RESEARCH PAPER

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The effect of morphine administration on GluN3B NMDA receptor subunit mRNA expression in rat brain

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Opioid addiction is critically dependent on the activation of N-methyl-D-aspartate (NMDA) receptors, which are widely found in the mesocorticolimbic system. Meanwhile, opioid addiction may affect the expression level of NMDA receptor subunits. The existence of GluN3 subunits in the NMDA receptor's tetramer structure reduces the excitatory current of the receptor channel. We evaluated the changes in the mRNA expression pattern of the GluN3B subunit of the NMDA receptor in rat brains following acute and chronic exposure to morphine. Chronic, escalating intraperitoneal doses of morphine or saline were administered twice daily to male Wistar rats for six days. Two other groups were injected with a single acute dose of morphine or saline. The mRNA level of the GluN3B subunit of the NMDA receptor in the striatum, hippocampus, and nucleus accumbens (NAc) was measured by real-time PCR. mRNA expression of the GluN3B subunit was considerably augmented (3.15 fold) in the NAc of animals chronically treated with morphine compared to the control group. The difference between rats that were chronically administered morphine and control rats was not statistically significant for other evaluated brain areas. In rats acutely treated with morphine, no significant differences were found for GluN3B subunit expression in the examined brain regions compared to the control group. It was concluded that chronic exposure to morphine notably increased the GluN3B subunit of the NMDA receptor in NAc. The extent of the impact of this finding on opioid addiction and its features requires further evaluation in future studies.

Key words: morphine, rat, NMDA receptor, GluN3B

INTRODUCTION

Opioid addiction is a chronic disorder that results from the interactions of several neurotransmitters in the brain, such as glutamate. Glutamate is released from excitatory synapses in various brain areas, including the reward pathway (D'Souza, 2015). The pathway is composed of different brain sites, such as the ventral tegmental area (VTA), nucleus accumbens (NAc), amygdala, and prefrontal cortex (PFC). Drugs of abuse can affect this pathway, and they all ultimately increase the concentration of dopamine (the primary neurotransmitter of reward) in the NAc. Transient elevation of dopamine concentration in NAc is the leading cause of pleasure induced by addictive drugs or behaviors

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(Adinoff, 2004; Nazari et al., 2022). Glutamatergic and dopaminergic neurons interact significantly in the reward pathway. The pathway is highly rich in glutamate receptors and synapses, which is the basis for the glutamatergic system's involvement in addictive disorders (Tzschentke and Schmidt, 2003).

Both ionotropic and metabotropic receptors play a role with glutamate in the addiction phenomenon. The most critical ionotropic receptor for glutamate is the N-methyl-D-aspartate (NMDA) receptor, assembled as a tetramer that is highly permeable to calcium ions. The tetramer arrangement is made up of different subunit categories: GluN1 (or NR1), GluN2A–D (also called NR2A–D), and GluN3A–B (also known as NR3A–B). The electrical and ionic conductance properties of different NMDA receptor structures depend on the receptor's subunit composition. For example, the presence of GluN3 subunits in the structure of the NMDA receptor reduces the passage of calcium through the receptor channel and inhibits the excitatory properties of the receptor (Willard and Koochekpour, 2013).

It has been shown that different features of opioid addiction are critically dependent on the normal function of NMDA receptors because inhibition of these receptors diminishes the tolerance and dependence aspects of opioid addiction (Inturrisi, 2005). Conversely, it has been reported that abuse of opioid drugs such as morphine may alter the NMDA receptor's subunit composition by up- or down-regulating particular subunits. For instance, previous studies have found that after chronic consumption of opioid drugs, transcription of the GluN1 subunit is elevated in some rat brain areas, such as the locus coeruleus and amygdala (Zhu et al., 1999; Turchan et al., 2003). However, the expression of GluN2 subunits is not altered in the brain following chronic opioid consumption (Zhu et al., 1999). When it comes to GluN3 subunits, the data about these subunits' expression changes after chronic opioid intake is minimal. We have previously shown that the expression of the GluN3A subunit of the NMDA receptor is up-regulated in rat PFC after chronic morphine injection (Vousooghi et al., 2016). Here, we have investigated mRNA expression changes of the GluN3B subunit of the NMDA receptor, as another member of the GluN3 family, after acute and chronic morphine administration.

METHODS

Animals

The animals used in the study were male Wistar rats (220–260 g) purchased from the Pasteur Institute in Tehran, Iran. Rats were kept as three per Plexiglas standard cage under a 12/12-h light-dark cycle (light beginning at 7:00 a.m.) with a controlled temperature of 22 ± 2°C and food and water *ad libitum*. Six animals were randomly selected for each experimental group, and each rat was tested only once. All trials were done in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996). The study's protocols and procedures were approved by the Research and Ethics Committee of the School of Medicine, Tehran University of Medical Sciences.

Morphine administration

As is demonstrated in Fig. 1, animals were randomly assigned to four groups (n=6 in each group). In group 1, animals were treated with a single i.p. dose of morphine (30 mg/kg). Group 2 was used as the control group for group 1, in which animals received a single i.p. injection of saline. In group 3, animals were exposed to chronic i.p. escalating doses of morphine. Group 4 was designed as the control group for group 3, and animals received i.p. injections of saline with the same protocol as group 3. The doses of morphine were progressively increased from 7 to 30 mg/kg over 6 days according to the method previously defined (Nieto et al., 2002). Briefly, animals received injections of the drug twice a day at 8:00 a.m. and 6:00 p.m. with doses written in parentheses, respectively, on the following days: the first day (7 and 10 mg/kg), the second day (15 and 20 mg/kg), the third day (25 and 30 mg/kg), the fourth and fifth days (30 and 30 mg/kg), and the sixth day (30 mg/kg, only at 8:00 a.m.). Control animals were injected with saline at similar time intervals as the morphine group.

Morphine sulfate was obtained from Temad Company (Tehran, Iran) and solubilized in sterile 0.9% saline just before intraperitoneal (i.p.) administration in a final volume of 0.1 ml per 100 g of body weight.

Brain tissue collection

Two hours after the last dose of morphine or saline injection, rats were rapidly decapitated, and their brain tissues were extracted. Three areas, including the NAc, striatum, and hippocampus, were dissected for RNA isolation later. The NAc is the ventral extent of the striatum, and in most studies investigating the neurobiology of addiction, samples are taken from both the dorsal striatum and the ventral part of the region (NAc) (Yager et al., 2015; Danielsson et al., 2021; Wright and Wesson, 2021) to monitor neural func-

tion and assess the molecular mechanism of addiction throughout the striatum. Thus, we first punched the NAc with a suitable device and then separated the striatum. To reduce RNA degradation, all dissection procedures were performed on a sterile, ice-cold plate, and samples were homogenized in RNA extraction buffer within 5 minutes of sacrifice.

Tissue RNA extraction and cDNA synthesis

The RNeasy Lipid Tissue Mini Kit (Qiagen) was used to isolate total RNA from tissue samples. The extracted RNA concentration in each sample was determined with spectrophotometry, and its integrity was confirmed by gel electrophoresis (1% agarose; Gibco/BRL). First-strand complementary DNA (cDNA) was synthesized using 1 µg of total RNA and the QuantiTect Reverse Transcription Kit (Qiagen) in a final volume of 20 µl.

Real-time PCR

To perform real-time PCR reactions, 2 µl of the first-strand cDNA were mixed with specific primers and Power SYBR® Green PCR Master Mix (Life Technologies) and ran on a StepOnePlus Real-Time PCR System (Applied Biosystems). The reactions of beta-actin and GluN3B genes were set up with an annealing temperature of 60°C. Melting curve analysis showed a single peak for each gene, which is an indicator of a PCR

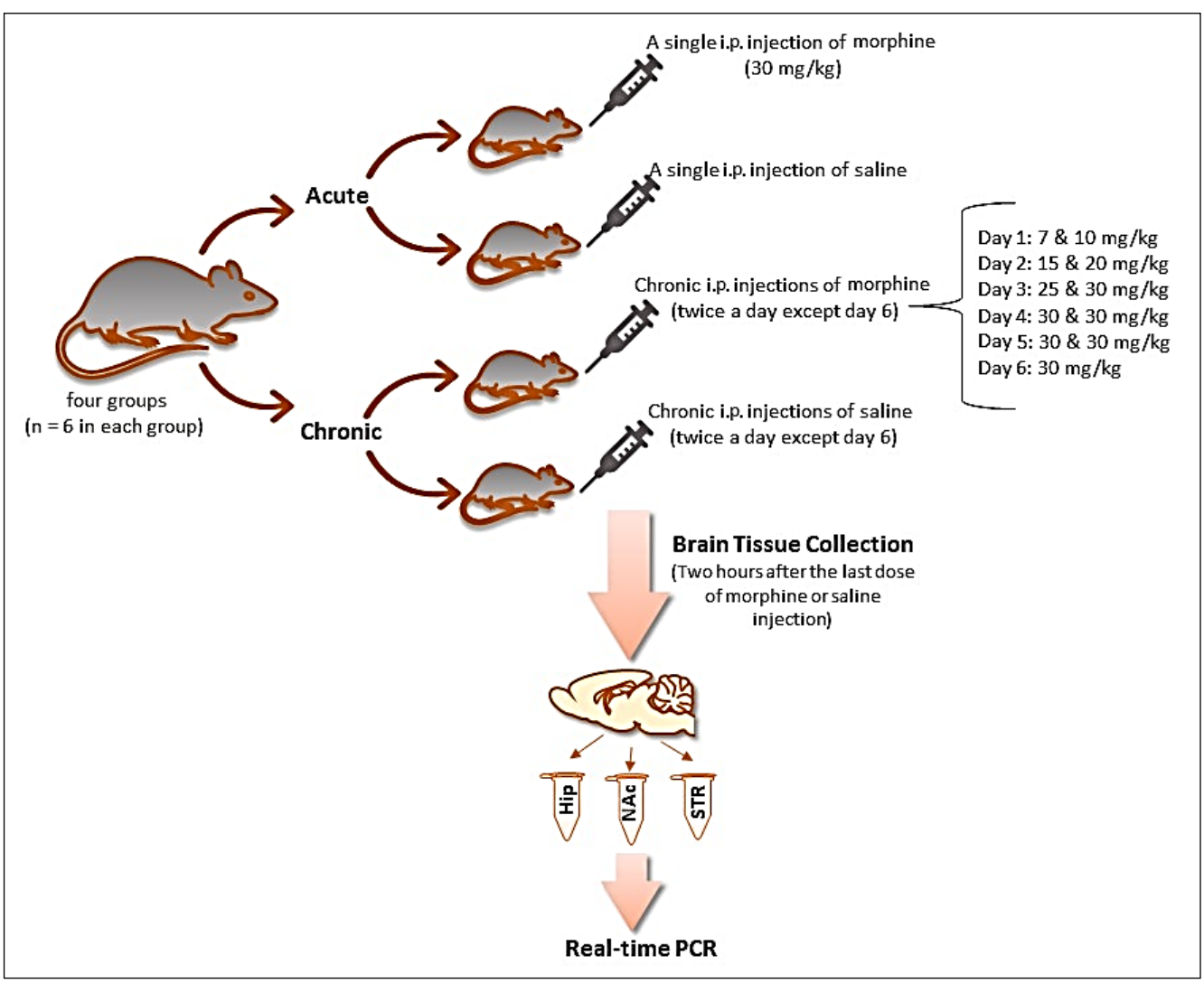

Fig. 1. The design of the study and protocols of drug administration. NAc: nucleus accumbens; Hip: hippocampus; STR: striatum.

product's specificity. To confirm PCR product lengths, we used 2.5% agarose gel and ran the amplicons, which were then visualized with ethidium bromide.

Beta-actin was used as the housekeeping gene to normalize the expression of the target gene in the present study. The oligonucleotide primers for beta-actin and GluN3B were bought from the primer bank of the Qiagen Company.

Data analysis

As we wanted to measure the amount of the target gene in prepared samples, it was necessary to have a standard series of dilutions of a cDNA sample in each run of the real-time PCR reaction. The cross point at which the sample fluorescence met a preset threshold was defined as the Cp of the sample and was referenced to the standard curve. As it was necessary to normalize the data of the treatment and control groups, beta-actin was used as the housekeeping gene. The samples were loaded into plate wells in duplicate, and the mean was used for data analysis. To calculate the statistically significant differences in the level of gene expression between the morphine-treated and control groups, we used the Relative Expression Software Tool (REST)-XL version 2. The software performs gene quantification and normalization at the same time (Pfaffl et al., 2002). A pair-wise fixed reallocation randomization test defines the significance of outcomes in REST-XL. Data are presented as fold differences of the mean normalized expression values ± standard error of the mean. $P<0.05$ was considered statistically significant.

RESULTS

Fig. 2 shows the comparison of the GluN3B subunit gene expression levels in the hippocampus (A), NAc (B), and striatum (C) in two groups of control and chronically morphine-treated rats. GluN3B mRNA levels were significantly elevated (3.15 fold) in the NAc of rats chronically administered morphine ($P<0.001$) compared to the control group. In other investigated brain regions, the gene expression of the GluN3B subunit was not significantly different between control and chronically morphine-injected rats ($P>0.05$).

In groups that received the drug acutely, the expression level of the GluN3B subunit was not statistically different between the morphine-treated and control (saline-administered) groups in any of the studied brain areas ($P>0.05$) (data not shown). The beta-actin expression did not differ for any of the examined groups (data not shown).

DISCUSSION

The N-methyl-D-aspartate receptor (NMDAR), as a critical subtype of ionotropic glutamate receptor, exerts a significant impact on various neuronal processes, including synaptic plasticity, learning and memory, and reward and addiction, as well as a spectrum of other essential neural functions (Fluyau et al., 2020; Dong et al., 2023). The receptor is mainly involved in the processes of long-term potentiation (LTP) and long-term depression (LTD), comprising the fundamental mechanisms of learning and memory (Dupuis et al., 2023). Furthermore, NMDARs play a significant role in brain development and regulate crucial processes such as neuronal migration, axon guidance, dendritic arborization, and synaptogenesis. They are also responsible for the formation of the complex architecture of neural connectivity (Petralia, 2012; Hou and Zhang, 2017; Perez-Rando et al., 2017; Dupuis et al., 2023). However, excessive NMDAR activation leads to cell death, disrupting the equilibrium between excitatory and inhibitory neural signaling and potentially resulting in neuronal degeneration (Yu et al., 2023; Zhou et al., 2023).

Within the realm of addiction, NMDARs play a crucial role by interacting with a variety of neurotransmitter systems, including dopamine, GABA, and acetylcholine (Hanania and Johnson, 1999; Tzschentke and Schmidt, 2003; Zweifel et al., 2008; Chartoff and Connery, 2014; Singh et al., 2016). The key synaptic changes in the brain's reward pathway depend on the crucial role of NMDARs in synaptic plasticity. This plasticity forms the essential basis for acquiring knowledge and memory associated with pleasurable experiences. Drugs of abuse can affect the function and expression of NMDARs, resulting in alterations in synaptic strength within reward regions (Lüscher and Malenka, 2011; Koob and Volkow, 2016). These adaptations collectively intensify drug cravings and exacerbate drug-seeking behaviors (Koob and Volkow, 2016; Hopf, 2017). Additionally, NMDARs significantly impact negative reinforcement in addiction (Covington et al., 2008; Hopf, 2017). NMDA receptor antagonists can inhibit the development of tolerance, reinforcement, and dependence on abusive drugs as well as the aversive symptoms of withdrawal (Gass and Olive, 2008; Fluyau et al., 2020; Montemitro et al., 2021; Hadizadeh et al., 2022).

Three groups of subunits, including GluN1, GluN2 (A–D), and GluN3 (A and B), form the NMDA receptor's functional tetramer. The receptor complex consists of the GluN1 subunit and the GluN2 and/or GluN3 subunits. Different assemblies of these subunits result in various NMDA receptors with different functional and biophysical properties (Cull-Candy and Lesz-

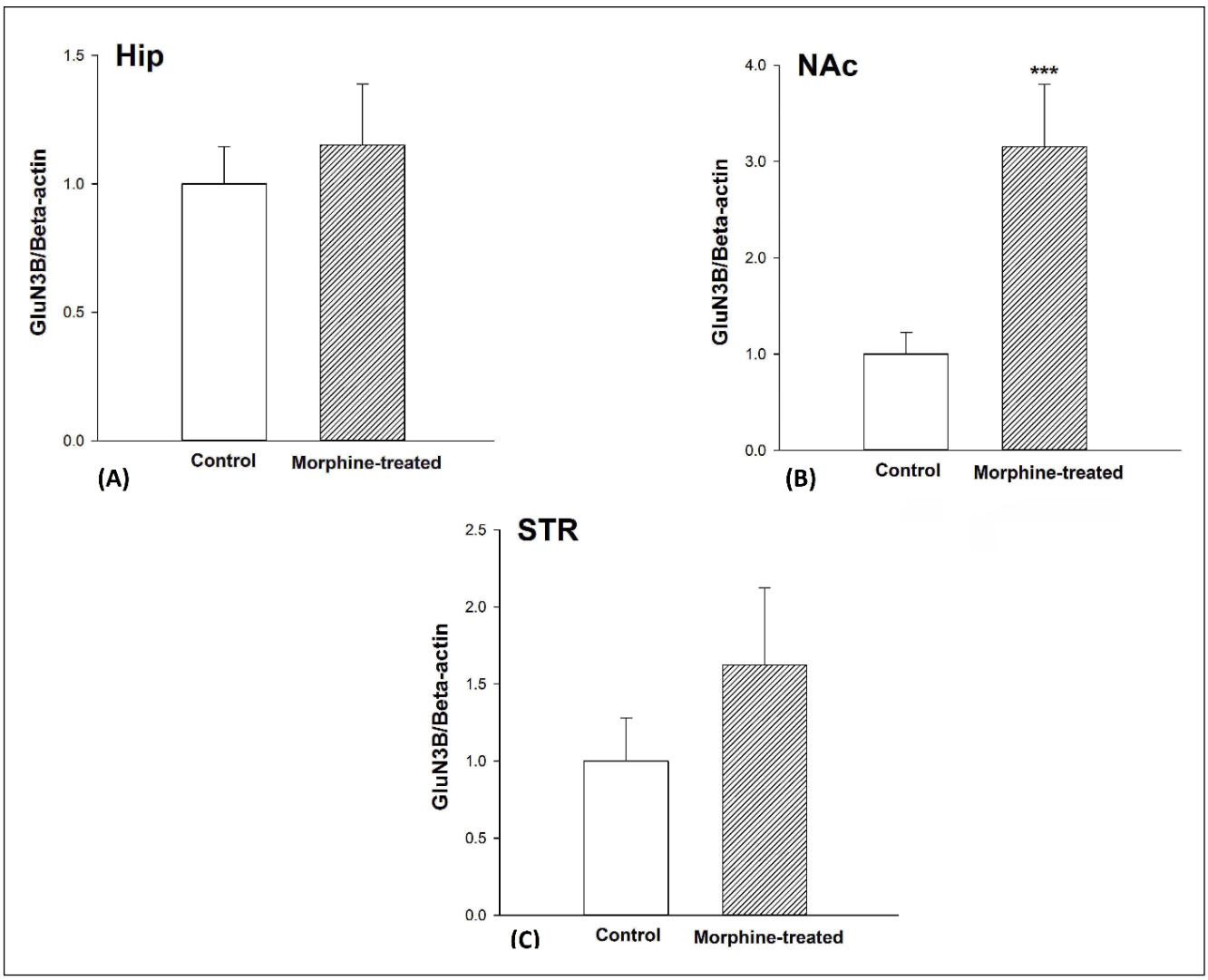

Fig. 2. Differences in the mRNA expression level of the GluN3B subunit of the NMDA receptor in the hippocampus (A), NAc (B), and striatum (C) of chronically morphine-treated rats compared to the control group. *** $P<0.001$ compared to the control group.

kiewicz, 2004; Paoletti and Neyton, 2007; Salussolia et al., 2011). The GluN3 subunits (also known as GluN3A and GluN3B) have been a subject of research interest. However, their specific role in addiction is not as well-established as the roles of other glutamate receptors and neurotransmitter systems (Beesley et al., 2020). GluN3 subunits have a unique expression pattern in the brain (Pachernegg et al., 2012). Areas that highly express the GluN3B subunit are the brainstem, spinal cord, and hippocampus (Nishi et al., 2001; Matsuda et al., 2002; 2003; Bendel et al., 2005). However, more studies have suggested that other regions express GluN3B, such as the NAc and striatum (Wee et al., 2008), which is in line with our study detecting GluN3B in these areas. Alterations in NMDA receptor function *via* changes in GluN3 subunit expression could influence the neural adaptations that occur during addiction. Evidence has indicated that long-term drug treatment alters glutamate-mediated synaptic transmission by modifying the composition of the NMDA receptor complex (Bajo et al., 2006; Crawley et al., 2022). It has been shown that the existence of the GluN3 subunit in the NMDA receptor complex reduces the activity of the receptor by decreasing the calcium permeability of the channel (Crawley et al., 2022; Hurley et al., 2022). GluN3A mRNA levels were found to be increased in the brains of individuals with alcohol dependence (Jin et al., 2014). In our previous study, we found that changes in GluN3A expression were associated with addiction to opioids (Roozafzoon et al., 2010). We also found that chronic morphine administration led to the up-regulation of the GluN3A

subunit in PFC (Vousooghi, Ghane et al., 2016). Another study found that chronic methamphetamine enhanced GluN3A expression, reducing cortical plasticity and impairing motor learning (Huang et al., 2017). Yuan et al. found that cocaine injection could drive the insertion of GluN3A-NMDARs at synapses in reward-related regions with subsequent recruitment of calcium-permeable AMPARs, a form of adaptive plasticity involved in relapse (Yuan et al., 2013). Although GluN3B has not been as extensively studied as the GluN3A subunit, a growing number of recent studies have investigated GluN3B in the context of addiction. In 2010 we showed increased GluN3B gene expression in both heroin-addicted and heroin-withdrawal patients (Sedaghati et al., 2010). Studies have shown that the variants of the GluN3B gene are associated with the development of addictive behaviors. A genetic polymorphism, GluN3B rs2240158, has been reported to be associated with heroin addiction in the Han Chinese population. By genotyping SNPs in GluN3B in male heroin addicts and normal control subjects, Xie et al. (2016) found that the genotype and allele frequency of rs2240158 were significantly different in the cases and controls. These results were confirmed by a recent study demonstrating that rs2240158 in GluN3B might be a susceptibility gene underlying heroin addiction, which was significantly associated with the ability to overcome heroin use (Huang et al., 2021). Of interest, a recent study implicated time-dependent changes in the plasma membrane expression of the GluN3 subunits of the NMDA receptor within the NAc core as important for incubated cocaine craving (Christian et al., 2021). These findings suggest a role for the GluN3 genes in drug addiction, acting as dominant-negative modulators of the NMDA receptor. To our knowledge, the current study is the first report measuring gene expression changes of the GluN3B subunit after acute and chronic morphine administration in the rat. We found that GluN3B subunit expression was markedly higher in the NAc of chronically morphine-treated rats than in the controls. It is currently unclear whether the increase observed in our study is a compensatory response to chronic morphine administration or the effect of the chronic morphine itself. Additional investigations are needed to study this issue.

The primary function of the NAc is the integration of cortical and midbrain dopaminergic information. This region receives excitatory glutamatergic innervations from the corticolimbic areas. Moreover, the NAc is densely innervated by dopaminergic inputs in the midbrain. Along with other vital areas such as the VTA, striatum, amygdala, and PFC, the NAc plays a crucial role in the reward pathway, addiction, and drug-seeking behavior (Zhang, 2005; Peciña and Berridge, 2013). The long-term use of addictive drugs can lead to pathological changes in glutamate and dopamine transmission in the NAc (Di Chiara et al., 2004; Quintero, 2013).

The role of glutamate, as a critical molecule in the NAc, in the neuroplasticity of the reward system is undeniable (Quintero, 2013). Chronic exposure to addictive substances can disrupt glutamatergic transmission in the PFC-NAc pathway, prominently affecting glutamate homeostasis in the NAc (LaLumiere and Kalivas, 2008; Reissner and Kalivas, 2010). The NMDA receptor is the critical glutamate receptor involved in this phenomenon (Kalivas and Volkow, 2005). Ca^{2+} permeability through the NMDA receptor channel, as well as the receptor's excitatory properties, can be reduced and inhibited due to the presence of the GluN3 subunit in the NMDA receptor (Cavara and Hollmann, 2008). Therefore, in our study, a significant up-regulation in the level of GluN3B may have led to a decrease in the activity of the NMDA receptor in the NAc of rats chronically administered morphine. Our results appear to agree with a study that showed that chronic morphine treatment could reduce glutamate transmission by altering NMDA receptor properties in the NAc (Martin et al., 1999). It has been claimed that cellular homeostasis may be altered following long-term morphine exposure. Chronic morphine administration can result in the down-regulation of mu-opioid receptors (MOR), stimulation of immediate early gene (IEG) expression, and an elevated level of cyclic AMP. cAMP has a crucial role in numerous biological pathways responsible for controlling cell homeostasis (Martin et al., 1999). There is evidence that a rise in cAMP levels may be regulated by an IEG, such as the one for cAMP response-binding protein (CREB) (Lane-Ladd et al., 1997; Martin et al., 1999). Furthermore, activation of the NMDA receptor could partially regulate the gene expression of IEGs such as c-jun, jun-B, and CREB (Cole et al., 1989; Szekely et al., 1990; Morgan and Linnoila, 1991; Martin et al., 1999). It can be suggested that NMDA receptors may control the enhanced level of cAMP after morphine consumption. Therefore, the decreased activity of the NMDA receptor may be considered a compensatory mechanism, reducing the NMDA receptor stimulation of CREB synthesis and the elevation of cAMP concentrations stimulated by chronic morphine administration (Martin et al., 1999).

Our findings did not identify marked changes in the expression level of GluN3B in the hippocampus and striatum after chronic morphine administration. Additionally, there was no marked difference in GluN3B subunit expression levels in groups that acutely re-

ceived morphine compared to controls in any of the three brain areas mentioned.

Future studies in addiction models are required to elucidate how GluN3 influences the molecular mechanism of drug addiction, leading to potential therapies for the disorder. Indeed, although developing safe and effective drugs that target GluN3 subunits is a complex challenge that requires further exploration, selective GluN3 modulation would help in understanding the functional role of GluN3-NMDARs in the pathophysiology of addiction and might be valuable for treating substance use disorders. GluN3 modulation may present an intriguing avenue for addiction research and potential therapeutic interventions. Understanding the precise role of GluN3 subunits in addiction-related processes and developing targeted pharmacological agents could offer new strategies for managing addiction and improving treatment outcomes. However, this field is still in its early stages, and further research is needed to fully elucidate its therapeutic potential.

Lastly, it should be mentioned that although the amount of morphine injected in acute treatment could affect the mRNA expression in a dose-dependent manner, as an acute dose, we had to choose an amount within a routine and accepted dose range according to the previous studies (Huang et al., 1997; Boronat et al., 2001; Rajaei et al., 2005; Zamanian et al., 2020) but that was also large enough to elicit possible effects of single-dose morphine on GluN3B expression. Thus, we injected 30 mg/kg morphine as our acute dose, which was equal to the highest dose of the drug used in the chronic escalating protocol. In addition, we have not shown the data for the acute dose of morphine as the focus and main goal of our study was on the chronic effects of morphine on GluN3B expression, which we are considering as a therapeutic target to be further explored for potential benefits in people suffering from opioid use disorder. We included the acute group only to confirm that the observed effects in chronically treated animals could not be induced after a single dose of morphine treatment, which confirms that the mechanistic pathways in acute and chronic morphine administration and the signaling routes are different.

Additionally, we did not isolate the PFC region in the present study because the expression of the GluN3B subunit of the NMDA receptor in the CNS is more limited compared to the GluN3A. Thus, we extracted the parts of the brain reward pathway in which GluN3B expression was indicated from previous studies. However, it would be of interest to examine the PFC site for expression of GluN3B after chronic drug administration in future studies.

CONCLUSION

Our study is the first report measuring the gene expression alteration of the GluN3B subunit after acute and chronic administration of morphine to rats. We found that the GluN3B subunit expression is markedly higher in the NAc of chronic morphine-treated rats than in the control group. However, we are unable to determine whether the observed effect is a compensatory response to chronic morphine or the effect of the chronic morphine itself. Future evaluations of selective GluN3 modulation would help understand the functional role of GluN3-NMDARs in the pathophysiology of addiction and might be valuable for treating substance use disorders. This field of study may open a window toward using agents effective on GluN3 subunits of NMDAR as potential therapeutic molecules for addiction.

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