, Zhang, Liu & Yu, 2012). Investigation on the adaptive change of the level of gene expression caused by chronic morphine treatment is crucial for further understanding of mechanisms underlying morphine addiction (Ammon-Treiber & Hollt, 2005; Zhu, Badisa, Palm & Goodman, 2012). Nevertheless, changes in expression of CaMKII α in morphine withdrawal have not been carefully examined. The aim of the present study was to examine gene expression profile of CaMKII α at mRNA level during 21 days of morphine withdrawal.

2. Methods

2.1. Subjects

Male Wistar rats initially weighing 220-250 g (Pasteur Institute, Iran) were used. They were housed in groups of 4 per cage, and kept in an animal house at a constant tem-

perature (22 ± 2 °C) under a 12 h light/dark cycle (light on at 7:00 a.m.). Animals had free access to food and water except during the experiments. All animals were handled for 5 min/day during the five days adaptation period before the experiments began. All experiments were carried out during the light phase between 8:00 and 12:00. Experimental groups consisted of either eight rats in behavioral experiments or six rats in gene expression experiments. Each animal was tested only once. All procedures were performed in accordance with international guidelines for animal care and use (NIH publication #85-23, revised in 1985).

2.2. Drugs

The drug used in this study was morphine sulfate (Temad Co., Tehran, Iran). The drug was dissolved in 0.9% saline and administered 1 ml/kg through an intraperitoneal (i.p.) route.

2.3. Induction of Morphine Tolerance

Morphine tolerance was induced by daily i.p. injection of morphine (10 mg/kg) for 7 consecutive days in rats. Twenty four hour after the last injection, morphine tolerance was examined by a hotplate test of analgesia.

2.4. Antinociception Test

The temperature of hot plate was maintained at 52 ± 0.1 °C. Animals were placed in a glass square on the hotplate apparatus (Armaghan Co., Iran) to prevent escaping from the hotplate. Then, the latency for licking of the hind paws or first jump was recorded as an index for pain reaction latency. A cutoff time of 80 s was defined as complete analgesia. Eight groups of either saline- or morphine- treated animals (n=8) were used to assess morphine analgesia. Four groups of these animals as controls (naïve) received repeated injections of saline (1 ml/kg), and the other four groups received repeated injections of morphine (10 mg/kg) for seven consecutive days. One day after the final repeated injections, all four groups of animals received saline (1 ml/kg) or different doses of morphine (5, 7.5 and 10 mg/kg). In 30 min, they were submitted to the hotplate test to have their pain reaction latencies examined.

2.5. Tissue Extraction and Preparation

To examine the gene expression profile during 21 days of withdrawal, in different groups of rats (n=5), brain excision were done on on days 1, 3, 7, 14 and 21 after the final repeated injection of morphine (induction

of morphine tolerance). Assessment of CaMKII α mRNA levels in the hippocampus was performed using 80-100 mg of the intended tissue. In brief, each rat was decapitated, the whole brain was quickly removed from the skull, the hippocampus was immediately separated from the whole brain on an ice-chilled sterile surface according to a method previously described (Chiu, Lau, Lau, So & Chang, 2007). After harvesting, the tissues were immediately submerged in RNAlater RNA stabilization reagent (QIAGEN) and, incubated overnight in the reagent at 4°C, then stored at -70°C until further analysis.

2.6. Total RNA Extraction and cDNA Synthesis

Total RNA extraction was performed using a Trizol method with some modification. Each tissue sample was transferred to a new tube containing 1 ml lysis buffer (RNX+ reagent, Cinagen, Tehran, Iran). The tissue was then homogenized for 60 sec (Silent Crusher S., Heidolph, Germany), and subjected to total RNA extraction by a high pure tissue RNA extraction protocol. Total RNA was separated through a 1% agarose gel to assess quality of 28s and 18s ribosomal RNA. The concentrations of total RNA were also measured by a Biophotometer (Ependorph, Germany). Then, a semi-quantitative RT-PCR method was used to assess gene expression (Marone, Mozzetti, De Ritis, Pierelli & Scambia, 2001). RT reaction was performed using Viva 2-step RT-PCR Kit according to manufacturer's protocol (Vivantis, Malaysia). In brief, the RT reaction was performed using 0.3 μ g/ μ l of total RNA as a template, random hexamer primer and M-MuLV reverse transcriptase enzyme. The reactions were incubated at 42°C for 60 min, and then inactivated at 85°C for 5 min.

The polymerase chain reaction (PCR) was used for amplification of β -actin (as an internal standard) and CaMKII α genes. Primers for both genes were designed at exon-exon junctions with the following sequences:

β -actin forward primer, 5'-

CTGGGTATGGAATCCTGTGGC-3'; β -actin reverse primer, 5'-

CAGGAGGAGCAATGATCTTGATC-3'; CaMKII α forward primer, 5'-

AGGAGGAACTGAAGGGAG -3'; CaMKII α reverse primer, 5'-

CAGGGTCGCACATCTTCGTG -3'.

PCR was carried out in a reaction volume of 20 μ l consisting of 10 μ l PCR Master Mix (Fermentase), 2 μ l cDNA, 1 μ l upstream and downstream mix of CaMKII α primers (10 μ M), 1 μ l upstream and downstream mix of β -actin primers (10 μ M) and nuclease free water up to 20 μ l. The reaction parameters were adjusted to obtain a condition with a linear relation between the number of PCR cycles and PCR products, and with linear relation between the initial amount of cDNA template and PCR product. Therefore, thermal cycling was initiated with a first denaturation step of 95°C for 3 min, followed by 29 cycles of thermal cycling of 94°C for 30 sec (denaturation), 60°C for 30 sec (annealing), 72°C for 30 sec (extension), followed by 10 min final extension at 72°C (C1000 Thermal Cycler, Biorad, USA). The PCR products were subsequently analyzed on 2% agarose gel electrophoresis, and respective bands were quantified by a densitometry method using an Image J software. Then, ratio of band density of CaMKII α / β -actin was used as an index of gene expression.

2.7. Profiling mRNA Level of CaMKII α in the hippocampus on days 1, 3, 7, 14 and 21 days of Morphine Withdrawal in the Hippocampus

Six groups of rats (n=6 per group) were used to assess CaMKII α gene expression in the hippocampus. The first group as control received saline (1ml/kg) for 7 days, and their brains were excised one day after the final repeated injection. Other five groups received a daily injection of morphine (10 mg/kg) for 7 consecutive days, and were submitted to the brain excision on days 1, 3, 7, 14 and 21 since the final injection, respectively.

2.8. Statistical Analysis

For the hotplate experiment data, one-way ANOVA was used to determine the analgesic effect of morphine in different groups. Two-way ANOVA was used to determine the main effect of morphine tolerance (as Factor A) and group (as Factor B), and their interaction in the hotplate test. Further post hoc comparisons were made using Tukey's test. To analyze the semi-quantitative RT-PCR data, the intensity of nucleic acid bands on agarose gel electrophoresis was converted to quantitative values using an Image J program. Subsequently, the results were analyzed by one-way ANOVA to investigate overall differences between groups. Upon presence of a significant F value, Tukey's test was used to assess 'between groups' paired comparisons. P<0.05 was considered as a significant statistical level throughout.

3. Results

3.1. Antinociception Test Revealed that Repeated Administrations of Morphine for 7 Days Induced Morphine Analgesic Tolerance

Based on one-way ANOVA, morphine altered pain reaction latency in control rats which received 7 days pretreatment of repeated administrations of saline [F (3, 28) = 13.51, $P < 0.001$]. Post hoc analysis with Tukey's test showed that morphine at the dose of 10 mg/kg, significantly induced analgesia in naïve rats as revealed by an increase in pain reaction latency as compared to saline control group. One-way ANOVA also showed that morphine could not alter pain reaction latency in rats which had received pretreatment of 7 days repeated administrations of morphine (10 mg/kg) [F (3, 28) = 0.5, $P > 0.05$]. Post hoc analysis with Tukey's test showed that the analgesic effect of morphine at the dose of 10 mg/kg in rats which received repeated injections of the drug was significantly attenuated as compared to the analgesic effect of the same dose of morphine in naïve rats which received saline during the repeated injections. The later results indicated morphine-induced analgesic tolerance (Fig. 1).

3.2. Effect of Morphine Tolerance on mRNA level of CaMKII α in the Hippocampus During 21 Days of Withdrawal

Analysis of the results with one-way ANOVA revealed that gene expression of CaMKII α in the hippocampus was significantly altered during 21 days of morphine withdrawal [one-way ANOVA, F (5, 24) = 2.7, $P < 0.05$]. Post hoc analysis with Tukey's test also revealed that gene expression of CaMKII α in the hippocampus on day 14 of withdrawal was significantly increased ($P < 0.05$). Meanwhile, there were no significant changes in CaMKII α gene expression on days 1, 3, 7 and 21 of withdrawal period compared to the control saline-treated group (Fig. 2).

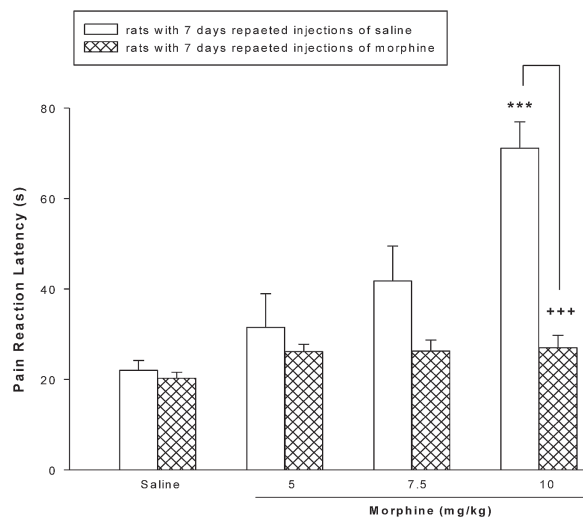
4. Discussion

The results of the present study showed that repeated injections of morphine for seven days induced morphine tolerance as revealed by a decrease in morphine analgesia in a hotplate test. This is similar to what has previously been reported (Sepehrizadeh et al., 2008a; Sepehrizadeh et al., 2008b). It has been proposed that repeated morphine injections can enhance agonist potency, hence increase receptor desensitization and promote tolerance (Bohn, Lefkowitz & Caron, 2002; Ingram, Macey, Fossum & Morgan, 2008). On the contrary, some studies

have suggested that morphine tolerance may derive from a lack of rapid desensitization, resulting in other adaptive, and potentially slowly reversible changes becoming dominant [for review see (Martini & Whistler, 2007)].

In the present study, after induction of morphine tolerance, we examined the gene expression profile of CaMKII α during 21 days of morphine withdrawal. According to the results, mRNA level of CaMKII α in the hippocampus was significantly increased on day 14 of morphine withdrawal. Our results indicated no significant difference in gene expression of CaMKII α in the hippocampus on days 1, 3, 7 and 21 of morphine withdrawal as compared to the saline control group. A return to a new set point in expression of CaMKII α after 21 days was observed. It is possible that the gene expression of CaMKII α in the hippocampus during repeated morphine injections which began to increase, would continue up to 14 days of morphine withdrawal, and then return to a new set point. Chen et al. (2008) has reported that chronic morphine treatment (20 mg/kg) for 6 days, but not a single injection of morphine, increased CaMKII α expression in the hippocampus (Chen et al., 2008). In contrast to this, we administered morphine at the dose of 10 mg/kg for 7 days, and did not observe a significant change in CaMKII α expression in the hippocampus on day 1 after the last morphine injection. A possible explanation for different results may be the difference in applied doses of morphine during repeated injections. Explanation of the variability in the hippocampal CaMKII α expression after morphine injection on days 1, 2, 3, 4, 5, 6 and 7 may be an interesting issue for which more experiments should be designed.

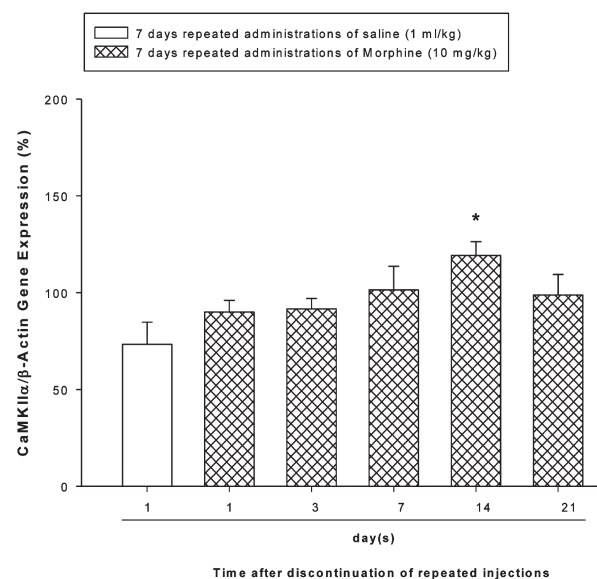
Chronic morphine administration has been shown to induce a down-regulation of mu-opioid receptors and number of receptors available (Bernstein & Welch, 1998; Lopez-Gimenez, Vilaro & Milligan, 2008; Takasaki, Nojima, Shiraki & Kuraishi, 2006; Zhu, Badisa, Palm & Goodman, 2012). Opioids have also been shown to modulate excitatory and inhibitory synaptic transmission in the hippocampus (McQuiston, 2007; Salmanzadeh, Fathollahi, Semnanian & Shafizadeh, 2003). Additionally, chronic treatment of three-week with methadone or morphine had no significant changes in phosphorylated CaMKII in the hippocampus at one hour and one week after treatment (Andersen, Klykken & Morland, 2012). It has been reported that subcutaneous implantation of morphine pellets for 6 days in mice increases the levels of CaMKII α mRNA in spinal cord tissue, which may suggest that CaMKII α expression in the spinal cord tissue might have contributed to



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Figure 1. Repeated injections of morphine induced analgesic tolerance to the drug. Eight groups of animals were used. Four groups of them received 7 days repeated administrations of saline, and 1 day after the final injection (day 8) they received saline (1 ml/kg) or one dose of morphine (5, 7.5 and 10 mg/kg) 30 min before the hotplate test. The other four groups of the animals received 7 days repeated administrations of morphine (10 mg/kg), and 1 day after the final injection (day 8) they received saline (1 ml/kg) or one dose of morphine (5, 7.5 and 10 mg/kg) 30 min before the hotplate test. Each bar represents mean±S.E.M. of pain reaction latency related to eight rats per group. Two-way ANOVA revealed significant effects of repeated injections of morphine (factor A) and groups (factor B), and their interaction [for factor A, $F(1, 56) = 17.59$, $P < 0.001$; for factor B, $[F(3, 56) = 14.16$, $P < 0.001$; for factor A * factor B, $[F(3, 56) = 10.83$, $P < 0.001$]. *** $P < 0.001$ compared to control group that received saline on the test day. +++ $P < 0.001$ compared to respective group of saline pretreated that received morphine 10 mg/kg on the test day.

the development of morphine tolerance in mice (Liang, Li & Clark, 2004). Based on the prespecified goals of the current investigation, we profiled gene expression of CaMKII α during 21 days of withdrawal, with the results showing a significant increase in CaMKII α gene expression at 14 days of withdrawal and no significant changes on days 1, 3 and 7. It is possible that CaMKII α expression in the hippocampus significantly increases after a time window (14 days) since the chronic morphine treatment ceased. In support of this result, studies using a chronic administration of morphine (20 mg/kg for 9 consecutive days) showed that this treatment did not significantly affect mRNA of CaMKII α in the spinal cord, brain stem and hippocampus one day after discontinuation of morphine treatment (Lou, Zhou, Wang & Pei, 1999). On the other hand, the results of the present



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Figure 2. Profiling the ratio of CaMKII α / β -actin mRNA expression in the hippocampus during 21 days of morphine withdrawal. Six groups of rats were used. One group of rats ($n=6$) as control group received seven days repeated injections of saline (1 ml/kg), and their brain were excised 1 day after the final injection to examine gene expression in the hippocampus. The other five groups of rats received seven days repeated injections of morphine (10 mg/kg), and their brains were excised on days 1, 3, 7, 14 and 21 after the final injection of morphine to examine gene expression profile in the hippocampus. Each bar represents mean±S.E.M. related to the ratio of CaMKII/ β -actin band density of 5 rats per group. * $P < 0.05$ compared to the saline treated control group.

study suggest that CaMKII α expression and subsequent changes in the hippocampus may contribute to behavioral signs of morphine withdrawal.

Of the CaM kinases, CaMKII has particularly been proposed as a candidate molecule for the long-term storage of information because of its ability to remain phosphorylated in the absence of CaM (Lisman, Schulman & Cline, 2002). Once activated, CaMKII can phosphorylate a number of intracellular targets including AMPA receptors (Poncer, Esteban & Malinow, 2002), NMDA receptors (Bayer, De Koninck, Leonard, Hell & Schulman, 2001; Bayer & Schulman, 2001), and L-type calcium channels (Dzhura, Wu, Colbran, Balser & Anderson, 2000). Based on the role of AMPA, NMDA, and L-type calcium channels in the development of be-

havioral sensitization, it is possible that an increase in the expression of CaMKII α during morphine injections and subsequently during withdrawal may activate these target molecules, and subsequently result in development of dependence and reward-related memory in the hippocampus. In support of the above suggestion, it has been reported that among four subunits of CaMKII namely α , β , γ and δ ; , the α -subunit plays a crucial role in learning and memory, LTP and neuronal plasticity (Hudmon & Schulman, 2002). Numerous studies have also indicated that the stimulation of Ca²⁺ permeable NMDA receptors in the hippocampal neurons results in translocation of CaMKII to the postsynaptic density in the spine head (Colbran, 2004). Moreover, CaMKII association with the postsynaptic density can be either transient or prolonged (Shen, Teruel, Connor, Shenolikar & Meyer, 2000). Therefore, chronic morphine treatment may increase gene expression of CaMKII α and its levels at postsynaptic density, which would in turn, improve or result in the rewarding memory for morphine up to 14 days of withdrawal. In support of the above hypothesis, it has been reported that chronic but not acute microinjection of CaMKII inhibitors into the hippocampus is able to prevent the development of opioid tolerance (Fan, Wang, Qiu, Ma & Pei, 1999). It is thus plausible that the withdrawal syndrome, drug craving, and relapse to morphine during withdrawal may at least partly result from the potentiated synaptic strength in the hippocampus, which may be affected indirectly by CaMKII α .

5. Conclusions

According to the obtained results in this study, there was an increase of CaMKII α expression during morphine withdrawal being significant on day 14 of withdrawal and then subjected to , a return to a new set point thereafter. Our study suggests a possible link between the CaMKII α expression in the hippocampus and behavioral changes associated with morphine withdrawal syndrome. Finally, a better understanding of such changes in CaMKII α expression in the brain may hopefully provide new targets for the treatment and control of opiate addiction.

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