Brain, Behavior, and Immunity xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

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

Full-length Article

Innately activated TLR4 signal in the nucleus accumbens is sustained by CRF amplification loop and regulates impulsivity

Irina Balan [a], Kaitlin T. Warnock [b], Adam Puche [c], Marjorie C. Gondre-Lewis [d], Laure Aurelian [a,*]

[a] Department of Pharmacology, University of Maryland School of Medicine, Baltimore, MD, USA
[b] Neuropsychopharmacology Laboratory, Department of Psychiatry and Behavioral Sciences, Howard University College of Medicine, Washington, DC, USA
[c] Department of Anatomy and Neurobiology, University of Maryland School of Medicine, Baltimore, MD, USA
[d] Department of Anatomy, Laboratory for Neurodevelopment, Howard University College of Medicine, Washington, DC, USA

ARTICLE INFO

Article history:
Received 25 August 2017
Received in revised form 31 October 2017
Accepted 12 November 2017
Available online xxxx

Keywords:
Activated TLR4 signal
PKA/CREB
CRF
$GABA_A$ α2
HSV siRNA vectors
Impulsivity

ABSTRACT

Cognitive impulsivity is a heritable trait believed to represent the behavior that defines the volition to initiate alcohol drinking. We have previously shown that a neuronal Toll-like receptor 4 (TLR4) signal located in the central amygdala (CeA) and ventral tegmental area (VTA) controls the initiation of binge drinking in alcohol-preferring P rats, and TLR4 expression is upregulated by alcohol-induced corticotropin-releasing factor (CRF) at these sites. However, the function of the TLR4 signal in the nucleus accumbens shell (NAc-shell), a site implicated in the control of reward, drug-seeking behavior and impulsivity and the contribution of other signal-associated genes, are still poorly understood. Here we report that P rats have an innately activated TLR4 signal in NAc-shell neurons that co-express the α2 $GABA_A$ receptor subunit and CRF prior to alcohol exposure. This signal is not present in non-alcohol drinking NP rats. The TLR4 signal is sustained by a CRF amplification loop, which includes TLR4-mediated CRF upregulation through PKA/CREB activation and CRF-mediated TLR4 upregulation through the CRF type 1 receptor (CRFR1) and the MAPK/ERK pathway. NAc-shell Infusion of a neurotropic, non-replicating herpes simplex virus vector for TLR4-specific small interfering RNA (pHSVsiTLR4) inhibits TLR4 expression and cognitive impulsivity, implicating the CRF-amplified TLR4 signal in impulsivity regulation.

1. Introduction

Cognitive impulsivity is a heritable trait generally defined as a tendency to act without thinking that correlates with drug addiction and is believed to represent the ethanol-seeking behavior, which precedes steady alcohol consumption (Beckwith and Czachowski, 2014; Oberlin and Grahame, 2009). Stressor-induced elevations in the corticotropin-releasing factor (CRF) system regulate impulsivity and play a key role in the transition to escalated drug taking, including excessive ethanol drinking (Gondre-Lewis et al., 2016; Lowery-Gionta et al., 2012). However, the genes that regulate the predisposition to initiate alcohol drinking, their potential interaction at distinct brain sites, and their contribution to impulsivity, if any, are still poorly understood.

Toll-like receptors (TLRs) are largely recognized as neuroimmune signals located in neurons and glial cells (Takeda and Akira, 2015). An extensive body of literature has associated one of the TLRs, TLR4, with a lifetime of alcohol consumption and adaptation during ethanol exposure, likely involving differentially activated neuronal and glial signaling pathways. This includes the findings that systemic injection of the TLR4-specific ligand, bacterial endotoxin lipopolysaccharide (LPS) increases voluntary alcohol consumption in mice, and human alcoholics have elevated levels of plasma LPS (Alfonso-Loeches et al., 2016; Blednov et al., 2011; Breese and Knapp, 2016; Crews et al., 2017; Leclercq et al., 2012; Pandey, 2012; Pascual et al., 2011). Pharmacologic and genetic studies suggested that alcohol induces CRF signaling in the central amygdala (CeA) and it plays a significant role in the maintenance of addiction, apparently via activation of the CRF1 receptor [CRFR1] (Dedic et al., 2017; Gondre-Lewis et al., 2016; Koob et al., 2014, Lowery-Gionta et al., 2012; Phillips et al., 2015).

We have previously shown that alcohol-preferring (P) rats, which fulfill most of the criteria for an animal model of human alcohol abuse (Bell et al., 2006), have a neuronal TLR4/monocyte chemoattractant protein 1 (MCP-1) signal located in the CeA and the ventral tegmental area (VTA) that controls the predisposition to initiate alcohol drinking and is regulated by the γ-aminobutyric $acid_A$ ($GABA_A$) receptor α2 subunit (α2). Significantly, however, this

* Corresponding author at: Department of Pharmacology, University of Maryland School of Medicine, 655 W. Baltimore St., BRB 4-023, Baltimore, MD 21201, USA.
E-mail address: LAurelian@som.umaryland.edu (L. Aurelian).

https://doi.org/10.1016/j.bbi.2017.11.008
0889-1591/

signal does not function in the ventral pallidum (VP), documenting the existence of dominant regulatory mechanisms at distinct brain sites (Liu et al., 2011). Furthermore, alcohol-induced CRF expression in the CeA and VTA upregulates TLR4 (June et al., 2015), establishing a potential link between stress and TLR4 expression at these brain sites. However, the role of the CRF/CRFR1 system and its interaction with the TLR4 signal in defining the predisposition of non-alcohol exposed rats to initiate alcohol drinking, particularly as it relates to impulsivity regulation, have not been investigated.

We report that in the nucleus accumbens shell (NAc-shell), a site implicated in the control of reward, drug-seeking behavior and impulsivity (Chaudhri et al., 2010; Feja et al., 2014) the levels of TLR4, CRF and α2, and the percentage of co-expressing neurons, are significantly higher in P, than non-alcohol preferring (NP) rats. The TLR4 signal is innately activated in P rats before exposure to alcohol, as evidenced by increased expression and nuclear localization of the phosphorylated transcription factor cAMP response element-binding protein (CREB). Activation is sustained by a CRF amplification loop that includes TLR4-induced CRF expression through protein kinase A (PKA)/CREB activation and CRF feedback regulation of TLR4 expression through CRFR1 and mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) activation. The TLR4 signal regulates impulsivity, as evidenced by the finding that it is markedly reduced through TLR4 inhibition by NAc-shell infusion of a neurotropic HSV vector for TLR4 siRNA (pHSVsiTLR4).

2. Materials and methods

2.1. Animals

Alcohol preferring (P) (n = 59; 3–4 months old; 250–550 g) and non-preferring (NP) rats (n = 14, 3–4 months old; 250–550 g) were obtained from the Alcohol Research Center, Indiana University School of Medicine. P rats perform an operant response for access to ethanol that is not performed by the NP rats, develop both tolerance and physical dependence following excessive intake, and upon removal, show signs of withdrawal following chronic consumption (Bell et al., 2006). Animals were individually housed, maintained at an ambient temperature of 21 °C and a reverse 12 h light/dark cycle and provided with food and water, ad libitum. Training and experimental sessions were conducted between 8:30 am and 5:30 pm. Treatment was approved by the IACUC of the Howard University College of Medicine and all procedures were conducted in strict adherence with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

2.2. Antibodies

The following antibodies were commercially obtained and used according to the manufacturer's instructions: mouse anti-GAPDH [catalog (Cat.) #sc-47724; RRID: AB_627678] and mouse anti-TLR4 monoclonal antibody (Cat. #sc-293072, RRID: AB_10611320) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Two CRF antibodies were used, with similar results: (i) mouse anti-CRF monoclonal antibody (2B11) (Santa Cruz Biotechnology, Cat. # sc-293187, RRID: AB_2687937) that recognizes amino acids (aa) 154–196 in the human protein and (ii) rabbit polyclonal anti-CRF antibody (Bioss Antibodies, Woburn, MA, USA; Cat. # bs-0246R, RRID: AB_10885735) that recognizes amino acids 176–196 in the human protein. Other used antibodies include rabbit phospho-CREB (pCREB; Ser133; Cat. # 9198, RRID: AB_2561044), rabbit phospho-PKA (pPKA; Thr197; Cat. # 4781, RRID: AB_2300165), and rabbit phospho-p44/42 MAPK (pERK1/2; Thr202/Tyr204; Cat # 4377, RRID: AB_331775) were from Cell Signaling Technology (Danvers, MA, USA). Other antibodies were goat anti-GAD67 (glutamic acid decarboxylase 1 (GAD1)) (LifeSpan BioSciences, Seattle, WA, USA, Cat. # LS-B3027-50, RRID: AB_1965223), rabbit anti-CRFR1 (Thermo Fisher Scientific, Waltham, MA, USA, Cat. # 720290, RRID: AB_2633242), rabbit anti-NF-kB p65 (Abcam, Cambridge, MA, USA, Cat. # ab32536, RRID: AB_776751), mouse beta Actin (β-Actin; Proteintech Group, Rosemont, IL, USA, Cat. # 66009-1-Ig, RRID: AB_2687938), Alexa Fluor 488 goat anti-rabbit or donkey anti-goat IgG (H + L; Cat. # A11034, RRID: AB_2576217 or Cat. # A11055, RRID: AB_142672, respectively, Thermo Fisher Scientific), and Alexa Fluor 546 goat anti-mouse or goat anti-rabbit IgG (H + L; Cat.# A11030, RRID: AB_2534089, or Cat. # A11035, RRID: AB_2534093, respectively, Thermo Fisher Scientific). Horseradish peroxidase-labeled secondary antibodies were anti-rabbit (Cat. # 7074, RRID: AB_2099233, Cell Signaling Technology) and anti-mouse IgG (Cat. # 170-6516, RRID: AB_11125547, Bio-Rad, Hercules, CA, USA). The generation and specificity of the rabbit-derived $GABA_A$ α2 antibody (W. Sieghart, Center for Brain Research, Medical University of Vienna; Vienna; Austria Cat# GABAA Receptor alpha 2, RRID: AB_2532077) was previously described; it recognizes amino acids 322–357 of the α2 protein (Liu et al., 2011). Antibody validation used the following criteria: (i) protein expression in some, but not other cell lines in the absence of non-specific reactivity, (ii) protein expression following transfection with the appropriate plasmid (positive control), (iii) loss of protein expression with siRNA knockdown (negative control), (iv) failure to inhibit protein expression with scrambled siRNA (siRNA control), and (v) above listed criteria applied to monoclonal and polyclonal antibodies that target distinct epitopes. The data are shown in Supplemental Information (SI) Figs. S1–S4 for TLR4, CRF and CRFR1 antibodies. Antibody specificity is further confirmed by data presented in the MS, notably restored protein expression upon loss of siRNA integrity (MS, Fig. 7B).

2.3. Cells, plasmids, transfection and reagents

SK-N-SH (human) and Neuro2a (mouse) neuroblastoma cells were from American Type Culture Collection (Manassas, VA, USA). SK-N-SH cells were grown in RPMI 1640 medium with 2 mM L-glutamine (Gibco, Gaithersburg, MD, USA), 10% fetal bovine serum (FBS; Gemini, West Sacramento, CA, USA), and 1% Penicillin/Streptomycin (Gibco). The Neuro2a cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% FBS and 1% Penicillin/Streptomycin. The $TLR4^{FLAG}$ plasmid (# 42646) is a gift from Scott Friedman, the $GABRA2^{phGFP}$ plasmid (# 49169) is a gift from Tija Jacob & Stephen Moss, and the pEMS1153-hCRF plasmid (#29068) is a gift from Elizabeth Simpson. The plasmids were from Addgene (Cambridge, MA, USA). They were incubated [(15 min, room temperature (RT)] with FuGENE 6 Transfection Reagent (Promega, Madison, WI, USA, Cat. # E2693) in antibiotic-free medium and added to 50–80% confluent cultures (20 μg/T-75 flask, 7 μg/T-25 flask or 2.6 μg/well of 6-well plate). The selective CRFR1 antagonist, antalarmin hydrochloride (15 nM; R & D Systems, Minneapolis, MN, USA, Cat. # 2778), the PKA-specific inhibitor H89 (10 μM; Cell Signaling Technology, Cat. # 9844), and the specific inhibitor of MAPK extracellular signaling regulated kinase (ERK) kinase (MEK), U0126 (20 μM; Promega, Cat. # V1121) were added to the cultures 24 h before cell collection. By inhibiting MEK1/2, U0126 prevents the activation of MAP kinases p42 and p44 (ERK1/2).

2.4. Immunofluorescence

Immunofluorescent staining was as previously described (Aurelian et al., 2016; June et al., 2015). Cells grown on poly-L-