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Expression of NR3B but not NR2D subunit of NMDA receptor in human blood lymphocytes can serve as a suitable peripheral marker for opioid addiction studies

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ABSTRACT

Glutamate is critically involved in opioid addiction. It has been suggested that neurotransmitter receptors expression in peripheral blood lymphocytes may reflect brain status. In the present study, using Real-time PCR, the mRNA expression of NR2D and NR3B subunits of NMDA glutamate receptor has been investigated in peripheral blood lymphocytes of four groups each comprising of 25 male individuals: opioid addicts, methadone maintained patients, long-term abstinent former opioid addicts, and non-addicted control subjects. We found that NR2D subunit mRNA expression was not changed in all three test groups in comparison to control subjects. However, the NR3B mRNA expression was significantly up-regulated by the factors 9.11 ($P<0.001$), 10.07 ($P<0.001$) and 4.08 ($P<0.05$) in abstinent, addicted and methadone maintained subjects, respectively relative to control group. As a conclusion, our data indicate that the transcriptional level of the NR2D subunit of NMDA receptor is not regulated by chronic opioid addiction. However, it seems that the over-expression of NR3B subunit of NMDA receptor is a long lasting result of opioid abuse. In addition, considerable decrease in the up-regulated state of the NR3B subunit by methadone may represent another benefit of methadone therapy for opioid addicts and may serve as a suitable marker to evaluate the successfulness of therapy.

1. Introduction

Glutamate as the main excitatory neurotransmitter in the brain plays a key role in opioid addiction. Glutamatergic projections from brain regions like frontal cortex to nucleus accumbens and ventral tegmental area are involved in the development of addiction and expression of addictive behaviors like drug-seeking or sensitization (Kalivas et al., 2009).

Ionotropic glutamate receptors including NMDA receptors are highly permeable to calcium. NMDA receptors are composed of three subunit groups; NR1, NR2 (NR2A-D) and NR3 (NR3A and B) which are assembled as NR1/NR2 or NR1/NR2/NR3. Incorporation of NR3 subunits in the NMDA receptor complex reduces the calcium permeability and the excitatory current transmission of the receptor (Cavara and Hollmann, 2008).

The critical role of NMDA receptors in opioid addiction has been confirmed using MK-801, an NMDA receptor antagonist, which is capable of inhibiting the tolerance to and dependence on morphine (Mao, 1999). MK-801 microinjection into the ventral tegmental area reduces morphine-induced conditioned place preference (Popik and Kolasiewicz, 1999) and heroin reinforcement (Xi and Stein, 2002).

NMDA receptors are also expressed in cells other than neurons like immune cells. It has been found that the NMDA receptor antagonists block T cell proliferation via inhibiting their activation. NR1 and NR2B subunits are expressed in human resting lymphocytes and Jurkat T cells, while NR2A and NR2D expressions have been observed in phytohemagglutinin-activated cells. NR2C expression in immune cells has not been detected yet (Miglio et al., 2005).

Opioid abuse has been reported to alter the expression pattern of NMDA receptor subunits in the brain. NR1 mRNA expression is increased in the rat locus coeruleus, hypothalamic paraventricular nucleus (Zhu et al., 1999), amygdala (Turchan et al., 2003), nucleus raphe magnus and medial thalamus (Zhu et al., 2003), but not in frontal cortex, caudate-putamen or nucleus accumbens after chronic opioid treatment. NR2A or NR2B mRNA expression in different parts of the

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brain is not affected by chronic morphine administration (Zhu et al., 1999), however, NR1 and NR2B protein levels are up-regulated in the nucleus accumbens (Bajo et al., 2006).

It is not possible to study these patterns directly in the living brain of opioid addicts; however, according to the peripheral marker hypothesis, the neurotransmitter receptors expression in peripheral blood lymphocytes may mirror their expression in the brain. For example, in amyotrophic lateral sclerosis disorder, a significant reduction in the mRNA expression of the metabotropic glutamate receptor 2 in lymphocytes has been reported which may be parallel to glutamatergic dysfunction in the central nervous system (Poulopoulou et al., 2005).

Previous studies have focused on NR1, NR2A and NR2B expressions by chronic opioid treatment (Bajo et al., 2006; Zhu et al., 1999, 2003) and less attention has been paid to other subunits. Therefore, we have investigated the mRNA expression of NR2D and NR3B subunits in peripheral blood lymphocytes of opioid addicted, methadone maintained, and long time abstinent subjects in comparison to non-addicted controls to evaluate the possibility of using the expression status of these subunits as a peripheral marker in opioid abusers.

2. Materials and methods

2.1. Subjects

4 groups, each comprising of 25 male individuals were included in the study: opioid addicts, methadone maintained patients, long-term abstinent former opioid addicts, and control subjects. Participants of the first two groups were recruited from the Iranian National Center for Addiction Studies, abstinent subjects from the Narcotics Anonymous organization and control subjects from Tehran University students and staff. It has been shown that drug addiction differs between male and female subjects. For example, the self-administered doses of drugs including opioids increase more rapidly in women and relapse following abstinence is more common in this group (Becker and Hu, 2008). On the other hand, previous studies have introduced the possibility that the expression pattern of NMDA receptor subunits is different between two sexes (Devaud and Alele, 2004; Devaud and Morrow, 1999). Thus, in order to diminish the effect of sexuality on opioid addiction and NMDA receptor subunits expression, only male individuals were recruited in our study. Opioid dependence was diagnosed according to the DSM-IV (American Psychiatric Institute, 1994) criteria. Exclusion criteria were: (1) dependence on other drugs of abuse such as alcohol, cocaine, amphetamine, etc., (2) consumption of other prescribed medications that could affect the central nervous system, (3) past or current major neurological, psychiatric, cardiovascular or endocrinological disorders, and (4) any current inflammatory or infectious disease including HCV, HBV and HIV infections. Control group was age-matched healthy individuals with no life-time history of substance abuse. Furthermore, we used "Multi-drug one step 10 drug screen test panel" (Acon, USA) which is designed to detect 10 different kinds of abused drugs (Cocaine, Amphetamine, Methamphetamine, Marijuana, Opiate, Phencyclidine, Barbiturates, Benzodiazepines, Methadone, and Tricyclic Antidepressants) in the urine. If any of these drugs were detected in the urine samples of abstinent or control subjects, the subjects were excluded. All subjects participated voluntarily and provided a written informed consent before enrollment. Protocols for this study were approved by the Ethics Committee of Tehran University of Medical Sciences. Demographics of the study participants are shown in Table 1.

2.2. Peripheral blood lymphocyte preparation

12 ml blood sample was collected by antecubital venopuncture into ethylenediaminetetraceticacid (EDTA)-containing tubes and then placed on a cell separation medium (Histoprep/BAG, Lich, Germany) and centrifuged for 35 min with 1200 *g* at room temperature in a horizontal rotor according to the manufacturer's protocol. The lymphocyte layer was collected and washed three times in calcium–magnesium–free phosphate-buffered saline (pH = 7.4). Separation of lymphocytes was done no later than 4 h after drawing blood.

2.3. Total RNA extraction and reverse transcription

The RNA gents® Total RNA isolation system (Promega, USA) was used to extract total RNA from lymphocytes. After determining the quantity and purity of extracted RNA by spectrophotometry and gel electrophoresis (1.2% agarose; Gibco/BRL), 1 µg of RNA was used for reverse transcription into first-strand cDNA. The process was done using a reverse transcription kit (QuantiTect®; Qiagen) according to the manufacturer's protocol in a final volume of 20 µl.

2.4. Oligonucleotide primers

In order to normalize each target gene expression, beta-actin was used as the housekeeping gene. All the primers used for real-time PCR amplification of NR2D and NR3B subunits of NMDA receptor and also beta-actin were ordered from Qiagen company primer bank.

2.5. Real-time PCR

In all of the performed real-time PCR reactions, 2 µl cDNA was used together with the SYBR Green I Master Mix (Quantifast®; Qiagen) on a RotorGene 6000 (Corbett Research, Mortlake, NSW, Australia). For all primer pairs, the annealing temperature was optimized which ranged from 58 to 60 ºC. The specificity of PCR products was confirmed by obtaining a single peak in melting curve analysis. Complementary length verification was done by visualizing PCR products on standard 2.5% agarose gel with ethidium bromide.

2.6. Data analysis

In order to measure the quantity of the target gene in each sample, it was first necessary to find the cycle at which the sample fluorescence reached a preset threshold which was appreciably above the background. The cycle number was then referred to a standard curve present in each run of amplification. The data obtained for all 4 groups of the study were normalized against the housekeeping gene (beta-actin). All samples were measured in duplicate and the mean was used for further analysis. REST-XL version 2 software (http://www.wzw.tum.de/gene-quantification) was used to analyze

Table 1
Demographics of study participants.

	Control ($n=25$)	Addict ($n=25$)	Methadone maintained ($n=25$)	Abstinent ($n=25$)
Age (years, means ± SD)	32.9 ± 5.3	32.2 ± 6.7	33.3 ± 4.8	31.5 ± 5.55
Duration of drug use (years, mean ± SD)	–	5.13 ± 2.96	7.48 ± 3.75	6.64 ± 4.28
Duration of methadone maintenance (years, mean ± SD)	–	–	4.2 ± 2.7	–
Duration of abstinence (years, mean ± SD)	–	–	–	4.64 ± 1.11
Dose of administered methadone (mg/day)	–	–	70 ± 10	–

data and detect significant differences in relative expression levels between samples and the control group. In order to use the software, the amount of PCR efficiencies (E) and the mean crossing point (CP) deviation between two groups are needed. Normalizing the target gene expression against the reference gene is done according to the following equation:

$$\text{Ratio} = \left(E_{\text{target}}\right)^{\Delta\text{CP target(MEAN control–MEAN sample)}} \div \left(E_{\text{reference}}\right)^{\Delta\text{CP reference(MEAN control–MEAN sample)}}$$

The software uses the "pair-wise fixed reallocation randomization test" to calculate the significance of results. Randomization tests have the advantage of making no distributional assumptions about the data, while remaining as powerful as more standard tests. Therefore, for each target gene (NR2D and NR3B), we performed three separate analyses (addicted, methadone maintained and abstinent groups in comparison to control group). $P<0.05$ is considered statistically significant and data are presented as Fold differences of Mean Normalized Expression values ± standard error of mean (S.E.M.).

3. Results

Fig. 1 is the result of comparison of NR3B subunit mRNA expression ratio in Peripheral blood lymphocytes between study groups. The NR3B mRNA expression was significantly up-regulated by the factors 9.11 ($P<0.001$), 10.07 ($P<0.001$) and 4.08 ($P<0.05$) in abstinent, addicted and methadone maintained subjects, respectively in comparison to control group. Furthermore, NR3B mRNA expression in methadone maintained subjects is significantly down-regulated in comparison to both addicted and abstinent groups ($P<0.05$).

Beta-actin expression level was not different among the groups tested (data not shown).

As shown in Fig. 2, NR2D subunit mRNA expression was down-regulated reaching 0.83, 0.88 and 0.91 the amount of the control group in abstinent, addicted and methadone maintained individuals, respectively. None of the groups was statistically different from control subjects.

4. Discussion

Previous studies have shown that the glutamate receptors are expressed in immune cells including thymocytes and T lymphocytes (Boldyrev et al., 2004; Ganor et al., 2003; Miglio et al., 2005; Storto et al., 2000), macrophages (Dickman et al., 2004), and natural killer cells (Kuo et al., 2001). It appears that the stimulation of these receptors affects cellular functions such as proliferation, growth and death (Miglio et al., 2007). To our knowledge, this may be the first report concerning the mRNA expression of NR3B subunit of NMDA glutamate receptor in human peripheral blood lymphocytes. Furthermore, our results show that chronic opioid abuse leads to significant mRNA up-regulation of this subunit in the peripheral blood lymphocytes of addicted subjects. This is not surprising as many lines of evidence indicate that chronic morphine treatment alters glutamate-mediated synaptic transmission by modifying the composition of the NMDA receptor complex (Martin et al., 1999a) which may represent a neuroadaptation to chronic drug use. It has been shown that the existence of NR3B subunit in the NMDA receptor complex reduces the activity of the receptor via decreasing the calcium permeability of the channel (Matsuda et al., 2002). Thus, significant up-regulation of NR3B subunit in peripheral blood lymphocytes of addicted individuals may result in the suppression of NMDA receptor transmission in these cells.

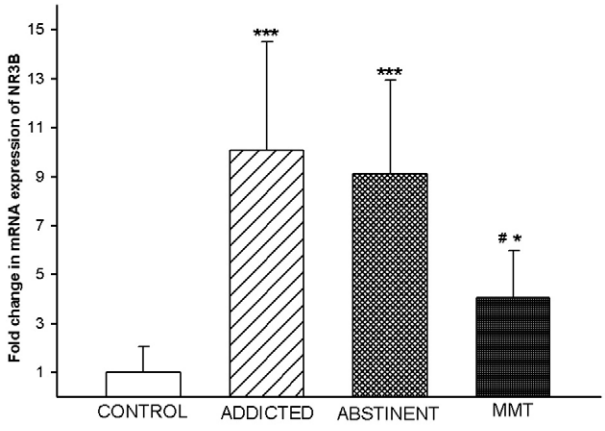

Fig. 1. Shows the mRNA expression of NR3B subunit of NMDA glutamate receptor in the lymphocytes of the control, abstinent, addicted and methadone maintenance treated (MMT) individuals. $^{*}P<0.05$, $^{***}P<0.001$ different from control subjects, $\#P<0.05$ different from addicted and abstinent groups.

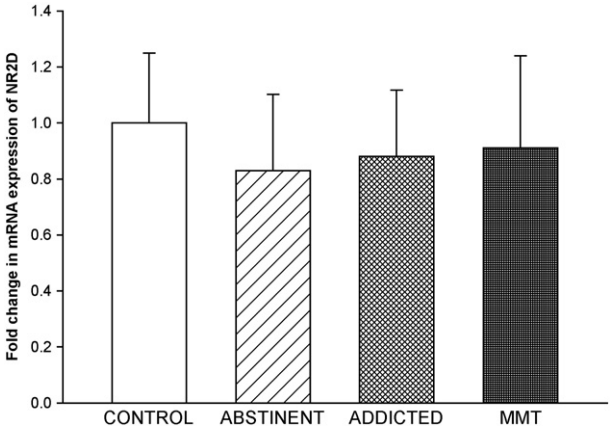

Fig. 2. Represents the difference of mRNA expression ratios of NR2D subunit of NMDA glutamate receptor in the lymphocytes of the control, abstinent, addicted and methadone maintenance treated (MMT) individuals.

There is a hypothesis called the "peripheral marker hypothesis" stating that the expression status of a neurotransmitter receptor in peripheral blood lymphocytes is a reflection of its status in the brain. For example, it has been found that the dopamine receptor expression in lymphocytes of schizophrenic patients is increased (Bondy and Ackenheil, 1987; Ilani et al., 2001) in correlation with clinical severity of the disorder (Kwak et al., 2001). Furthermore, in Parkinson's disease, the dopamine receptor expression in peripheral blood lymphocytes is down-regulated which is parallel to the dopaminergic neurotransmission decrease in the striatum and can indicate the clinical severity of the disease (Nagai et al., 1996) (see also Introduction). According to the above hypothesis, one may assume that the NR3B subunit of the NMDA receptor is much more expressed in the brain of addicted subjects in comparison to controls which may influence the receptor activity there. It was first suggested that NR3B expression is highly restricted to certain nuclei in the brainstem and motor neurons in the spinal cord (Matsuda et al., 2002; Nishi et al., 2001). However, recent studies have detected other regions expressing NR3B such as the cerebellum, cortex and hippocampus of the adult rat central nervous system (Wee et al., 2008). NR3B mRNA is also highly expressed in the human hippocampal formation and adjacent neocortex (Bendel et al., 2005). The data regarding the effect of chronic opioid treatment on NMDA receptor-mediated excitatory currents in the brain have shown either inhibition (Martin et al., 1999a,b, 2004) or enhancement (Chen and Huang, 1991; Yoshikawa et al., 2008) depending on factors such as preparations used in the studies. Unfortunately, there is no report concerning the effect of chronic opioid administration on NR3B subunit expression in the

brain. Thus, whether the up-regulation observed in our study is a compensatory response to chronic opioid abuse or the effect of the drug itself, is not clear at the moment. This is an interesting subject for further studies in the future.

Our data also indicate that a considerable long duration of abstinence is not capable of restoring the up-regulated state of NR3B subunit expression in peripheral blood lymphocytes to normal levels in long-term abstinent former opioid addicts. In agreement with previous findings (Hope et al., 2007; Schoffelmeer et al., 1995; Spijker et al., 2004; Tjon et al., 1995; Vanderschuren and Kalivas, 2000), this may be another piece of evidence showing that some neuroadaptations resulted from drug addiction may persist for long times after quitting the drug use. However, one may speculate that the observed up-regulation in abstinent people is related to a hereditary matter rather than being only a consequence of drug use. Further investigations are needed to confirm the above statement.

Methadone as a relatively long acting opioid with oral bioavailability is among the most widely used medications for treatment of heroin addiction. The effectiveness of the drug is believed to be the result of reducing or diminishing heroin use and craving (Kreek and Vocci, 2002). It can provide the stability necessary for patients to initiate lifestyle changes and discontinue heroin use. It has been shown that methadone maintenance therapy is able to normalize the functions of various human systems including the immune system (Kreek, 2000).

The present data indicate that NR3B mRNA expression in methadone maintained subjects is also up-regulated relative to control individuals. Besides, further analysis demonstrates significant decrease in NR3B expression level in methadone treated group in comparison to addicted and abstinent subjects. Here arises an important question: why the NR3B subunit expression level in methadone maintained group who are exposed to a considerable amount of opioid each day is less than half of that observed in addicted or long-term abstinent subjects of our study? The possible explanation coming to mind is based upon the unique characteristic of this drug among other opioid agonists. In contrast to morphine and other opioids, methadone is capable of antagonizing NMDA receptor within its therapeutic concentration ranges (Callahan et al., 2004). This dual effect of methadone may be responsible for reducing the up-regulated state of NR3B expression in opioid addiction because a previous study has demonstrated that when LY274614, a competitive NMDA receptor antagonist, is co-administered with morphine, it can inhibit the NR1 mRNA expression changes induced by chronic morphine administration (Zhu et al., 2003). Thus, it seems that another mechanism by which methadone alleviates the deleterious effects of chronic opioid addiction may be via reducing the over-expressed state of NR3B subunit in abusers. However, this suggestion needs to be further investigated in the future.

Our results also show that the mRNA expression level of NR2D subunit of NMDA receptor in peripheral blood lymphocytes of addicted, methadone maintained and abstinent subjects is not statistically different from control individuals. NR2D is found mainly in mid brain structures (Laurie et al., 1997). In the striatum, the subunit is exclusively expressed in interneurons (Standaert et al., 1996). NR2D subunit expression is also found in the murine hippocampus and it has been reported that the NR1/NR2A/NR2D and NR1/NR2B/NR2D complexes of NMDA receptors are present in the rat thalamus and cortex (Dunah et al., 1998). The presence of the NR2D subunit in NMDA receptor complex is suggested to result in considerable increase of excitatory postsynaptic currents, which may result in remarkable physiological consequences (Thompson et al., 2002). The data obtained in our study as a reflection of brain status suggest that the NR2D subunit mRNA expression in the brain is not affected by opioid drugs consumption. Although we did not find any report concerning the mRNA expression changes of NR2D subunit in the brain after chronic opioid treatment, previous studies have found that in nucleus accumbens, as a major part of the reward system, neither NR2A nor NR2B mRNA levels are affected after chronic morphine administration (Hemby, 2004; Zhu et al., 1999). Thus it seems that the transcriptional level of NR2 subunits of NMDA receptor is not regulated by chronic morphine treatment.

According to the peripheral marker hypothesis, we suggest that our observed findings in peripheral blood lymphocytes may also occur in the brain in addiction process. However, this correlation cannot be extended much at the moment because there is not any report concerning the mRNA expression of NR3B and NR2D subunits in the brain of opioid addicts. Performing post mortem studies in the brain of addicted individuals or investigating animal brains after chronic opioid administration may help to test the correlation of NR3B and NR2D subunits expression between immune and central nervous systems in the future.

In conclusion, drug addiction may be a brain disease with multiple genetic and environmental aspects. It seems that the transcriptional level of the NR2D subunit of NMDA receptor is not a subject of regulation by opioid addiction. However, our findings suggest that the over-expression of NR3B subunit of NMDA receptor is a long lasting result of chronic opioid abuse. Furthermore, methadone maintenance therapy considerably decreases the over-expression state of the subunit in peripheral blood lymphocytes which may serve as a suitable peripheral marker to evaluate the successfulness of therapy. However, to clarify the exact mechanism(s) involved, further experiments are required in the future.

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