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NMDA-induced ERK signalling is mediated by NR2B subunit in rat cortical neurons and switches from positive to negative depending on stage of development

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ABSTRACT

It is known that NMDA receptor stimulation can activate or inhibit the extracellular signal-regulated kinase (ERK) signalling cascade, a key pathway involved in neuronal plasticity and survival. However, the specific subtype(s) of NMDA receptor that exert bi-directional regulation of ERK signalling is under debate. Here we show that in young neurons (7–9 days in vitro, DIV), NMDA activated ERK signalling. In mature neurons (14–16 DIV), NMDA-evoked, in coincidence with a concentration-dependent increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), an increase in ERK phosphorylation at low concentrations (1–30 μM) while an inhibition at high concentrations (30 μM-250 μM). In more mature neurons (21–23 DIV) NMDA inhibited ERK signalling. Both activation and inhibition of ERK signalling were fully reversed by the selective NR2B receptor antagonists Ro 25-6981 and ifenprodil. Thus, the NR2B subunit can be both negatively or positively coupled to ERK signalling in rat cortical neurons, depending on their stage of development.

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1. Introduction

The N-Methyl-D-aspartate (NMDA) receptor is a ligand-gated ion channel activated by glutamate and glycine that plays a critical role in excitatory neurotransmission, synaptic plasticity and neuronal cell death in the mammalian central nervous system (Dingledine et al., 1999; Kemp and McKernan, 2002). Functional NMDA receptors are heteromeric tetramers composed of at least one obligatory NR1 subunit, and one or more NR2 or NR3 subunits. The well-characterized glutamate- and glycine-responsive NMDA receptor requires both NR1 and NR2 subunits, whereas the NR3 subunit can complex with NR1 subunits to form a glycine-responsive excitatory receptor that does not require glutamate (Smothers and Woodward, 2009). Four glutamate-binding NR2 subunits (NR2A-D) are expressed in a regionally and developmentally regulated manner. NR2A and NR2B are predominant in the hippocampus and neocortex, NR2C is highly enriched in cerebellum, and NR2D in the brain-stem (Cull-Candy et al., 2001; Monyer et al., 1994; Sheng et al., 1994). In cortex and hippocampus, only NR2B subunits are expressed prenatally, whereas a perinatal shift in expression occurs in favour of NR2A at the synapse. Thus, during brain maturation, NR2A-containing receptors are added to synapses and partially replace NR2B-containing receptors; however, NR2B-containing receptors remain the predominant subtype at extrasynaptic sites (Li et al., 1998; Liu et al., 2004; Mizuta et al., 1998; Stocca and Vicini, 1998; Tovar and Westbrook, 1999; Zhong et al., 1994). Ternary complexes containing NR1/2A/2B have been consistently observed in native tissues, particularly in the cortex, where they represent a significant fraction of the NMDA receptor population (Luo et al., 1997; Chazot and Stephenson, 1997). The developmental change in subunit

Abbreviations: CP-101,606,1-[(1S,2S)-2-hydroxy-2-(4-hydroxyphenyl)-1-methyl ethyl]-4-phenyl-4-piperidinol; ERK, extracellular signal-regulated protein kinase; FLIPR, fluorometric imaging plate reader; HBSSH, Hank's balanced salt solution supplemented with HEPES; HEK, human embryonic kidney; ifenprodil, (1R,2S)-erythro-2-(4-Benzylpiperidino)-1-(4-hydroxyphenyl)-1-propanol; memantine, 3,5-Dimethyl-tricyclo[3.3.1.13,7]decan-1-amine hydrochloride; (+)-MK-801, (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine; NMDA, N-methyl-D-aspartic acid; NVP-AAM077, [(R)-{[(1S)-1-(4-bromophenyl)ethyl]amino}(2,3-dioxo-1,2,3,4-tetrahydro-5-quinoxalinyl)methyl]phosphonic acid; probenecid, 4-(dipropylsulfamoyl)benzoic acid; PSS, Physiological Salt Solution; Ro 25-6981, (aR,bS)-a-(4-Hydroxyphenyl)-b-methyl-4-(phenylmethyl)-1-piperidinepropanol maleate; U0126, 1,4-diamino-2,3-dicyano-1,4-bis (2-amino-phenylthio)butadiene.

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composition confers to the NMDA receptor distinct pharmacological and kinetic properties and potentially modifies the receptors ability to interact with intracellular signalling pathways (Cull-Candy and Leszkiewicz, 2004; Köhr, 2006; Paoletti and Neyton, 2007; Williams et al., 1993).

The ERK1/2 (extracellular signal-regulated kinase 1/2) signalling pathway is activated by calcium influx through NMDA receptors, and represents a key component contributing to cell survival and neuronal plasticity (Grewal et al., 1999; Impey et al., 1999; Sweatt, 2004). Previous studies have shown that NMDA receptors bi-directionally modulate ERK activity by coupling to opposing stimulatory and inhibitory pathways (Chandler et al., 2001). It has been proposed that NMDA receptor subunit composition differentially regulates ERK response, with the suggestion that NR2A-containing receptors promote ERK phosphorylation, whereas NR2B-containing subunits are mainly involved in the inhibition of ERK activity (Kim et al., 2005; Paul and Connor, 2010). However, in contrast, it has also been reported that NMDA receptor-dependent ERK activation is mainly mediated by NR2B-containing receptors through a direct interaction with RasGRF1, a Ca^{2+}/calmodulin-dependent Ras-guanine-nucleotide-releasing factor (Krapivinsky et al., 2003). Thus, the subunit pharmacology of NMDA receptor regulation of ERK activity remains unclear. Furthermore, it remains unclear whether subunit composition and/or spatial distribution (synaptic vs. extrasynaptic) of NMDA receptors determinates the direction of ERK signalling (Ivanov et al., 2006).

In this study we investigated the contribution of NR2A- and NR2B-containing receptors in mediating ERK signalling in rat cortical neurons in vitro at different stages of neuronal development. We took advantage of distinct non-selective NR2A/NR2B antagonists such as the pore blocker MK-801 or the competitive antagonist NVP-AAM077 (which shows only ~10-fold selectivity for NR2A- over NR2B-containing receptor, Neyton and Paoletti, 2006) in comparison to highly potent and selective NR2B subunit antagonists: ifenprodil (Williams, 1993), its derivative, Ro 25-6981 (Fischer et al., 1997), and an unrelated compound, CP-101,606 (Mott et al., 1998). We show that the ERK response is completely mediated by NR2B subunit and that NR2B subunits can mediate both activation and inhibition of ERK signalling depending on the stage of neuron maturation.

2. Materials and methods

2.1. Drugs

NMDA, Ro 25-6981 maleate, ifenprodil hemitartrate and U0126 were purchased from Tocris Bioscience, Ellisville, MO; (+)-MK-801 hydrogen maleate and memantine hydrochloride were obtained from Sigma–Aldrich, Milano, Italy; CP-101,606 and NVP-AAM077 diammonium salt were synthesised by the Medicinal Chemistry department, GlaxoSmithKline (Verona, Italy). All antagonists were dissolved in dimethyl sulfoxide (DMSO) and further diluted in the assay medium to give a final DMSO concentration not exceeding 0.5%.

2.2. Primary neuronal cultures

Cortical neuronal cultures were obtained from embryonic day 18–19 Sprague-Dawley rats (Charles River, Como, Italy). Animal manipulations were performed according to Italian law (art.7, Legislative Decree No.116, 27 January 1992), which acknowledged the European Directive 86/609/EEC, and GlaxoSmithKline policy on the care and use of laboratory animals and related codes of practice. Rat brains were dissected out and cortices were quickly isolated at 4 °C in Hank's Balanced Salt Solution supplemented with HEPES (HBSSH buffer), containing 5 mM KCl, 0.4 mM KH_2PO_4, 138 mM NaCl, 0.3 mM Na_2HPO_4, 5.5 mM D-glucose, 26 mM phenol red, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES at pH 7.3. Cortical cells were treated with HBSSH buffer added with Trypsin 0.1% at 37 °C for 10 min. In the last 5 min of incubation, 166 μg/ml of DNAse I (Sigma–Aldrich) was added. After a single wash with HBSSH buffer containing 10% FBS (Invitrogen, Milano, Italy) and two additional washes with HBSSH buffer, cells were mechanically dissociated by triturating with Pasteur pipettes and placed in Poly-L-lysine coated 384-well plates (Greiner Bio-one, Frickenhausen, Germany) at the density of 8,000 cells/well in serum-free Neurobasal medium (Invitrogen) with B27 supplement (Invitrogen), 500 μM Glutamine, 100 U/ml Penicillin and 100 μg/ml Streptomycin. Cells were grown at 37 °C, 5% CO2. Addition of fresh medium was made after 3, 10 and 17 days until the cells were used.

2.3. FLIPR/$[Ca^{2+}]_i$ assay

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurements were made using the FLIPR^{TETRA} fluorescent imaging plate reader (Molecular Devices, Sunnyvale, CA). At the assay day, cultures (15 DIV) were washed two times with Physiological Salt Solution (PSS; composition 145 mM NaCl, 1 g/L glucose, 5 mM KCl, 2 mM $CaCl_2$, 20 mM HEPES) supplemented with 10 μM glycine and 2.5 mM probenecid (Sigma–Aldrich) using the EMBLA 384 instrument (Molecular Devices). Then, cells were added with the cytoplasmic calcium indicator, Fluo-4 (Invitrogen) in the acetoxylmethyl (AM) ester form (2 μM) in PSS containing 5 mM probenecid and 0.04% Pluronic F-127 (Sigma–Aldrich). After 40 min at 37 °C cells were washed 3 times as above and transferred to the FLIPR^{TETRA}. $[Ca^{2+}]_i$ responses were measured using a cooled CCD camera with excitation at 470–495 nM and emission at 515–575 nM. Camera gain and intensity were adjusted for each plate to yield a minimum of 300 relative light units (RLU) baseline fluorescence. To assess the effect of NMDA receptor antagonists on the NMDA-triggered $[Ca^{2+}]_i$ response, culture were exposed to different concentrations of test drugs for 10 min, then exposed to a sub-maximal concentration of NMDA (EC_{80}, 10 μM) for 180 s. Prior to addition of antagonists, 5 fluorescence readings were taken to measure baseline. $[Ca^{2+}]_i$ responses were quantified as area under the curve of the time-response data over baseline.

2.4. pERK1/2 measurement

Cortical cells were plated at a density of 15,000 cells/well in 384-well plates. The day of the experiment, 10× solutions of drugs dissolved in medium were added to 80 μl medium that was left in each well. Antagonists were pre-incubated for 10 min at 37 °C before the addition of NMDA. Stimulation with NMDA was stopped after 6 min by removal of medium with drugs and cell lysis. pERK1/2 was measured by using the p-ERK 1/2 SureFire® assay kit (PerkinElmer, Boston, MA), a non-Western blot-based proprietary technique that relies on the energy transfer-based AlphaScreen (Amplified Luminescent Proximity Homogeneous Assay Screen) concept (Osmond et al., 2005) and measures the phosphorylation of endogenous cellular ERK 1/2 at Thr202/Tyr204 by MEK1/2. Cell lysates were prepared and processed according to the manufacturer's protocol. In brief, a 5 μl sample of processed cell lysate was transferred to a white 384-well ProxiPlate, and 7 μl of a 1:1 mix of Protein A AlphaScreen donor and acceptor beads was added to each well. Plates were incubated for 2 h at 22 °C in the dark. An Envision reader (PerkinElmer) was used to excite the donor beads and to measure the emission of light from the acceptor beads after energy transfer. Previous pERK1/2 kinetic experiments performed after different times in culture (between 7 and 24 DIV) showed that the maximum change in pERK1/2 response induced by 30 μM NMDA was observed after 5–7 min, over a period of 30 min of incubation. Hence, all the pERK1/2 experiments were readout after 6 min of NMDA stimulation.

2.5. Western blot

Cultured neurons at 8, 15, and 22 DIV from plating were analyzed for NR2A and NR2B subunit expression by western blot. Cells at the various time-points were rinsed in PBS and then scraped and homogenate in 0.1% SDS, 0.5% NP40, 0.5% sodium deoxicolate, 150 mM NaCl, protease inhibitors Complete mini (Roche, Indianapolis, IN, USA) phosphatase inhibitors cocktail I and II (Sigma–Aldrich) in 50 mM Tris–HCl, pH 7.5. Homogenates were centrifuged at 10,000 × *g* for 20 min to pellet the insoluble fraction. The supernatant (total extract) was analyzed for total protein content with Micro BCA kit (Pierce, Rockford, IL, USA). Equal amounts (15 μg/lane) of total proteins were loaded onto 4–12% Novex Bis-Tris Pre-Cast Mini Gels (Invitrogen). After separation, proteins were transferred to polyvinylidene difluoride membranes (Amersham/GE Healthcare, Buckinghamshire, UK), and blocked for 1 h at room temperature in 5% low fat skimmed milk, 0.1% Tween-20 in Tris Buffered Saline (TBS). Membranes were subsequently processed with primary antibodies rabbit anti-NR2A (Ab77980 from Abcam, Cambridge, MA, USA; 1:2000), rabbit anti-NR2B (Ab109 from Abcam, 1:1000) and mouse anti-GAPDH (SC-32233 from Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:10000), washed in 0.1% Tween-20 in TBS, and incubated again with the peroxidase-conjugated secondary antibodies goat anti-rabbit IgG–HRP and goat anti-mouse IgG–HRP (both from Santa Cruz; 1:5000). Chemiluminescence reaction was developed with ECL Plus (Amersham) and detected with Luminescent Image Analyzer LAS-4000mini (Fujifilm, Tokyo, Japan). Optical densities were determined by densitometric analysis with Quantity One software (Bio-Rad, Hercules, CA, USA). GAPDH (40 kDa) was used as a reference protein for normalization purpose.

2.6. Whole cell patch clamp electrophysiology in HEK cells expressing NR1/NR2B subunits

Human embryonic kidney (HEK) cells permanently transfected with Macrophage Receptor Scavenger type 2 (MRS II), were transiently transfected with cDNAs