

## Full length article

# Evaluation of dynorphin and kappa-opioid receptor level in the human blood lymphocytes and plasma: Possible role as a biomarker in severe opioid use disorder

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## ABSTRACT

**Background:** The dynorphin (DYN)/kappa opioid receptor (KOR) system plays an important role in the development of addiction, and dysregulation of this system could lead to abnormal activity in the reward pathway. It has been reported that the expression state of the neurotransmitters and their receptors in the brain is reflected in peripheral blood lymphocytes (PBLs).

**Methods:** We have evaluated the PBLs and plasma samples of four groups: 1) subjects with severe opioid use disorder (SOD), 2) methadone-maintenance treated (MMT) individuals, 3) long-term abstinent subjects having former SOD, and 4) healthy control subjects (n = 20 in each group). The mRNA expression level of pre-prodynorphin (ppDYN) and KOR in PBLs has been evaluated by real-time PCR. Peptide expression of PDYN in PBLs has been studied by western blot, and DYN concentration in plasma has been measured by ELISA.

**Results:** The relative expression level of the ppDYN mRNA and PDYN peptide in PBLs were significantly up-regulated in SOD, MMT, and abstinent groups compared to control subjects. No significant difference was found in the plasma DYN concentration between study groups. The expression level of the KOR mRNA in PBLs was significantly decreased in all three study groups compared to the control subjects.

**Conclusion:** the expression changes in the DYN/KOR system after chronic exposure to opioids, including methadone, seems to be stable and does not return to normal levels even after 12 months abstinence. These long-time and permanent changes in PBLs may serve as a biomarker and footprint of SOD development in the periphery.

## 1. Introduction

Severe opioid use disorder (SOD) is a progressive brain disorder, involving changes in neuronal pathways with complex neuroadaptive mechanisms that result in dependence, craving, and relapse. All these states could lead to the continuation of drug use whose biological

elements have not yet been reasonably described. The reward system of the brain is the common characteristic of all types of addictive drugs. The mesocortical dopamine system is the principal part of the reward system, which has a critical role in drug addiction (Sadat-Shirazi et al., 2018a, b). It is believed that all addictive drugs increase the amount of extracellular dopamine in the nucleus accumbens (NAc) as a central

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part of the reward system. This increase could be either directly (e.g., psychostimulant drugs such as cocaine and amphetamine directly affect dopamine transporters), or indirectly (e.g., opioids inhibit GABA interneurons in the ventral tegmental area (VTA), resulting in the stimulation of dopamine neurons) which leads to the “high” sensation in abusers (Noble and Marie, 2018). Cumulative evidence proposes that susceptibility to drug abuse and addiction may be dependent not only on environmental but also on genetic features (Goldman et al., 2005; Lessov et al., 2004).

The endogenous opioid system is critically involved in the development of addictive disorders. Studies have proposed that molecular dysregulations in this system may be a cause for beginning and maintenance of addiction (Bazov et al., 2013; Gieryk et al., 2010).

Dynorphins (DYN), a class of endogenous opioid peptides derived from the precursor protein prodynorphin (PDYN), have the affinity for all three types of opiate receptors. However, these peptides show considerable preference for the kappa-opioid receptor (KOR) (Schwarzer, 2009) and are claimed to mediate the aversive properties of addictive drugs because KOR agonists can induce place aversion in animals (Karkhanis et al., 2017; Mucha and Herz, 1986; Shippenberg and Herz, 1986; Zhang et al., 2005) and dysphoria in humans (Bazov et al., 2018; Shippenberg et al., 2007; Walsh et al., 2001). Aversion and dysphoria occur because KOR stimulation could result in a decrease in dopaminergic neurotransmission (Nestler, 2004).

In addition to the central nervous system (CNS), it has been demonstrated that opioid peptides including DYN, its precursor, and its related opioid receptor (KOR) are expressed by a number of cells of the immune system like lymphocytes (Chuang et al., 1995; Machelska, 2003; Mousa et al., 2004). According to a hypothesis named “peripheral marker hypothesis”, it has been claimed that the expression level of neurotransmitter receptors in the peripheral blood lymphocytes (PBLs) is in correlation with their expression changes in the brain. For example, it has been reported that the expression level of dopamine receptors in schizophrenic patients’ PBLs is increased in parallel to the high activation of central dopaminergic neurotransmission (Carlsson et al., 1999; Ilani et al., 2001; Zvara et al., 2005). In a reverse state like Parkinson’s disease, which is resulted from the reduced striatal transmission of dopamine, it has been shown that dopamine receptors are also down-regulated in PBLs, which is related to the severity of the disorder (Nagai et al., 1996; Pontieri and Colosimo, 2010). These and many other pieces of research (Amidfar et al., 2017; Liu et al., 2016; Zhang et al., 2017) have persuaded investigators that PBLs could serve as a suitable mean to assess the changes of neurotransmitter receptors in neuropsychiatric and neurological diseases and monitor the outcomes of therapeutic strategies. We have previously reported that the expression level of splice variants of the mu-opioid receptor in PBLs may change with the opioid consumption state (Vousooghi et al., 2009). We have shown these changes in other PBLs receptors like dopamine (Goodarzi et al., 2009), NMDA receptor subunits (Roozafzoon et al., 2010; Sedaghati et al., 2010), and even in behavioral addictions (Sadat-Shirazi et al., 2018a, b; Vousooghi et al., 2015). According to the critical role of KOR and its endogenous ligand DYN in SOD and their pharmacotherapeutic implications for it (Butelman et al., 2012), we have aimed in the present study to investigate whether the expression level of this opioid receptor and its related endogenous peptide in PBLs

would be altered in the process of SOD. Besides, in order to assess the trend of changes of KOR and DYN in SOD treatment, we have also aimed to study subjects in methadone maintenance treatment (MMT) and narcotics anonymous (NA) programs as two main methods of treatment of SOD. Since its introduction in 1965, methadone has been the most broadly accepted and well-investigated maintenance treatment for SOD. The key concept of maintenance treatment is replacement of opioid drug such as heroin with an opioid agonist (methadone) (Ali et al., 2017). NA group, based upon abstinence approach, is a self-help group with a supportive setting that encourages members to consume no addictive drugs and recover their personalities by a method named the 12-step program (Best et al., 2001). Therefore, we designed the present study to investigate preprodynorphin (pPDYN) mRNA, PDYN peptide, and KOR expression level in PBLs, and DYN concentration in the plasma of SOD group, MMT patients, and long-term abstinent subjects having former SOD (NA group) compared to healthy control subjects to evaluate whether these peptides and receptor could serve as peripheral markers for studying the development of SOD, effects of different therapeutic interventions, and finding vulnerable people for SOD.

## 2. Materials and methods

### 2.1. Subjects

Eighty male subjects were involved in the study: twenty SOD and twenty MMT patients were recruited from a few clinics in Tehran, twenty long-term abstinent cases were selected from Narcotics Anonymous organization (NA) (thus, from now on, NA group means abstinent subjects), and twenty control individuals were chosen from Tehran University students and staff. SOD was defined according to the DSM-5 criteria assessed by the structured clinical interview. Exclusion criteria were (1) dependence to other drugs like cocaine, amphetamine, alcohol, marijuana, or benzodiazepines (five SOD and six MMT patients were nicotine smokers but had not the criteria of nicotine dependence according to the DSM-5), (2) consuming any other legal or illegal medications that could alter the function of the CNS, (3) having a history or current major psychiatric or neurological disorder, (4) being currently affected by inflammatory or infectious disorders including HCV, HBV and HIV infection.

The control group was age-matched healthy subjects who had no background of drug dependence as assessed by clinical interview. Besides, we checked the urine samples of abstinent and control subjects using ACON 10 Panel Drug Test which is made to detect ten different types of addictive drugs (Cocaine, Amphetamine, Methamphetamine, Marijuana, Opiate, Phencyclidine, Barbiturates, Benzodiazepines, Methadone, and Tricyclic Antidepressants) in the urine. If any of the mentioned compounds were identified in the urine samples, the subject was excluded from the study. The nutritional state of the groups was not checked, but having a major general health issue was taken into account, and such subjects were not included in the study. All subjects contributed voluntarily and signed written informed consent before admission. Protocols of the project were approved by the Ethics Committee of Tehran University of Medical Sciences. Demographics of the study participants are shown in Table 1.

**Table 1**  
Demographics of study participants.

	Control	Severe opioid use disorder	Methadone maintained	Abstinent
Age (years, mean $\pm$ SD)	28.15 $\pm$ 5.25	29.7 $\pm$ 3.82	29.95 $\pm$ 5.03	32.95 $\pm$ 4.82
Duration of drug use (months, mean $\pm$ SD)	—	22.25 $\pm$ 8.85	34.61 $\pm$ 7.36	45.73 $\pm$ 9.93
Duration of methadone maintenance (months, mean $\pm$ SD)	—	—	29.45 $\pm$ 15.55	—
Duration of abstinence (months, mean $\pm$ SD)	—	—	—	12.85 $\pm$ 5.09
Dose of administered methadone (mg/day)	—	—	54 $\pm$ 19.4	—
Hepatitis C, B and AIDS	0	0	0	0

## 2.2. Isolation of PBLs

Eight ml blood sample was taken from each subject by antecubital venipuncture and transferred into an ethylenediaminetetraacetic acid (EDTA)-containing tube. All the blood samples were taken between 8 to 11 a.m. in all four groups. Blood plasma was separated for next ELISA tests, and the remaining part of the sample was transferred on a cell separation medium (Histoprep/BAG, Germany) and centrifuged in a horizontal rotor at room temperature with 1200 g for 35 min according to the manufacturer's instructions. The lymphocyte cells were isolated and washed with phosphate-buffered saline (pH = 7.4). The elapsed time between taking the blood samples and lymphocyte isolation was not more than four h. Lymphocytes were then kept in a -80 °C refrigerator until the next day in which the total RNA and protein of lymphocytes were extracted as is described in the next steps.

## 2.3. RNA and protein extraction and cDNA synthesis

Total RNA and protein of lymphocytes were extracted using the All-In-One DNA/RNA/Protein Miniprep Kit (Bio Basic, Canada) according to the company protocol. Extracted RNA and protein samples were immediately transferred to -80 °C refrigerator and kept there until the performance of the next steps. The concentration and purity of extracted RNA and protein were determined using Picodrop (GB 881 3758 91, Microliter Spectrometer Picodrop, Saffron Walden, UK). The integrity of isolated RNA was checked by 1% denaturing gel electrophoresis. In order to synthesize the first-strand cDNA from extracted RNA, one µg RNA of each sample was reverse transcribed using QuantiTect® reverse transcription kit (Qiagen) according to the manufacturer's instructions in a final volume of 20 µl.

## 2.4. Oligonucleotide primers for real-time PCR

In order to normalize the expression data of target genes, beta-actin was selected as the housekeeping gene in real-time PCR. The forward and reverse primers for amplification of pPDYN, KOR, and beta-actin were purchased from Qiagen company primer bank.

## 2.5. Real-Time PCR

All real-time PCR runs were set up using 2 µl of the cDNA sample and Power SYBR® Green PCR Master Mix (Life Technologies, Grand Island, NY, USA), according to the manufacturer's manual on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a final volume of 25 µl. The optimized annealing temperature of all primer pairs was 60 or 61 °C. The achieved data was normalized using the beta-actin as the reference gene. The samples were all loaded in duplicate, and the mean data were considered for statistical analysis. Quantification of target genes in cDNA samples was performed by the standard curve method. The specificity of PCR products was confirmed when a single peak was observed in melting curve plots. To make sure about the amplicon lengths, all PCR products were visualized on standard 2.5% agarose gel.

## 2.6. ELISA of DYN

The separated plasma of blood samples was used for ELISA analysis. The concentration of DYN in plasma samples in all four groups was evaluated by Human Dynorphin ELISA Kit (Cusabio Biotech Co., Wuhan, P.R. China) according to the company instructions using an ELISA reader to record optical density. The exact concentrations of DYN in plasma samples were calculated using a standard curve created using kit standards. Each sample was tested in triplicate, and the mean value was used for data analysis.

## 2.7. Western Blotting of PDYN

In order to confirm that the pPDYN mRNA in lymphocyte cells has been translated to PDYN peptide, we performed western blot analysis using extracted proteins from lymphocyte cells. Protein aliquots were mixed with loading buffer and subjected to denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed with a mini protean II electrophoresis cell (Bio-Rad, Hercules, CA, USA) under reduced conditions. The resolved proteins were subsequently electro-transferred onto a polyvinylidene fluoride (PVDF) membrane in transfer buffer at 4 °C overnight in a mini transblot cell (Biorad, Hercules, CA, USA). The membrane was then blocked at room temperature in Tris-Buffered-Saline (TBS)-Tween with 5% nonfat dry milk for one h. After three times washing in TBS-Tween for 10 min, the membranes were incubated by the primary antibody of human anti-PDYN (Abcam, 1:1000) at 4 °C overnight. After several times of washing, the membrane was treated with the secondary antibody [goat anti-rabbit IgG – H&L (HRP) (1:3000) in TBST milk] at room temperature for one h. The resulted bands were detected using ECL (Amersham, Little Chalfont, UK) chemiluminescence. Densitometry of western blot data was performed by image j software (Bakhtzad et al., 2016).

## 2.8. Data analysis

The data of western blot and ELISA tests were evaluated by one-way analysis of variance (ANOVA) using SPSS software (version 21). Post-hoc analysis (Tukey's test) was performed to determine particular group comparisons. Western blot and ELISA data are depicted as the mean ± standard error of the mean (SEM).  $P < 0.05$  was considered statistically significant.

Real-time PCR data analysis was done using the standard curve method. The target gene amount of each sample was found from the cycle at which the sample fluorescence crossed the threshold line (CP). The cycle number was then referred to a standard curve existing in each amplification run. We used REST-XL software (Pfaffl et al., 2002) to evaluate the data and finding statistically significant differences in the relative expression level of target genes between our study groups. REST software uses the "pair-wise fixed reallocation randomization test" to define the significance of data. The software needs the values of PCR efficiencies (E) and crossing points (CP) to calculate the expression ratio. Normalization of target gene expression against the reference gene is done using the following equation:

$$\text{Ratio} = \frac{(E_{\text{Target}})^{\Delta\text{CP}_{\text{target}} (\text{Mean control} - \text{Mean sample})}}{(E_{\text{Reference}})^{\Delta\text{CP}_{\text{reference}} (\text{Mean control} - \text{Mean sample})}}$$

$P < 0.05$  was considered statistically significant. The data are shown as fold differences of mean normalized expression values ± SEM.

## 3. Results

Fig. 1 demonstrates the relative mRNA expression level of the pPDYN gene in PBLs in control, NA, MMT, and SOD groups. pPDYN mRNA was significantly up-regulated in all three groups (NA, MMT, and SOD) in comparison to control volunteers ( $P < 0.001$  for all three groups). The order of pPDYN mRNA expression level in PBLs was SOD > MMT > NA. Besides, the amount of pPDYN mRNA in the SOD group was significantly more than the NA ( $P < 0.001$ ) and MMT subjects ( $P < 0.001$ ). No changes were found in the expression of beta-actin in experimental groups (data not shown).

Fig. 2 shows the relative expression level of PDYN peptide in PBLs samples of NA, MMT, and SOD in comparison to control subjects. The data analysis revealed that the peptide expression level in all three groups (NA, MMT, and SOD) was significantly more than the control

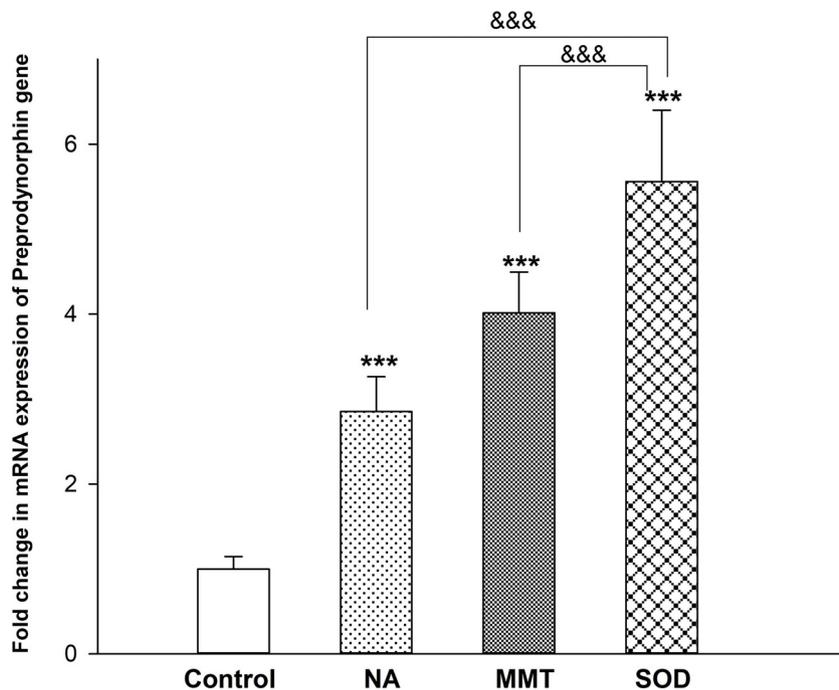


Fig. 1. Fold changes of the pPDYN mRNA expression in the PBLs of the control, NA, SOD, and MMT groups. \*\*\*P < 0.001 from the control and &&&P < 0.001 from the indicated group.

individuals (P < 0.05 for NA and MMT group and P < 0.001 for SOD patients). Moreover, the PDYN peptide level in the SOD group was significantly higher than the NA subjects (P < 0.001). The trend of PDYN peptide expression in PBLs in study groups was similar to the order of pPDYN mRNA expression: SOD > MMT > NA.

Fig. 3 illustrates the concentration of DYN peptide in the plasma samples of NA, MMT, and SOD subjects in comparison to the control group. The data analysis revealed that no significant difference existed in the plasma DYN concentration between all the study groups (P > 0.05).

Fig. 4 shows the relative mRNA expression level of the KOR gene in PBLs in control, NA, MMT, and SOD groups. KOR gene was significantly

down-regulated in all three study groups (NA, MMT, and SOD) compared to the control individuals (P < 0.001 for all three groups). The trend of KOR mRNA expression level in PBLs in study groups was opposite to the order of pPDYN mRNA level in the mentioned groups and was as follows: NA > MMT > SOD. Furthermore, the mRNA expression level of the KOR gene in NA (P < 0.05) and MMT groups (P < 0.05) was significantly higher than the amount in SOD patients.

#### 4. Discussion

Peripheral marker hypothesis has caught attention in recent years. In agreement with the potential clinical applications of this hypothesis,

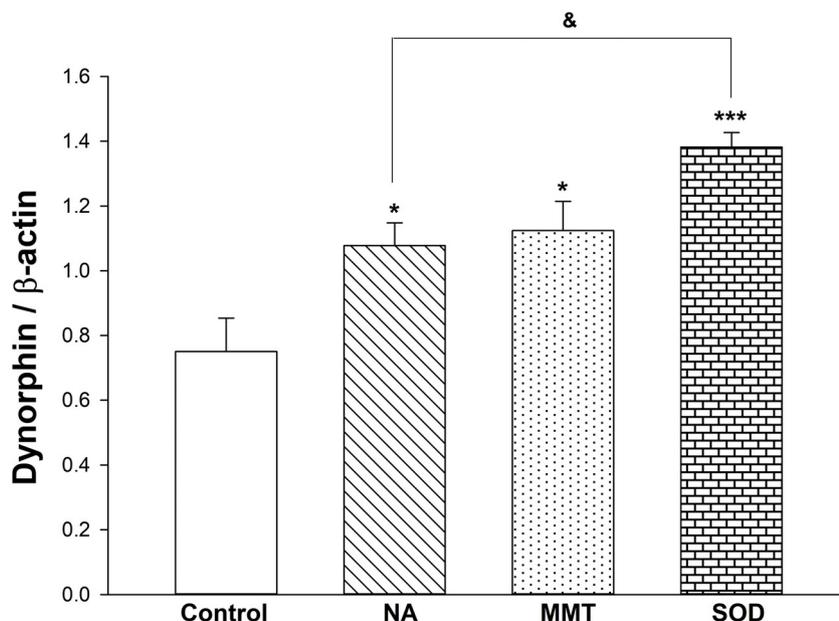


Fig. 2. Fold changes of the PDYN peptide expression in PBLs of the control, NA, SOD, and MMT individuals. \*P < 0.05 and \*\*\*P < 0.001 from the control group; & P < 0.05 from the indicated group.

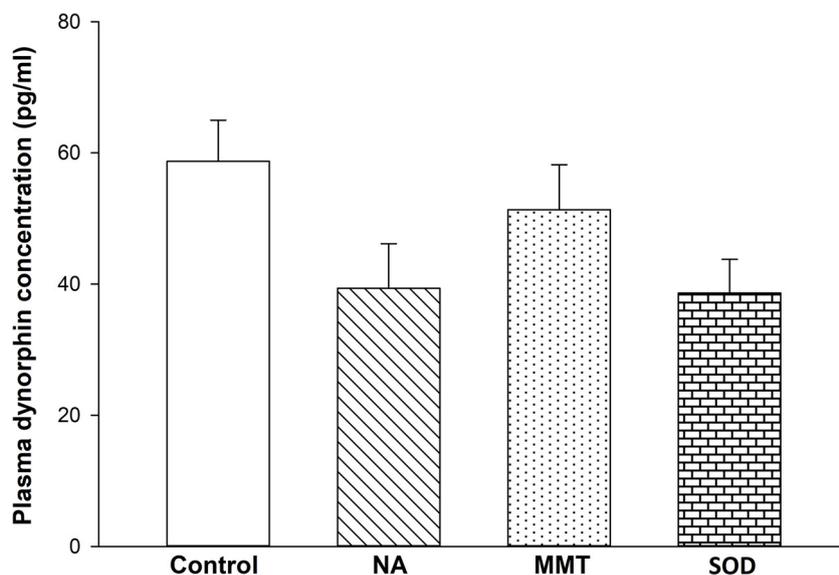


Fig. 3. Plasma DYN concentration in control, NA, SOD, and MMT groups.

Malafoglia et al., 2017 and colleagues hypothesized in 2017 that certain qualitative or quantitative features of opioid receptors on the PBLs, related to a chronic pain state, could be used as markers of pathology. The authors have claimed that the expression level and functionality of these receptors could be applicable for personalized drug therapy. They have added that using PBLs as an accessible and noninvasive diagnostic strategy could help clinicians to provide the most suitable treatment with the lowest cost, dose, stress and anxiety, and time duration which could lead to attenuation of tolerance and addiction to opioid drugs. Here, we aimed to evaluate whether the DYN / KOR system in PBLs and plasma could serve as a peripheral marker in SOD. The DYN/KOR system has been considered in the development of SOD. It has been proposed that dysregulation of this system may contribute to abnormal activity in brain sites that affect the initiation and maintenance of drug abuse (Bazov et al., 2013; Tejada et al., 2012; Tejada and Bonci, 2018).

The findings of the current study demonstrated that the expression of pPDYN mRNA and PDYN peptide in the PBLs of SOD, MMT, and NA groups are elevated in comparison to control subjects. Although similar studies in PBLs are minimal, however, there are some reports about the expression of pPDYN mRNA and PDYN peptide in various parts of the brain after chronic administration of addictive drugs. For example, preclinical data have revealed that the level of PDYN mRNA and dynorphin peptide is increased in the rodent and primate caudate putamen (Caputi et al., 2014; Daunais and McGinty, 1994, 1995; Hurd et al., 1992; Romualdi et al., 1996; Schlussman et al., 2005; Sivam, 1989; Spangler et al., 1993, 1996; Spangler et al., 1997; Turchan et al., 1998; Werme et al., 2000; Yuferov et al., 2001; Zhang et al., 2013; Ziolkowska et al., 2006), striatum (Adams et al., 2003; Daunais et al., 1993; Fagergren et al., 2003; Tzaferis and McGinty, 2001), and nucleus accumbens (NAc) (Caputi et al., 2014; Daunais and McGinty, 1994;

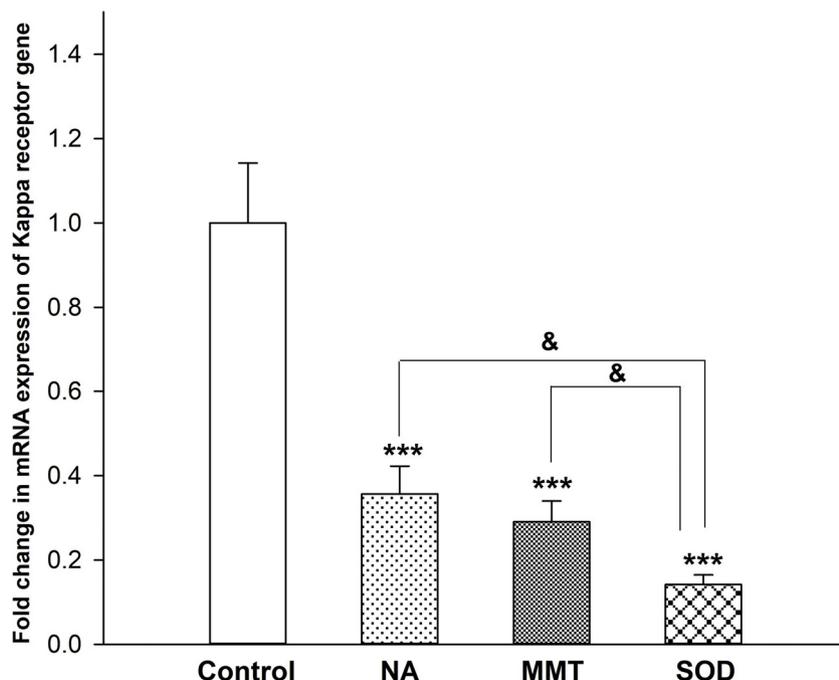


Fig. 4. The fold changes in the mRNA expression level of KOR in PBLs of the control, NA, SOD, and MMT subjects. \*\*\*P < 0.001 compared to the control and & P < 0.05 from the indicated groups.

Daunais et al., 1995; Fagergren et al., 2003; Hurd et al., 1992; Mathieu-Kia and Besson, 1998; Turchan et al., 1998; Ziolkowska et al., 2006) after acute and chronic administration of cocaine or amphetamine. Chronic consumption of opioids like morphine (McClung et al., 2005; Nylander et al., 1995a, b; Przewlocka et al., 1996; Rattan et al., 1992; Trujillo et al., 1995; Turchan et al., 1997; Wan et al., 1998) and heroin (Solecki et al., 2009; Weissman and Zamir, 1987) has also been reported to up-regulate the level of PDYN-derived peptides in brain sites like the striatum, NAc, globus pallidus, hypothalamus, hippocampus, central nucleus of amygdala, locus coeruleus, and ventral tegmental area (VTA) in rodents. It has been reported that direct stimulation of the mu-opioid receptor can modulate the up-regulation of the PDYN gene (Gonzalez-Nicolini et al., 2003; Horner and Keefe, 2006). Besides, it has been shown that long-time alcohol exposure could also lead to the elevation of DYN system in the brain. Intra-gastric binge-like alcohol consumption for one day is enough to increase PDYN mRNA expression in the rat amygdala and prefrontal cortex (PFC) (D'addario et al., 2013). More prolonged durations of alcohol exposure have also up-regulated DYN expression in the rat hippocampus (Kuzmin et al., 2013), hypothalamus (Chang et al., 2007, 2010), NAc (Lindholm et al., 2000; Przewlocka et al., 1997), central nucleus of the amygdala (Chang et al., 2010; Kissler et al., 2014), and medial PFC (Chang et al., 2010). In addition to the state of drug abuse, there are some other conditions that drugs, including opioids, are used chronically in a non-dependent context such as chronic pain. Opioids have become the backbone of treatment for several forms of acute and chronic pain (Patanwala et al., 2008; Rozet et al., 2014). It has been reported that chronic exposure to systemic or spinal morphine or spinal DAMGO could lead to the increased dynorphin amount in the lumbar cord and immunoreactivity for PDYN (Gardell et al., 2002; Liang et al., 2013; Sahbaie et al., 2016; Vanderah et al., 2000, 2001). It has been claimed that chronic opioid exposure could affect the PDYN gene through epigenetic mechanisms and increase the levels of acetylated H3K9. This effect could raise the expression of PDYN within the CNS (Descalzi et al., 2015; Geranton and Tochiki, 2015; Liang et al., 2013, 2015).

Various Human studies are also by the statement that chronic drug abuse may lead to the up-regulation of DYN system in the brain. For example, it has been reported that the pPDYN mRNA and DYN level is elevated in the caudate, putamen, ventral pallidum, cingulate, and dorsal lateral PFC of human methamphetamine and cocaine users (Frankel et al., 2007, 2008; Hurd and Herkenham, 1993; Peckys and Hurd, 2001). In post-mortem samples taken from alcoholics, it has been detected that PDYN mRNA and dynorphins in dorsolateral-PFC and hippocampus were elevated (Bazov et al., 2013). A history of marijuana use has also been reported to be associated with elevated expression of DYN system in the cingulate and dorsal lateral PFC (Peckys and Hurd, 2001). These reports, together with our data, may serve as another evidence for peripheral marker hypothesis, which claims that the expression pattern of neurotransmitters and their receptors in the brain could be reflected in PBLs.

Dynorphins and the KORs are expressed in many parts of the dopaminergic mesolimbic-mesocortical pathway and carry out a modulatory function in drug reward, probably by affecting the basal and drug-induced dopaminergic tone (Kreek et al., 2002). Dynorphin peptide reduces dopamine amount in many parts of the dopaminergic reward pathway. DYN/KOR system may perform a counter-modulatory role in the brain after drug-induced dopaminergic stimulation (Zhang et al., 2005). Thus, the up-regulation of pPDYN mRNA and PDYN peptide in the brain after chronic consumption of addictive drugs like opioids may be a compensatory mechanism and negative feedback to counteract the overstimulation of mesolimbic dopaminergic system (by increasing the production of endogenous ligands of the KORs) (Koob, 2008). Our data showed that the pPDYN mRNA and PDYN peptide in PBLs was up-regulated in MMT and NA groups in addition to the SOD subjects. It seems that methadone is not considerably different from other addictive opioids concerning the effect on DYN system, although

the up-regulation in the MMT group was less than the SOD subjects. Besides, these data demonstrated that the up-regulated state of pPDYN mRNA and PDYN peptide in PBLs was maintained in the NA group, which were drug-abstinent for a considerable period. This finding may confirm some previous reports that neuro-adaptations induced by chronic drug abuse are stable and may remain for a long time after discontinuing the drug. It has been proposed that in subjects with severe drug use disorder, the augmented DYN tone may lead to the reduced transmission of dopamine, develop dysphoria and depressive-like behaviors, and contribute to the elevated drug intake (Bazov et al., 2018). These changes may be a possible mechanism for evaluating and relapse in drug-abstinent people, which needs to be further evaluated in future studies. Another finding of our study was the trend of pPDYN mRNA and DYN peptide expression in PBLs as SOD > MMT > NA > control. Although the expression level in all three groups was significantly higher than the control subjects, however, the amount in NA and MMT groups was considerably less than the SOD patients. This finding may indicate the reflection of the effect of treatment strategies in PBLs, which could be used to follow the therapeutic effectiveness of different approaches in the management of SOD. These data demonstrated that although NA and MMT approaches did not succeed in restoring the expression level of DYN gene to normal quantities, however, they were significantly different from the SOD group and closer to normal levels.

Although our data of PBLs at the level of mRNA and peptide were consistent (similar to the previously reported positive and extremely significant correlation between PDYN mRNA and PDYN-derived peptides in the human striatum (Sarkisyan et al., 2015)), our plasma data were not in line with PBLs findings. Plasma DYN concentration in MMT, NA, and SOD groups was not statistically different from control subjects. This discrepancy may result from the fact that the quantitation of neuropeptides in plasma may not be much accurate because of their instability resulted from degradation by some proteases. Metabolism of dynorphins in human plasma by specific proteolytic enzymes (Chou et al., 1994; Muller and Hochhaus, 1995; Oizumi and Hayakawa, 1991) may alter the real concentration of the intact peptide released into the plasma. Other implications would seem to be that either a) proteases are more active in the opioid groups to reduce higher dynorphin levels in tissues to the same level in plasma or b) that blood proteases maintain dynorphin plasma levels at a set level independent of tissue levels or c) tissue (CNS / PBLs) levels are not correlated with plasma levels. Besides, the DYN peptide could also be metabolized in the CNS and cerebrospinal fluids (Csuha et al., 1995; Nyberg et al., 1986). The transport of the peptide and its metabolites from the CNS to the plasma and vice versa across the blood-brain barrier has been reported (Terasaki et al., 1989; Turner et al., 1998). All this evidence may explain the discrepancy between the data obtained from PBLs and plasma. It seems that as a peripheral biomarker in SOD, we can only count on the findings of PBLs and not the plasma data. Confirmation of our suggestions needs further evaluation in the future.

We also evaluated the expression level of KOR mRNA in PBLs in all four groups of the study. KOR in the reward pathway is located on the VTA dopamine terminals in NAc that allows dynorphins to prevent dopamine release. Stimulation of KOR leads to dysphoria in humans (Pfeiffer et al., 1986; Wadenberg, 2003) and place aversions and depressive-like behaviors in animals (Di Chiara and Imperato, 1988; Mucha and Herz, 1985). Our data demonstrated that the expression level of KOR in PBLs of SOD, MMT, and NA groups was significantly down-regulated in comparison to control subjects. According to the peripheral marker hypothesis, we searched to find out whether the expression level of KOR is also reduced in the brain after chronic consumption of addictive drugs. Previous studies have shown that the level of KOR mRNA is down-regulated in the rodent NAc and VTA after acute and chronic administration of alcohol and cocaine or amphetamine (Rosin et al., 1999; Turchan et al., 1998). Another study has reported that the KOR level in the dorsal striatum is reduced after acute or

chronic cocaine exposure (Turchan et al., 1998). These changes in KOR may be a compensatory down-regulation in response to the up-regulation of PDYN, which occurs after chronic consumption of addictive drugs like opioids. Our data showed that KOR was reduced in all three experimental groups. Thus, treatment with methadone or being in the abstinence state (like NA group) was not enough to restore the KOR expression amount to levels of the control group. The stable changes of DYN/KOR system in the abstinent group could represent a perpetuating factor for craving and relapse in this group of individuals. Besides, our further analysis showed that although the expression level of KOR in PBLs in all three experimental groups was significantly lower than the control subjects, however, the expression amount in NA and MMT groups was considerably higher than the SOD and closer to control value. This trend could be evidence of possible partial recovery of normal functioning with NA and MMT approaches.

At last, as previous studies have claimed that nicotine could affect the endogenous opioid system (Kishioka et al., 2014), we analyzed our data once again after omitting the non-dependent smokers' data from our study groups. We found that the results and the trend of expression changes in groups were similar to the primary analysis. It seems that occasional smoking in these subjects has not significantly affected the results of this study.

## 5. Conclusion

Showing the brain changes of different neurotransmitter systems including DYN / KOR in PBLs could have some beneficial effects: 1) it can help us to better understand the molecular mechanisms of addiction and the neuro-adaptations that occur after chronic drug consumption, 2) the correlated alterations in PBLs can serve as a peripheral biomarker in order to follow up the process of treatment and evaluate the usefulness of potential therapies from molecular aspect, 3) the observed changes in PBLs can guide us in the direction of designing new drugs and strategies for treatment of drug abuse, and give us new ideas about how we can manage this critical social problem via manipulation of endogenous neurotransmitter systems by drugs.

Our data showed that the chronic consumption of opioids, including methadone, could up-regulate the pPDYN mRNA and PDYN peptide; and down-regulate the KOR in the PBLs. Also, in abstinent subjects with a history of SOD, the mentioned alterations in DYN/KOR system at the level of mRNA and protein seem to be stable and significantly different from control subjects. It may be hypothesized that these long-time changes could have a role in mediating the craving, relapse, and stress reactivity that is observed after chronic consumption of addictive drugs, including opioids. Our data also revealed that using MMT or NA approaches for SOD treatment could have some partial recovery effects towards normal levels of the DYN / KOR system in PBLs. These changes in PBLs can also serve as a biomarker and footprint of addiction development in the periphery. Confirmation of these suggestions needs further evaluation in the future.

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## Contributors

KS conducted the experiments. NV Designed the experiments, contributed in analyzing and interpreting the data, and wrote the manuscript. FG contributed in conducting the experiments. AM contributed in revising the manuscript. PB contributed in analyzing the data. MSSS contributed in conducting the experiments. NBA contributed in

conducting the experiments. AS contributed in conducting the experiments. MRZ contributed in interpreting the data and editing the manuscript. All the authors contributed to and approved the final version of the manuscript.

## Declaration of Competing Interest

No conflict declared.

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