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NR2B-containing NMDA receptors promote neural progenitor cell proliferation through CaMKIV/CREB pathway

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ABSTRACT

Accumulating evidence indicates the involvement of *N*-methyl-D-aspartate receptors (NMDARs) in regulating neural stem/progenitor cell (NSPC) proliferation. Functional properties of NMDARs can be markedly influenced by incorporating the regulatory subunit NR2B. Here, we aim to analyze the effect of NR2B-containing NMDARs on the proliferation of hippocampal NSPCs and to explore the mechanism responsible for this effect. NSPCs were shown to express NMDAR subunits NR1 and NR2B. The NR2B selective antagonist, Ro 25-6981, prevented the NMDA-induced increase in cell proliferation. Moreover, we demonstrated that the phosphorylation levels of calcium/calmodulin-dependent protein kinase IV (CaMKIV) and cAMP response element binding protein (CREB) were increased by NMDA treatment, whereas Ro 25-6981 decreased them. The role that NR2B-containing NMDARs plays in NSPC proliferation was abolished when CREB phosphorylation was attenuated by CaMKIV silencing. These results suggest that NR2B-containing NMDARs have a positive role in regulating NSPC proliferation, which may be mediated through CaMKIV phosphorylation and subsequent induction of CREB activation.

1. Introduction

Neural stem/progenitor cells (NSPCs) are characterized by their self-renewing and multipotent differentiation capacity in the central nervous system [1]. Although the functional properties of NSPCs have been studied extensively, researchers have only begun to understand how the self-renewal of NSPCs is regulated. It has been reported that NSPCs are receptive to neurotransmission signals that regulate self-renewal in vivo and in vitro [2,3].

N-Methyl-D-aspartate receptors (NMDARs) are inotropic receptors of the neurotransmitter glutamate. NMDARs have critical roles in modulating the proliferation of neural stem cells, although the results have varied significantly [2,4,5]. It is now well established that this variability can be accounted for by different receptor subunit compositions. Multiple subtypes of NMDARs exist, each displaying distinct pharmacological and biophysical properties. These properties are largely determined by the type of regulatory NR2 subunit (NR2A, -B, -C, or -D) incorporated in the heteromeric NR1/NR2 complex [6–9]. Indeed, the NR2 subunit confers a particularly rich pharmacology with distinct recognition sites for exogenous and endogenous allosteric ligands and glutamate [10,11]. The NR2A and NR2B are the subunits most commonly expressed in the mammalian forebrain; especially the NR1/NR2B receptor subtype may mediate long-term Ca^{2+} influx [12]. The regulatory role of NR2B in modulating the proliferation of NSPCs and the mechanism underlying is much less well defined.

It has been demonstrated that NMDARs can mediate postnatal Müller glia-derived retinal progenitor proliferation and cAMP response element binding protein (CREB) phosphorylation [13]. Also, activated CREB is highly expressed in immature dividing cells in adult mouse and zebrafish brains, and that CREB regulates the proliferation of NSPCs in embryonic mouse and zebrafish brains [14–16]. These observations suggest that phosphorylated CREB is an attractive candidate to facilitate NSPC proliferation after NMDAR activation. To date, reports about the link between NMDARs and CREB in NSPC proliferation are still lacking.

Calcium/calmodulin-dependent protein kinase IV (CaMKIV) has emerged as the most important Ca^{2+}-activated CREB kinase [17]. The CaMKIV/CREB pathway functions to maintain neuroblastoma cells in an undifferentiated proliferative state [18]. Furthermore, in mature neurons, activation of NMDARs results in Ca^{2+} influx followed by activation of CaMKIV/CREB [19]. Generally, the

Abbreviations: ANOVA, analysis of variance; CaMKIV, calcium/calmodulin-dependent kinase IV; CCK-8, cell counting kit-8; CREB, cAMP response element binding protein; LSD, least significant difference; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; NSPCs, neural stem/progenitor cells; PBS, phosphate buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA; TBST, Tris-buffered saline with 0.1% Tween 20.

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NR2B, CaMKIV and CREB are considered to be memory related genes [20] and synaptic plasticity genes [21]. Whether CaMKIV plays a role in NSPCs has not been studied so far.

In this study, we examined the role of NR2B-containing NMDARs in the proliferation of NSPCs and tested the hypothesis that the CaMKIV/CREB cascade is involved in the regulation of NR2B-containing NMDAR-mediated proliferation. We provided a better understanding of how NR2B-containing NMDARs (and NMDARs in general) operate in NSPC proliferation.

2. Materials and methods

2.1. Hippocampal NSPC culture

2.1.1. Primary cell culture

NSPCs were prepared from hippocampi derived from Sprague Dawley rats at postnatal day 0 as described previously, with slight modifications [22,23]. The experimental procedures were approved under the local regulations on ethics in experiments on animals. Briefly, hippocampi were carefully isolated in ice-cold high-glucose DMEM (Invitrogen). After removal of the meninges, the freshly prepared hippocampal cells were dissociated by 0.25% trypsin at 37 °C for 15 min. The cells were then subsequently filtered through 400 μm screen mesh and centrifuged. Cell pellet was resuspended in DMEM/F12 (Invitrogen) supplemented with 2% B27 (Invitrogen), 20 ng/ml epidermal growth factor (PeproTech) and 20 ng/ml basic fibroblast growth factor (PeproTech). Cells were planted at 5×10^5 cells/ml and incubated at 37 °C in 5% CO_2. The medium was half-replaced on the 3rd day.

2.1.2. Single cell prepared from the neurospheres

After 5 days cultured in vitro, the primary neurospheres (most of them were 120 μM in diameter) were collected, enzymatically dissociated with Accutase (Invitrogen) for 10 min at 37 °C, and mechanically triturated with fire-polished glass pipettes. The single cells were resuspended at a density of 5000 cells/ml and incubated at 37 °C in 5% CO_2. Such cells were processed for the experiment described below.

2.2. Cell proliferation assay

Cell proliferation assay was assessed by the cell viability assay and neurosphere number counting assay. Single cells were treated with NMDA, Ro 25-6981 or MK-801 (all from Sigma). Subsequently, for the cell viability assay, 100 μl per well of these NSPCs were seeded in 96-well plates. After 24 h, 10 μl of cell counting kit-8 (CCK-8) solution (Dojindo) was added to the well. Following a 4 h incubation at 37 °C, the absorbance was measured at 450/600 nm by microplate-reader (Multiskan spectrum, Thermo labsystems). For neurosphere counting, such cells were seeded into uncoated 24-well microplates and cultured for an additional 72 h. The number of spheres (>50 μm) was counted under the microscope (100×) from 10 randomly chosen fields of five different wells.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from neurospheres by using TRIzol reagent (Invitrogen). One microgram of RNA was reverse transcribed into cDNA and amplified using RT-PCR (Promega). All procedures for total RNA isolation and RT-PCR were performed according to the procedure suggested by the manufacturers. Primers for PCR were as follows: NR1 (5′-GGTACCCATGTCATCCCAAA-3′, 5′-CATCATTCCGTTCCACTCCT-3′); NR2B (5′-GCAAGCTTCTGTCATGCTCAACATC-3′, 5′-GCTCTGCAGCTTCTTCAGCTGATTC-3′); β-actin (5′-CCCATCTATGAGGGTTACGC-3′, 5′-TTTAATGCACGCACGATTTC-3′). The PCR cycles for NR1 consisted of an initial incubation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min, and a final extension at 72 °C for 1 min. The PCR cycles for NR2A consisted of an initial incubation at 95 °C for 10 min, 30 cycles of denaturation at 94 °C for 45 s and annealing at 67 °C for 1 min, and a final extension at 72 °C for 1 min. The PCR cycles for β-actin consisted of an initial incubation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s and annealing at 61 °C for 30 s, and a final extension at 72 °C for 45 s. The RT-PCR products ware analyzed by 1% agarose gel electrophoresis and visualized with ethidium bromide.

2.4. Immunocytochemistry

Neurospheres attached on poly-L-lysine-coated coverslips were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) for 20 min. After washing the samples three times with PBS, cells were blocked in PBS with 5% bovine serum albumin for 1 h. Samples were then incubated with mouse-anti-NR1 (1:500, BD Pharmingen) and rabbit-anti-NR2B (1:1000, Sigma) overnight at 4 °C. Then the cells were stained by FITC conjugated or TRITC conjugated antibody (Santa Cruz) as the secondary antibody for 1 h at room temperature. Their nuclei were stained with Hoechst 33258 for 10 min. Results were visualized by using a laser confocal microscope (Fluoview FV10i, Olympus).

2.5. Western blot

Total cell lysates were prepared using lysis buffer (Beyotime) supplemented with a protease and phosphatase inhibitor cocktail (Pierce). Equal amounts of proteins were separated by 7% or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred to NC membrane. After blocking for 3 h in Tris-buffered saline with 0.1% Tween 20 (TBST) and 3% bovine serum albumin, the following primary antibodies were used overnight at 4 °C in TBST: mouse-anti-NR1 (1:1000), rabbit-anti-NR2B (1:1000), rabbit-anti-Ser^{133}-CREB (1:500, Abcam) or mouse-anti-β-actin (1:1000, Santa Cruz). Blots were subsequently incubated with differentially labeled species-specific secondary antibodies [anti-rabbit IRDyeTM 800CW (green) and anti-mouse Alexa 680 (red), Li-COR Biosciences]. pCREB and β-actin antibodies were subsequently stripped by NewBlot Nitro Stripping Buffer (Li-Cor Biotechnology) for 4 min. The removal of antibodies was verified by rescanning the membrane. The membrane was then reprobed with rabbit-anti-CREB antibody (1:500, Cell signaling).

2.6. Immunoprecipitation

After exposing to drugs for the desired time, NSPCs were collected and processed for immunoprecipitation by standard procedures described previously [23,24]. Briefly, sample proteins (400 μg) were diluted fourfold with HEPES buffer supplemented with a protease and phosphatase inhibitor cocktail (Pierce). The supernatants were incubated with 2 μg of rabbit-anti-CaMKIV (1:200) overnight at 4 °C. After the addition of protein A/G–Sepharose (Santa Cruz), the mixture was incubated 4 °C for an additional 2 h. Samples were triple washed with HEPES buffer and eluted by sodium dodecyl sulfate–polyacrylamide gel electrophoresis loading buffer and then boiling at 100 °C for 5 min. Supernatants were used for western blot, which was subsequently detected by anti-mouse-phospho-Ser/Thr antibody (1:500) and rabbit-anti-CaMKIV antibody (1:400).