Accepted Manuscript

**Title:** Acute Tramadol-induced cellular tolerance and dependence of ventral tegmental area dopaminergic neurons: An in vivo electrophysiological study.

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To appear in: Basic and Clinical Neuroscience
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Please cite this article as:
DOI: 10.32598/bcn.9.10.180
Abstract:

Introduction: Ventral tegmental area (VTA) is a core region of brainstem that contributes in different vital bio-responses such as pain and addiction. The dopaminergic (DA) cellular content of the VTA revealed have major roles in different functions. The cellular effect of the tramadol on the putative VTA-DA neurons evaluated in this study.

Methods: Wistar rats classified into three control, sham, and tramadol-treated groups. Animals anesthetized and VTA-DA neuronal activity acquisitioned under controlled stereotaxic surgery. The firing rate of the neurons extracted according principle component analysis by Igor pro software and analysed statistically with \( p<0.05 \). Tramadol (20 mg/kg) infused intraperitoneally.

Results: Overall, 121 putative VTA-DA neurons isolated in all groups. In tramadol-treated rats, inhibition of the neuronal firing as tolerance and constitutive excitation period as dependence or withdrawal observed. The inhibition lasted up to 50.34±10.17 minutes and 31% of neurons abolished firing and silenced for 24±3 min but the remaining lowered their firing up to 43% to 67% of their baseline firing. All neurons showed the excitation period that lasted about 56.12±15.30 min and the firing of neurons raised up their firing from 176% up to 244% of their baseline or pre-injection period.

Conclusion: The tolerance and dependence effects of tramadol relate to the change of neuronal firing rate at the putative VTA-DA neurons. The acute injection of tramadol can initiate neuroadaptation on the opioid and non-opioid neurotransmission to mediate these effects.

Keywords: Tramadol, Dopamine, Ventral Tegmental Area, Firing Rate, Tolerance, Dependence.
1. Introduction:

Many international policy makers in the field of pain management involve in the exploring of different perspectives of analgesic effects and/or side effects to marketing safer analgesics. On the other hand, pain modulation is a highly focused research topic for drug design and development. Analgesic agents have changed rapidly to decrease side effects, improve analgesia, and lowering addiction in pain therapy especially chronic pain (Yaksh, 2002). Chronic pain dreadfully is increasing worldwide but analgesic drugs for chronic pain is reportedly inadequate due to their side effects, often intractable and maybe irreversible. Neuroplasticity of nociception in the brain depict the rational for pharmacological researches for therapy of pain (Fornasari, 2014). The opiate-based compounds have been very common in the field of analgesia for several decades, so the drugs that effect on mu opioid receptors (MOR) considered as major analgesics. Synthetic agonists and/or agonist-antagonists of MORs formulated for pain control widely (Pasternak, 2010; Trescot, Datta, Lee, & Hansen, 2008).

Tramadol (ULTRAM) marketed recently for the pharmacotherapy of the chronic pain such as neuropathic pain. It is commonly used as a comedication or alone for pain alleviating with greater safety. Its public distribution due to lower susceptibility to addiction, make it as one of the most chosen analgesic in many pharmacopeias (Grond & Sablotzki, 2004). Tramadol has less side effects and dependence than equianalgesic doses of strong opioids such as morphine (Flor, Yazbek, Ida, & Fantoni, 2013; Moron Merchante et al., 2013; Pergolizzi, Taylor, & Raffa, 2011; Rosenberg, 2009; Savoia, Loreto, & Scibelli, 2000). Tramadol has affinity to the morphine opioid receptors (MORs) but it is 6000 times less than that of morphine (Raffa et al., 2012). Tramadol and opioids used for third-line treatment of neuropathic pain, in which lowering outcomes of first-and second-line agents and obtaining the safety in long-term administration (Amescua-Garcia et
al., 2017; O'Connor & Dworkin, 2009). Beside its action on MORs, tramadol has antidepressive effects in case of reuptake inhibition of serotonin and noradrenaline (Barber, 2011; Bloms-Funke, Dremencov, Cremers, & Tzschtentke, 2011; Caspani, Reitz, Ceci, Kremer, & Treede, 2014). It may be more effective than methadone in control of opioid withdrawal symptoms and considered as a potential replacement for methadone therapy (Zarghami, Masoum, & Shiran, 2012).

In addition to safety of tramadol than morphine, there are some side effects in case of therapeutic or overdose administration. Post sub-acute and chronic tramadol toxicities, seizure is clinically a profound side effect that may occur. The social-based prevalence of tramadol-induced seizure is 8% to 14% but the hospitalized data are reported 15% to 55% with mostly one episode (Emamhadi, Sanaei-Zadeh, Nikniya, Zamani, & Dart, 2012; Matthiesen, Wohrmann, Coogan, & Uragg, 1998; Spiller et al., 1997). The tramadol-induced seizure not prevented by naloxone, a mu opioid receptor antagonist (Eizadi-Mood, Ozcan, Sabzghabaee, Mirmoghtadaee, & Hedaiaty, 2014) but in the rats with serotonin depletion, the tramadol-induced seizure was enhanced (Fujimoto et al., 2015). These reports showed that there are a strict link between tramadol and brain monoamines. The analgesic effects of tramadol remain controversial; the contribution of micro-opioid receptors (microORs) along with MOR is evaluated (Minami, Sudo, Miyano, Murphy, & Uezono, 2015). Tramadol may change ion channels conductance by G-protein coupled receptors (GPCRs) which can affect monoamine transporters (Minami, Ogata, & Uezono, 2015). Tramadol has rewarding effects (mediated by MOR activation) on a rat with sciatic neuropathic pain. The dopamine level in the rat's nucleus accumbens is significantly increase by either tramadol or its metabolite (Nakamura et al., 2008). In human cases, there are some reports on the rewarding and withdrawal effects of tramadol (Reeves & Cox, 2008) but its use for depression treatment is limited due to reported side effects (Ansermot, Chocron, Herrera, & Eap, 2015).
The overlapping chronic pain and depression is evidenced by some epidemiologic studies (Bair, Robinson, Katon, & Kroenke, 2003) in which the cortical and subcortical structures involved in the perception of different aspects of pain (Bushnell, Ceko, & Low, 2013). The mesolimbic dopaminergic neurons have the modulatory effect on pain perception. There are evidence of dysfunction of the nucleus accumbens and ventral tegmental area dopaminergic (VTA-DA) neurons to initiation excessive pain in animal model (Saade, Atweh, Bahuth, & Jabbur, 1997). The VTA contains dopamine, gamma amino butyric acid (GABA), glutamate releasing neurons and a few other types of neurons. The major population of its neurons are dopaminergic (about 65%), but the GABAergic is second (about 30%) and others are remaining (Holly & Miczek, 2016; Nair-Roberts et al., 2008; Yamaguchi, Wang, Li, Ng, & Morales, 2011). The VTA-DA neurons as A10 group project to nucleus accumbens, amygdala, hippocampus, prefrontal cortex, and limbic system (Albanese & Minciacchi, 1983; Fallon, 1988) to make two major projecting systems, mesolimbic and mesocortical systems. Mesolimbic system regulates motivational processes but the mesocortical related to cognitive and motor functions (Bjorklund & Dunnett, 2007; Le Moal & Simon, 1991; Merrill, Friend, Newton, Hopkins, & Edwards, 2015; O'Connell & Hofmann, 2011; Pierce & Kumaresan, 2006). The VTA-DA neurons is a key neuronal substrate of limbic system to rewarding, motivational, addiction, and neuropsychiatric illness (Luscher & Malenka, 2011; Nestler & Carlezon, 2006; Wise, 2004). The VTA-DA neurons have mu-opioid receptors that are involved in the disinhibition of dopamine in the nucleus accumbens (Johnson & North, 1992).

Although the rewarding effects of the tramadol documented but the precise mechanism of its action is not well known. Tramadol could mimic the opioid effects but it seems that its side effects related to its opioid and non-opioid effects. This study designed to evaluate cellular effect of acute intraperitoneal application of the tramadol on the firing rate of VTA-DA neurons with extracellular
single neuron recording techniques. The units with stable spontaneous tonic activity without pattern changes studied.
2. Material and Methods:

2.1. Ethical Approval

Urmia Medical Science Research Ethics Committee (UMSREC) reviewed all procedures and experiments as a local referral biomedical committee for research ethics. Protocols and guidelines carried on in accordance with the National Institutes of Health (NIH) for the care and use of experimental animals. The Animal Laboratory Center of Urmia University of Medical Sciences precisely outlined these protocols and guidelines. Supervision of animal protocols carried out continually according to the research design.

2.2. Animals

Healthy male Wistar rats (Purchased from Pasteur Institute, Tehran, Iran, weight 180-220 gr.) were housed (three in a cage) at a 12h light/dark cycle (7:00am-7:00pm, light on at 7:00 am) and controlled temperature (22±2 °C) with the food and water ad libitum. The animals divided into 3 control, sham, and tramadol treated groups (20 mg/Kg, single dose, i.p.). The firing of putative VTA-DA neurons recorded under urethane anesthesia. Single dose of urethane (1.2 gr/kg) which is usually sufficient for the entire recording, but the booster doses (15-25% of the initial dose) used if there was any discomfort sign. Animal body temperature continuously monitored to maintaining at ~37 °C during the experiment. Finally, the stereotaxic coordinates for bregma and interaural line were calculated from the bregma zero-zero (BZZ) plane and a burr hole for electrode placement was drilled to access the VTA according to the Paxinos and Watson rat brain atlas (Paxinos & Watson, 2007).

2.3. In vivo Extracellular Electrophysiological Recording of VTA-DA neurons
Extracellular multi-unit electrophysiological activity of the putative VTA-DA neurons recorded under urethane anesthesia as described previously. Briefly, after anesthesia the rat mounted into the stereotaxic frame (Steolting, USA), the skull exposed, and the stereotaxic landmarks were determined for opening a burr hole on the BZZ plane. Glass microelectrodes (in vitro impedance 3-6 MΩ, at 1 kHz) were filled with pontamine sky blue (2.0%) in sodium acetate solution (0.5 M) and lowered into the VTA (Bregma= -6.84, ML= ±0.5 and DV= 8.6 mm at the BZZ plane in adjusted coordinates, angle=15° coronally). Signals amplified (10,000×) and filtered (300-3000 band pass, sample rate=50,000) for acquisition through a high speed, isolated USB port on a PC running Windows 7.0 Premium Pro. Only spontaneous active right putative VTA-DA neurons recorded and analyzed. The criteria explained in previous articles included in analysis software protocol. The main criteria were low discharge rate (<10 spike/sec), long-duration of spike (2.5 ms) and a triphasic (+/-+) or biphasic spike commonly with a notch in the positive component (Hyland, Reynolds, Hay, Perk, & Miller, 2002; Nimitvilai, Arora, McElvain, & Brodie, 2012). Only units with stable firing recorded while non-stable ones ignored. The first 10 minutes recorded as baseline spiking after stability. In the sham group, the drug vehicle (fresh normal saline) infused intraperitoneally then records continued to investigate the confounding effects. In the control group, the firing of putative VTA-DA neurons recorded up to 120 minutes to evaluate recording condition and stability. Units isolated with Igor Pro 6.0 (Wavemetrics Inc. USA) and Principle Component Analysis (PCA). Peri-Stimulus Time Histogram (PSTH) calculated off-line for all recordings and the average of firing rate in the pre- and post-infusion periods used for each recording in statistical analysis.

2.4. Data Analysis
The Kolmogorov–Smirnov (K-S) test used as a goodness of fit test for statistical probability distribution of data for parametric or non-parametric statistical tests. The one-way repeated measures ANOVA used for comparing intra- and inter-groups mean firing rates with $p<0.05$ as the least statistical significance level. The information presented as Mean± SEM and data analysis implemented with Igor Pro 6.0 software.

2.5. Histological Verification

At the end of the experiments, the recording site marked by passing -20µA electrical current through the recording electrode for 10 minutes to deposit pontamine sky blue dye. The rats deeply anesthetized, perfused transcardinally with phosphate buffered (10%) formalin solution (4%) and the brains removed and fixed in perfused solution. 40-μm coronal sections took with a microtome (SLEE, London) to exploring the electrode tip location and the trajectory path under a light microscope.

2.6. Drugs and Materials

Drugs and chemicals used are as follows: tramadol, formalin, Pontamine Sky Blue, fast cresyl violet, urethane (Sigma-Aldrich, USA), sodium acetate and sodium chloride (Merck, Darmstadt, Germany), polyethylene microtube (A-M system, USA), Hamilton micro-syringes (Hamilton Bonaduz AG, Switzerland).
3. Results:

3.1. Summary:

Briefly, a single dose of the tramadol infused intraperitoneally and the acute tramadol effect on the putative VTA-DA neurons studied. Tramadol infusion could inhibit the neuronal firing of putative VTA-DA neurons (acute neuronal tolerance) in a short time but the firing of neurons return to baseline for a while and increase up to double rate of baseline (acute neuronal dependence or withdrawal). The average of firing rate of the putative VTA-DA neurons can explain some of side effects of acute tramadol administration.

3.2. The putative VTA-DA neuronal spike signature.

The putative VTA-DA neurons with stable and tonic spiking isolated in all recordings. Totally 125 VTA-DA neurons obtained in all groups and recorded for up to about 3 hours maximally. The spike shapes of the extracted putative VTA-DA neurons had a three phasic mode commonly. In Fig.01, a typical spike signature showed. Mean spike amplitude in the recordings was 520±87.5 microvolts peak-to-peak. Putative VTA-DA neurons also showed low spontaneous firing rate (<10 spikes/sec). As shown in the Fig.01 the main spike signature are the low firing rate and long-duration.

3.3. The histogram of the putative VTA-DA neurons in control and sham groups.

The firing rates of putative VTA-DA neurons in the rats of control (naïve) and sham groups showed in the Fig.02 and 03. Fig.02 shows the firing of a sample putative VTA-DA neuron in control group. The mean firing rate of this neuron was 4.4±0.61 spikes/sec during 120 min. The mean firing in each minute calculated from the averaged firing in each second in every minute. The
Fig. 02 shows the stable firing without any pattern change. Fig. 03 shows firing of a sample of putative VTA-DA neuron in sham group, in the pre- and post-vehicle injection (normal sterile saline as vehicle of tramadol), in the 10th minute of recording. The sample neuronal firing rate was $6.13 \pm 0.82$ spikes/sec. Saline had no any significant effect on the neuronal firing rate.

3.4. The histogram of the putative VTA-DA neurons in tramadol-treated group.

The acute effect of the intraperitoneal (i.p.) application of tramadol (20 mg/Kg, single dose) on putative VTA-DA neurons firing evaluated. Fig. 04 shows a typical firing of a putative VTA-DA neuron to application of tramadol. The baseline pre-injection firing rate of the neuron was $5.84 \pm 0.17$ spikes/sec. Tramadol (20 mg/kg, i.p) injected in the 10th minute of recording. The recording of neuronal firing continued until the firing of post-injection period returned to the rate of the pre-injection period. In all selected recordings of tramadol-treated animals, neuronal firings showed three district phases. In a short time after tramadol injection, the neuronal firing decreased. This period called inhibition period or the tolerance to tramadol. In the showed sample, the neuron was silent about 13 min and in the inhibition period, the mean of firing was $1.83 \pm 1.94$ spikes/sec. The inhibition period calculated from minute that the mean of firing rate decreased statistically significant from previous consecutive minute. The termination of the inhibition period calculated as the same procedure as for inhibition that firing rate returned to the pre-injection one. In the sampled neuron, the duration of inhibition was 103 min. The inhibition of neurons followed by an increase of the firing rate. This period was called as excitation period or withdrawal sign (dependence period). The duration of excitation phase calculated by statistical significant difference of the post-inhibition firing in the minute that firing returned to the pre-injection period and then increased. The mean firing rate of excitation period of the showed sample in the Fig. 04 was $8.33 \pm 1.87$ spikes/sec and maximum rate was 11.4 spikes/sec. The final baseline firing
calculated and continued for about 15 min. The duration of the excitation period was 87 min. The final baseline firing was 5.2±0.16 spikes/sec.

Fig.05 depicts the mean firing rate of control group neurons (n=33) in 120 min of recording. The mean firing of control groups was 5.82±0.83 spikes/sec. The mean firing rate of the sham group neurons (n=31) was 6.13±0.82 spikes/sec and did not change until 120 min of recording. The mean neuronal firing of tramadol treated group were 5.24±0.57, 1.53±0.94, 8.33±1.27, 5.2±0.66 in the pre-injection, inhibition period, excitation period, and final baseline respectively. Statistical comparison of control and sham group’s neuronal firing rate showed there are no any significant effect of vehicle in putative VTA-DA neuronal firing and pre-injection and final period in the tramadol treated groups. The neuronal firing rates decreased in the inhibition period (cellular tolerance) and increased in the excitation period (cellular withdrawal or dependence) in comparison with pre-injection and final baseline firing of tramadol treated groups, also control, and sham groups.

Totally 57 putative VTA-DA neurons isolated in the tramadol-treated group. Overall, tramadol-induced inhibition of neurons began 6±0.5 minutes after the injection and lasted up to 50.34±20.17 minutes. Thirty-one % of neurons abolished firing and silenced for 24±3 min but remaining lowered their firing about 43% to 67% of their baseline firing in average. The inhibited neurons showed withdrawal response to the tramadol with increasing the firing. In the excited period, the neurons showed excitation that lasted about 56.12±15.30 min. In this period, the neurons raised up their firing from 176% up to 244% of their baseline or pre-injection period.
4. Discussion

In this study, the results showed that the acute effect of tramadol on some putative VTA-DA neurons has two consecutive inhibitory and excitatory effects. We referred the inhibitory effect as cellular tolerance and excitatory response as cellular withdrawal or dependence. The inhibitory response lasted about 50±20 min and the excitatory period lasted about 56±15 min. The biphasic response of the recorded neurons showed that some of the dopaminergic neurons of the VTA might be involve in the exploring of the neuronal adaptation to acute effects of tramadol. The results of this study is the first report about the putative VTA-DA neurons to tramadol at the extracellular electrophysiological levels. The indirect evidence can explain the basis of the responses but the pharmacological evidences need to elucidate the tramadol effects on these neurons.

4.1. Tolerance and dependence: neuroadaptation of the reward system

The substance use disorder (SUD) is a neurochemical dysregulation of the brain reinforcement system. In the chronic use, the brain structural and functional abnormalities lead to different somatic and psychologic outcomes (Russo et al., 2010). The abused drugs associated with SUDs could elevate dopamine to supraphysiological levels and subsequent pre- or post-synaptic events occurred based on receptor post-signaling cascade. The genomic and non-genomic effects related to the type and duration of drug use. The involvement of cyclic adenosine mono-phosphate (cAMP) via G-proteins are the key focal point to the events (Nestler, 2012). The contribution of the VTA in the reward circuitry evidenced by its roles in the conditional and/or non-conditional aspects of SUDs (Volkow et al., 2010). The potency of drugs to activate or inhibit the neuronal elements of the reward circuitry can produce positive subjective effects to use a given substance. Drug craving has originated from continued desire produce the tolerance and withdrawal signs that
may produce physical and psychological problems. In the chronic drug consumption, the synaptic plasticity can produce mal-adaptation, which is responsible for the tolerance and withdrawal. The intensity of these SUDs symptoms varies greatly across different classes of substances but undoubtedly contributes to continuation of use. The treatment of withdrawal-induced alterations needs the understanding of the biochemistry and neuroadaptations to chronic or acute use (Everitt, 2014). Drug-induced neuroadaptations in the repeated use initiate two clinical consequences events, drug tolerance and withdrawal. The need to achieving proper effects with incremental doses express the tolerance and a syndrome that occur at disruption of drug called withdrawal. The neuronal basis of tolerance and withdrawal is overlapped and withdrawal appeared in the tolerated patients. In tolerance, the responsiveness of the neuronal receptors to stimulation by drugs gradually decrease and the greater amounts of drug might use to produce demanded effect. The cellular mechanism for tolerance related to the neurotransmitter receptors, cAMP, protein kinas A (PKA), and cAMP response element-binding (CREB) signaling molecules. The genomic alteration could prolong and enhance the tolerated responses in parallel with neurotrophic factors (Trujillo, 2002). Opiate tolerance provides a well example to show association of neuroadaptations of tolerance and withdrawal. The locus coeruleus (LC) is expressed important changes in opiate tolerance and withdrawal. Noradrenergic LC efferents project to prefrontal cortex, VTA, brainstem, and some subcortical area to excite them. Opioids that bind to mu opioid receptors on the LC neurons, suppress them and loss the stimulation on its target. In the presence of chronic opiate, LC neurons compensate the suppressive effect in case of enhancing the intracellular metabolic machinery to produce noradrenaline. The sudden abrupt of opiates kindle the enhanced system to initiate the withdrawal symptoms (Trujillo, 2002). At the cellular level, the tolerance of the LC neurons to opiates is coincident with lowering and withdrawal response to increasing the
firing rate (Rasmussen, 1991; Rasmussen, Beitner-Johnson, Krystal, Aghajanian, & Nestler, 1990). The ablation of the LC from its afferents *in vivo* or *in vitro* showed that the withdrawal-induced activation of the LC neurons is dependent to its afferents (Rasmussen & Aghajanian, 1989). It can postulated that the tolerance and withdrawal in the cellular level related to the decrease and increase of firing rate.

4.2. Tramadol: new analgesic, new challenge.

Tramadol is commonly used in many countries despite its potential for mis- or abusing. The use of tramadol is uncontrolled in some countries. It may have high abuse-risk potential in the long run (H. Zhang & Liu, 2013), the fact that makes its limitation in the pharmacopeia recently (Patt & McDiarmid, 2005). Tramadol as an opioid drug may acts via a multiple mechanisms to changing the behavioral responses. The acute and chronic use of tramadol showed different effects on the behavioral processes. The mechanism of the tramadol action in analgesia and addiction is not well established (Minami, Ogata, et al., 2015). Tramadol can easily pass the blood brain barrier to make a central effect on behavior such as depression and motivation (Bertaina-Anglade, Enjuanes, Morillon, & Drieu la Rochelle, 2006; Tetsunaga et al., 2016; Tetsunaga, Tetsunaga, Tanaka, & Ozaki, 2015). The low susceptibility of addiction of tramadol propels its use without any consideration to other perspectives of its side effects. In addition to typical withdrawal symptoms of opiates, the tramadol withdrawal symptoms include in other atypical symptoms such as hallucinations, paranoia, extreme anxiety, panic attacks, confusion and unusual sensory experiences such as numbness and tingling in one or more extremities (Senay et al., 2003). Unfortunately, recent reports of the World Health Organization warns about increase the misuse and abuse of tramadol in Africa and Western Asia (WHO, 2014). Although the addiction to tramadol can interfere with other medications but for any clinical trials may important the fluent
documentation to choose the right medication in patients with tramadol misuse (Pathak, Kumar, & Rastogi, 2017). The main challenge in the field of tramadol is the hidden part of the tramadol function in the body especially in the brain. There are documents about the enhancing the addition potency of tramadol to initiation to other addictive drugs and vice versa. The opioid dependent persons may have experiences more susceptible to tramadol misuse (Liu et al., 1999; Naslund & Dahlqvist, 2003). The tramadol-induced seizure is a high risk for its long-term use with increase the incidence of serotonin syndrome due to its serotonin reuptake inhibition (McNicol, Midbari, & Eisenberg, 2013).

4.3. Tolerance to acute administration of tramadol

In this study, tramadol decrease the firing rate of putative VTA-DA neurons in the early phase of response. The VTA-DA neurons that had regular firing with low rate showed cellular tolerance as inhibition and cellular dependence as excitation. Tramadol could inhibit the neurons about 50 min with after-excitation lasted about 56 min.

At the cellular and molecular levels, tolerance and dependence mediated by different signaling mechanisms. The chronic administration of tramadol can produce the tolerance due to its effect as the same as of opiates that explained previously. The mechanism of chronic–induced tolerance, dependence, and withdrawal to tramadol are probably the same as other opiates. The chronic form of abused drug could produce the global brain changes in the regions that mediate addiction directly. The mal-adaptation and dis-regulated neuronal responses could produce the profound somatic or psychologic symptoms. Molecular and cellular modifications of reward system in the chronic administration of the opiates reflect the memory formation for many opiates effects (Rosen, Sun, Rushlow, & Laviolette, 2015).
The acute administration of the opiates are very common to management the post operation pain. The acute tolerance is an important issue because of the efficacy of analgesics. Ming et al showed that the co-administration of the tramadol with dihydroetorphine in rats could produce profound synergistic analgesia, which could delay the onset and developing of the tolerance to tramadol (Ming, Wang, Han, & Luo, 2005). The acute tolerance, which is rapidly developed, related to the interaction of the receptors of the abused drug. Receptor desensitization and/or internalization are perhaps the important reply of the neuron to acute exposure to the opiate. The neuroadaptations at different levels can explain the acute tolerance. Receptor tolerance referred to the loss of responsiveness of the target receptor occupation over the time. The loss of cell surface receptors or functional coupling may produce inhibition of the neuron. The involvement of the potassium channels to producing the decrease of the neuronal activity are proposed (Williams, Christie, & Manzoni, 2001). Recent studies documented the role of intracellular regulators of receptor signaling (RGS) molecules. The mice with knockout of RGS9-2 showed an increase in sensitivity and delayed tolerance with exacerbated physical dependence to morphine in acute and chronic administration (Zachariou et al., 2003). The knockout of the RGS9 but not RGS2 in mice, increase potency and duration of the opioid analgesia. The single effective dose of morphine could not showed acute tolerance and the development of tolerance after a daily intracerebroventricular injection of the opioid for 4 days (Garzon, Rodriguez-Diaz, Lopez-Fando, & Sanchez-Blazquez, 2001).

Actually, the tramadol as an opiate analogue provides the induction of acute tolerance by modification of the opioid receptors and/or other mechanisms. The involvement of opioid receptors with interactions of other neurotransmitter receptors could explain some side effects or mal-outcomes of the tramadol in a single dose of administration. The clinical outcomes of the
Tramadol is better than some opiates and due to its low side effects, it marketed as an Over-The-Counter (OTC) drug in some countries but the precise mechanisms and side effects of the tramadol remains to be elucidated.

4.4. Cellular activity of the VTA-DA neurons to acute administration of tramadol.

The in vivo extracellular neuronal activity is a well-known technique to achieving the cellular effects of the agents. The firing rate alteration is the most neuronal response can conduct other studies to evaluating the cell signaling. Sevcik et al showed that tramadol and its main metabolite, O-Desmethyltramadol (O-DT), inhibits the spontaneous discharge of locus coeruleus neurons in prepared slices in a concentration-dependent manner. The effects of (-)-tramadol was abolished in the presence of rauwolscine (an alpha 2-adrenoceptor antagonist) while the effects of (+)-O-DT virtually disappeared in naloxone presence (an opioid antagonist). (+)-Tramadol and (-)-O-DT became inactive only in naloxone and presence of rauwolscine. (-)-Tramadol and (+)-O-DT can hyperpolarize membrane potential of LC neurons and thus could inhibit spontaneous firing. This effect abolished by rauwolscine. (+)-O-DT-induced hyperpolarization abolished by naloxone. The hyperpolarizing effects of noradrenaline can be potentiated in (-)-tramadol presence, not in (+)-O-DT's. There is no potentiation of the noradrenaline effect when the cells are hyperpolarized by current injection to an extent similar to that produced by (-)-tramadol. Both noradrenaline and (-)-tramadol decrease input resistance. These data confirm that the analgesic effects of tramadol included in both opioid and non-opioid components. It appears that (-)-tramadol inhibits noradrenaline reuptake and via a subsequent increase in the concentration of endogenous noradrenaline, stimulates alpha 2-adrenoceptors indirectly. (+)-O-DT seems directly stimulate opioid micro-receptors. The effects of (+)-tramadol and (-)-O-DT consist of combined micro-opioid and alpha 2-adrenergic components (Sevcik, Nieber, Driessen, & Illes, 1993).
Koga et al showed that mono-O-dimethyl-tramadol (M1) superfusion could inhibit substantia gelatinosa neurons \textit{in vitro} in whole-cell patch-clamp recordings. In 41% of neurons, M1 produces outward current at -70 mV that reverses at potential close to $E_K$. The hyperpolarizing current persists for more than 30 minutes and hardly declines after its washout. This current correlates with DAMGO-induced (a MOR agonist) current in amplitude and is largely reduced by CTAP, an MOR antagonist, not by yohimbine. Noradrenaline produces outward current at -70 mV in a neuron where M1 has no effect on holding currents. M1 can produce persistent hyperpolarization by activating MOR in adult rat SG neurons (Koga, Fujita, Totoki, & Kumamoto, 2005).

Haeseler et al showed that sufentanil, fentanyl and tramadol reversibly suppress sodium inward currents at high concentrations when depolarizations started from hyperpolarized holding potential but morphine does not. Short depolarizations inducing fast-inactivation and long induction of pre-pulses slow-inactivation significantly increase blocking potency of tramadol and morphine. Sufentanil, fentanyl and tramadol block voltage-gated sodium channels (Haeseler et al., 2006).

Tramadol can suppress the amplitude of delayed rectifier K+ current and shift its steady-state inactivation to more negative membrane potential in the NG108-15 neuronal cell line. This effect can stabilize resting membrane potential and reduce the firing rate of neurons. The effects of tramadol on blocking these channels can explain its antidepressant action (Tsai, Tsai, Wu, & Liu, 2006).

Although tramadol, whose precise mechanism remains to be elucidated, because of relation to opioid analgesics, commonly use due to low dependence, tolerance and side effects vis-à-vis some opioid analgesics (Casali, Lepri, Cantini, Landi, & Novelli, 2000; Caspani et al., 2014). While MOR activation and monoamine reuptake inhibition proposed in the presence of tramadol, there is no evidence for direct action of tramadol on MORs. It may act on ion channels and G protein-
coupled receptors (GPCRs) along with monoamine transporters (Minami, Sudo, et al., 2015). It is believed that advantageous antidepressant-like properties of tramadol is related to its monoaminergic reuptake inhibition (Barber, 2011; Caspani et al., 2014; Ferrari, Tiraferri, Palazzoli, & Licata, 2014). The interaction of tramadol with MORs is well established. Tramadol, morphine and buprenorphine can produce conditioned place preference (CPP) dose-dependently due to mechanism may mediate by MORs. Tramadol, morphine and buprenorphine together also produce CPP sub-effectively, but adjuvantly to potentiate rewarding effects of morphine or buprenorphine (M. Zhang et al., 2012). Nalbuphine, a kappa opioid and mu opioid partial agonist can block Tramadol-induced CPP. CPP and antinociception effects of tramadol related to the dopamine level of VTA-DA neurons (Abdel-Ghany, Nabil, Abdel-Aal, & Barakat, 2015).

As noticed, there are evidence on the interaction between tramadol, MOR, and non-opioid receptors. In heterozygous and homozygous MOR knockout (MOR-KO) rats, tramadol-induced antinociception reduced, significantly. This antinociception is not greatly affected by methysergide, a serotonin receptor antagonist, but is partially blocked by yohimbine, an adrenergic alpha-2 receptor antagonist, and both naloxone, a non-selective opioid receptor antagonist, and yohimbine. It suggested that MOR and the adrenergic alpha-2 receptor mediate most of analgesic properties of tramadol (Ide et al., 2006). Along with these findings, some studies showed that different opioid and non-opioid agents could change the dopamine level of the brain. The main brain area that could influence by these agents are VTA-DA neurons. These neurons can change the firing rate to affect their targets (Chenu, El Mansari, & Blier, 2009; Rodriguez-Landa, Contreras, Gutierrez-Garcia, & Bernal-Morales, 2003; Werkman et al., 2004; Zhou, Bunney, & Shi, 2006).
In summary, the effect of tramadol on VTA-DA neurons partly related to the tolerance and dependence of these neurons in cellular level. Tramadol alone can produce cellular dependence and withdrawal. It may contribute the MORs and other neurotransmission to produce the cellular tolerance and dependence. The results of this study is the first report about the effect of acute tramadol on the VTA-DA neuronal firing for explaining its tolerance and dependence function at the cellular level. Extracellular recording can explore the total neuronal response and this report showed that some of VTA-DA neurons could produce the neuronal signaling to tolerance and dependence (withdrawal). The precise evaluation of the putative VTA-DA neurons to the acute effects of tramadol needs the different cellular and molecular studies to find the key factors that effective on these neurons.
Acknowledgment and Author’s contribution:

Firouz Ghaderi Pakdel was responsible for the study concept, design, student mentorship. Khodayari Sh contributed to the acquisition of animal data. Naderi S performed the Igor analysis blindly. Shahabi assisted with data analysis and interpretation of findings. All authors critically reviewed content and approved final version for publication. Urmia Research and Technology Vice Chancellor Grant (Grant No 1961) sponsored this study and the practical works done at Danesh Pey Hadi Company lab.
References:


Figure 01: A typical multiunit recording of the VTA dopaminergic neurons in control group. A) A trace of the multiunit recording. B) A typical spike signature of VTA dopaminergic neuron isolated from the recording. The VTA dopaminergic neurons have commonly a tri-phasic extracellular spike shape with the firing rate less 10 spikes/sec. The showed neuron had 3 phasic (+/-/+) shape with a notch in the beginning of the first + phase. Mean amplitude of the showed neuron was about 800 microvolts peak-to-peak. VTA dopaminergic neurons showed low spontaneous firing rate (<10 spikes/sec).
Figure 02: A sample of the multiunit firing of the putative VTA dopaminergic neurons in the control group. A) A sample trace of the multiunit firing of the VTA dopaminergic neurons in the control group. B) The histogram of the firing of the VTA dopaminergic neurons of the control group. In control group (naïve) animals, the VTA dopaminergic neuronal firing under standard condition recorded up to 120 min. The mean firing rate of the neurons in the control group was $4.4 \pm 0.61$ spikes/sec. The mean firing in each minute calculated from the averaged firing in each second in every minute. Data has shown as Mean±SD.
Figure 03: The firing of the putative VTA dopaminergic neurons in the sham group. The tramadol vehicle (sterile normal saline) applied intraperitoneally, pre- and post-injection firing rate of the selected neurons evaluated. A) The firing of sample VTA dopaminergic multiunit neurons in the sham group. In sham groups, the putative VTA dopaminergic neuronal firings under standard condition recorded up to 120 min. Injection of the tramadol vehicle (sterile normal saline, in 10th min) done by gage 30 syringe. B) The histogram of the neuronal firing rate of the recorded neurons. Vehicle had no significant effect on the neuronal firing. The statistical paired student *t*-test used for statistical analysis.
Figure 04: The histogram of the mean firing of a typical putative ventral tegmental area dopaminergic (VTA-DA) neuron to infusion of tramadol (20 mg/Kg, single dose, i.p., in 10th minute). The pre- and post-injection neuronal firing were recorded until the last firing return to baseline of pre-injection one. The typical decrease or inhibition of firing and post-consecutive excitation were seen in the figure. The recorded neuronal firing were acquisitioned and averaged in 1 second in each minute with 1 msec bin size for the evaluation of pattern change. The peri-stimulus time histogram compared in the pre- and post-injection to evaluation statistical significance. The recording of neuronal firing continued until the firing of post-injection period returned to the pre-injection period rate. In all recordings of tramadol treatment, neuronal firings showed two district phases in the firing rate. In a short time after tramadol injection, the neuronal firing decreased. This period called inhibition period or neuronal tolerance. In the showed sample, the neuron was silent about 13 min and in the inhibition period, the mean of firing was 1.83±1.94 spikes/sec. In this neuron, the duration of inhibition was 93 min. The second phase began with increase the firing rate as excitation or dependence period. The mean firing rate of excitation period was 8.33±1.87 spikes/sec and maximum rate was 11.4 spikes/sec. The duration of the excitation period was 87 min. The
final baseline firing was 5.2±0.16 spikes/sec. The firing rate of the baseline, inhibition, and excitation periods had difference significantly (one-way analysis of variance, Tukey’s post-hoc test, \( p<0.001 \)).
Figure 05: The bar chart of the firing rates of the putative ventral tegmental area dopaminergic (VTA-DA) neurons. The chart shows the comparison of the averaged neuronal firing rate of the control and sham groups with the separated periods of responses to tramadol injection (pre-injection, post-inhibition, post-excitation, and final baseline). Tramadol injected in the 10th minute and the neuronal firings were acquisition until the pre-injection firing returned. The mean firing rate of pre- and post-injection calculated for determination of the statistical difference. The figure shows there was a significant statistical difference between pre- and post-injection of tramadol. The two distinct phases of tramadol effects (inhibition as tolerance and excitation as dependence) seen in the firing rates of the neurons. (one-way ANOVA, Tukey’s post hoc test, *** p<0.001.).