Nitric Oxide Synthase 1 Adaptor Protein, a Protein Implicated in Schizophrenia, Controls Radial Migration of Cortical Neurons

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ABSTRACT

BACKGROUND: Where a neuron is positioned in the brain during development determines neuronal circuitry and information processing needed for normal brain function. When aberrations in this process occur, cognitive disorders may result. Patients diagnosed with schizophrenia have been reported to show altered neuronal connectivity and heterotopias. To elucidate pathways by which this process occurs and become aberrant, we have chosen to study the long isoform of nitric oxide synthase 1 adaptor protein (NOS1AP), a protein encoded by a susceptibility gene for schizophrenia.

METHODS: To determine whether NOS1AP plays a role in cortical patterning, we knocked down or co-overexpressed NOS1AP and a green fluorescent protein or red fluorescent protein (TagRFP) reporter in neuronal progenitor cells of the embryonic rat neocortex using in utero electroporation. We analyzed sections of cortex (ventricular zone, intermediate zone, and cortical plate [CP]) containing green fluorescent protein or red fluorescent protein TagRFP positive cells and counted the percentage of positive cells that migrated to each region from at least three rats for each condition.

RESULTS: NOS1AP overexpression disrupts neuronal migration, resulting in increased cells in intermediate zone and less cells in CP, and decreases dendritogenesis. Knockdown results in increased migration, with more cells reaching the CP. The phosphotyrosine binding region, but not the PDZ-binding motif, is necessary for NOS1AP function. Amino acids 181 to 307, which are sufficient for NOS1AP-mediated decreases in dendrite number, have no effect on migration.

CONCLUSIONS: Our studies show for the first time a critical role for the schizophrenia-associated gene NOS1AP in cortical patterning, which may contribute to underlying pathophysiology seen in schizophrenia.

Keywords: Cortical development, Cortical neuron migration, in utero Electroporation, NOS1AP, Rodent model, Schizophrenia

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Proper brain development and function require the correct migration and placement of neurons. Abnormal neuronal migration may cause abnormal cortical function. There is increasing evidence that altered neuronal connectivity seen in schizophrenia may be due to factors during neurodevelopment in utero [reviewed in (1,2)]. Here, we study the role of nitric oxide synthase 1 adaptor protein (NOS1AP), a protein encoded by a schizophrenia susceptibility gene (3–7), during cortical neuron migration.

NOS1AP was first identified in the rat as a binding partner of neuronal nitric oxide synthase (nNOS) (8). It competes with postsynaptic density protein 95 (PSD-95) for nNOS binding and presumably reduces *N*-methyl-D-aspartate (NMDA) receptor signaling via PSD-95 and nNOS. There are at least three isoforms of NOS1AP (long [L], short [S], and short') that have been reported to have altered protein expression in the cortex of individuals diagnosed with schizophrenia (9). Specifically, NOS1AP-L protein expression, normalized to the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is elevated approximately 10-fold, whereas the other two isoforms are elevated approximately 100-fold (9). In addition, NOS1AP-L messenger RNA expression is significantly decreased by 40% in postmortem brain tissue from patients treated with antipsychotics when compared with untreated patients with the same psychiatric diagnosis (4). All three NOS1AP isoforms contain a C-terminal PDZ-binding domain, which is responsible for the interaction of NOS1AP with nNOS. NOS1AP-L contains a phosphotyrosine binding (PTB) domain (8), which binds to DexRas1, synapsin, and Scribble (10–12). In addition, we recently showed that NOS1AP-L interacts with carboxypeptidase E by a domain contained in its middle region (amino acids 181–307) and that NOS1AP regulates dendrite morphology through this interaction (13).

While overexpression of NOS1AP-L, the isoform affected by administration of antipsychotic drugs, has been reported by our group (13) to alter dendrite number and by another group (12) to alter spine development, an understanding of how

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NOS1AP, specifically NOS1AP-L, regulates synaptic connectivity in vivo has not been fully elucidated. Here, we show that NOS1AP-L negatively controls radial migration during cortical development. In addition, we identify the PTB domain as the main region of the protein involved in this role. These results suggest that upregulation of NOS1AP protein due to schizophrenia-associated alleles may promote altered connectivity seen in individuals with schizophrenia.

METHODS AND MATERIALS

Antibodies

Rabbit polyclonal anti-NOS1AP antibodies (sc-9138) were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Mouse monoclonal anti-GAPDH antibody was from Millipore (Billerica, Massachusetts), rabbit polyclonal anti-hrGFP antibody was from Agilent Technologies (Santa Clara, California), and chicken polyclonal anti-enhanced green fluorescent protein (EGFP) antibody was from AVES (Tigard, Oregon). Anti-chicken secondary antibody conjugated to Alexa-Fluor 488 was from Jackson ImmunoResearch (West Grove, Pennsylvania).

DNA Constructs

pCAG-GFP plasmid was obtained by subcloning the sequence of EGFP from pEGFP-C1 plasmid from Clontech (Mountain View, California) into a vector containing the CMV–actin–β-globin (CAG) promoter (gift from Dennis O'Leary, Salk Institute). Complementary DNA encoding human NOS1AP-L (referred to as NOS1AP or NP for this article), NOS1AP-213-end (NPΔPTB), and NOS1AP-181-307 ($NP_{181\text{-}307}$) were subcloned into pCAG-GFP plasmid to obtain N-terminally labeled GFP fusion constructs. Complementary DNA encoding NOS1AP and NOS1AP-1-487 (NPΔPDZ) were subcloned in pCAG-IRES-EGFP (pCIG, gift from Gabriella D'Arcangelo, Rutgers University) and pCAG-IRES-TagRFP plasmid (pCIR, gift from Marie-Catherine Tiveron, Institut de Biologie du Développement de Marseille).

For short hairpin RNA (shRNA) constructs, oligonucleotides were ligated into the pGE2hrGFPII vector (Agilent Technologies). The sequence of the RNA interference target for NOS1AP was 5′-GGGTGACAGTTTGGATGAT-3′ [shNP (13)]. As negative control, we used a sequence that did not align with any mammalian gene: 5′-GAGCATTTGTATGAGCGCG-3′ (shControl, against glutathione S-transferase; gift from Estela Jacinto, Rutgers Medical School). We have previously reported the sequences of NOS1AP shRNA, human NOS1AP-L, and mouse/rat NOS1AP-L in Figure 2C of Carrel *et al.* (13).

Transfection of Cultured Cells

COS-7 cells were cultured in six well plates and transfected at 30% to 50% confluency with a 3:1 ratio of shNP:NOS1AP to test for shRNA efficiency for NOS1AP knockdown using Lipofectamine 2000 (Life Technologies, Carlsbad, California) following the manufacturer's protocol. (A 1:1 ratio also resulted in significant NOS1AP knockdown.)

Western Blotting

COS-7 cells were cultured in 60 mm dishes and transfected using Lipofectamine 2000. Cells were collected 2 days after transfection and lysed, and expression of NOS1AP was detected by immunoblotting after resolving proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). After blocking with 2% bovine serum albumin in Tris-buffered saline containing .1% Tween-20, membranes were incubated with primary antibodies overnight at 4°C: 1:250 for anti-NOS1AP (Santa Cruz Biotechnology), 1:1000 for anti-GAPDH (Millipore), or 1:1000 for anti-humanized Renilla green fluorescent protein (Agilent Technologies). After washing, horseradish peroxidase linked secondary antibody was applied at 1:5,000 for 1 hour at room temperature. Immunoreactive bands were visualized using HyGlo quick spray (Denville Scientific, Inc., South Plainfield, New Jersey) and quantified using Image Pro software (Media Cybernetics, Rockville, Maryland).

In Utero Electroporation

Cells were transfected in vivo by in utero electroporation. Pregnant Sprague-Dawley rats at embryonic day 16 (E16) were anesthetized with ketamine/xylazine (75 mg/kg/10 mg/kg mixture). The abdominal cavity was opened to expose the uterine horns. Plasmids (1–3 μL of a 1.5–2 μg/μL solution) with 1 mg/mL Fast Green (Sigma, St Louis, Missouri) were microinjected through the uterus into the lateral ventricles of embryos by pulled glass capillaries (Drummond Scientific, Broomall, Pennsylvania). Electroporation was performed by placing heads of the embryos between tweezer-type electrodes. Square electric pulses (70 V, 50 msec) were passed five times at 1-second intervals using a CUY21 EDIT electroporator (Nepagene; Bulldog Bio, Inc., Portsmouth, New Hampshire). Embryos were allowed to develop in utero for 4 days after electroporation (until E20).

All animals used in this study were handled in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Rutgers, the State University of New Jersey, and in compliance with national and international laws and policies (Council directives no. 87-848, 19 October 1987, Ministère de l′Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale).

Histological Procedures and Microscopy

Embryonic rat brains (E20) were dissected and fixed for 48 hours in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4°C. Postnatal day (P) 14 rat brains (P14) were fixed by transcardial perfusion of 4% PFA in PBS and postfixed for 3 hours in 4% PFA in PBS. Brains were then cryoprotected in 30% sucrose in PBS, frozen in OCT compound (Sakura, Tokyo, Japan) and sectioned coronally at 16 μm (E20 brains) and 30 μm and 80 μm (P14 brains) using a cryostat.

For immunofluorescence staining, sections were incubated for 1 hour in antibody buffer (5% normal goat serum, .1% Triton X-100 in PBS) before overnight incubation with anti-EGFP antibody (1:1000). Following washes, sections were incubated with A488 anti-chicken antibody (1:1000) for 2 hours at room temperature. Sections were then washed, incubated for 10 minutes with Hoechst 33342 (Thermo Scientific, Rockford, Illinois) for nuclear staining and mounted using Fluoromount G (Southern Biotechnology, Birmingham, Alabama).

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