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Neurogranin in the nucleus accumbens regulates NMDA receptor tolerance and motivation for ethanol seeking

Ashlie N. Reker [a], Alfredo Oliveros [b], John M. Sullivan III [a], Lailun Nahar [a], David J. Hinton [b], Taehyun Kim [b], Robert C. Bruner [b], Doo-Sup Choi [b], Nicholas E. Goeders [a], Hyung W. Nam [a, *]

[a] Department of Pharmacology, Toxicology, and Neuroscience, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA
[b] Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine, Rochester, MN 55905, USA

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ABSTRACT

Dysfunction of N-methyl-D-aspartate receptor (NMDAR) signaling in the nucleus accumbens (NAc) has been implicated in the pathophysiology of alcohol use disorders (AUD). Neurogranin (Ng), a calmodulin-binding protein, is exclusively expressed in the post-synapse, and mediates NMDAR driven synaptic plasticity by regulating the calcium-calmodulin (Ca^{2+}-CaM) pathway. To study the functional role of Ng in AUD, we administrated behavior tests including Pavlovian instrument transfer (PIT), operant conditioning, and rotarod test using Ng null mice ($Ng^{-/-}$ mice). We used adeno-associated virus (AAV)-mediated Ng expression and pharmacological manipulation to validate behavioral responses in $Ng^{-/-}$ mice. The results from our multidisciplinary approaches demonstrated that deficit of Ng increases tolerance to NMDAR inhibition and elicit faster cue reactivity during PIT without changes in ethanol reward. Operant conditioning results demonstrated that $Ng^{-/-}$ mice self-administered significantly more ethanol and displayed reduced sensitivity to aversive motivation. We identified that ethanol exposure decreases mGluR5 (metabotropic glutamate receptor 5) expression in the NAc of $Ng^{-/-}$ mice and pharmacological inhibition of mGluR5 reverses NMDAR desensitization in $Ng^{-/-}$ mice. Together these findings specifically suggest that accumbal Ng plays an essential role in the counterbalance between NMDAR and mGluR5 signaling; which alters NMDAR resistance, and thereby altering aversive motivation for ethanol and may ultimately contribute to susceptibility for alcohol addiction.

1. Introduction

Alcohol use disorders (AUD) contribute greatly to the global economic burden of mental health disorders and cause 88,000 deaths in the United States every year (Stahre et al., 2014). A growing body of evidence suggests that pharmacological and genetic regulation of N-methyl-D-aspartate receptors (NMDAR) alters both neural and behavioral effects of ethanol, as glutamate dysregulation is associated with AUD (Chen and Holmes, 2009). Moreover, hyper-glutamatergic NMDAR regulation within the nucleus accumbens (NAc) contributes to the physiological response to alcohol and addiction development (Di Ciano et al., 2001; Kalivas, 2009). However, causality between the effect of ethanol exposure and the development of ethanol seeking as it pertains to the NMDAR-intracellular mechanism in AUD pathology remains unclear.

Neurogranin (Ng) is ideally situated to modulate downstream NMDAR function (Chakravarthy et al., 1999). Ng is a postsynaptic neuron-specific protein and is involved in the regulation of the calcium-calmodulin complex (Ca^{2+}-CaM) in the Ca^{2+}/CaM-dependent protein kinase II/IV (CaMKII/IV) pathway (Miyakawa et al., 2001; Pak et al., 2000). Ng accumulates in the postsynaptic region by binding to phosphatidic acid, and features an IQ CaM-binding motif and is phosphorylated (S36) by the metabotropic

Abbreviations: NMDAR, of N-methyl-D-aspartate receptors; Ng, Neurogranin; AUD, alcohol use disorders; mGluR5, metabotropic glutamate receptor 5; NAc, nucleus accumbens; AAV, adeno-associated virus; CaM, calmodulin; PIT, Pavlovian instrument transfer; CPP, conditioned place preference; GFP, green florescent protein; FR, fixed ratio; oNg, overexpression neurogranin; dnNg, dominant negative neurogranin.

* Corresponding author. Department of Pharmacology, Toxicology, and Neuroscience, 1501 Kings Highway, LSU Health Sciences Center, Shreveport, LA 71130, USA.
E-mail address: hnam@lsuhsc.edu (H.W. Nam).

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glutamate receptor 5 (mGluR5) mediated PKCγ signaling pathway (Dominguez-Gonzalez et al., 2007; Krueger and Nairn, 2007). Ng null ($Ng^{-/-}$) mice do not show any obvious developmental or neuroanatomical abnormalities, but do exhibit a deficit in spatial learning and memory when compared to wild-type littermates (Miyakawa et al., 2001; Pak et al., 2000). Electrophysiology studies also demonstrate that Ng expression may enhance postsynaptic sensitivity and increase synaptic strength in a Ca^{2+}-dependent manner downstream of NMDAR and mGluR5, suggesting an essential role for this protein in synaptic plasticity (Hayashi, 2009; Zhong et al., 2009).

Previously decreased NMDAR-Ng regulation in the nucleus accumbens (NAc) has been linked with phenotypes associated with alcohol drinking under NMDA mediated hyper-glutamatergic states (Nam et al., 2011). Consequently, inhibition of NMDAR glutamate receptor signaling in the NAc has been shown to enhance the sensitivity to conditioned cue and decrease cue-induced drug seeking (Backstrom et al., 2004; Backstrom and Hyytia, 2007; Kumaresan et al., 2009). Therefore, Ng signaling in the NAc may contribute to “cue response” for reinforcement, or underlie the motivation to “wanting” ethanol reinforcement and finally lead to susceptibility of sensitization and addiction (Robinson and Berridge, 2001, 2003; Yager and Robinson, 2010).

In this study, we tested whether Ng is associated with any predisposition toward AUD by examining ethanol tolerance, ethanol reward, and ethanol reinforcement using $Ng^{-/-}$ mice and NAc specific AAV-mediated Ng knockdown or overexpression. To understand Ng mediated glutamate neuroadaptation that may be caused by ethanol, protein expression and neural morphology analysis after ethanol exposure in $Ng^{-/-}$ mice were used. Our study shows the potential role of Ng in regulating Pavlovian instrument transfer (PIT) and operant behaviors mediated by the counterbalance between NMDAR- and mGluR5-glutamate receptor signaling. Together, this work elucidates a novel mechanism through which the transition to excessive ethanol seeking in mice may develop, and has important translational implications for the human AUD patient population.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice and $Ng^{-/-}$ mice (C57BL/6J background, Jackson Laboratories, Bar Harbor, ME) aged 8–16 weeks were used. Mice were group-housed in standard plexiglas cages under a 12h light/dark cycle (lights on at 6:00 a.m.) at a constant temperature (24 ± 0.5 °C) and humidity (60 ± 2%) with food and water available *ad libitum*. For two-bottle drinking experiments, mice were singly housed. The animal care and handling procedures were in accordance with LSUHSC and Mayo Clinic institutional and National Institutes of Health (NIH) guidelines.

2.2. Adeno-associated virus (AAV)-mediated Ng expression

AAV vectors using AAV Helper-Free system (Stratagene, La Jolla, CA) were produced by transfection of plasmids. Ng mouse cDNA clone (NM_022029.1) purchased from Origene was used to induce overexpression of Ng (oNg). The Serine 36 (ATG) site was transformed to Glycine 36 (GGT) *via* QuickChange site-directed mutagenesis kit (Stratagene) to induce dominant negative Ng (dnNg). Then, both plasmids were sub-cloned using pAAV-MCS vector. The AAV vector system contains the necessary genes from adenovirus (pHelper vector) to induce the lytic phase of AAV producing recombinant, replication-defective AAV virus ready to deliver a gene of interest to target cells. Briefly, pAAV2-Ng plus the pRC vector encoding Rep and Cap proteins and the pHelper vector encoding the adenovirus gene products were used to transfect 293T cells at an 80% confluence stage. At 3 days after transfection, AAV vector-producing 293T cells were harvested for vector purification. The cells were lysed by freeze and thaw cycling, followed by ultracentrifuge concentration (~400,000 g for 2 h) through iodixanol gradient centrifugation. The resulting AAV vectors were desalted and concentrated using Amicon Ultra-15 100k filtration (Amicon, Billerica, MA) before being resuspended in PBS. The titers of pAAV were determined using real-time PCR analysis by calculating the viral genome copy number.

2.3. Microinjection of AAV-mediated Ng expression into the NAc

Stereotactic injections of AAV viruses were conducted as described previously (Nam et al., 2013). Mice were anesthetized with ketamine/xylazine (100 and 15 mg/kg, i.p.) and placed in a digital stereotactic alignment system (Model 1900; David Kopf Instruments). Two holes for bilateral injection were drilled above the target brain region. The injector (33 Ga; Plastics One) was connected to a Hamilton syringe (25 μl) and virus infusion was controlled by syringe pump. To infuse the viruses, 1 μl of AAV viruses (AAV-oNg or AAV-dnNg) was bilaterally injected into either the NAc (AP: 1.34 mm; ML: ±1.3 mm; DV: 3.5 mm) or medial prefrontal cortex (mPFC) (AP: 1.94 mm; ML: ± 0.3.mm; DV: 2.0 mm) at a 0.1 μl/min rate. Injectors remained in place for an additional 5 min per each infusion side. All animals with a misplacement of the needle tract were excluded from the study.

2.4. Golgi-cox staining and Sholl analysis for dendritic morphology

Ethanol (2 g/kg, *i.p.*, 1h) or saline treated mice were anesthetized with pentobarbital (80 mg/kg) and their brains were rapidly dissected out. Brains were immediately immersed in Golgi-Cox impregnation solution from FD Rapid GolgiStain™ Kit (FD NeuroTechnologies, Columbia, MD) (Peca et al., 2011). The solution was stored at room temperature for two weeks in the dark and then rinsed for sectioning. 100 μm brain slices were cut on a cryostat (Leica) at −20 °C and mounted on gelatin-coated microscope slides. Slides were rinsed and dehydrated according to the manufacture's protocol and a cover slip with Permount mounting solution was applied. NeuronJ and Sholl Analysis Plugin for NIH ImageJ software (https://imagej.nih.gov/ij/index.html) were utilized to facilitate the tracing and quantification of dendrite branching in the neuron.

2.5. Operant self-administration of sucrose and ethanol

Mice were placed on a food restriction schedule to maintain 85% of their free-feeding weight and trained to respond for a 20% sucrose reward. All operant training procedures were performed in computer-controlled mouse operant chambers (Model ENV-307W; Med Associates) equipped with house light. All operant training programs, schedules of reinforcement, cue lights, and syringe pump activations were controlled by Med PC-IV (Med Associates). To train mice to associate a tone with reward delivery, random presentations of tones (1s duration) were paired with delivery of 100 μl of a reward solution during 30-min magazine training sessions. To test the Pavlovian conditioned behavior, natural reward (20% sucrose) was paired with a tone conditioned stimuli (CS+) and the latency time to retrieve that reward (beam break in fountain) following CS + presentation was measured (Hall et al., 2001). This was expanded to a modified, 6-day differential reward of low rate (DRL) schedule (Young and McNaughton, 2000), which measures cue responding time by forcing mice to wait to retrieve a reward after the CS + presentation. To test operant conditioned behaviors,