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INHIBITION OF N-METHYL-D-ASPARTATE RECEPTOR ACTIVITY RESULTED IN ABERRANT NEURONAL MIGRATION CAUSED BY DELAYED MORPHOLOGICAL DEVELOPMENT IN THE MOUSE NEOCORTEX

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Abstract—Embryonic and neonatal neocortical neurons already express functional *N*-methyl-D-aspartate (NMDA) receptors before they form synapses. To elucidate the role of NMDA receptors in neuronal migration in the developing neocortex, we visualized radially migrating neurons by transferring the enhanced green fluorescent protein (EGFP) gene into the ventricular zone (VZ) of the mouse neocortex using *in utero* electroporation at E15.5. Two days later, we prepared neocortical slices and examined the EGFP-positive cells using time-lapse imaging in the presence of the NMDA receptor antagonist Cerestat. The EGFP-positive cells generated in the VZ in the control slices exhibited a multipolar morphology, but within several hours they became bipolar (with a leading process and an axon-like process) and migrated toward the pial surface. By contrast, many of the multipolar cells in the Cerestat-treated slices failed to extend either process and become bipolar, and frequently changed direction, although they ultimately reached their destination even after Cerestat-treatment. To identify the molecules responding for mediating NMDA signaling during neuronal migration and the changes in morphology observed above, we here focused on Src family kinases (SFKs), which mediate a variety of neuronal functions including migration and neurite extension. We discovered that the activity of Src and Fyn was reduced by Cerestat. These findings suggest that NMDA receptors are involved in neuronal migration and morphological changes into a bipolar shape, and in the activation of Src and Fyn in the developing neocortex.

Key words: development, corticogenesis, Src family kinases, NMDA receptor antagonist, *in utero* electroporation.

Neuronal migration is an important process in brain development. Newborn neurons are influenced by several factors that regulate their morphology, mobility, and destination during migration, and recent studies have shown that neurotransmitters, including glutamate and GABA, have a crucial modulatory effect on neuronal migration (Luján et al., 2005; Heng et al., 2007). For example, the granule cell migration in the cerebellum depends on the activity of *N*-methyl-D-aspartate (NMDA) receptors (Komuro and Rakic, 1993). NMDA receptor antagonists and $GABA_A$ receptor antagonists affect the morphology of the migrating neurons in the hippocampus in a manner that severely impairs neuronal migration (Manent et al., 2005). Neurons in the developing cerebral cortex migrate in one of two directions. The excitatory projection neurons generated near the ventricle move to the developing cortical plate (CP) through a radial mode of migration (Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic, 1972), whereas the GABA-containing interneurons originate in ganglionic eminences and enter the developing CP through a tangential mode of migration (Marin and Rubenstein, 2001; Nakajima, 2007). Interestingly, Yozu et al. (2008) found that a subpopulation of medial ganglionic eminence (MGE)-derived cells in the neocortex stopped migrating or changed their direction of migration in response to AMPA, but that neither MGE-derived cells migrating in the subcortical territory nor radially migrating cells in the neocortex were affected by exposure to AMPA. These results indicate that the activation of AMPA receptors directly affects tangential migration in the neocortex. While many studies, including our own previous study (Hirasawa et al., 2003), have shown that the inhibition of NMDA receptors with an antagonist suppresses radial migration (Behar et al., 1999; Hirai et al., 1999; Simonian and Herbison, 2001), Kihara et al. (2002) observed that the stimulation of NMDA receptors also inhibited radial migration. Since the investigators in all the above-mentioned studies used 5-bromo-2′-deoxyuridine (BrdU) or [^{3}H]thymidine pulse-labeling experiments to examine neuronal migration, providing conclusive evidence of NMDA receptor-mediated neuronal migration has been difficult since neuronal migration was never investigated directly.

In the present study, we visualized migrating neurons by transferring an enhanced green fluorescent protein (EGFP)-encoding plasmid into the ventricular zone (VZ) of the murine embryonic cerebrum using *in utero* electroporation and directly observed the migrating cells in living neocortical slices using time-lapse imaging. These time-

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Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; BSA, bovine serum albumin; CP, cortical plate; EGFP, enhanced green fluorescent protein; FAK, focal adhesion kinase; IMZ, intermediate zone; MLA, methyllycaconitine; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; SFKs, Src family kinases; TBS-T, tris-buffered saline containing 0.1% Tween20; VZ, ventricular zone.

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lapse observations revealed that the migratory profile during the inhibition of NMDA receptors differed considerably from the radial migration observed in the control mice. We also found that the activity of Src family kinases, including Src and Fyn, was significantly reduced after exposure to an NMDA antagonist. These results provided evidence that NMDA receptors play an important role in the radial migration that occurs in the developing neocortex.

EXPERIMENTAL PROCEDURES

Crl:CD-1 (ICR) mice (Clea Japan, Tokyo, Japan) were used in this study. All experimental procedures were approved by The Animal Care and Use Committee of the National Institute of Neuroscience.

Gene transfer using in utero electroporation

Gene transfer using *in utero* electroporation was performed as previously described (Nakahira and Yuasa, 2005), but with a slight modification of the original procedures (Tabata and Nakajima, 2001). On embryonic day (E) 15.5 pregnant ICR mice were deeply anesthetized with diethyl ether (Wako, Osaka, Japan), and their uterine horns were exposed. Plasmid DNA pCAGGS-EGFP that had been purified using the CsCl/Ethidium Bromide equilibrium density-gradient centrifugation method was dissolved to a final concentration of 5 μg/μL in phosphate-buffered saline (PBS) and 0.05% Fast Green. Approximately 1–2 μL of the plasmid DNA solution was injected into a lateral ventricle of each embryo with a glass micropipette prepared from a microcapillary tube (GD-1; Narishige, Tokyo, Japan). The head of the embryo in the uterus was placed between the tips of a tweezers-type electrode (CUY650-5; Nepa Gene, Chiba, Japan), and electronic pulses (35 V, 50 ms) were discharged five times at intervals of 950 ms with an electroporater (CUY21E; Nepa Gene). The uterine horns were then replaced in the abdominal cavity to allow the embryos to continue normal development.

Time-lapse imaging

Organotypic coronal brain slices (200 μm thick) from the anterior third of the forebrain were prepared using a vibratome (Dosaka, Japan, Kyoto, Japan) at 2 days after electroporation, then placed on a Millicell-CM (pore size, 0.4 μm; Millipore, Bedford, MA, USA), mounted in collagen gel (Nitta Gelatin, Osaka, Japan), and cultured in Neurobasal medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal calf serum (HyClone, Logan, UT, USA), 0.5 mM L-glutamine and B27 supplement (Invitrogen, San Diego, CA, USA) with or without the NMDA receptor antagonist, 50 μM Cerestat (CNS-1102; Sigma, St Louis, MO, USA) or 10 μM Memantine (Sigma), or α7 nicotinic acetylcholine receptor (nAChR) antagonist, 10 nM methyllycaconitine (MLA, Sigma). The dishes were then mounted in a CO_2 incubator chamber (5% CO_2, at 37 °C) fitted onto a confocal microscope (FV300; Olympus, Tokyo, Japan), and the dorsomedial region of the neocortex was examined. Approximately 10–20 optical Z sections were acquired automatically every 30 min, and ~20 focal planes (~50 μm thick) were merged to visualize the entire shape of the cells.

Tissue preparation and immunohistochemistry

The brain of neonate mouse was dissected out and postfixed in 4% paraformaldehyde (PFA) in PBS for 16 h. After washing in PBS for 1 h, the brain was successively equilibrated in 5%, 10%, and 20% sucrose in PBS, then embedded in optimal cutting temperature compound (Sakura, Tokyo, Japan) and frozen in dry ice. The frozen brain was coronally sectioned at 50 μm with a cryostat (CM-3000; Leica, Nussloch, Germany), and the brain sections were subjected to immunohistochemistry using the floating method. First, the sections were immunostained at 4 °C for 16 h with rabbit polyclonal anti-EGFP antibody (1:500; Invitrogen) in PBS containing 0.1% bovine serum albumin (BSA), 1% normal goat serum, and 0.1% Triton X-100. After successive washes with PBS, the sections were then immunostained at room temperature for 1 h with Alexa Fluor 488 goat anti-rabbit IgG (H+L) (1:1000; Invitrogen) in PBS containing 0.1% BSA, 1% normal goat serum, and 0.1% Triton X-100. In addition, living cultured slices were fixed with 4% PFA at 4 °C for 16 h and subjected to immunohistochemistry using the floating method described above. The slices were immunostained with rat monoclonal anti-nestin antibody (1:500; BD Biosciences, San Jose, CA, USA) coupled to Alexa Fluor 594 goat anti-rat IgG (H+L) (1:1000; Invitrogen) and mounted on silane-coated glass slides with PermaFluor (Thermo Shandon, Pittsburgh, PA, USA). Fluorescence images were obtained with a fluorescence microscope (AX70; Olympus) or a confocal laser microscope (FV1000; Olympus).

Preparation of protein samples from neocortex and immunoblot analysis

At E17.5, pregnant ICR mice were i.p. injected with the NMDA receptor antagonist, Cerestat (5 mg/kg body weight) or Memantine (10 mg/kg body weight; Sigma) or with the same volume of PBS as a control. The mice were then killed 1 h or 1 day after the injection, and the neocortex of the embryos was dissected out and homogenized in lysis buffer (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM sodium orthovanadate, and a protease inhibitor cocktail [Roche, Mannheim, Germany]) using a Polytron homogenizer. After removing the nuclei and debris by centrifugation (2000×g for 10 min at 4 °C), the protein concentration of the supernatant was determined using BCA Protein Assay Reagent (Pierce, Rockford, IL, USA), and the supernatant was stored at −80 °C until use.

The neocortical proteins were separated by electrophoresis through an SDS polyacrylamide gel (Daiichi, Pure Chemical, Tokyo, Japan) and then electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were blocked by incubation at room temperature for 1 h with 1% skim milk, 3% skim milk, or 1% BSA in Tris-buffered saline containing 0.1% Tween20 (TBS-T) or StartingBlock T20 Blocking Buffer (Pierce), then incubated at room temperature for 1 h with one of the following primary antibodies in 1% skim milk, 3% skim milk, or 1% BSA in TBS-T or StartingBlock T20 Blocking Buffer: anti-Src-pY418 antibody (1:1000; Biosource International Inc., Camarillo, CA, USA), mouse monoclonal anti-Fyn antibody (1:250, BD Biosciences), mouse monoclonal anti-Yes antibody (1:500, BD Biosciences), mouse monoclonal anti-Src antibody (1:1000; Upstate Biotechnology, Lake Placid, NY, USA), rabbit polyclonal anti-Lyn (H-70) antibody (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or mouse monoclonal anti-$\tilde{\beta}$actin antibody (1:1000; Abcam, Cambridge, UK). After three washes in TBS-T, the membranes were incubated at room temperature for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:1500; Sigma) or horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:1500; GE Healthcare, UK Ltd., Buckinghamshire, UK), and washed three times with TBS-T. Immunoreactive bands were visualized using a chemiluminescence detection system (ECL; GE Healthcare, UK Ltd.).

Immunoprecipitation assay

Neocortical proteins (500~1000 μg) were incubated at 4 °C for 2 h with 1 μg of antibody bound to protein A- and G-Sepharose beads (GE Healthcare, UK Ltd). The immunoprecipitates were washed three times with lysis buffer, and after elution by boiling in SDS