

Effect of Morphine Treatment on mRNA Expression of GluN3A Subunit of the NMDA Receptor in Rat Brain

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Abstract

N-methyl-D-aspartate (NMDA) receptors are mainly involved in opioid addiction and are highly expressed in the brain reward pathway. Presence of GluN3A subunit in the composition of NMDA receptor decreases conductance of receptor channel. Opioid administration may change the expression pattern of NMDA receptor subunits. Here we have investigated the mRNA expression alterations of GluN3A subunit of the NMDA receptor in the rat brain after acute and chronic morphine administration. Male Wistar rats received chronic intraperitoneal injections of escalating doses of morphine twice daily for 6 days. Control animals received saline instead of morphine with the same protocol. Two other groups received acute single dose of morphine (30 mg/kg) or saline, respectively. The mRNA expression of GluN3A subunit of NMDA receptor in prefrontal cortex, hippocampus, striatum, and nucleus accumbens was evaluated using real-time PCR method. The mRNA expression level of GluN3A subunit was significantly increased (1.5 fold) in prefrontal cortex in chronic morphine administered rats compared to control group. No statistically significant difference was observed between control and chronic morphine treated rats in other sites of the brain. In acute morphine administered animals, no significant difference was observed in GluN3A subunit expression in evaluated sites of the brain compared to the control group. It is concluded that chronic morphine administration leads to the up-regulation of GluN3A subunit of NMDA receptor in prefrontal cortex. Involvement of this alteration in features of opioid addiction needs to be further studies in the future.

Keywords

Morphine, Rat, Addiction, NMDA receptor, GluN3A subunit

Introduction

Glutamate, the main neurotransmitter of the excitatory synapses in the brain, is essentially involved in opioid addiction. The neurotransmitter may be found in considerable amounts in multiple sites of the brain including the mesocorticolimbic pathway [1]. The pathway originates mainly from the ventral tegmental area and projects to regions such as nucleus accumbens, prefrontal cortex, amygdala and hypothalamus. Addictive drugs are able to stimulate this pathway and lead to dopamine release in the nucleus accumbens which results

in euphoria and reward [2]. Glutamatergic synapses and receptors are commonly detectable in different regions of the reward pathway. For example, glutamatergic projections from the amygdala, hippocampus, and frontal cortex are received in the nucleus accumbens which supports the hypothesis of interaction between dopaminergic and glutamatergic systems in the development of addiction disease [3].

Glutamate interacts with both types of ionotropic and metabotropic receptors. The N-methyl-D-aspartate (NMDA) receptor is one of the ionotropic glutamate receptors and is extremely permeable to calcium ions. The receptor has a tetramer structure composed of seven different subunits: GluN1 (also known as NR1), GluN2A-D (also known as NR2A-D), and GluN3A-B (also known as NR3A-B). It has been shown that incorporation of GluN3 subunit in the arrangement of NMDA receptor leads to the reduction of calcium penetrance and ultimately attenuation of excitatory current conduction of the receptor [4].

The critical role of the NMDA receptors in opioid addiction became apparent when researchers observed that NMDA receptor antagonists are able to block both the tolerance to and dependence on morphine. This finding has been confirmed in many animal models of addiction such as conditioned place preference [5]. On the other hand, researchers have also observed that chronic consumption of opioid drugs may change the molecular structure of NMDA receptors in the brain by altering the expression pattern of receptor subunits. For example, it has been shown that chronic administration of opioids increases the mRNA level of GluN1 subunit in the rat amygdala [6] and locus coeruleus [7]. However, long term opioid treatment did not induce significant changes in mRNA levels of GluN2 subunits in different areas of the brain [7]. Although GluN3 subunits of the NMDA receptor are found in many parts of the brain reward pathway [8] and have a potential role in the modulation of the receptor channel conductance, data concerning their up or down-regulation after opioid consumption is lacking. Therefore, we decided to investigate the mRNA expression alterations of GluN3A subunit of the NMDA receptor in different parts of the rat brain after acute and chronic morphine administration.

Methods

Animals

Male Wistar rats (Pasteur institute, Tehran, Iran) weighting 220–260 g were used. The animals were housed three in a standard rat cage and had access to food and water *ad libitum*. They were kept in a temperature-controlled (22 ± 2 °C) environment under a 12/12-h light–dark cycle (light beginning at 6:00 A.M.). Each animal was tested only once and there were six rats per each experimental group. All experimental protocols and procedures were approved by the Research and Ethics Committee of the School of Medicine, Tehran University of Medical Sciences. All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996).

Chemicals

Morphine sulfate was purchased from Temad Company (Tehran, Iran). The drug was dissolved in sterile 0.9% saline just before intraperitoneal (i.p.) injection in a final volume of 0.1 ml/100 g body weight.

Morphine administration

Rats were randomly divided into two main groups of acute and chronic treatment ($n = 12$). In acute treatment group, animals received either single i.p. dose of morphine (30 mg/kg) or saline ($n = 6$ in each sub-group). In chronic treatment group, animals received either chronic i.p. escalating doses of morphine or saline ($n = 6$ in each sub-group). The doses of morphine were gradually increased from 7 to 30 mg/kg in a period of 6 days according to the method described earlier [9]. Briefly, the drug was injected two times a day at 8:00 A.M. and 6:00 P.M. with doses indicated in the parentheses respectively on following days: first day (7 and 10 mg/kg), second day (15 and 20 mg/kg), third day (25 and 30 mg/kg), fourth and fifth days (30 and 30 mg/kg), and sixth day (30 mg/kg, only at 8:00 A.M.). In the control group, rats received saline injections at time intervals similar to the morphine group.

Brain tissue collection

Rats were sacrificed by rapid decapitation 2 h after the last injection of morphine or saline. The brains were detached from the skull and four areas including prefrontal cortex, hippocampus, striatum and nucleus accumbens were dissected with tissue corers for RNA extraction. In order to minimize RNA degradation, dissections were done on a sterile ice-cold plate and tissues were homogenized in RNA extraction buffer within 5 min after sacrifice.

Total RNA extraction and reverse transcription

Total RNA was extracted from brain samples using the RNeasy Lipid Tissue Mini Kit (Qiagen). Quantity of extracted RNA was measured with spectrophotometry and its purity was determined by gel electrophoresis (1.2% agarose; Gibco/BRL). In order to synthesize first-strand complementary DNA (cDNA), 1 μ g of total RNA was reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen) in a final volume of 20 μ l.

Real-time PCR primers

Beta-actin gene was used as the housekeeping gene to normalize target gene expression. The oligonucleotide primers of beta-actin and GluN3A were purchased from Qiagen company primer bank.

Real-time PCR

Two μ l of the first strand cDNA was used in all real-time PCR reactions together with specific primers and Power SYBR® Green PCR Master Mix (Life Technologies) on a StepOnePlus Real-Time PCR System (Applied Biosystems). The annealing temperature of beta actin and GluN3A gene was 60 °C. Specificity of PCR products was verified by obtaining a single peak for each gene in melting curve analysis. PCR products were also ran on standard 2.5% agarose gel and

visualized with ethidium bromide in order to confirm the amplicon lengths.

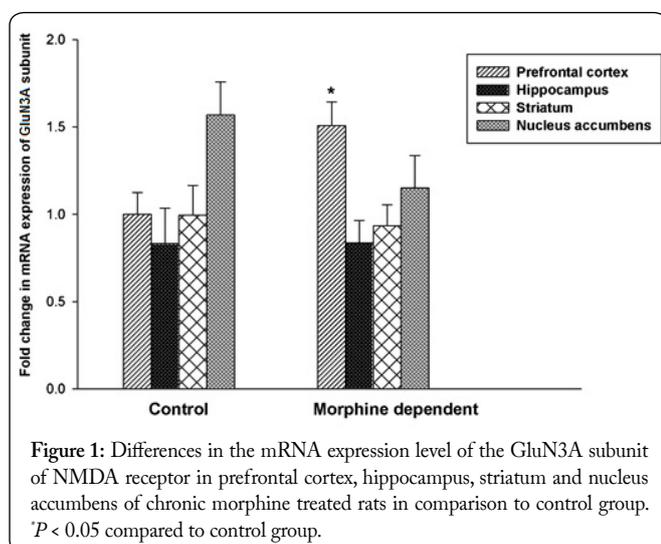
Data analysis

With the aim of measurement of the quantity of target gene in each sample, a standard series of cDNA dilutions were present in each run of amplification. The cycle at which the sample fluorescence crossed a preset threshold was referred to the standard curve. The data of treatment and control group were normalized using the housekeeping gene, beta-actin. All of the samples were measured in duplicate and the mean was used for data analysis. Relative Expression Software Tool (REST)-XL version 2 [10] was used to determine significant differences in relative expression levels of genes between treatment and control group. The software simultaneously performs both gene quantification and normalization. Significance of results in REST-XL is defined by pair-wise fixed reallocation randomization test. Data are shown as fold differences of mean normalized expression values \pm standard error of mean. $P < 0.05$ was considered statistically significant.

Results

Figure 1 indicates comparison of the mRNA expression ratio of GluN3A subunit in prefrontal cortex, hippocampus, striatum, and nucleus accumbens in two groups of control and morphine dependent rats. The mRNA expression of GluN3A was significantly up-regulated (1.5 fold) in prefrontal cortex in morphine dependent rats ($P < 0.05$) in comparison to control group. The mRNA level of GluN3A gene was not significantly different between control and morphine treated rats in other sites of the brain ($P > 0.05$).

In acute treatment experiments, no significant difference in the mRNA expression level of GluN3A subunit was observed between morphine treated and control (saline-administered) group in all the examined brain sites ($P > 0.05$) (data not shown). Beta-actin expression did not differ in all the tested groups (data not shown).



Discussion

Many previous studies were focused on the functional role of NMDA receptor transmission in the development and expression of opiate dependence, tolerance, and withdrawal [11]; and have proved NMDA receptor-dependent modulation of dopamine transmission within the mesocorticolimbic system. For example, several antagonists of NMDA receptor, including MK-801 and ketamine, are able to decrease or prevent the development of tolerance and somatic symptoms of physical dependence to opiates in rodents [12]. In addition, it has been demonstrated that NMDA receptor antagonists block the aversive symptoms of opiate withdrawal measured by conditioned place aversion in mice [13]. Similarly, it has been reported that the features of naloxone-precipitated morphine withdrawal in mutant mice lacking an NMDA receptor subunit is very weak [14]. The receptor complex is composed of three major families of subunits: GluN1, GluN2 (A–D) and GluN3 (A and B). It has been claimed that one or more GluN1 subunits co-assemble with various combinations of the GluN2 (A–D) or GluN3 (A–B) subunits to form functional receptors [4]. The combination of different receptor subunits may confer diverse biophysical and functional properties on these receptor subtypes. Furthermore, it has been shown that the expression pattern of different subunits of NMDA receptor in the CNS is highly region specific. In the case of GluN3A subunit, areas with the highest level of expression are spinal cord, thalamus, hypothalamus, brainstem, CA1 of hippocampus, amygdala, and certain parts of the cortex [15]. Our results also showed that the expression level of GluN3A subunit in the control group was not statistically different among four studied parts of the brain which is consistent with previous reports [16].

Chronic opiate exposure is associated with alterations in the expression of NMDA receptor subunits within various parts of the mesocorticolimbic system [7, 17, 18]. It has been shown that disturbance in glutamatergic transmission via these NMDA receptors may not only be involved in neuroplastic adaptations observed after chronic opiate exposure (which lead to the dependence and aversive effects of withdrawal), but also results in increased vulnerability to the rewarding and addictive properties of opiates during early exposure [19]. To our knowledge, this is the first report evaluating the mRNA expression changes of the GluN3A subunit after chronic morphine administration in rat. Our data showed that the mRNA level of GluN3A subunit is significantly up-regulated in rat prefrontal cortex after repeated intermittent injection of morphine. The presence of GluN3A subunit in the NMDA receptor complex leads to decreased Ca^{2+} permeability and excitatory function of the receptor [4]. Thus, it seems that the up-regulated state of GluN3A subunit together with previously reported unchanged [7] or decreased [20] expression of other excitatory subunits like GluN1, GluN2A, and GluN2B will lead to the reduction of NMDA receptor function in the prefrontal cortex of morphine-dependent rats. Reduction of overall expression of excitatory subunits of the NMDA receptor has also been observed in chronic ethanol-treated monkeys. As subtle changes in glutamate-related signaling may disrupt the information processing in prefrontal cortex [21], our data may propose a new mechanism for

cognitive deficiency which is observed in rats [22] and humans [23] after chronic morphine consumption. Furthermore, many findings have suggested that the prefrontal cortex, an important source of excitatory amino acid projections to ventral tegmental area and nucleus accumbens [3], is critical for sensitization phenomenon, i.e., progressive augmentation of response which is observed after repeated intermittent exposure to opiates [24]. Lesion studies have indicated that prefrontal cortex projections provide the glutamatergic tone in the ventral tegmental area that is required for induction of sensitization [25, 26]. In addition, these studies have also shown that the responsiveness of midbrain dopamine neurons to prefrontal cortex stimulation is altered in sensitized rats [27]. Changes in glutamate and dopamine transmission in the prefrontal cortex may contribute to sensitization. It has been reported that NMDA receptors strongly affect the balance of dopamine and glutamate concentration within this region of the brain [28]. NMDA receptor hypofunction in prefrontal cortex has been reported to intensely increase the sensitivity to the rewarding properties of both systemic and intra-ventral tegmental area opiates, and the abuse liability of psychotropic drugs via dopamine dependent mechanisms. Intra-prefrontal cortex administration of NMDA antagonists may potentiate opiate-related dopaminergic signals from the ventral tegmental area to the nucleus accumbens, which may in turn amplify the associative reward signals converging on the nucleus accumbens from prefrontal cortex and the ventral tegmental area [19]. Thus, it seems that the up-regulation of GluN3A subunit in the prefrontal cortex (which may lead to NMDA receptor hypofunction) may possibly be involved in the sensitization phenomenon. This claim needs to be further investigated by other experiments in the future.

Our results did not show significant changes in the expression of GluN3A subunit in the hippocampus, striatum and nucleus accumbens after chronic morphine administration. However, it has been shown that the NMDA receptor function is altered in these regions in addicted rat [20] which may highlight the importance of other NMDA receptor subunits in these brain regions.

Conclusion

Chronic morphine administration may alter the mRNA expression level of NMDA receptor subunits in the brain reward pathway. Up-regulation of GluN3A subunit of NMDA receptor in prefrontal cortex of chronic morphine-treated rats may be involved in the alteration of NMDA receptor function in this area and may take part in some aspects of chronic opioid consumption such as sensitization and cognitive function changes. Further studies are needed to evaluate this hypothesis in the future.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors Contributions

N. Vousooghi, Co-author of: principles, methods, implementation of research, calculation, interpretation and discussion of results and manuscript. Author of: query in the international literature, hypotheses, and preparation of the manuscript. F. Hosseini Ghane, Co-author of: implementation of research, and manuscript. M.S. Sadat-Shirazi, Co-author of: methods, implementation of research, calculation, and manuscript. P. Safavi, Co-author of: implementation of research. M.R. Zarrindast, Co-author of: principles, methods, interpretation and discussion of results and manuscript. All authors reviewed and approved the manuscript.

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References

1. Gass JT, Olive MF. 2008. Glutamatergic substrates of drug addiction and alcoholism. *Biochem Pharmacol* 75(1): 218-265. doi: 10.1016/j.bcp.2007.06.039
2. Spanagel R, Weiss F. 1999. The dopamine hypothesis of reward: past and current status. *Trends Neurosci* 22(11): 521-527. doi: 10.1016/S0166-2236(99)01447-2
3. Tzschentke TM, Schmidt WJ. 2003. Glutamatergic mechanisms in addiction. *Mol Psychiatry* 8(4): 373-382.
4. Cavara NA, Hollmann M. 2008. Shuffling the deck anew: how NR3 tweaks NMDA receptor function. *Mol Neurobiol* 38(1): 16-26. doi: 10.1007/s12035-008-8029-9
5. Popik P, Kolasiewicz W. 1999. Mesolimbic NMDA receptors are implicated in the expression of conditioned morphine reward. *Naunyn Schmiedebergs Arch Pharmacol* 359(4): 288-294. doi: 10.1007/PL00005354
6. Turchan J, Maj M, Przewlocka B. 2003. The effect of drugs of abuse on NMDAR1 receptor expression in the rat limbic system. *Drug Alcohol Depend* 72(2): 193-196. doi: 10.1016/S0376-8716(03)00193-5
7. Zhu H, Jang CG, Ma T, Oh S, Rockhold RW, et al. 1999. Region specific expression of NMDA receptor NR1 subunit mRNA in hypothalamus and pons following chronic morphine treatment. *Eur J Pharmacol* 365(1): 47-54. doi: 10.1016/S0014-2999(98)00861-9
8. Nilsson A, Eriksson M, Muly EC, Akesson E, Samuelsson EB, et al. 2007. Analysis of NR3A receptor subunits in human native NMDA receptors. *Brain Res* 1186: 102-112. doi: 10.1016/j.brainres.2007.09.008
9. Nieto MM, Wilson J, Cupo A, Roques BP, Noble F. 2002. Chronic morphine treatment modulates the extracellular levels of endogenous enkephalins in rat brain structures involved in opiate dependence: a microdialysis study. *J Neurosci* 22(3): 1034-1041.
10. Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30(9): e36. doi: 10.1093/nar/30.9.e36
11. Trujillo KA. 2000. Are NMDA receptors involved in opiate-induced neural and behavioral plasticity? A review of preclinical studies. *Psychopharmacology (Berl)* 151(2-3): 121-141.
12. González P, Cabello P, Germany A, Norris B, Contreras E. 1997. Decrease of tolerance to, and physical dependence on morphine by, glutamate receptor antagonists. *Eur J Pharmacol* 332(3): 257-262. doi: 10.1016/S0014-2999(97)01099-6
13. Kawasaki Y, Jin C, Suemaru K, Kawasaki H, Shibata K, et al. 2005. Effect of glutamate receptor antagonists on place aversion induced by

- naloxone in single-dose morphine-treated rats. *Br J Pharmacol* 145(6): 751-757. doi: 10.1038/sj.bjp.0706228
14. Miyamoto Y, Yamada K, Nagai T, Mori H, Mishina M, et al. 2004. Behavioural adaptations to addictive drugs in mice lacking the NMDA receptor epsilon1 subunit. *Eur J Neurosci* 19(1): 151-158. doi: 10.1111/j.1460-9568.2004.03086.x
15. Goebel DJ, Pooch MS. 1999. NMDA receptor subunit gene expression in the rat brain: a quantitative analysis of endogenous mRNA levels of NR1Com, NR2A, NR2B, NR2C, NR2D and NR3A. *Brain Res Mol Brain Res* 69(2): 164-170. doi: 10.1016/S0169-328X(99)00100-X
16. Tang W, Wesley M, Freeman WM, Liang B, Hemby SE. 2004. Alterations in ionotropic glutamate receptor subunits during binge cocaine self-administration and withdrawal in rats. *J Neurochem* 89(4): 1021-1033. doi: 10.1111/j.1471-4159.2004.02392.x
17. Bhargava HN, Reddy PL, Gudehithlu KP. 1995. Down-regulation of N-methyl-D-aspartate (NMDA) receptors of brain regions and spinal cord of rats treated chronically with morphine. *Gen Pharmacol* 26(1): 131-136. doi: 10.1016/0306-3623(94)00147-F
18. Bajo M, Crawford EF, Roberto M, Madamba SG, Siggins GR. 2006. Chronic morphine treatment alters expression of N-methyl-D-aspartate receptor subunits in the extended amygdala. *J Neurosci Res* 83(4): 532-537. doi: 10.1002/jnr.20756
19. Bishop SF, Lauzon NM, Bechard M, Gholizadeh S, Laviolette SR. 2011. NMDA receptor hypofunction in the prelimbic cortex increases sensitivity to the rewarding properties of opiates via dopaminergic and amygdalar substrates. *Cereb Cortex* 21(1): 68-80. doi: 10.1093/cercor/bhq060
20. Murray F, Harrison NJ, Grimwood S, Bristow LJ, Hutson PH. 2007. Nucleus accumbens NMDA receptor subunit expression and function is enhanced in morphine-dependent rats. *Eur J Pharmacol* 562(3): 191-197. doi: 10.1016/j.ejphar.2007.01.027
21. Acosta G, Hasenkamp W, Daunais JB, Friedman DP, Grant KA, et al. 2010. Ethanol self-administration modulation of NMDA receptor subunit and related synaptic protein mRNA expression in prefrontal cortical fields in cynomolgus monkeys. *Brain Res* 1318: 144-154. doi: 10.1016/j.brainres.2009.12.050
22. Spain JW, Newsom GC. 1991. Chronic opioids impair acquisition of both radial maze and Y-maze choice escape. *Psychopharmacology (Berl)* 105(1): 101-106.
23. Dolan SL, Bechara A, Nathan PE. 2008. Executive dysfunction as a risk marker for substance abuse: the role of impulsive personality traits. *Behav Sci Law* 26(6): 799-822. doi: 10.1002/bsl.845
24. Babbini M, Davis WM. 1972. Time-dose relationships for locomotor activity effects of morphine after acute or repeated treatment. *Br J Pharmacol* 46(2): 213-224.
25. Li Y, Hu XT, Berney TG, Vartanian AJ, Stine CD, et al. 1999. Both glutamate receptor antagonists and prefrontal cortex lesions prevent induction of cocaine sensitization and associated neuroadaptations. *Synapse* 34(3): 169-180.
26. Wolf ME, Dahlin SL, Hu XT, Xue CJ, White K. 1995. Effects of lesions of prefrontal cortex, amygdala, or fornix on behavioral sensitization to amphetamine: comparison with N-methyl-D-aspartate antagonists. *Neuroscience* 69(2): 417-439. doi: 10.1016/0306-4522(95)00248-H
27. Tong ZY, Overton PG, Clark D. 1995. Chronic administration of (+)-amphetamine alters the reactivity of midbrain dopaminergic neurons to prefrontal cortex stimulation in the rat. *Brain Res* 674(1): 63-74. doi: 10.1016/0006-8993(94)01439-O
28. Moghaddam B, Adams B, Verma A, Daly D. 1997. Activation of glutamatergic neurotransmission by ketamine: a novel step in the pathway from NMDA receptor blockade to dopaminergic and cognitive disruptions associated with the prefrontal cortex. *J Neurosci* 17(8): 2921-2927.