Neuroscience Letters 686 (2018) 133–139

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Memantine attenuated alcohol withdrawal-induced anxiety-like behaviors through down-regulating NR1-CaMKII-ERK signaling pathway

Ji Yuanyuan[a], Zhu Junyan[b], DeJi Cuola[a], Cui Jingjing[a], Shi Yuhui[a], Xu dan[a], Dang Wei[a,b,c], Zhu Yongsheng[a,c,*]

[a] College of Forensic Science, School of Medicine, Xi'an Jiaotong University, No. 76, Yanta West Road, Xi'an, Shaanxi 710061, China
[b] Xi'an Mental Health Center, Xi'an, Shannxi, China
[c] Key Laboratory of Environment and Genes Related to Diseases, Ministry of Education, Xi'an, Shaanxi 710061, China

ARTICLE INFO

Keywords:
Anxiety
N-methyl-D-aspartic acid receptor
Memantine

ABSTRACT

Alcohol abuse and anxiety disorders often occur concurrently, but their underlying cellular mechanisms remain unclear. N-methyl-D-aspartic acid receptors (NMDARs) have recently received attention from those interested in the neurobiology of anxiety. A chronic alcohol exposure rat model (28 consecutive days of 20% alcohol intake and 6 h of withdrawal) was established. Here, we investigated the NMDAR1 (NR1), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and extracellular signal-regulated kinases (ERK) pathway in the modulation of anxiety-like behaviors in rats exposed to an open field and elevated plus maze (EPM) through systematic injections of memantine (a NMDAR inhibitor). We found that the NR1-CaMKII-ERK signaling pathway was activated after alcohol withdrawal in medial prefrontal cortex (mPFC) and nucleus accumbens shell (NAcSh) but not core (NAcC). Memantine treatment greatly ameliorated anxiety-like behavior in the rats experiencing alcohol withdrawal. Moreover, memantine uniformly suppressed the phosphorylation of NR1-CaMKII-ERK pathway induced by alcohol withdrawal. Our results suggest that activation of the NR1-CaMKII-ERK pathway in the mPFC and NAcSh is an important contributor to the molecular mechanisms underlying alcohol withdrawal-induced anxiety behaviors. NMDAR signaling pathway inhibitors are thus potential therapeutics for treating alcohol abuse.

1. Introduction

Chronic alcohol treatment induces addiction. Addiction is defined by the emergence of somatic and affective withdrawal signs. In human, alcohol withdrawal syndrome (AWS) typically includes anxiety, seizures, delirium tremens, depression [1], *etc.* especially 6 h after the last drink in rats [2]. The relapse rate is near 100% due to AWS. Alleviation of withdrawal-induced negative-affective states becomes a primary motivation for continued alcohol use [3]. AWS triggers persistent molecular and cellular modifications in the medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) [4,5].

The mPFC is centered in the prelimbic cortex. Recent evidence indicates that the mPFC is critical for the extinction of both fear and drug-seeking behaviors [6]. The mPFC represents a common node in the extinction circuit for these behaviors; treatments that target this region may help alleviate symptoms of both anxiety and addictive disorders [6]. The NAc is a point of convergence for excitatory afferents arising from limbic and cortical regions, including the mPFC. Notably, the NAc is necessary for triggering drug reward, as well as negative-affective states and some somatic signs associated with drug withdrawal [7]. The shell of the NAc is involved in the cognitive processing of rewards, and the core is involved in the cognitive processing of motor function related to reward and reinforcement. The inputs from the PFC to the NAc modulate outputs to motor relay circuits that oversee motor actions and outcomes [8].

The *N*-methyl-D-aspartate-type glutamate receptors (NMDARs) are heteromeric complexes that incorporate the NMDAR1 (NR1), NR2, and NR3 subunits. Without NR1, NMDAR complexes are not functional [9]. NR1 subunits are also important determinants of alcohol sensitivity [10]. It has been postulated that alcohol binds to the third transmembrane domain (TM3) of the NR1 subunit [11]. NMDARs can activate the mitogen-activated protein kinase (MAPK) pathway directly through a Ca^{2+}-dependent signaling pathway, which is critical for long-lasting behavioral plasticity [12]. Our previous data showed that levels of phospho-ERK are decreased, while those of phospho-NR1 are elevated in the rat PFC and NAc following long-term alcohol exposure [2]. The altered NR1 and NR2A could have potential impact on anxiety-like behavior in the adult offspring exposed to prenatal stress [13]. Studies

* Corresponding author at: No.76 Yanta west road, Xi'an, Shaanxi 710061, China.
E-mail address: aiwapeter@163.com (Z. Yongsheng).

https://doi.org/10.1016/j.neulet.2018.09.006
Received 10 August 2018; Received in revised form 1 September 2018; Accepted 4 September 2018
Available online 10 September 2018
0304-3940/

have shown that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) could directly activate the Ras/Raf1/ERK pathway [14] and induce MEK1 phosphorylation by CaMKII [15]. Our results indicated that region-specific activation of CaMKII-ERK signaling in the hippocampal CA1 and DG regions [16] and activation of NR1-CaMKII-ERK signaling in the nucleus accumbens shell (NAcSh) but not core (NAcC) contributed to ethanol consumption and chronic ethanol-related negative emotional states [17]. Memantine is used to treat moderate to severe Alzheimer's disease. It acts on the glutamatergic system by blocking NMDAR. Memantine has been studied in generalized anxiety disorder, as an augmentation therapy for anxiety disorders [18].

To address this issue, we determined the NR1 subunit and CaMKII isoform involved in ERK activation during alcohol withdrawal-induced anxiety behaviors. The elevated plus maze (EPM) and open field test (OFT) were used to assess anxiety-like behavior. Phosphorylation of NR1-CaMKII-ERK signaling molecules was tested in the mPFC, NAcSh and NAcC. Memantine was used to determine signaling pathway inhibition and to ameliorate negative-affective states.

2. Materials and methods

2.1. Animals

Eight-week-old male Sprague-Dawley rats (Laboratory Animal Center of Xi'an Jiaotong University, China) weighing 260–280 g were habituated and handled in housing conditions 1 week before testing. Animals were housed 3–4 per cage on a 12-h light-dark cycle (light on at 08:00) in a temperature-controlled room. Rats were allowed access to food and water in their home cages throughout the experiments. All conditioning and testing experiments were carried out during the light phase of the cycle. All experimental procedures were performed according to the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

2.2. Ethanol and antibodies

Anhydrous ethanol (Huada Pharmaceutical Factory, Guangdong, China) was prepared in sterile water at concentrations ranging from 2% to 20%. Phospho-NR1 (Ser890), phospho-CaMKII (Thr286), phospho-ERK1/2 (Thr202/Tyr204), NR1, CaMKII (pan) α/β and ERK1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). β-actin monoclonal antibody and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Memantine was purchased from Sigma-Aldrich (Sigma-Aldrich, Darmstadt, USA).

2.3. Experimental procedures

Experiment 1: Withdrawal after chronic alcohol exposure. The experiment was conducted as described previously [2,19] with some adjustments (Fig. S1A). Rats were randomly assigned to two experimental groups: a water exposure group (n = 8) and an alcohol exposure group (n = 8), receiving water and alcohol solution, respectively. Solutions were administered at concentrations ranging from 2% to 20% (v/v) during the first 10 days for adaptation, then administered at 20% alcohol (v/v) as the only liquid source for a consecutive 28 days. After 28 days of treatment, the alcohol solution was replaced by water for withdrawal-related behavioral tests, including the OFT and EPM.

Experiment 2: To examine behavioral and molecular changes after blocking NR1 receptors, rats were randomly assigned to four experimental groups: water-saline (n = 8), alcohol-saline (n = 8), water-memantine (n = 8), and alcohol-memantine (n = 8) (Fig. S1B). After 28 days of alcohol exposure, rats were injected with saline (10 ml/kg, i.p.) or memantine (4 mg/kg, i.p.) 30 min before behavioral tests.

2.4. Open field test (OFT)

The OFT was used to measure locomotion and anxiety-like behavior in rats. A black square arena (100 cm × 100 cm × 45 cm) was used for the test. The arena was illuminated with three 30 W fluorescent bulbs placed 2 m above it in a sound-attenuating room. Rats (n = 8/group) were placed in the corner of the arena equipped with a computerized video-tracking system (SMART, Panlab SL, Barcelona, Spain) and allowed 15 min of exploration. We divided the arena virtually into two areas: a peripheral zone (PZ) and a central zone (CZ) (41% of the arena). The data of total distance traveled and the percent of time spent in the center of the arena were analyzed.

2.5. Elevated plus maze test (EPM)

According to the method of previous studies [2,20], the EPM was conducted to assess anxiety-like behavior. Our maze was made of medium-density fiberboard with a black surface and consisted of four arms (two open without walls and two enclosed by 30 cm high walls) 50 cm long and 10 cm wide. The rat elevated plus maze was placed close to the center of the room and had similar levels of illumination on both open and closed arms. The initial 5 min behaviors of the rats were recorded by the video-tracking system. The number of entries into the open arms and closed arms and the time spent in the open arms and closed arms were measured. An entry was scored when the limbs or more than 80% of bodies of rats entered an arm. The measures used to indicate fear/anxiety memory were the number of the open arm entries and the percentage (%) of time spent in the open arms.

2.6. Western blotting

The rats were sacrificed by decapitation after the behavioral tests. The mPFC and the core and shell regions of the NAc of the brain were quickly removed for western blotting. The tissue was homogenized with an ultrasound homogenizer in a precooled RIPA buffer (50 mM Tris−HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 10 mM EGTA, 2 mM sodium pyrophosphate, 4 mM paranitrophenylphosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl uoride, 2 mg/ml aprotintin, 2 mg/ml leupeptin and 2 mg/ml pepstatin). The homogenates were incubated on ice for 30 min and centrifuged at 12,000 × *g* for 15 min at 4°C. The protein content was determined using the bicinchoninic acid (BCA) method (PPLYGEN, Beijing, China). The protein samples were subjected to 12% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% (w/w) non-fat dried milk in Tris-buffered saline (TBS) (500 mM NaCl and 20 mM Tris−HCl pH 7.4) containing 0.05% Tween-20 and incubated overnight with one of the following antibodies at 4 °C: anti-phospho-NR1 (Ser890), phospho-CaMKII (Thr286), phospho-ERK1/2 (Thr202/Tyr204), NR1, CaMKII (pan) α/β or ERK1/2. On the following day, the membranes were washed three times with 0.1% Tween-20 TBS (pH 7.4) and incubated with horseradish peroxidase-conjugated secondary antibodies for 70 min. An enhanced chemiluminescence kit (Millipore, MA, USA) was used to detect immunoreactive protein bands. The protein band intensities were analyzed by the Quantity One software (Bio-Rad, Hercules, USA) to calculate the ratio of target protein/internal control (β-actin 1:1000).

2.7. Statistical analysis

All data were expressed as the means ± SEM. Statistical analysis was performed using a two-way analysis of variance (ANOVA) or Student's unpaired *t*-test in accordance with the experimental design. Specifically, unpaired t-tests were used to assess the data in Experiment 1 (Fig. S1A). A two-way ANOVA was used to analyze the effects of alcohol withdrawal and memantine injection in Experiment 2 (Fig. S1 B). Multiple comparisons were performed using Bonferroni's *post hoc*