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Tex261 modulates the excitotoxic cell death induced by *N*-methyl-D-aspartate (NMDA) receptor activation

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Abstract

N-methyl-D-aspartate (NMDA) receptor is a calcium-permeable ionotropic glutamate receptor and plays a role in many neurologic disorders such as brain ischemia through its involvement in excitotoxicity. We have performed differential display PCR to identify changes in gene expression that occur in the hippocampus of the mouse brain after intraperitoneal injection of NMDA and identified a gene, Tex261 as an inducible gene by NMDA stimulation in vivo. Tex261 mRNA was gradually induced in response to NMDA and reached about 4.5-fold at 24 h. When HEK 293 cells are transfected with NMDA receptors, the cells die in a manner that mimics excitotoxicity in neurons. HEK 293 cells transfected with the combination of Tex261 and the NMDA receptors NR1/NR2A produced the greater cell death compared with the cells transfected with the NMDA receptors alone. These findings suggest that Tex261 modulates the excitotoxic cell death induced by NMDA receptor activation.

Keywords: Differential display; Tex261; NMDA; Excitotoxicity

L-Glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system. It mediates its effects via interaction with the glutamate receptors, two main classes of which have been distinguished on the basis of their transduction mechanisms; namely the G-protein-coupled metabotropic and the fast-acting, ionotropic glutamate receptors. Within the ionotropic glutamate receptors, the *N*-methyl-D-aspartate (NMDA) receptor is a fast-acting ligand-gated cation channel with a high permeability for Ca^{2+} and has been implicated both in the induction of long-term potentiation, an activity that may underlie learning and memory, and in neuronal degeneration. NMDA receptor plays a role in many neurologic disorders such as brain ischemia through its involvement in excitotoxicity [1]. Primary neurons grown in culture die when exposed to glutamate because of excess excitation through glutamate receptors [2]. The NMDA receptor is composed of NR1, NR2A–NR2D and NR3A-B subunits. The combination of an NR1 and an NR2 subunit is required for production of a fully functional receptor in mammalian systems [3,4]. When HEK 293 cells are transfected with NMDA receptors, the cells die in a manner that mimics excitotoxicity in neurons. HEK 293 cells are normally transfected in the presence of serum, which contains micromolar concentrations of glutamate and glycine leading to receptor activation and thus cell death [3,5].

Differential display PCR allows the identification of differentially expressed genes by using PAGE to display PCR-amplified cDNA fragments between two or more mRNA populations. We have performed differential display PCR to identify changes in gene expression that occur in the hippocampus of the mouse brain after intraperitoneal injection of NMDA and identified a gene, Tex261 as an inducible gene by NMDA stimulation. Tex261 is a gene cloned from a subtractive cDNA library from 10-day postnatal mouse testis. Tex261 is preferentially expressed in germ cells during and after meiotic differentiation [6].

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HEK 293 cells transfected with the combination of Tex261 and the NMDA receptors NR1/NR2A produced the greater cell death compared with the cells transfected with the NMDA receptors alone. These findings suggest that Tex261 modulates the excitotoxic cell death induced by NMDA receptor activation.

Materials and methods

Animals. Wild-type Std-ddY, 6-week-old adult male mice, weighing between 27 and 33 g, were purchased from Sankyo Laboratories, Toyama, Japan. The mice were housed in metallic breeding cages in a room with a light:dark cycle of 12 h and humidity of 55 ± 2% at 25 ± 1 °C, with food and water available ad libitum for more than 1 day before use. Animal care was conducted in accordance with the Guidelines of Animal Experimentation of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. All efforts were made to minimize the number of animals used. Animals were injected intraperitoneally (i.p.) with phosphate-buffered saline (PBS) (pH 7.4) or 100 mg/kg NMDA (Sigma) in a volume of 0.1 ml.

Differential display analysis. Differential display analysis was described previously [7]. Total cellular RNA was isolated from frozen tissue using Isogen (Nippon Gene, Japan). Total RNA samples used in differential display experiments were treated with RQ1 RNase-free DNase (Promega) to remove contaminating chromosomal DNA. Total RNA was extracted from the hippocampi 6 h after NMDA or PBS injection and differential display-polymerase chain reaction (DD-PCR) was performed using Fluorescence Differential Display Kit (TaKaRa, Shiga, Japan). The DD-PCR products were electrophoresed on denaturing urea 6% polyacrylamide gel.

Northern blotting. Total cellular RNA was extracted from mice hippocampus using Isogen and precipitated by isopropyl alcohol. The extracted RNA (2.5 μg) was resolved on 1% formaldehyde/agarose gels and transferred onto positively charged nylon transfer membranes (Hybond N+, Amersham Biosciences, UK). Hybridizations were carried out using DIG-labeled riboprobe for Tex261, c-fos, c-jun and GAPDH. Northern blot analysis for c-fos, c-jun and GAPDH was described previously [8]. DIG-labeled riboprobe for Tex261 was prepared from full-length Tex261 cDNA (a gift from Dr. Jesu's del Mazo at Department of Cell and Developmental Biology, Centro de Investigaciones Biologicas, Spain). Images were quantified with Image J software. The intensity of each lane was quantified and the data were expressed and normalized as a ratio of mRNA expression for each gene over GAPDH. To examine the NMDA receptor mediated induction, mice were pretreated with MK801 (10 mg/kg) intraperitoneally, the NMDA receptor antagonist, 10 min before NMDA injection.

In situ hybridization. In situ hybridization for c-fos and c-jun was described previously [8]. Cryosections (10 μm) were mounted on silanized slides (DakoCytomation, Japan) in RNase-free conditions. The sections were fixed with 4% PFA, followed by HCl, proteinase K and acetic anhydride treatment. After prehybridization, the sections were hybridized with 500 ng/ml DIG-labeled Tex261, c-fos or c-jun riboprobe at 65 °C for 16 h. The slides were treated with RNase A, blocked with 1.5% blocking buffer and incubated with anti-DIG-AP-Fab fragments (Roche, Germany) at 4 °C overnight. The sections were developed by 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium (BCIP/NBT).

Cell culture and transfections. N1E-115 neuroblastoma cells and HEK 293 cells were grown in DMEM supplemented with 5% fetal bovine serum (FBS). cDNA encoding myc tagged Tex261 protein prepared from full-length Tex261 cDNA was cloned into pcDNA 3.1 (Invitrogen). N1E-115 cells were transfected with the expression vector by the calcium phosphate method and allowed to differentiate by culturing in the medium containing 2.5% FBS in the presence of 2% dimethyl sulfoxide (DMSO) for 2 days [9]. For fluorescence immunocytochemistry, transfected N1E-115 cells were fixed with 4% formaldehyde in PBS (pH 7.4) at 4 °C for 20 min, permeabilized with methanol at room temperature for 20 min and incubated with the anti-Myc antibody (9E10). Cytoplasmic and nuclear soluble fractions were prepared as described previously [10]. Each extract (20 μg protein) was separated by SDS–PAGE, transferred to Immobilon membrane, and immunoblotted with the anti-myc antibody, anti-β-tubulin antibody (Sigma) and anti-lamin B antibody (Santacruz Biotechnology). Protein concentrations were determined by the Bradford method (Bio-Rad).

Expression vectors for NR1a and NR2A were kindly provided from Dr. Jon Johnson at Department of Neuroscience, University of Pittsburgh. HEK 293 cells were transfected with 1:3 ratio of NR1a and NR2A subunits by the calcium phosphate method and 48 h later, the cells were incubated with 2 μM EthD-1 (Molecular Probes) in PBS at 37 °C for 30 min. EthD-1-positive cells were counted by choosing five random fields per each dish and expressed as a percentage. Statistical significance was tested using Student's *t*-test.

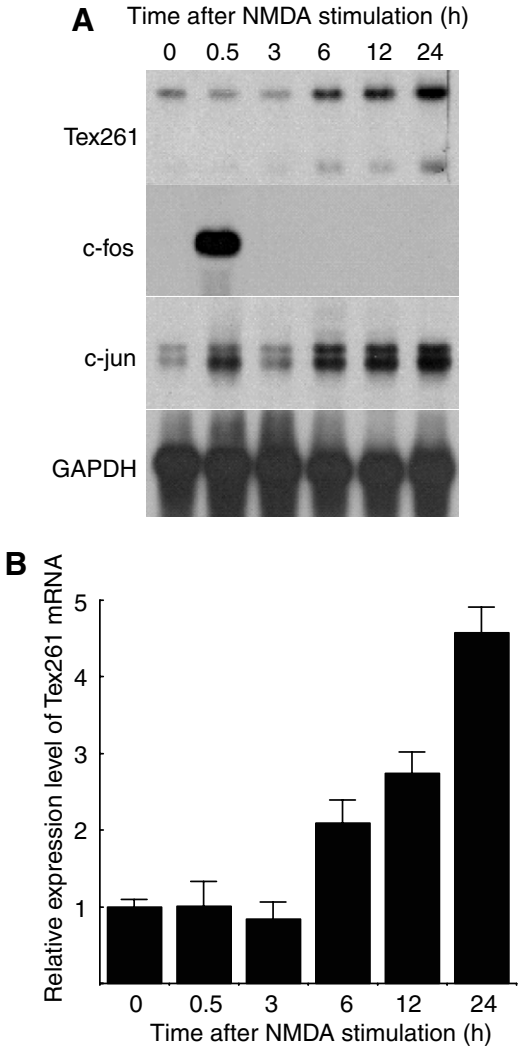

Fig. 1. Northern blot analysis. (A) Total cellular RNA was extracted from the hippocampi at the time as indicated after NMDA injection. Total RNA (2.5 μg) was resolved on 1% formaldehyde/agarose gels and Tex261, c-fos, c-jun and GAPDH mRNAs were detected by DIG-riboprobes. GAPDH was used as an internal standard. (B) Quantification of the Tex261 mRNA level shown in (A). Levels of Tex261 mRNA were measured, normalized with mRNA levels of GAPDH and represented as fold change over control at 0 h (mean ± SEM; $n = 3$).