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Interleukin-6 reduces NMDAR-mediated cytosolic Ca^{2+} overload and neuronal death via JAK/CaN signaling

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ABSTRACT

Cytosolic Ca^{2+} overload induced by N-methyl-D-aspartate (NMDA) is one of the major causes for neuronal cell death during cerebral ischemic insult and neurodegenerative disorders. Previously, we have reported that the cytokine interleukin-6 (IL-6) reduces NMDA-induced cytosolic Ca^{2+} overload by inhibiting both L-type voltage-gated calcium channel (L-VGCC) activity and intracellular Ca^{2+} store release in cultured cerebellar granule neurons (CGNs). Here we aimed to show that NMDA-gated receptor channels (i.e., NMDA receptors, NMDARs) are an inhibitory target of IL-6 via a mediation of calcineurin (CaN) signaling. As expected, IL-6 decreased NMDAR-mediated cytosolic Ca^{2+} overload and inward current in cultured CGNs. The NMDAR subunits, NR1, NR2A, NR2B and NR2C, were expressed in CGNs. Blocking either of NR2A, NR2B and NR2C with respective antagonist reduced NMDA-induced extracellular Ca^{2+} influx and neuronal death. Importantly, the reduced percentages in extracellular Ca^{2+} influx and neuronal death by either NR2B or NR2C antagonist were weaker in the presence of IL-6 than in the absence of IL-6, while the reduced percentage by NR2A antagonist was not significantly different between the presence and the absence of IL-6. AG490, an inhibitor of Janus kinase (JAK), abolished IL-6 protection against extracellular Ca^{2+} influx, mitochondrial membrane depolarization, neuronal death, and CaN activity impairment induced by NMDA. The CaN inhibitor FK506 reduced these IL-6 neuroprotective properties. Collectively, these results suggest that IL-6 exerts neuroprotection by inhibiting activities of the NMDAR subunits NR2B and NR2C (but not NR2A) via the intermediation of JAK/CaN signaling.

1. Introduction

Cytosolic Ca^{2+} is an important signaling molecule involved in various aspects of neuronal physiology and development [1]. However, excessive cytosolic Ca^{2+} is associated with some neurological disorders, such as excitotoxicity evoked by neuronal excessive release of glutamate, an excitatory neurotransmitter [2]. Glutamate has three receptor subtypes that bear the names of their selective agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate. NMDA binds to NMDA receptors (NMDARs) on neuron, which allows Ca^{2+} and Na^{+} to enter the cell and as a result induces neuronal cell death [3]. Most NMDARs are tetramers thought to be composed of two NMDAR1 (NR1) and two NR2 subunits [4]. Both NR1 and NR2 subunits are necessary to form functional glutamate-gated NMDARs in mammalian systems [4]. NR2 has been found to have the four isoforms, NR2A, NR2B, NR2C and NR2D, which are differentially expressed in different developmental stages of the central nervous system (CNS) and different brain regions [5,6]. NR2C is expressed in the cerebellum and highly expressed in cerebellar granule cell layer [6,7]. Therefore, expression of the four principal NMDAR subtypes, NR1/NR2A, NR1/NR2B, NR1/NR2C and NR1/NR2D, is highly regulated and varies by brain region, developmental stage, experience, and disease state [8–10]. However, it is unclear whether

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CaN, calcineurin; CGNs, cerebellar granule neurons; CNS, central nervous system; Ifen, ifenprodil; IL-6, interleukin-6; IP_3R, inositol 1,4,5-trisphosphate receptor; JAK/STAT3, Janus kinase/signal transducer and activator of transcription-3; MK-801, dizocilpine maleate; NFAT, nuclear factor of activated T cells; NMDA, N-methyl-D-aspartate; NMDARs, NMDA receptors; NR1, NMDAR1; NR2A, NMDAR2A; NR2B, NMDAR2B; NR2C, NMDAR2C; NVP, NVP-AAM077; PPDA, (2R*,3S*)-1-(phenanthrenyl-2-carbonyl)piperazine-2,3-dicarboxylic acid; TG, thapsigargin; VGCCs, voltage-gated calcium channels.

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NMDAR subtypes are regulated by the cytokine interleukin-6 (IL-6) in cerebellar granule neurons (CGNs).

IL-6, originally described as an immune cell mediator in the periphery, has been involved in the modulation of neurological functions [11]. IL-6 expression is low in the brain under normal conditions, but it increases dramatically in some neurological disorders, such as stroke, brain damage and seizures [12–15]. IL-6 role in the brain is complicated. It exerts neurotrophic property and also is a mediator of acute inflammation [16]. For example, IL-6 reduces ischemic brain damage as well as glutamate and NMDA excitotoxic injuries [17–19]. On the other hand, endogenous or exogenous IL-6 increase in the brain contributes to pathogenesis of some neurodegenerative diseases due to neuroinflammation it mediates [20,21]. We have previously shown that IL-6 reduces NMDA-induced cytosolic Ca^{2+} overload and thereby inhibits neuronal apoptosis and necrosis, demonstrating a neuroprotection of IL-6 [22,23]. It is well known that NMDA-induced cytosolic Ca^{2+} overload involves the three mechanisms: Ca^{2+} influx through NMDA-gated receptor channels; Ca^{2+} influx through voltage-gated calcium channels (VGCCs) activated by membrane depolarization; Ca^{2+} release from cytoplasmic Ca^{2+} stores [24]. Recently, we have reported that IL-6 exerts neuroprotection against NMDA-induced cytosolic Ca^{2+} overload by inhibiting both L-type VGCC activity and cytoplasmic Ca^{2+} store release in CGNs [25,26]. Thus, it remains to be clarified that NMDA-gated receptor channels (i.e., NMDARs), especially NMDAR subtypes, are inhibitory targets for IL-6 neuroprotection.

A canonical intracellular signaling pathway transducing IL-6 action is Janus kinase (JAK)/signal transducer and activator of transcription-3 (STAT3). Binding of IL-6 to its receptor complex activates JAK and in turn, it phosphorylates STAT3 [27]. This JAK/STAT3 signaling has been shown in our previous work to be involved in IL-6 protection against NMDA-induced neuronal Ca^{2+} overload and death [22,23,28]. Nevertheless, the link between JAK/STAT3 signaling and NMDARs is unknown. We hypothesized that calcineurin (CaN), a serine/threonine protein phosphatase activated by calcium/calmodulin, is a negative connecter that transmits JAK/STAT3 signaling to NMDARs in neurons. CaN is highly expressed in the brain [29] and is involved in a negative feedback regulation of Ca^{2+} entry through VGCCs [30]. It can also modulate NMDAR activity by desensitization of NMDARs [31].

Accordingly, here we firstly confirm that IL-6 reduces NMDAR-mediated cytosolic Ca^{2+} overload and inward current; secondly we establish that NR1/NR2B and NR1/NR2C subtypes are major inhibitory targets for IL-6 reduction of neuronal Ca^{2+} overload and death; and lastly we propose that CaN is a mediator that transduces JAK/STAT3 signaling to a negative regulation of the NMDAR subtypes in IL-6 neuroprotection.

2. Materials and methods

2.1. Cell culture

Primary cultures of neonatal rat CGNs were prepared according to a reported protocol with minor modifications [25]. Sprague-Dawley infant rats of postnatal day 8 (The Center of Experimental Animals, Nantong University, China) were anesthetized by hypothermia via placing them in an ice bath for 2–4 min. The cells were dispersed by digestion with trypsin (Amresco, USA) and DNase I (Worthington, USA) and were resuspended in the following culture medium: basal Eagle's medium (Sigma, USA), 10% fetal bovine serum (Amresco, USA), 25 mM KCl, 0.1 g/L gentamicin, 2.2 g/L $NaHCO_3$, 2.385 g/L HEPES and plated onto poly-L-lysine-coated glass slides (0.32×10^6 cells/ml) for electrophysiological recording or seeded at a density of 0.8×10^6 cells/ml in 96 wells for calcium imaging and 2.0×10^6 cells/ml in 6 wells for immunoblot, respectively. The sample was incubated at 37 °C with a humidified 5% CO_2/95% air atmosphere in an incubator (ESPEC BNA-311, Japan). Cytosine arabinoside (10 μM) was added 18–24 h after the cell planting to inhibit glial cells proliferation. Cultures were used at 8–9 days in vitro.

2.2. Drug treatments

The CGN cultures were pretreated with rat recombinant IL-6 (R&D, USA) at a concentration of 120 ng/ml for 24 h as described previously [25]. NMDA was dissolved in Locke's solution containing 10 μM glycine (Amresco), which was applied to the 8-day cultures at a concentration of 100 μM at room temperature for 6 min for measurement of dynamic intracellular Ca^{2+} signals or for 30 min for examination of neuronal death, expression of NMDAR subunits, and CaN expression and activity. Thapsigargin (TG, Sigma), dizocilpine maleate (MK-801, Sigma), [(R) -[(S)-1-(4-bromophenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]- phosphonic acid (NVP-AAM077, Sigma), Ifenprodil (Ifen, Sigma) and (2R*, 3S*)-1-(phenanthrenyl-2-carbonyl) piperazine-2, 3-dicarboxylic acid (PPDA, Tocris) [35], the antagonists for endoplasmic reticulum Ca^{2+}-ATPase, NMDAR, NR2A, NR2B and NR2C, respectively, was applied to Locke's solution 25 min prior to measurement of dynamic intracellular $Ca2^{+}$ fluorescence intensity or applied to extracellular solution 10 s prior to recording of whole-cell current at a concentration of 5, 10, 0.1, 10, and 0.5 μM, respectively [18,32–35]. AG490 (Sigma) and FK506 (sigma), the antagonists for JAK and CaN, was added to cell cultures 1 h before IL-6 incubation at a concentration of 5 μM and 0.1 μM, respectively [36,37].

2.3. Calcium imaging

Dynamic changes of intracellular Ca^{2+} levels were determined by confocal laser scanning microscope (CLSM, Leica TCS SPE, Germany) as described previously with modification [25]. Briefly, cells were loaded with Fura-3 AM (5 μM, Calbiochem) for 40 min at 37 °C and washed twice with Locke's solution. The Locke's solution was composed of 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5 mM HEPES, 5.6 mM glucose, 2.3 mM $CaCl_2$. Inhibitors [5 μM TG, 10 μM MK-801, 0.1 μM NVP-AAM077 (NVP), 10 μM Ifen, 0.5 μM PPDA] were added to the loading solution for 25 min before NMDA stimulation. 5 μM AG490 or 0.1 μM FK506 was added to cell cultures 1 h before IL-6 incubation. Successive images were collected at 5 s intervals for about 7.5 min in xyt scan mode. Fura-3 fluorescence was excited at 488 nm and emitted light was measured at 530 nm. All the above and the quantification of the fluorescence intensity were performed using TCS-SPE software. In one-scanned visual field, 30 neurons were randomly selected to obtain their dynamic intracellular Ca^{2+} levels. Neuronal basal Ca^{2+} fluorescence intensity before NMDA stimulation was firstly recorded for about 90 s and was set as baseline 1.0. Neuronal maximal Fura-3 fluorescence intensity after NMDA stimulation was defined as peak fluorescence intensity. Each experiment was repeated a minimum of three times with comparable results.

2.4. Patch clamp

NMDA-activated currents were recorded using Axopatch 200B patch-clamp amplifier interfaced to 1440A (Axon Instruments, USA) at room temperature (20–22 °C). For whole-cell recording of NMDA-activated currents, the extracellular solution had the following composition (in mM): NaCl 140, KCl 5, $CaCl_2$ 2.5, HEPES 10, glucose 10, glycine 0.01, tetrodotoxin (TTX) 0.001, pH adjusted to 7.4 with NaOH, and the pipette solution contained (in mM): K-gluconate 120, NaCl 5, TEACl 10, $CaCl_2$ 1, $MgCl_2$ 2, HEPES 10, EGTA