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Tramadol Treatment Induces Change in Phospho-Cyclic Adenosine Monophosphate Response Element-Binding Protein and Delta and Mu Opioid Receptors within Hippocampus and Amygdala Areas of Rat Brain

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Original Article

Abstract

Background: Tramadol induces its unique effects through opioid pathways, but the exact mechanism is not known. The study aims to evaluate changes in the level of mu-opioid receptor (μOR), delta-opioid receptor (δOR), and phosphorylated cyclic adenosine monophosphate (cAMP) response element-binding protein (p-CREB) in the hippocampus (HPC) and amygdala (AL) areas of tramadol-treated rats.

Methods: For this purpose, a total of 36 male rats were divided into two main groups for chronic or acute tramadol exposure. The animals were then exposed to 5 $mg.kg^{-1}$ of tramadol, 10 $mg.kg^{-1}$ of tramadol, and normal saline. The HPC and AL areas of the animals were dissected upon completion of the period. The levels of p-CREB and μOR were quantified using the western blotting technique. The data were subjected to analysis of variance (ANOVA) followed by Tukey's post-hoc analysis. The differences with the P-value lower than 0.05 were considered as significant.

Findings: In the HPC and AL areas of the brain, the level of μOR was decreased by acute tramadol exposure, while no significant difference was observed by chronic tramadol exposure. Moreover, results showed that the level of p-CREB dose-dependently increased by acute and chronic tramadol exposure.

Conclusion: HPC and AL are essential in the control of tramadol abuse. Tramadol abuse affects gene expression and transcription factors such as CREB. With acute drug tramadol treatments, the level of cAMP response element-binding protein (CREB) rapidly increases, while by chronic tramadol treatment, "peak and trough pattern is observing". The activation of the rewarding mechanism is a precise instance of addictive behavior in tramadol-treated individuals.

Keywords: Tramadol; Hippocampus; Amygdala; Cyclic adenosine monophosphate response element-binding protein A; Opioid receptor

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Introduction

Tramadol [(1*RS*,2*RS*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride] is an orally active substance that clinically has been used for several decades.[1,2] Tramadol is considered as one of the most prescribed analgesic medications in the world.[3] It has been shown that tramadol is metabolized in the liver to O-desmethyl tramadol (M1) and N-desmethyl tramadol (M2) by cytochrome enzymes.[4,5] Tramadol induces its unique effects through opioid and non-opioid pathways. However, the exact mechanism of action is not known.[6] Moreover, the analgesic effect of opioid drugs such as tramadol is mediated through opioid receptors (ORs).[7]

ORs are a member of a superfamily called G-protein-coupled receptors (GPCRs) and the three subtypes, including mu (μ), delta (δ), and kappa (κ), which have strong homology. δOR functionally is very close to μOR and also induces reward and antinociception.[8] Moreover, morphine has a strong binding with μOR, while the agonist for δOR showed less binding, which makes δOR a new target for developing analgesic compounds.[8]

The analgesic effect of tramadol is attributed to the activation of μOR by the M1 metabolite.[2,9] However, tramadol's analgesic action has a low affinity with δ and κ ORs.[2,10] Another evidence shows that tramadol's superior analgesic mechanism over pure opioids could be due to a unique combination of two mechanisms: monoamine reuptake inhibitor and OR agonist.[10] Furthermore, tramadol is a weak inhibitor of serotonin and norepinephrine (NE) reuptake.[11]

Tramadol dependence is rare but can happen.[12] Evidence shows that tramadol users with no history of abuse have great abuse potential under the long-term and infrequent abuse of high doses.[13] Accumulative evidence suggests dependence potential as well as clinical benefits.[13-16]

Moreover, it is reported that tramadol, buprenorphine, and morphine dose-dependently induce conditioned place preference in rats due to the mechanism that could be medicated by μORs.[17] The results from behavioral studies revealed that tramadol had excellent potential to induce psychological and physical dependence in animal models.[13,18,19] It is suggested that tramadol usage must be monitored to prevent possible abuse in the future.[12,19]

Addiction is characterized by broad cellular and molecular adaptations; evidence supports the involvement of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) in the expression and phenotype of brain regions to drug reward and seeking behavior.[20] Moreover, the role of CREB in the mediation of long-term plasticity is not well known, which also could be associated with the development of addiction.[21]

Furthermore, tramadol changes anxiety-related and depression-associated behaviors in rats.[22] However, based on our knowledge, changes in the level of μOR, δOR, and phosphorylated CREB (p-CREB) in the brain of tramadol-treated rats rarely have been discussed.[23] In the present study, we utilized the western blotting technique to examine the changes in the level of δOR, μOR, and the downstream transcription factor, CREB, in the hippocampus (HPC) and amygdala (AL) regions of tramadol-treated rats.

Methods

Animals and drugs: The male Wistar rats weighing 200-220 g were purchased from [Omitted due to ongoing blind review]. The animals were housed in plexiglass (4 per cages) with free access to clean water and food. The animals were maintained in the animal laboratory at the Iranian National Center for Addiction Studies, Tehran, Iran, at constant temperature (22 ± 2 °C) and 12-hour light/dark cycle (07:00 am-07:00 pm). Tramadol hydrochloride (HCL) (Omitted due to ongoing blind review) was dissolved in normal saline (0.9%) and was injected intraperitoneally (IP).

Experimental design: A total of 36 animals were divided into two main groups (n = 18, in each group) for acute and chronic tramadol exposure. For chronic tramadol exposure, the animals (n = 18) were exposed to 5 $mg.kg^{-1}$ of tramadol (n = 6), 10 $mg.kg^{-1}$ of tramadol (n = 6), and normal saline (n = 6) for 14 days. For acute tramadol exposure, the animals (n = 18) were exposed to 5 $mg.kg^{-1}$ of tramadol (n = 6), 10 $mg.kg^{-1}$ of tramadol (n = 6), and normal saline (n = 6) for one hour. The animals which received normal saline were considered as the control group.

Tissue extraction: One hour after the last injection, the animals were sacrificed, and the HPC and AL regions of the brain were extracted using the Paxinos coordinates.[24]

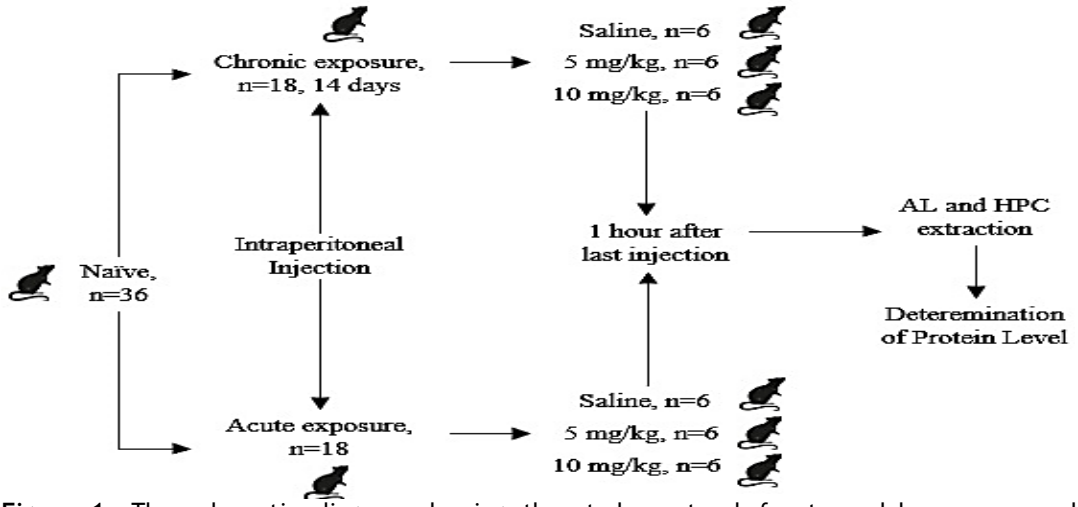

Figure 1. The schematic diagram showing the study protocol for tramadol exposure and subsequent molecular analysis

Tissue samples were collected using a micro-punch. The extracted tissues were immediately preserved in the liquid nitrogen at -70 °C for the change in the protein level using the western blot technique. The schematic view of the experiment design is illustrated in figure 1.

Western blotting: The level of proteins in the tissues was quantified using immunoblot analysis according to the technique that was described earlier.[23] The tissues were homogenized in the tris-sodium chloride (tris-NaCl), which contains protease inhibitor (Protease K, Sigma, USA), ethylenediaminetetraacetic acid (EDTA) (1 mM), and ethylene glycol tetraacetic acid (EGTA) (1 mM). The suspension was centrifuged at 12000 rpm for 10 minutes at 4 °C. The total protein concentration was determined using a spectrophotometer (Picodrop, UK). Denatured samples (60 µg per lane) together with Laemmli sample buffer that contains 5% beta-mercaptoethanol (BME) were electrophoresed on polyacrylamide sodium dodecyl sulfate (SDS) gel at 120 V for 120 minutes on 12.5% gradient gels (Bio-Rad Laboratories, USA). The proteins were transferred to the polyvinylidene fluoride (PVDF) membranes. The membranes' nonspecific area was blocked in blot (5% skim-milk, 150 mM NaCl, and 20 mM tri-HCL) for one hour at room temperature. Then, the blocked membrane was incubated with the primary antibodies, including p-CREB, total CREB, δOR, and µOR (Abcam Co., USA, 1/1000) overnight at 4 °C. Then, the membranes were washed three times with tris-buffered saline with Tween 20 (TBST) and then incubated with the secondary antibody (Abcam Co., USA, 1/5000). After washing the blots three times with TBST, they were developed using the ECL advanced kit (Amersham Biosciences Co., USA). The PVDF membranes were stripped and reused using an anti-actin antibody (AAA) (Abcam Co., USA, 1/5000) to normalize protein loading and transfer. The membranes were exposed, and the protein bands were detected using an X-ray film. The ImageJ software was used for densitometry analysis of protein bands.

The obtained data were imported to the SPSS software (version 22, IBM Corporation, Armonk, NY) for statistical analysis. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis. The differences were considered statistically significant when the P-values were less than 0.05. Moreover, all bonds were densitometric using ImageJ software.

Results

Tramadol changes the level of µOR, δOR, and p-CREB in the HPC: The effect of acute and chronic tramadol exposure on the level of µOR within the HPC is illustrated in figure 2-A. Results revealed that acute tramadol exposure (10 mg.kg^{-1}) significantly reduced the level of µOR in the HPC ($F_{2,15}$ = 9.41, P = 0.014). However, chronic tramadol exposure and 5 mg.kg^{-1} of acute tramadol exposure had no significant effect on level of µOR ($F_{2,15}$ = 0.833, P = 0.470). The effect of acute and chronic tramadol exposure on the level of p-CREB within the HPC is illustrated in figure 2-B.

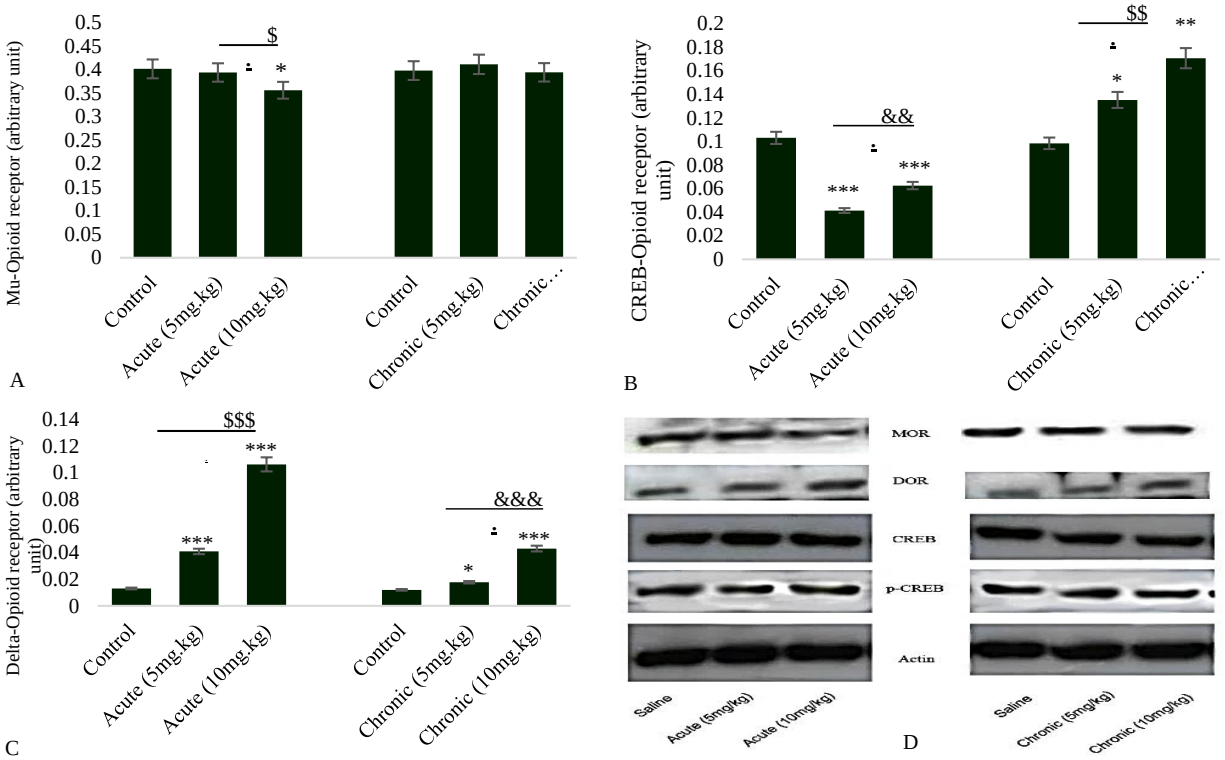

Figure 2. The effect of acute and chronic tramadol exposure on the level of the mu-opioid receptor (μOR), delta-opioid receptor (δOR), and cyclicadenosine monophosphate (cAMP) response element-binding protein (CREB) within the hippocampus (HPC). The total density of protein for chronic and acute tramadol exposure in the μOR (A), CREB (B), and δOR (C) was measured by ImageJ software. The protein level retrieved from western blot analysis for the μOR, δOR, and CREB is presented in figure D. The bars represent the mean ± standard error of mean (SEM) of protein level. In comparison with control groups, a significant difference with a P-value lower than 0.05 (*), 0.01 (**), or 0.001 (***) is presented with an asterisk. Significance difference between two concentrations of acute tramadol injection with a P-value lower than 0.05 (&), 0.01 (&&), or 0.001 (&&&) is presented with an ampersand. Significance difference between two concentrations of chronic tramadol injection with a P-value lower than 0.05 ($), 0.01 ($$), or 0.001 ($$$) is presented with a dollar sign.

Results revealed that acute tramadol exposure significantly decreased the level of p-CREB in the HPC ($F_{2,15}$ = 98.89, P < 0.0001). Moreover, results showed that 10 mg.kg^{-1} of acute tramadol exposure significantly had lower suppressive potential than 5 mg.kg^{-1} of acute tramadol exposure. Furthermore, the results showed that chronic tramadol exposure significantly elevated the level of p-CREB in the HPC ($F_{2,15}$ = 19.74, P = 0.0023). Furthermore, 10 mg.kg^{-1} of chronic tramadol exposure significantly led to a higher level of p-CREB compared to 5 mg.kg^{-1} of acute tramadol exposure, suggesting that the effect of chronic tramadol exposure on the level of p-CREB is dose-dependent.

The effect of acute and chronic tramadol exposure on the level of δOR within the HPC is illustrated in figure 2-C. Results showed that acute tramadol exposure significantly elevated the level of δOR in the HPC ($F_{2,15}$ = 351.90, P < 0.0001). Furthermore, 10 mg.kg^{-1} of acute tramadol exposure significantly had higher elevation potential than 5 mg.kg^{-1} of acute tramadol exposure that suggests the effect of acute tramadol exposure on level of δOR to be dose-dependent. The results also showed that chronic tramadol exposure significantly elevated the level of δOR in the HPC ($F_{2,15}$ = 164.90, P < 0.0001). Moreover, 10 mg.kg^{-1} of chronic tramadol exposure significantly led to a higher level of δOR compared to 5 mg.kg^{-1} of acute tramadol exposure, suggesting that the effect of chronic tramadol exposure on the level of δOR is dose-dependent. The western blotting technique quantified the protein levels of μOR, δOR, and p-CREB in the HPC of the animals. The results are represented in figure 2-D. The scatter plot of protein quantification and corresponding blot for the level of μOR, δOR, and CREB within the HPC region is illustrated in figure 3.

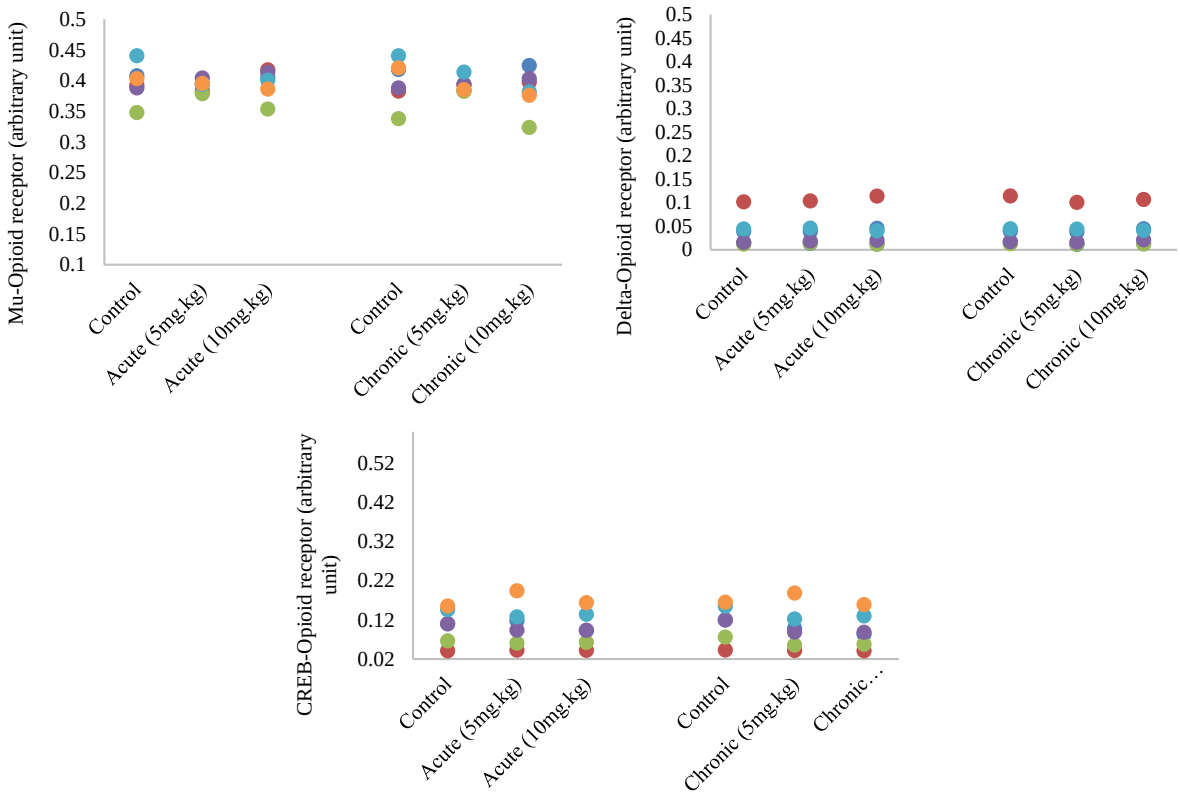

Figure 3. The scatter plot of protein quantification and corresponding blot for the effect of acute and chronic tramadol exposure on the level of mu-opioid receptor (μOR), delta-opioid receptor (δOR), and cyclicadenosine monophosphate (cAMP) response element-binding protein (CREB) within the hippocampus (HPC)

Tramadol changes the level of μOR, δOR, and p-CREB in the AL: The results revealed that acute tramadol exposure significantly decreased the level of μOR within the AL ($F_{2,15}$ = 10.07, P = 0.012). Results showed that the suppressive effect of 10 mg.kg^{-1} of acute tramadol was equal to 5 mg.kg^{-1} of acute tramadol exposure, and no significant difference between various concentrations of acute tramadol exposure was observed (Figure 4-A). Moreover, results showed that in comparison to the control group, the chronic tramadol exposure elevated the level of μOR in the AL, but the difference was not statistically significant ($F_{2,15}$ = 1.30, P = 0.330) (Figure 4-A).

The results showed that acute tramadol exposure significantly elevated the level of δOR in the AL. However, no significant difference between the elevation potential of various acute tramadol exposure concentrations was observed (Figure 4-B). Furthermore, the results showed that chronic tramadol exposure significantly elevated the level of δOR in the AL. Moreover, 10 mg.kg^{-1} of chronic tramadol exposure significantly led to a higher level of δOR in comparison to 5 mg.kg^{-1} of acute tramadol exposure, which suggests that the effect of acute tramadol exposure on the level of δOR is dose-dependent (Figure 4-B).

Moreover, the results revealed that acute tramadol exposure significantly elevated the level of p-CREB in the AL ($F_{2,15}$ = 330.20, P < 0.0001). Furthermore, 10 mg.kg^{-1} of acute tramadol exposure significantly had higher elevation potential than 5 mg.kg^{-1} of acute tramadol exposure, which suggests that the effect of acute tramadol exposure on the level of p-CREB is dose-dependent (Figure 4-C). Moreover, the results showed that 10 mg.kg^{-1} of chronic tramadol exposure significantly increased the level of p-CREB within the AL ($F_{2,15}$ = 169.90, P < 0.0001). However, 5 mg.kg^{-1} of chronic tramadol exposure had no significant effect on the expression of p-CREB, similar to the control group (Figure 4-C). The protein levels of μOR, δOR, and p-CREB in the AL region of tramadol-treated rats were quantified by the western blotting technique represented in figure 4-D.

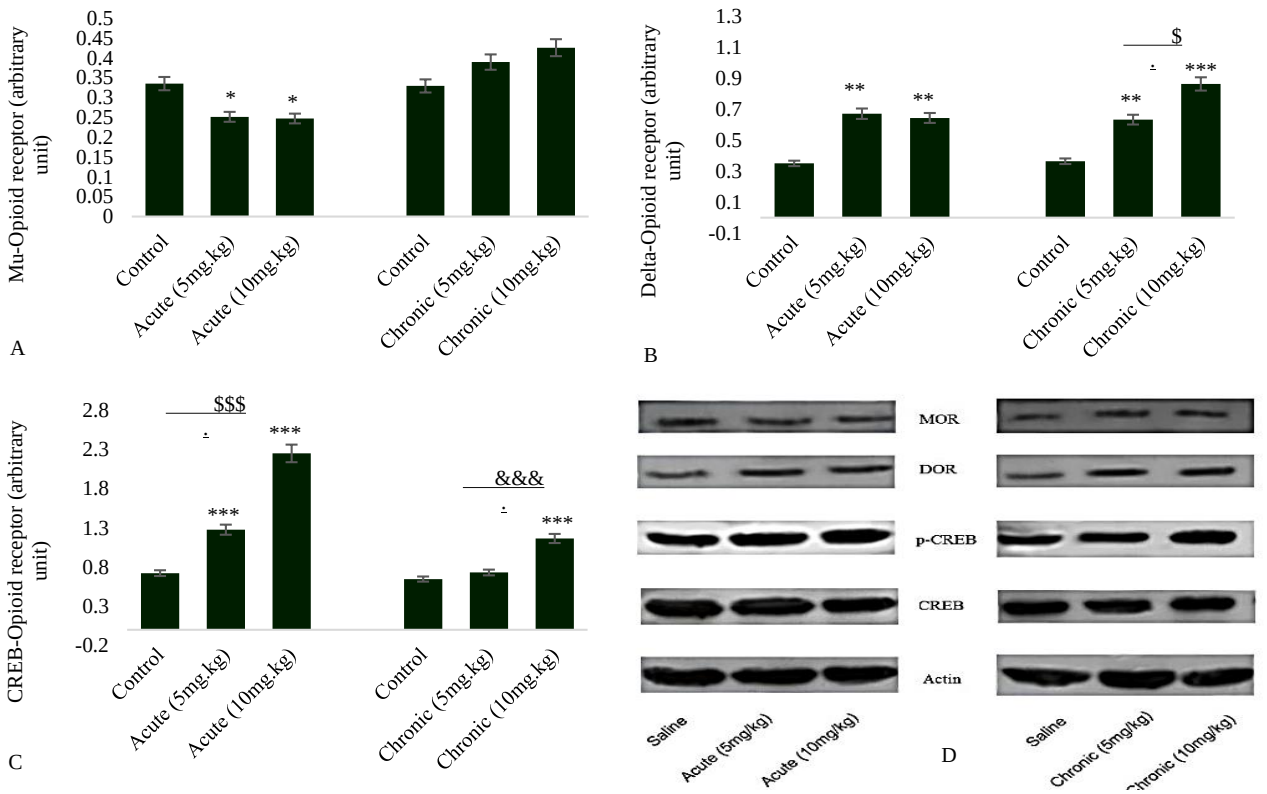

Figure 4. The effect of acute and chronic tramadol exposure on the level of mu-opioid receptor (μOR), delta-opioid receptor (δOR), and cyclicadenosine monophosphate (cAMP) response element-binding protein (CREB) within the amygdala (AL). The total density of protein for chronic and acute tramadol exposure in the μOR (A), δOR (B), and CREB (C) was measured by ImageJ software. The protein level retrieved from western blot analysis for μOR, δOR, and CREB is presented in figure D. The bars represent the mean ± standard error of mean (SEM) of protein level. In comparison with control groups, a significant difference with a P-value lower than 0.05 (*), 0.01 (**), or 0.001 (***) is presented with an asterisk. Significance difference between two concentrations of acute tramadol injection with a P-value lower than 0.05 (&), 0.01 (&&), or 0.001 (&&&) is presented with an ampersand. Significance difference between concentrations of chronic tramadol injection with a P-value lower than 0.05 ($), 0.01 ($$), or 0.001 ($$$) is presented with a dollar sign.

The scatter plot of protein quantification and corresponding blot for the level of μOR, δOR, and CREB within the AL region is illustrated in figure 5.

Discussion

Tramadol is a synthetic opioid and analogue of codeine, making it an excellent analgesic medication.[25,26] Pain management and analgesics in medical science are essential, and tramadol is one of the most prescribed pain killers.[3,27] Moreover, accumulative evidence suggests that tramadol has a clear risk of abuse under the long-term and infrequent high-dose abuse.[3,13,28-31] The effect of tramadol on the level of μOR, δOR, and p-CREB in the brain rarely has been discussed. Although μOR is reported as a gateway to drug addiction, understanding its function is an essential agenda in developing addiction therapies.[32,33] The study's main objective was to assess the effect of tramadol exposure on the level of μOR, δOR, and p-CREB in the different brain areas related to addiction neurology.

Tramadol exposure changes the level of μOR: In the previous study, probably for the first time, we assessed tramadol's effect on the ORs within the nucleus accumbens (NAC) and prefrontal cortex (PFC). The result showed that within the NAC and PFC, the level of μOR was elevated in the acute tramadol exposure [Omitted due to ongoing blind review]. In the current study, we observed that the level of μOR within the HPC and AL significantly decreased by acute tramadol exposure. Moreover, results revealed that the level of μOR in the NAC and PC was elevated by chronic tramadol exposure [Omitted due to ongoing blind review].

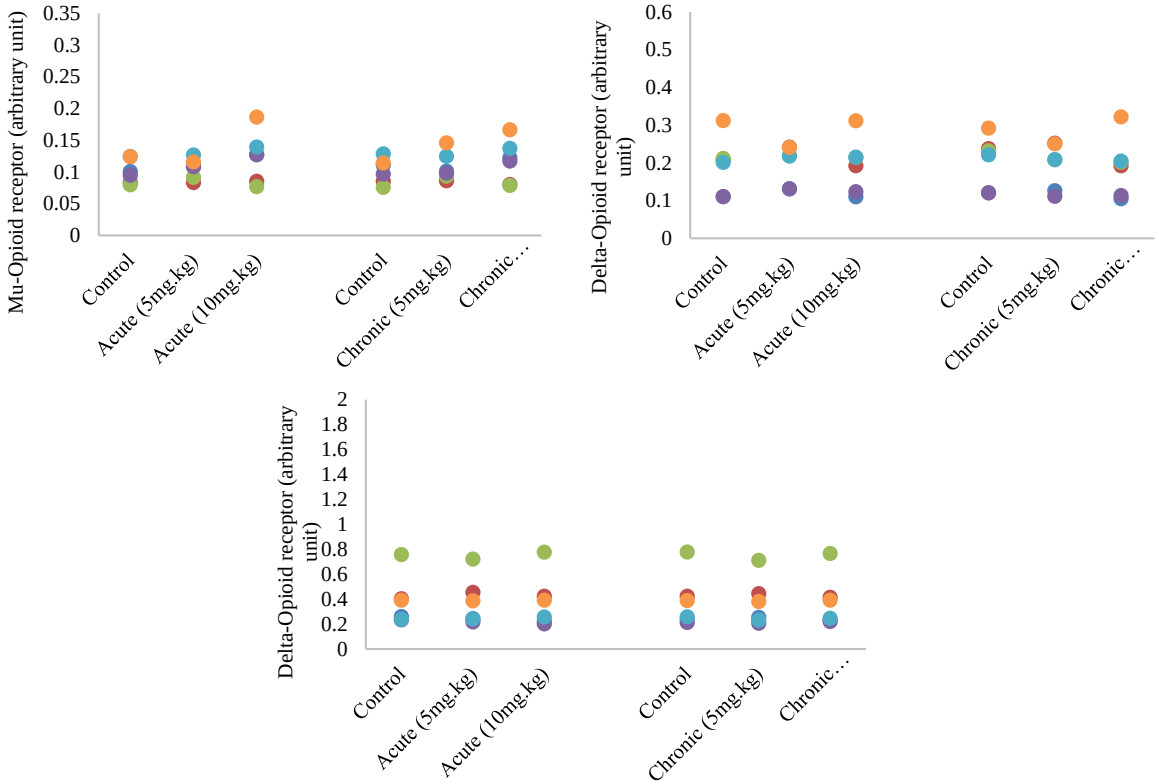

Figure 5. The scatter plot of protein quantification and corresponding blot for the effect of acute and chronic tramadol exposure on the level of mu-opioid receptor (μOR), delta-opioid receptor (δOR), and cyclicadenosine monophosphate (cAMP) response element-binding protein (CREB) within the amygdala (AL)

However, in the current study by chronic tramadol exposure, no significant difference was observed in the level of μOR in the HPC and AL.

The brain areas that mediate the craving and rewarding properties of the drug are NAC, PFC, AL, and ventral tegmental area (VTA). Dopamine is commonly secreted in the NAC upon drug abuse, resulting in the drugs' direct action on a dopaminergic neuron or modifying neurons such as gamma-aminobutyric acid (GABA)ergic neurons that interact with dopaminergic neurons.[34] Moreover, the evidence showed that acute cocaine exposure led to an increase of μOR messenger ribonucleic acid (mRNA) in the frontal cortex, AL, and NAC but not in the HPC.[35] Results showed that μOR contributed to analgesia, withdrawal symptoms, reward, and excitatory effect of opiates.[36-38] It is reported that μOR promotes recreational drug use and adaptation to chronic activation, which would lead to tolerance and dependence.[39] Another evidence revealed that the binding of μOR increased in the several brain regions of the cocaine users, including AL and PFC, that is positively correlated with the prevalence of relapse and cocaine craving.[40-42]

Altogether, an increase in the level of μOR in the AL would be related to developing a rewarding mechanism in the tramadol-treated animals.

Tramadol exposure changes the level of δOR: In the current study, we observed that the level of δOR in the HPC and AL was significantly elevated by acute tramadol exposure. Furthermore, results showed that chronic tramadol exposure led to significant elevation in the level of DeltaFosB (ΔFosB) in the NAC and PFC, which is a sustained molecular switch that converts acute drug response into persistent adaptation.[23,43,44] Similar results were observed by both chronic and acute tramadol exposures in the level of δOR in the HPC and AL areas.

Understanding the function of δOR is more limited than other ORs, because the specific techniques for study on δOR only recently became available.[45] The evidence revealed that δOR expression was elevated in neuropathic pain, while expression of μOR was elevated in inflammation.[46]

The results showed that tramadol had a low affinity with μOR and an even lower affinity with κOR and δOR.[10] The evidence revealed that lack of δOR in the δOR knockout mice lead to an elevation of anxiety-like behavior.[45] Moreover, the addicted individual suffers from impairment in reward sensitivity, and δORs are involved in this process.[47,48] Moreover, evidence revealed that the animal model with δOR gene knockout developed drug-related phenotype but failed to develop dependence or tolerance.[49]

Altogether, an increase in the level of δOR might impact emotional state and drug-related phenotype in the tramadol-exposed animal.

Tramadol exposure changes the level of p-CREB: Results showed that both acute and chronic tramadol exposures lead to a significant elevation in the level of p-CREB in the NAC and PFC.[23] Similar results were observed by both acute and chronic tramadol exposure in the HPC and AL areas.

Evidence revealed that chronic opioid administration by increasing the adenylate cyclase (AC) might lead to the up-regulation of the cAMP system.[50,51] Moreover, CREB may play an essential role in the opioid-induced increase in AC activity.[51-53] Another evidence showed that opioid withdrawal symptoms could be less intense in the CREB-deficient animal model.[20,51] CREB is rapidly phosphorylated (activated) upon acute drug exposure.[54] Besides, increasing the level of p-CREB in NAC after opioid exposure was well described previously.[55,56] Although, the enhancement in the levels of CREB leads to increase dynorphin expression and reduces morphine and cocaine sensitivity and caused tolerance.[57] Moreover, evidence shows that the drug of abuse activates CREB by phosphorylation which leads to increases the self-administration of drugs.[44,58]

Tramadol abuse changes gene expression and affects transcription factors such as CREB.[43] With acute drug tramadol treatments, the level of CREB rapidly increases, while by chronic tramadol treatment, "peak and trough pattern is observing".[59,60] The activation of a rewarding mechanism and behavioral adaptation mechanism is a precise instance of addictive behavior in the tramadol-treated individuals.[23]

Altogether, an increase in the level of p-CREB in the tramadol-exposed animal would be related to the activation of a rewarding mechanism in the tramadol-treated animals.

Conclusion

Tramadol is one of the most prescribed analgesic medications in the world. The results confirmed that CREB and δOR play an essential role in memory impairment by tramadol. Moreover, the results indicated the importance of HPC and AL in the control of tramadol abuse. Altogether, an increase in p-CREB and δOR in the tramadol-exposed animal would be related to activation of rewarding mechanisms and development of behavioral adaptation, which is a precise instance of addictive behavior in the tramadol-treated animals.

Conflict of Interests

The Authors have no conflict of interest.

Acknowledgements

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Authors' Contribution

Responsible for the analyses and reports of the study results: HAM and MSSS; responsible for writing and review: SA and RV; responsible for recruitment and data collection: SK; supervisor: MRZ. All authors read and approved the final manuscript.

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تأثیر تیمار ترامادول بر تغییرات گیرنده‌های اپیوئیدی میو، دلتا و فسفوکرب در قسمت‌های آمیگدالا و هیپوکمپ مغز موش صحرایی

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مقاله پژوهشی

چکیده

مقدمه: ترامادول اثر ویژه‌ای بر روی مسیر اپیوئیدی القا دارد، اما ساز و کار آن شناخته شده نیست. هدف از انجام مطالعه حاضر، ارزیابی تغییرات در سطح گیرنده اپیوئیدی میو (Mu)، دلتا و فسفوکرب در ناحیه هیپوکمپ و آمیگدالا در موش‌های تیمار شده با ترامادول بود.

روش‌ها: به این منظور، ۳۶ موش صحرایی در دو گروه صورت مزمن و حاد تحت تیمار ترامادول قرار گرفتند. حیوانات تحت تیمار ۵ میلی‌گرم در کیلوگرم و ۱۰ میلی‌گرم در کیلوگرم ترامادول قرار گرفتند و گروه سالین به عنوان تیمار شاهد مورد بررسی قرار گرفت. پس از تیمار، ناحیه هیپوکمپ و آمیگدالا جدا گردید و سطح گیرنده‌های اپیوئیدی میو، دلتا و فسفوکرب با استفاده از روش Western blot ارزیابی گردید. نتایج با استفاده از آزمون ANOVA و مقایسه میانگین به روش Tukey مورد تجزیه وتحلیل قرار گرفت. $P < ۰/۰۵$ به عنوان سطح معنی‌داری در نظر گرفته شد.

یافته‌ها: در ناحیه هیپوکمپ و آمیگدالا، سطح گیرنده اپیوئیدی میو به صورت معنی‌داری در هنگام تیمار حاد ترامادول کاهش پیدا کرد؛ در حالی که تغییر معنی‌داری با تیمار مزمن ترامادول مشاهده نگردید. بر اساس نتایج به دست آمده، مقدار فسفوکرب وابسته به دز بود و در هر دو تیمار حاد و مزمن به صورت معنی‌داری افزایش یافت.

نتیجه‌گیری: نتایج پژوهش حاضر تأیید نمود که ناحیه آمیگدالا و هیپوکمپ، در کنترل مصرف ترامادول اهمیت زیادی دارد. همچنین، سوء مصرف ترامادول، سطح بیان ژن‌ها و فاکتورهای رونویسی مانند کرب را تغییر می‌دهد. در هنگام مصرف داروی ترامادول، سطح کرب به صورت حاد افزایش می‌یابد؛ در حالی که در هنگام مصرف دارو به صورت مزمن، سطح کرب به صورت پیک تغییر می‌نماید. تغییرات در مکانیسم پاداش، نمونه دقیقی از رفتارهای اعتیادآور در افراد تحت تیمار با ترامادول می‌باشد.

واژگان کلیدی: ترامادول؛ هیپوکمپ؛ آمیگدالا؛ پروتئین متصل شونده به عناصر پاسخ دهنده به آدنوزین مونوفسفات حلقه‌ای؛ گیرنده اپیوئیدی

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