Original Article

The Effect of Orexin-2 and Endocannabinoid-1 Antagonists on Neuronal Activity of Hippocampal CA1 Pyramidal Neurons in Response to Tramadol in Rats

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Abstract

Background: CA1, as a major structure involved in learning and memory, has been shown to be affected by tramadol addiction. Both orexin and endocannabinoid receptors express in CA1 and play an important role in drug dependency. The aim of this study was to evaluate the modulatory effects of orexin-2 (OX2R) and endocannabinoid-1 (CB1R) receptors on neuronal activity in CA1, in response to tramadol in rats. **Materials and Methods:** Male Wistar rats were divided into 8 groups (n = 6–7); saline-dimethyl sulfoxide (DMSO), tramadol-DMSO, saline-TCS-OX2-29, saline-AM251, tramadol-TCS-OX2-29, tramadol-AM251, saline-TCS-OX2-29-AM251, tramadol-TCS-OX2-29-AM251. Tramadol was injected intraperitoneally, and then, AM251 (1 nmol/0.3 μL), CB1R antagonist and TCS-OX2-29 (1 nmol/0.3 μL), OX2R antagonist, were microinjected individually or concurrently into the CA1. Using *in vivo* extracellular single-unit recording, the firing of CA1 pyramidal neurons was investigated. **Results:** Tramadol decreased neuronal activity in CA1 ($P < 0.01$) but increased it after micro-injection of DMSO. TCS-OX2-29 increased neuronal activity in saline group ($P < 0.05$) but decreased it in tramadol group. AM251 had no effect on saline group but decreased neuronal activity in tramadol group ($P < 0.05$). Concurrent micro-injection of TCS-OX2-29 and AM251 had no effect on saline group but decreased neuronal activity in tramadol group ($P < 0.05$). **Conclusions:** Our findings suggest that neural activity in CA1 is rapidly affected by acute use of tramadol, and some of these effects may be induced through the endocannabinoid and orexin systems. Thus, the function of endocannabinoid and orexin systems in CA1 may play a role in tramadol addiction.

Keywords: *Cannabinoids, electrophysiology, hippocampus, neurons, orexin receptors, tramadol*

Introduction

Tramadol hydrochloride is a synthetic analgesic that acts on the central nervous system.[1] This drug is showing opioid and nonopioid properties and is widely used for treating moderate to severe pain. It has become one of the most popular prescribed opioids, worldwide.[2] Studies have shown that tramadol can induce addiction, which is associated with physical and psychological dependence.[3] Previous studies have shown that the cellular mechanism of tramadol addiction and its analgesic effects is through binding to the μ-opioid receptor as well as inhibiting the reuptake of noradrenaline and serotonin (5-HT).[4,5] Numerous studies have reported that tramadol inhibits the activity of acetylcholine receptors that play an important role in memory.[6] Tramadol can affect different parts of the brain, such as the ventral tegmental area (VTA) and nucleus accumbens (NAc), involved in the reward circuits. There is a link between CA1 and reward regions of the brain, and this region has been shown to mediate morphine reward effects.[7] A functional loop exists between the CA1 and VTA that is one of the sources of dopaminergic neurons to the hippocampus. Therefore, long-term usage of tramadol may create dependence and tolerance through affecting CA1, in addition to the other part of reward circuits.[8]

Learning and memory can be affected by drugs such as tramadol.[9] CA1 is part of the hippocampus and an important area that plays a role in learning and memory.[10] The main neurons of the CA1 area are pyramidal cells.[11] These cells receive excitatory and inhibitory inputs; glutamatergic afferents of the entorhinal cortex and CA3 supply most of the excitatory inputs to CA1. Furthermore, the

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major targets of septal cholinergic neurons are pyramidal cells.[12,13] Glutamatergic neurons are principal cells in the CA1 and have an important role in regulating the activity of pyramidal cells.[14] About 7%–11% of CA1 cells are GABAergic interneurons that express the μ-opioid receptor (MOR) receptor.[9,13]

Orexinergic and cannabinoid systems have been shown to mediate morphine and nicotine addictions.[15,16] Orexin/hypocretin neurons (including two types of orexin, A and B) originate in the lateral hypothalamus and are involved in reward, pain, and nutrition.[17] Orexins have two types of receptors, OX1R and OX2R, which bind to the G protein, and both receptors are expressed in CA1.[18] It has been shown that orexin can mediate the response to morphine in CA1, by affecting serotonergic, dopaminergic, adrenergic, and cholinergic systems.[19]

Endocannabinoids have two types of G protein-coupled receptors (CB1R, CB2R).[20] CB1Rs are functional in the brain and are widely expressed in the dorsal hippocampus.[21] Both CB1 and MORs are present on the presynaptic membrane, in the different brain areas, and overlap in the reduction of the neurotransmitter release.[22] These receptors are involved in the primary rewarding effects of drugs.[23]

The interaction between orexinergic and cannabinoid systems in some areas of reward circuits, in response to some addictive substances such as nicotine, has already been demonstrated.[24,25] Therefore, due to the similarity of the action of tramadol and morphine, as well as the lack of sufficient evidence regarding the mechanism of action of tramadol, in this study, our aim was to evaluate the acute effects of tramadol on CA1 neuronal activity as well as to explain the possible role of OX2 and CB1 receptors in the effects of tramadol.

Materials and Methods

Animals

Experiments have been done on male Wistar rats, weighing 250–300 g (acquired from Isfahan University of Medical Sciences, Isfahan, Iran). Animals were maintained in an animal house, under controlled temperature and scheduled illumination conditions (12-h light/12-h dark cycle, lights on at 07:00 am) with water and food available *ad libitum*.

All experiments were approved by the Animal Ethics Committee of Isfahan University of Medical Sciences (IR.MUI.MED.REC.1398.588), and performed in strict accordance with the directive, regarding care and use of animals for experimental procedures and use of laboratory animals (National Institutes of Health, Publication No. 85–23), revised 2010. We tried to minimize the number and suffering of animals to achieve statistically significant results.

Drugs

Tramadol hydrochloride (Alborz Drug Company, Iran) was injected intraperitoneally (i.p., 25 mg/kg).[26] TCS-OX2-29 (Tocris Bioscience, Bristol, UK), as an OX2R antagonist, and AM251 (Sigma-Aldrich, USA), as a CB1R antagonist, were dissolved in dimethyl sulfoxide (DMSO; 10% v/v) and saline (0.9%), and a drop of Tween 80 was used as a vehicle.[27]

CA1 single-unit recordings and data collection

Rats were deeply anesthetized by injection of urethane (1.6 g/kg, i.p) and placed in a stereotaxic instrument (Stoelting Co. USA). Animal body temperature was continuously monitored and maintained at 37°C, using an electrically heating pad (LSI, Spain). Surgery was performed, and a hole (roughly 3 mm in diameter) was made to permit positioning of a two-barrel micropipette (one barrel for drug microinjection, and the other for extracellular action potential recording), into the CA1 (AP = –3.2 mm; L = ±2.2 mm; DV = –2.4 mm).[28]

Single-unit activities of neurons of CA1 were recorded extracellularly with fine tip (1–3 μm) glass micropipettes, filled with 2 M sodium chloride solution. Micropipettes were gently pulled into the CA1 area, using a manual microdrive. Recorded signals were presented as a rate histogram. We recorded the extracellular electrical activity of one to four neurons from each animal. Recorded extracellular signals were filtered (300 Hz to 3 kHz bandpass), and digitized, using a commercial analog to the digital data acquisition system. Data analysis was performed by the related software tools, eLab (Science Beam Institute, Iran).

When steady firing rate was identified, the baseline was recorded for 15 min, and then, tramadol (25 mg/kg) was injected intraperitoneally, and 30 min later, TCS-OX2 (1 nmol/0.3 μL), AM251 (1 nmol/0.3 μL), or both of them were microinjected in the related groups,[24,29] and recording continued for other 60 min. In the control groups, DMSO was microinjected into the CA1, as a vehicle. Examinations were done on 12–18 neurons in 6–7 rats, in each experimental group.

Histological verification

After each experiment, rats were kept anesthetized and perfused transcardially with normal saline, followed by 10% buffered formalin. Then, brains were removed and sectioned coronally at 55 μm thickness, and recording and injection sites were histologically verified under a microscope, and compared to the rat brain Atlas [Figure 1].[28]

Data analysis

Data were analyzed, using the SPSS version 23 for windows. The spontaneous firing rate over 15 min was defined, as the

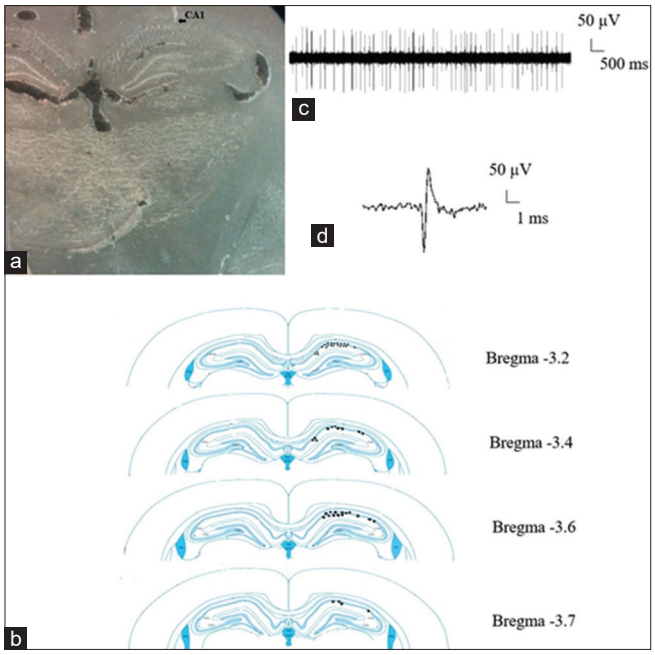

Figure 1: (a) The coronal photomicrograph of the recording and microinjection site in the CA1. (b) A representative image, displaying the microinjection and recording sites in the CA1. (c) A representative pattern of neuronal electrical activity recorded from the CA1. (d) An expanded waveform of a spike recorded from a CA1 neuron

baseline firing rate (in spikes/second). An increase/decrease of firing rates beyond the mean ± two-fold of the SD of the baseline firing rate was considered as an excitatory/inhibitory response, respectively.[16,24] The percent changes of the firing rate with respect to the baseline firing rate between the groups were analyzed, using the one-way ANOVA, followed by a *post hoc* Tukey's test and unpaired Student's *t*-test, and the Chi-square test for comparing cells with excitatory or inhibitory responses, between different groups. Data are expressed as mean ± standard error of the mean (n = 6–7 rats). $P < 0.05$ was considered statistically significant.

Results

CA1 neuronal spontaneous discharge

The putative pyramidal neurons were isolated based on the firing rate and waveform characteristics; neurons with a firing rate <8 Hz and spike duration more than 1.3 ms were chosen, therefore, according to the previous studies,[30,31] we assumed that our target neurons were pyramidal neurons [Figure 1]. After ensuring the stability of neuronal activity and baseline recording (15-min), tramadol was injected interperitoneally and 30 min later, DMSO or antagonists were microinjected into the CA1.

A Chi-square test, for comparing the difference in the proportion of neurons with excitatory/inhibitory or no-responses, revealed significant differences between the proportion of neurons in the tramadol group, compared to the saline group [$P < 0.01$; Figure 2a]. Furthermore, intraperitoneal injection of tramadol (25 mg/kg) had inhibitory effects on the majority of CA1 neurons, with respect to the baseline activity [$P < 0.01$; Figure 2b].

Neuronal response to the blockade of OX2R and CB1R following the tramadol administration

After microinjection of DMSO as a vehicle within CA1, following i.p. injection of saline or tramadol, neuronal firing showed an insignificant decrease in the saline group and an increase in the tramadol group. However, the percentage of changes varied between the groups, significantly [$P < 0.05$; Figure 3].

Furthermore, neuronal responses after intraperitoneal injection of saline or tramadol and then microinjection of antagonists in CA1 were similar to the baseline in the same group, and the cumulative effect of the two injections prevented a significant change in primary neuronal activity. However, in the comparison between the groups, different percentage changes were observed.

The OX2R blockade increased the firing rate, compared to the saline-DMSO group [$P < 0.05$; Figure 3] but prevented the increase of firing rate in the tramadol group, and it had no significant difference with respect to the saline-DMSO group [Figure 3]. The CB1R blockade in CA1 alone had no effect, and there were no significant differences between the saline-DMSO and the saline-AM251 groups. However, microinjection of AM251 prevented the increase of firing rate in the tramadol group significantly, with respect to the tramadol-DMSO group [$P < 0.05$; Figure 3].

Concurrent blockade of OX2R and CB1R in the CA1 prevented the increase of firing rate following OX2R blockade alone, and there was no significant difference with respect to the saline-DMSO group [Figure 3]; also, concurrent blockade of receptors decreased the neuronal firing rate significantly, with respect to the tramadol-DMSO group [$P < 0.05$; Figure 3].

A comparison of the difference in the proportion of neurons, excitatory/inhibitory, or no responses revealed significant differences between the proportion of neurons in the saline-TCS-OX2-29 ($P < 0.05$) and saline-AM251 groups ($P < 0.05$), with respect to the saline-DMSO group but no significant differences between the other experimental groups [Table 1].

Discussion

The results of the present study showed that systemic injection of tramadol can acutely reduce neuronal activity in the CA1 region of the hippocampus. According to the characteristics of the selected neurons,[31] this decrease in firing rate was related to the pyramidal neurons in this area.

The hippocampus, as a part of the limbic system, plays a significant role in learning and memory, and its role in drug addiction has been demonstrated.[32] The CA1 region that is under the influence of opioids contains

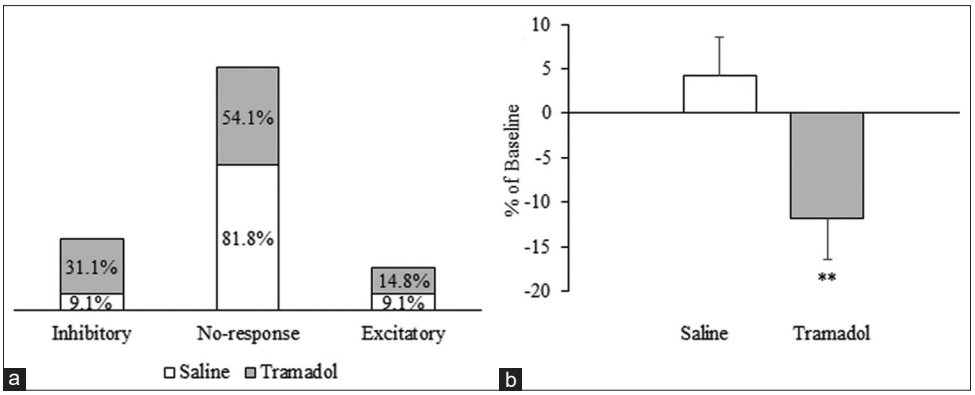

Figure 2: The effect of tramadol systemic administration, on (a) the proportion of neurons with excitatory/inhibitory or no-responses, and (b) the percentage difference of firing rate of neurons, within the CA1. Data are expressed as mean ± standard error of the mean (*n* = 116 neurons). ***P* < 0.01 is different from the saline group

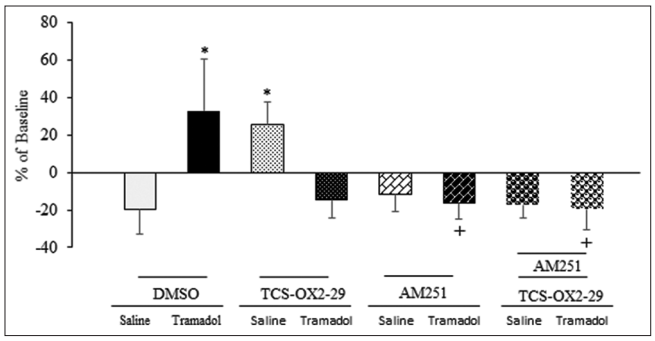

Figure 3: The effects of OX2R and CB1R antagonists, on the percentage difference of firing rate of neurons within the CA1, following the tramadol systemic administration. Data are expressed as mean ± standard error of the mean (*n* = 12–18 neurons in 6–8 rats in each experimental group). **P* < 0.05 is different from the saline-DMSO group. +*P* < 0.01 is different from the tramadol-DMSO group

several neurotransmitters, including glutamate, GABA, acetylcholine, dopamine, and monoamines.[33,34]

Glutamate receptors are abundantly expressed in the hippocampus and play a key role in synaptic plasticity and memory formation. Evidence shows that glutamate in pyramidal cells induces transmission of GABA and facilitates storage of DA, 5HT, and Ach in vesicles.[35] In the CA1 area, there is an interaction between the opioid and glutamatergic systems. Opioids can inhibit glutamate release in the CA1 and cause memory damage.[36] Tramadol probably through affecting opioid receptors can reduce glutamate transmission in CA1 and may have inhibitory effects on glutamate receptors.[36] Therefore, tramadol may reduce neural activity by affecting glutamate transmission.

Tramadol has also been shown to affect cholinergic neurotransmission, which is important for normal hippocampal function and learning and memory processes.[6] It has been demonstrated that tramadol can increase the expression of acetylcholinesterase in the CA1 and reduce the release of acetylcholine by the presynaptic terminal.[37]

Tramadol prevents the reabsorption of serotonin, noradrenaline, and increases the concentration of these neurotransmitters in the extracellular area.[38] It has been shown that stimulation of serotonin receptors (HT1A-5) inhibits the activity of CA1 pyramidal cells and may indirectly affect the release and activity of glutamate and acetylcholine.[39,40] Serotonin receptor stimulation has been shown to inhibit glutamate release in CA1 and prevent the release of acetylcholine in the forebrain.[39,41] Furthermore, studies have reported that stimulation of adrenergic receptors by tramadol can increase potassium conduction, hyperpolarization, and inhibition of spontaneous firing in locus coeruleus.[42] Furthermore, tramadol through the induction of oxidative stress[43] as well as disrupting Na/K ATPase activity can cause cell damage and cell death.[37]

In another part of this study, the mediating effects of the two endocannabinoid and orexin systems were evaluated by blocking CB1 and OX2 receptors in CA1 in response to tramadol. Blocking any of these receptors alone could prevent the acute effects of tramadol on neuronal activity.

The orexinergic system is essential for the effects of drugs such as morphine and cocaine, and studies have shown that injection of OX2R antagonist into the CA1 can reduce morphine absorption and reward.[44] It has been demonstrated that blockade of orexin receptors in VTA, significantly reduced the effects of morphine and cocaine.[45,46] Therefore, it is possible that by a similar mechanism, the OX2R blockade prevented the absorption and effects of tramadol in CA1.

The OX2R blockade in the saline group increased the firing rate. This is in line with studies that have shown that orexin has a stimulant effect in most areas of the brain.[17] However, orexin can also inhibit some neurons through presynaptic mechanisms, for example, OX2R may increase potassium current by activating Gi/Go.[17]

In the present study, the CB1R blockade in CA1 in the saline group had no effect on neuronal activity. However, microinjection of AM251 in CA1 prevented the increase of firing rate in the tramadol group. It has been revealed that CB1R can mediate the effects of tramadol in the

Table 1: The effects of OX2R and CB1R antagonists, on the neuronal activity within the CA1, following tramadol systemic administration

Percent changes in neuronal firing	Groups							
	Saline-DMSO	Saline-TCS-OX2-29	Saline-AM251	Saline-TCS-OX2-29-AM251	Tramadol-DMSO	Tramadol-TCS-OX2-29	Tramadol-AM251	Tramadol-TCS-OX2-29-AM251
Inhibitory	37.5	0	15.4	21.4	38.5	26.7	25	53.8
No response	31.3	66.7	76.9	64.3	30.8	53.3	50	38.5
Excitatory	31.3	33.3	7.7	14.3	30.8	20	25	7.7
P	-	*	*	-	-	-	-	-

*$P<0.05$ is a significant difference from the saline-DMSO group (TCS-OX2-29 as OX2R antagonist; AM251 as CB1R antagonist). The data show the percentage of changes in neuronal firing, compared to the baseline in each group after treatments. The criterion for increasing/decreasing activity was changed in firing rates beyond the mean±two-fold of the SD of the baseline firing rate. The Chi-square test was performed to compare cells with excitatory/inhibitory or no-responses (n=12–18 neurons in 6–8 rats in each experimental group). SD: Standard deviation

NAc.[47] Both CB1 and μ receptors are present on the presynaptic membrane and by inhibition of adenylate cyclase, a decrease of Ca^{2+} conduction and an increase of K conduction reduce the release of neurotransmitters, such as acetylcholine, serotonin, and noradrenaline.[22,48] Furthermore, stimulation of CB1 can reduce the release of GABA on pyramidal neurons in the hippocampus.[48] Thus, blockade of this receptor in the basal state may have no effect on neuronal activity, but when an effective drug such as tramadol has affected this area, it can prevent the effects of tramadol by inhibiting GABA release.

We observed that the concurrent blockade of OX2R and CB1R in the CA1 of the saline group prevented the increase of firing rate, following the OX2R blockade alone. Furthermore, concurrent blockade of the receptors prevented the increased neuronal firing rate in tramadol-treated rats but no more than blockade of each receptor alone.

OX2R and CB1R are widely expressed in different brain areas,[49] and recent studies have shown the interaction of OX2R and CB1R in the effects of drugs, such as nicotine.[16] Various studies have shown the interaction of Type 1 receptors of the two neuropeptides, and there is ample evidence about the interaction of these two neuropeptide systems. In the postsynaptic region, retrograde activation of OX1R inhibits the release of GABA by cannabinoids at the presynaptic terminal.[50] AM251 has been shown to reverse the effects of orexin in the PAG region and reduce the analgesic effects of orexin,[50] so it can be inferred that CB1 modulates the effects of orexin.

Conclusions

Therefore, it can be concluded that tramadol acutely reduces the neuronal activity in the hippocampus by affecting neuronal circuits in the CA1. These effects are probably mediated by two neuropeptide systems, endocannabinoids, and orexin, and by blocking their receptors, the effects of tramadol on this area can be largely prevented.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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