Cellular/Molecular

NOS1AP Regulates Dendrite Patterning of Hippocampal Neurons through a Carboxypeptidase E-Mediated Pathway

Damien Carrel,[1]* Yangzhou Du,[1]* Daniel Komlos,[1,2] Norell M. Hadzimichalis,[1] Munjin Kwon,[1,3] Bo Wang,[1] Linda M. Brzustowicz,[4] and Bonnie L. Firestein[1]

[1]Department of Cell Biology and Neuroscience, [2]Neuroscience Graduate Program, [3]Molecular Biosciences Graduate Program, and [4]Department of Genetics, Rutgers University, Piscataway, New Jersey 08854-8082

During neuronal development, neurons form elaborate dendritic arbors that receive signals from axons. Additional studies are needed to elucidate the factors regulating the establishment of dendritic patterns. Our work explored possible roles played by nitric oxide synthase 1 adaptor protein (NOS1AP; also known as C-terminal PDZ ligand of neuronal nitric oxide synthase or CAPON) in dendritic patterning of cultured hippocampal neurons. Here we report that the long isoform of NOS1AP (NOS1AP-L) plays a novel role in regulating dendrite outgrowth and branching. NOS1AP-L decreases dendrite number when overexpressed at any interval between day *in vitro* (DIV) 0 and DIV 12, and knockdown of NOS1AP-L results in increased dendrite number. In contrast, the short isoform of NOS1AP (NOS1AP-S) decreases dendrite number only when overexpressed during DIV 5–7. Using mutants of NOS1AP-L, we show that neither the PDZ-binding domain nor the PTB domain is necessary for the effects of NOS1AP-L. We have functionally narrowed the region of NOS1AP-L that mediates this effect to the middle amino acids 181–307, a region that is not present in NOS1AP-S. Furthermore, we performed a yeast two-hybrid screen and identified carboxypeptidase E (CPE) as a binding partner for the middle region of NOS1AP-L. Biochemical and cellular studies reveal that CPE mediates the effects of NOS1AP on dendrite morphology. Together, our results suggest that NOS1AP-L plays an important role in the initiation, outgrowth, and maintenance of dendrites during development.

Introduction

The proper development of dendrite morphology is crucial for normal brain function. Incorrect formation of stereotypical dendritic arborization patterns results in improper neuronal networking (Vetter et al., 2001; Schaefer et al., 2003). As such, various neuropsychiatric disorders are associated with disturbed dendrite development and maintenance. For example, in patients with schizophrenia, dendritic field size is smaller in layer V pyramidal cortical neurons (Black et al., 2004). Moreover, decreased dendrite number and/or branching is seen in a number of developmental disorders, such as autism, Rett syndrome, Down syndrome, and unclassified mental retardation (Zoghbi, 2003). Over the past few years, significant progress has been made in our understanding of the intracellular mechanisms regulating dendritic branching. For example, Rho GTPases (Li et al., 2000; Chen and Firestein, 2007), the guanine deaminase cypin (Akum et al., 2004), glutamate receptor-interacting protein (GRIP) (Hoogenraad et al., 2005), and novel transcription factors identified in *Drosophila* (Parrish et al., 2007) have been reported to influence the development and maintenance of dendrites. However, the involvement of these pathways in neuropsychiatric disorders has been elusive. Our current study examines the role played by nitric oxide synthase 1 adaptor protein (NOS1AP), also termed CAPON, a gene linked to schizophrenia (Brzustowicz et al., 2004; Xu et al., 2005; Zheng et al., 2005; Kremeyer et al., 2009; Wratten et al., 2009) in regulating dendrite morphology.

NOS1AP was first identified in the rat as a binding partner of neuronal nitric oxide synthase (nNOS) (Jaffrey et al., 1998). It competes with PSD-95 for nNOS binding and presumably reduces NMDA receptor signaling via PSD-95 and nNOS. Later studies in human revealed that there are at least two alternately expressed forms of NOS1AP (Xu et al., 2005). Transcription of all 10 NOS1AP exons gives rise to a cDNA that can be translated into a 501 aa protein, designated as NOS1AP-long (NOS1AP-L). When only the last two exons are transcribed, a protein of 125 aa, designated as NOS1AP-short (NOS1AP-S), is produced (Xu et al., 2005). NOS1AP-S has 12 unique N-terminal amino acids, followed by 113 aa that are shared with the C terminus of NOS1AP-L. NOS1AP-L protein contains two distinguishable domains, the C-terminal PDZ-binding domain, which is responsible for the interaction of NOS1AP with nNOS, and a phosphotyrosine-binding (PTB) domain (Jaffrey et al., 1998). The PTB domain of NOS1AP-L binds to Dexras1 and synapsin (Fang et al., 2000; Jaffrey et al., 2002). Interestingly, Dexras1 activity is regulated by nNOS-elicited nitrosylation. Subsequent studies showed that Dexras1 regulates iron metabolism (Cheah et al., 2006). Importantly, NOS1AP-L mRNA expression is signifi-

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*D.C. and Y.D. contributed equally to this work.

Correspondence should be addressed to Dr. Bonnie L. Firestein, Department of Cell Biology and Neuroscience, Rutgers University, 604 Allison Road, Piscataway, NJ 08854-8082. E-mail: firestein@biology.rutgers.edu.

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cantly decreased by 40% in postmortem brain tissue from subjects treated with antipsychotics when compared with untreated patients (Xu et al., 2005). In contrast, NOS1AP-S mRNA is unaffected by antipsychotic drugs, but is expressed at significantly higher levels in tissue from individuals with schizophrenia and bipolar disorder when compared with psychiatrically normal controls. However, the question of how alterations in NOS1AP expression affect brain function has not been fully elucidated. The previous studies on NOS1AP, plus previous reports revealing that dendrite bifurcation in motor neurons is severely reduced in nNOS knock-out mice (Inglis et al., 1998), prompted us to start analyzing whether NOS1AP, a nNOS binding partner, may influence dendrite morphology in hippocampal neurons.

In the present study, we report a new role for NOS1AP in the regulation of dendrite morphology. Interestingly, this function of NOS1AP is mediated by a novel domain within the NOS1AP protein sequence. Furthermore, we have identified a downstream mediator of NOS1AP action. Our work presents a possible mechanism for explaining the pathological significance of NOS1AP-L overexpression in schizophrenia and bipolar disorders.

Materials and Methods

Antibodies and reagents. Two different polyclonal NOS1AP antibodies and mouse anti-secretogranin II (SGII 9G3/3) were from Santa Cruz. Rabbit anti-NOS1AP (R-300) recognizes the C-terminal amino acids 304–501 of NOS1AP-L; goat anti-NOS1AP (S-17) recognizes the N terminus of NOS1AP-L. Rat anti-green fluorescent protein (GFP) was a kind gift from Dr. Shu-Chan Hsu of Rutgers University. Mouse anti-microtubule-associated protein 2 (MAP2) and mouse anti-carboxypeptidase E (CPE) were purchased from BD PharMingen. Mouse anti-syntaxin, monoclonal anti-HA antibody, and nNOS inhibitor *N*(ω)-nitro-L-arginine-methyl ester (L-NAME) were purchased from Sigma. Rabbit anti-synaptophysin was from Zymed, and mouse antibody against nNOS was from Transduction Laboratories. Cyanine (Cy) 2-, Cy3-, and Cy5-conjugated secondary antibodies were purchased from Jackson ImmunoResearch.

DNA constructs. cDNAs encoding human NOS1AP and several mutant forms of human NOS1AP were cloned into the expression vector pCMV6-XL4 (Origene). These plasmids are referred to directly as NOS1AP (or NOS1AP mutants) without indicating the vector. In addition, both human and mouse fusion proteins GFP-NOS1AP-L (GFP-NPL), GFP-NOS1AP-S (GFP-NPS), and GFP-NOS1AP-181–307 (GFP-NOS1AP-M, GFP-NPM) were constructed in the pEGFP-C1 vector (Clontech).

RNA interference. We designed small interference RNA (siRNA) targeting the CPE transcript (5′-GTTTGTCCGTGACCTTCAA-3′), the NOS1AP transcript (5′-GGGTGACAGTTTGGATGAT-3′), and an unrelated sequence as a negative control (5′-GCTTGGTTACTCCTGGATT-3′). Each siRNA was converted to shRNA and its DNA was subcloned into the pSuper-GFP vector (Oligoengine) according to the manufacturer's instructions. Oligonucleotides were synthesized and purchased from Bio-Synthesis.

Hippocampal neuron cultures. Neuronal cultures were prepared from hippocampi of rat embryos at 18 d gestation as described previously (Firestein et al., 1999). The hippocampi were dissociated, and cells were plated on poly-D-lysine-coated glass coverslips (12 mm diameter) at a density of 1800 cells/mm^2. Cultures were maintained in Neurobasal media supplemented with B27, penicillin, streptomycin, and L-glutamine. Cells were grown for 5–21 d *in vitro* (DIV) and used for specific experiments as indicated below.

Transfection of cultured cells. Cultured hippocampal neurons were transfected with the appropriate cDNA constructs as follows. Neurons were transfected on DIV 9 or 10 using EFFECTENE following the manufacturer's protocol (Qiagen). Neurons were transfected on DIV 2, 5, or 7 using Lipofectamine-2000 following the manufacturer's protocol (Invitrogen). The Amaxa Nucleofector kit for rat neurons (Amaxa, kit VPG-1003) was used to transfect newly dissected neurons (DIV 0) before plating.

For examining shRNA-mediated knockdown, COS-7 cells were cultured in 60 mm dishes and transfected using LIPOFECTAMINE-2000 following the manufacturer's protocol. Cells were collected 2 d after transfection, lysed, and expression of NOS1AP was detected by immunoblotting.

Immunocytochemistry. Neurons were fixed in 4% paraformaldehyde in PBS for 30 min and then incubated in blocking solution (PBS containing 0.1% Triton X-100, 2% normal goat serum, and 0.02% sodium azide) for 1 h. All antibodies used were diluted in blocking solution. For GFP and NOS1AP or GFP and HA double immunostaining, dilutions of 1:1000 for rat anti-GFP and 1:500 for rabbit anti-NOS1AP, or 1:500 for goat anti-NOS1AP, or 1:1000 for mouse anti-HA (CRP) were used. All incubations with primary antibodies were performed at 4°C overnight. Neurons were then washed with PBS three times. Secondary antibody consisted of a 1:250 dilution of Cy2-conjugated anti-rat IgG and Cy3-conjugated donkey anti-rabbit, anti-goat, or anti-mouse IgG. Coverslips were then mounted onto frosted glass microscope slides using Fluoromount G (Southern Biotechnology). Labeled cells were visualized by immunofluorescence on an Olympus Optical IX50 microscope with a Cooke Sensicam charge-coupled device cooled camera fluorescence imaging system and Image Pro software (Media Cybernetics).

Assessment of dendrite number. Primary and secondary dendrite numbers were counted by hand with the experimenter blinded to the conditions (Akum et al., 2004). Pictures of neurons were contrast-enhanced and the numbers of primary and secondary dendrites for each neuron were recorded. Primary dendrites are processes longer than 3 μm and originate directly from the cell body. Secondary dendrites are branches of primary dendrites. *n* values indicate the number of neurons counted.

Quantification of fluorescence intensity. Hippocampal neurons were prepared, cultured, and transfected as above. The neurons were immunostained with monoclonal antibody against CPE (1:1000). Fluorescence intensities of CPE were measured using Image Pro software. Cell bodies for each neuron were traced, and intensities were measured as average pixel intensity within the selected region. Fluorescence was visualized using a 20× objective. To quantitate the fluorescence levels of tagged proteins, images of neurons were captured by CCD as above using a constant gain and exposure time for all samples. Images were corrected for coverslip fluorescence by subtracting a background image generated using an 11 × 11 erosion filter. The experimenter was blinded to the condition when taking images and assaying fluorescence intensities.

Immunoprecipitation and Western blotting. One rat brain was homogenized in 10 ml of TEEN (25 mM Tris, 1 mM EGTA, 1 mM EDTA, and 100 mM NaCl, pH 7.4) + 1 mM PMSF. Triton X-100 was added to a final concentration of 1% for immunoprecipitation of NOS1AP, and proteins were extracted for 1 h at 4°C. The extract was centrifuged at 10,000–13,000 × *g* to remove insoluble material. The supernatant was precleared with Protein A- or Protein G-Sepharose for 1 h. Lysates were immunoprecipitated with 5 μg/ml monoclonal anti-human carboxypeptidase E (R&D Systems), or lysates were diluted fivefold with TEEN and incubated with rabbit anti-NOS1AP (R-300) at 4°C overnight. Protein A or G beads were added for 1 h of incubation. Beads were washed three times with TEEN containing 0.2% Triton X-100. Immunoprecipitated proteins were eluted with protein loading buffer and subjected to Western blot analysis.

SDS-PAGE gels were used for Western blots. After electrophoresis, resolved proteins were transferred to PVDF membranes (Immobilon-P). The blots were probed with the indicated antibodies. After blocking with 3% bovine serum albumin (BSA) in PBS or TBST, membranes were incubated with primary antibodies overnight at 4°C: polyclonal anti-Capon R-300 (1:250; Santa Cruz), rabbit polyclonal anti-CPE (1:2000; Millipore), monoclonal anti-CPE (1:500; BD PharMingen), monoclonal anti-actin (1:2000, Sigma-Aldrich), or monoclonal anti-GAPDH (1:1000, Millipore). After washing, horseradish peroxidase linked secondary antibody was applied at 1:2000 for 1 h at RT. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) system (GE Healthcare).

Glycerol gradient of synaptosomes. Fractions from synaptosome prepa-

rations sedimented through glycerol gradients (5–25%) (Bellocchio et al., 1998) were a kind gift from E. Bellochio (University of California, San Francisco, San Francisco, CA). Briefly, glycerol gradients (5–25%) were prepared in (in mM) 10 HEPES-NaOH, pH 7.4, 150 NaCl, 1 EGTA, and 0.1 $MgCl_2$ with protease inhibitors over a pad of 2 M sucrose. Synaptosome preparations were layered onto the glycerol gradients and centrifuged at 195,600 × g_{max} in an SW 50.1 rotor (Beckman) for 1 h at 5°C. Fractions were collected from the top of the gradient. The top fraction (cytosolic) was discarded, and the 10 other fractions were resolved by SDS-PAGE and used for Western blot as described above.

Results

NOS1AP-L reduces dendrite branching

If NOS1AP plays a role in neuronal development, it should be expressed in the developing brain. To address this question, we performed Western blot analysis on extracts of proteins from rat forebrain (hippocampus and cortex) from different ages. As seen in Figure 1*A*, both NOS1AP-L and NOS1AP-S expression increase from E15 to P14. We were unable to perform immunostaining for either isoform of NOS1AP since the antibody used for Western blotting does not recognize endogenous NOS1AP in neurons or cell lines (i.e., COS-7 cells) that express NOS1AP (data not shown). We attempted to raise antibodies against NOS1AP in both rat and rabbit, but our attempts to produce an antibody suitable for immunocytochemistry were futile. Thus, based on our immunoblotting results, NOS1AP-L and NOS1AP-S may play an important role in neuronal development.

NOS1AP mRNA is increased in the dorsolateral prefrontal cortex of patients with schizophrenia and bipolar disorder (Xu et al., 2005). As of yet, the physiological effect of NOS1AP overexpression is unknown. Since the absence of nNOS, which binds to the PDZ-binding domain of NOS1AP, has been shown to reduce the secondary branching of motor neurons in mice (Inglis et al., 1998), we asked whether NOS1AP is involved in regulating dendrite patterning. We performed our studies in cultured hippocampal neurons since (1) the cultures are relatively homogenous and show characteristic dendrite branching patterns (Akum et al., 2004; Charych et al., 2006; Chen and Firestein, 2007), and (2) this region of the brain has been implicated in schizophrenia (Heckers and Konradi, 2002). We began addressing possible roles of NOS1AP by examining whether overexpression of the two different isoforms, NOS1AP-S and NOS1AP-L, in neurons affects dendrite branching. Cultured hippocampal neurons were transfected with vectors containing GFP, or cotransfected with GFP and NOS1AP-L or GFP and NOS1AP-S on DIV 10. After 48 h, the cells were fixed and immunostained for GFP and NOS1AP. Primary and secondary dendrite numbers of GFP-positive neurons were analyzed. In the case of double transfection, only neurons transfected with both GFP and NOS1AP (Long or Short) were monitored. Interestingly, while overexpression of NOS1AP-L significantly reduced primary and secondary dendrite numbers, NOS1AP-S did not alter branching (Fig. 1*B–D*), demonstrating an isoform-specific role for NOS1AP in regulating dendrite morphology. Furthermore, overexpression of the mouse NOS1AP-L also decreased dendrite number (data not shown). Together, our data suggest that NOS1AP-L may act to maintain immature dendritic morphology in hippocampal neurons.

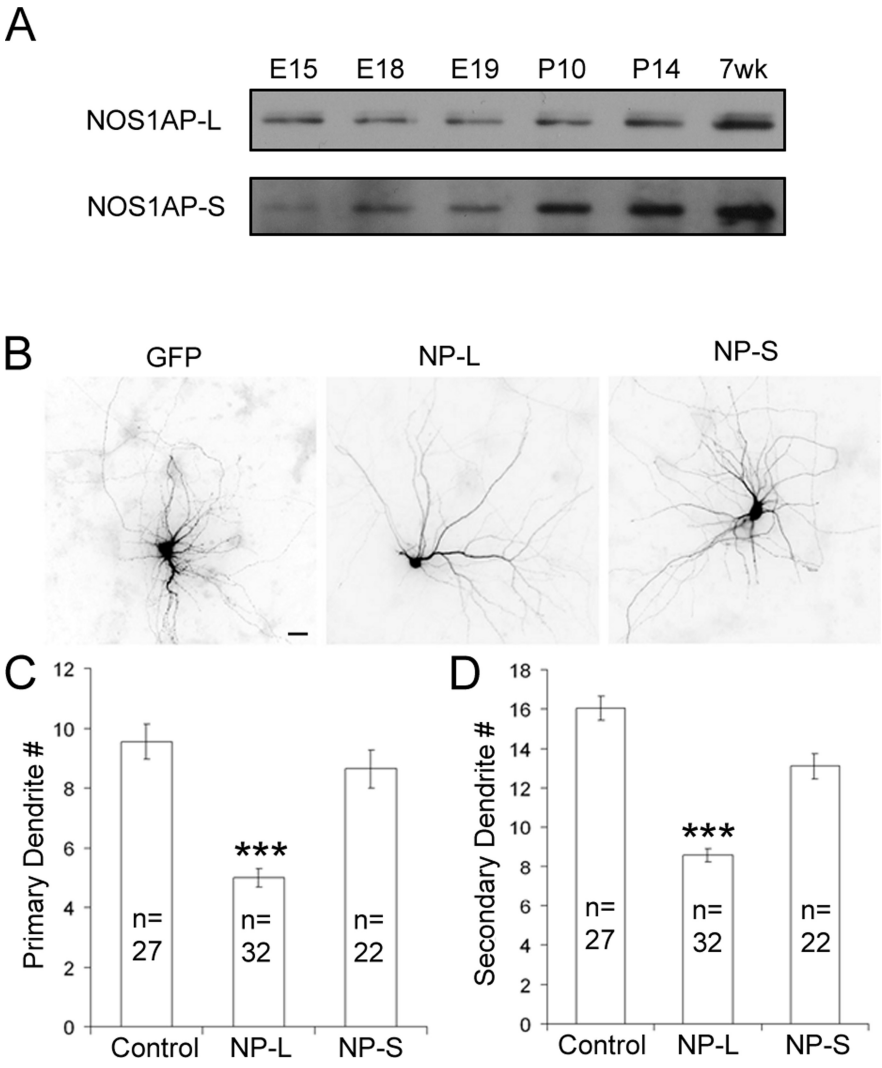

Figure 1. NOS1AP-L reduces dendrite number while NOS1AP-S has no effect. ***A***, Forebrain was dissected from brains of rat embryos or pups at the indicated ages. Proteins (12.5 μg) were resolved by SDS-PAGE and transferred to PVDF. Western blotting was performed using a pan NOS1AP antibody. Both NOS1AP-L and NOS1AP-S expression increase as the animals mature. ***B***, GFP images of representative hippocampal neurons cotransfected at DIV 10 with GFP and NOS1AP-L (NP-L) or NOS1AP-S (NP-S), and then fixed and immunostained for dendrite counting at DIV 12. Scale bar, 10 μm. ***C***, ***D***, Average number of primary and secondary dendrites in transfected neurons. ***$p < 0.001$, by nonparametric ANOVA followed by Dunn's analysis comparing cotransfected groups with GFP.

Knockdown of NOS1AP-L results in increased dendrite number

To determine whether NOS1AP-L does in fact play a role in dendrite branching, we constructed an shRNA against NOS1AP. As seen in Figure 2, *A* and *B*, coexpression of this shRNA with mouse NOS1AP-L in COS cells for 48 h results in a 60% decrease in NOS1AP-L expression as evidenced by Western blotting using a scrambled shRNA as a control. Furthermore, knockdown of

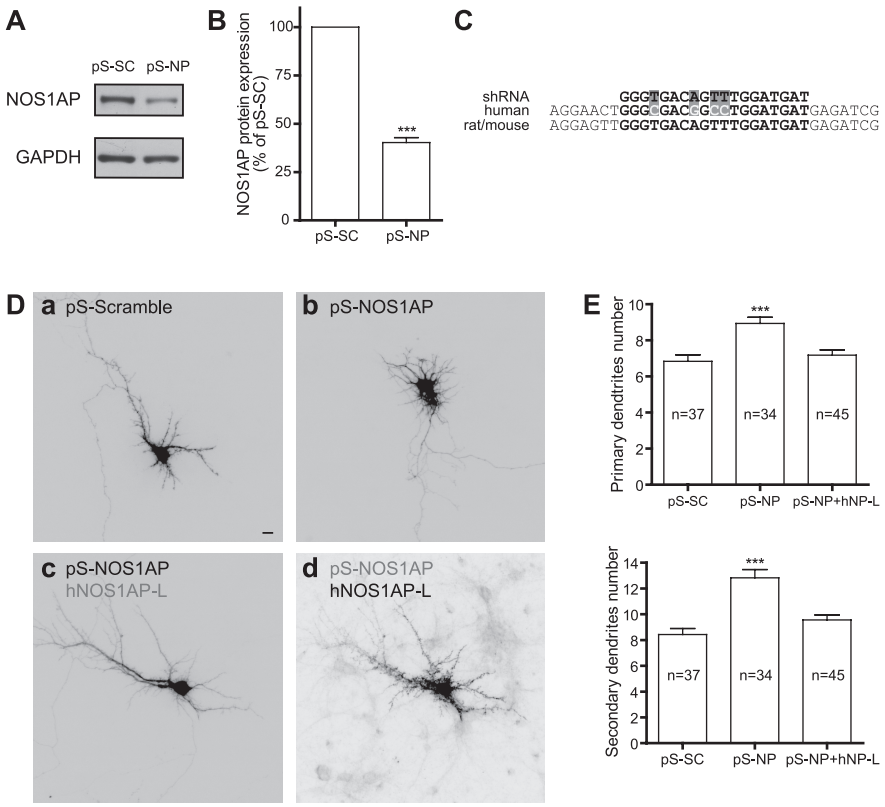

Figure 2. Expression of an shRNA against NOS1AP-L results in an increase in dendrite number. ***A***, COS-7 cells were cotransfected with pEGFP-NOS1AP-L (mouse) and either pSUPER-scramble shRNA (pS-SC, negative control) or pSUPER-NOS1AP shRNA (pS-NP). After 48 h, cells were lysed, and protein extracts were resolved using SDS-PAGE and probed with antibodies to NOS1AP and GAPDH. ***B***, Quantification of NOS1AP levels (normalized with GAPDH expression). pS-NP results in a 60% decrease in NOS1AP-L expression (data represent average of three independent experiments; $***p < 0.001$ by Student's *t* test). ***C***, Sequences of NOS1AP shRNA, human NOS1AP-L (hNP-L), and mouse/rat NOS1AP-L. Human NOS1AP-L contains four bases that differ from those in mouse/rat NOS1AP-L in the shRNA target sequence. ***D***, Representative images of hippocampal neurons transfected with pS-SC, pS-NP, or cotransfected with pS-NP and hNP-L at DIV 9 and fixed at DIV 12. GFP fluorescence (***Da–Dc***) was used to determine dendrite number. Cotransfected neurons were identified by immunostaining for exogenous NOS1AP (***Dd***). ***E***, Average number of primary and secondary dendrites in transfected neurons. $***p < 0.001$ by one way ANOVA followed by Bonferroni multiple-comparisons test versus pS-SC. Scale bars, 10 μm.

NOS1AP-L by expression of this shRNA in hippocampal neurons from DIV 9–12 results in increased primary and secondary dendrite numbers (Fig. 2*D*,*E*). To demonstrate the specificity of our shRNA construct, we coexpressed it with human NOS1AP-L in neurons. As human NOS1AP-L contains four differences in the sequence corresponding to our shRNA construct for mouse/rat NOS1AP (Fig. 2*C*), it should rescue NOS1AP-L expression and function. As expected, dendrite number returned to basal levels when we coexpressed NOS1AP shRNA with the human form of NOS1AP-L (Fig. 2*D*,*E*). Together with the overexpression data, these data strongly suggest that NOS1AP-L plays a role in regulating dendrite number and branching in developing hippocampal neurons.

Blocking nNOS activity attenuates the effects of NOS1AP on dendrite branching

Since both NOS1AP isoforms are nNOS binding partners, we next examined whether nNOS is involved in regulating the actions of NOS1AP on dendritic morphology. We used the nNOS inhibitor, L-NAME, to block nNOS activity. Although blocking nNOS with 500 μM L-NAME immediately after transfection did not fully inhibit the effects of NOS1AP-L, it did elicit a significant attenuation of NOS1AP-L-induced decreases in primary dendrite number (Fig. 3*A*,*B*). Similarly, secondary dendrite number in neurons overexpressing NOS1AP-L alone and NOS1AP-L neurons treated with L-NAME were marginally different (Fig. 3*A*,*C*). Thus, our results suggest that nNOS may act to modulate the effects of NOS1AP-L but that NOS1AP-L does not act solely via nNOS to decrease dendrite number.

NOS1AP mutants and their effects on dendrite branching

In addition to nNOS binding to the PDZ-binding domain of NOS1AP-L, Dexras1 and synapsin were identified as binding proteins to the PTB domain (Fang et al., 2000; Jaffrey et al., 2002). To explore the possible involvement of these proteins in the actions of NOS1AP-L in neurons, we constructed NOS1AP lacking or containing binding sites for nNOS, Dexras1, or synapsin. We generated five constructs encoding mutant forms of NOS1AP-L (Fig. 4*A*), and these mutants were cloned into the CMV6-XL4 vector. NOS1AP-HA-20–180 is the PTB domain tagged with HA (peptide YPYDVPDYA); NOS1AP-ΔPDZ lacks the PDZ-binding domain; NOS1AP-181-C lacks the N terminus and the PTB domains; NOS1AP-181-ΔPDZ lacks both the PTB and PDZ-binding domains; and NOS1AP-HA-181–307 is HA-tagged NOS1AP-L without the PTB domain and the C-terminal section identical to NOS1AP-S.

Cultured hippocampal neurons were cotransfected with GFP and the NOS1AP mutant constructs at DIV 10. Cells were fixed and immunostained for GFP and either NOS1AP or HA at DIV 12. Neurons transfected with cDNA encoding NOS1AP-L, NOS1AP-S, NOS1AP-181-C, NOS1AP-ΔPDZ, or NOS1AP-181-ΔPDZ were recognized by the rabbit anti-NOS1AP (R-300 for amino acids 304–501) antibody. Neurons transfected with cDNA encoding NOS1AP-HA-20–180 or NOS1AP-HA-181–307 were immunostained with mouse anti-HA. Dendrite branching was assessed for neurons that were positive for both GFP and NOS1AP (or mutant NOS1AP) (Fig. 4*B*). Overexpression of NOS1AP-ΔPDZ produced a similarly partially attenuated reduction in dendrite branching as the L-NAME treatment, further supporting the idea that nNOS is not the primary modulator of this activity. NOS1AP-HA-20–180 had no effect on dendrite numbers, suggesting that neither Dexras1 nor synapsin is involved in regulation of dendrite morphology by NOS1AP-L. This hypothesis was further strengthened by the fact that overexpression of NOS1AP-181-C presented the same level of reduction in dendritic branching as did overexpression of NOS1AP-L. In fact, neither the PDZ-binding domain nor the PTB domain appears to be essential for mediating the effects of NOS1AP-L on branching. In contrast, the middle region of NOS1AP, amino acids 181–307 (designated NOS1AP-middle, NOS1AP-M), plays an essential role in mediating NOS1AP-L actions (Fig. 4*C*,*D*).

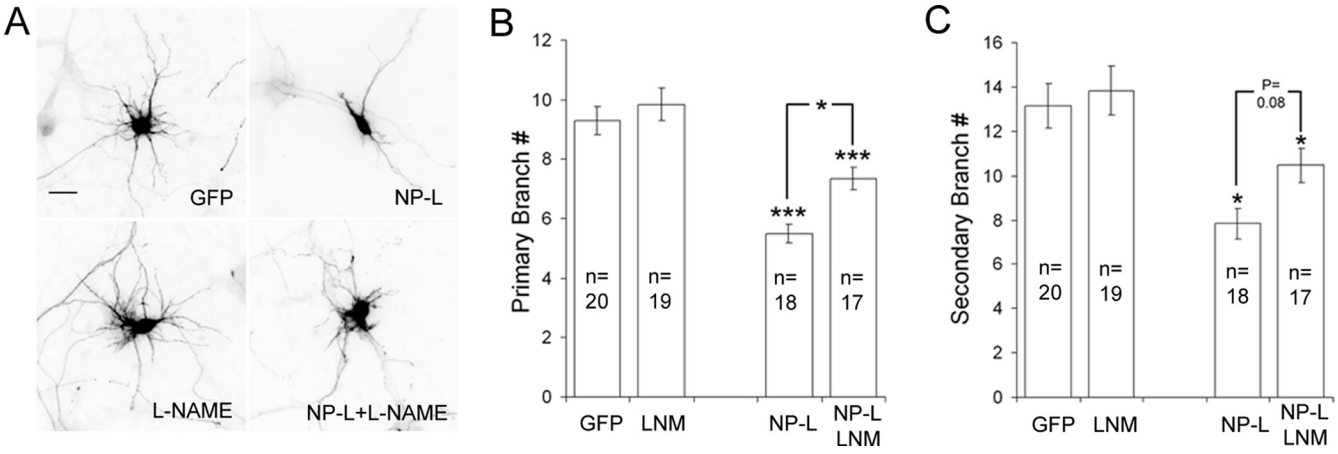

Figure 3. L-NAME reduces the effects of NOS1AP-L on dendrite number. NOS1AP-L (NP-L) was coexpressed with GFP in hippocampal neurons from DIV 10–12. L-NAME (LNM, 500 μM) was added to the cultures right after the start of transfection. After 48 h, neurons were fixed and immunostained for dendrite counting. ***A***, GFP images of representative neurons. Scale bar, 10 μm. ***B***, ***C***, Average number of primary and secondary dendrites in transfected neurons. *$p < 0.05$, by nonparametric ANOVA followed by Dunn's analysis comparing NP-L with NP-L plus LNM. ***$p < 0.001$, by nonparametric ANOVA followed by Dunn's analysis comparing cotransfected groups with GFP.

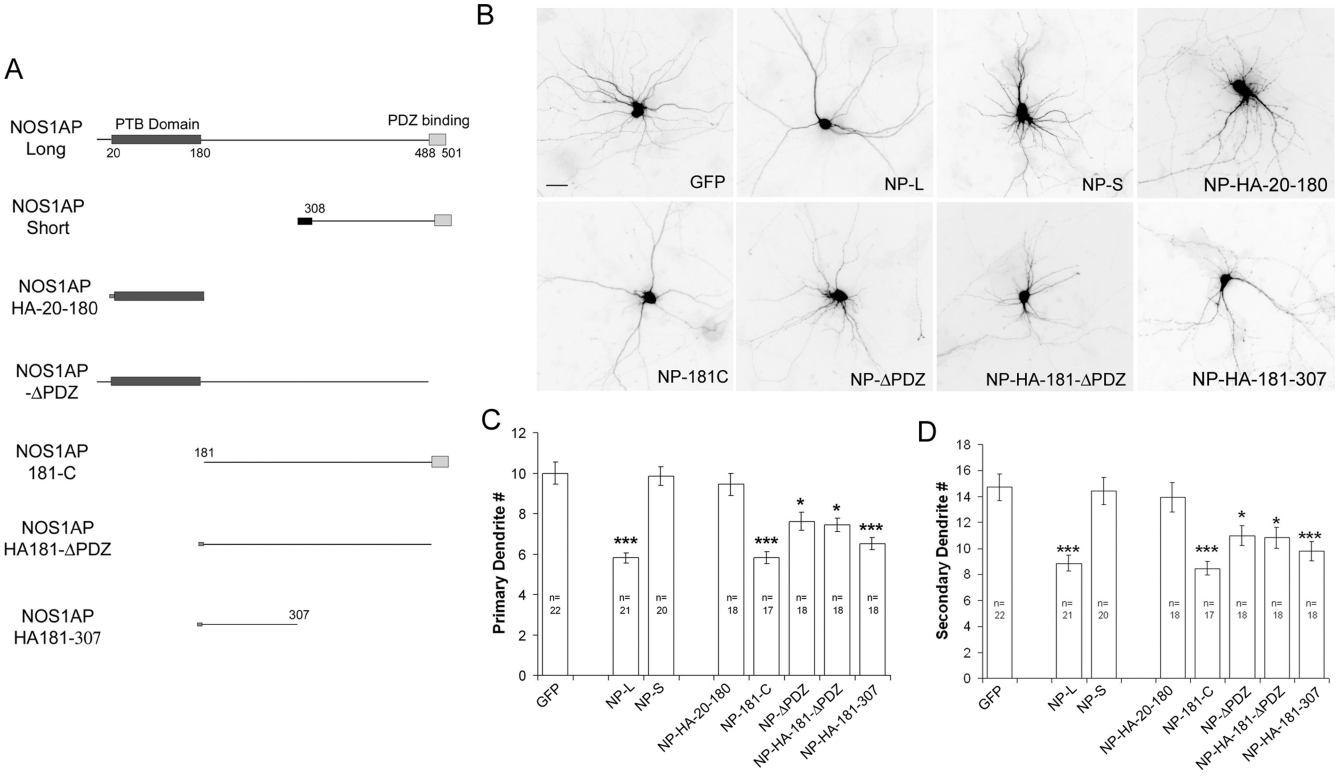

Figure 4. Effects of overexpression of mutant forms of NOS1AP on dendrite number. ***A***, Five constructs encoding mutant forms of NOS1AP-L are illustrated. ***B***, NOS1AP-L, NOS1AP-S, or mutant NOS1AP was coexpressed with GFP in hippocampal neurons from DIV 10–12. Neurons were fixed and immunostained for dendrite counting at DIV 12. GFP images of representative neurons were illustrated. Scale bar, 10 μm. ***C***, ***D***, Average number of primary and secondary dendrites in transfected neurons. *$p < 0.05$, ***$p < 0.001$, by nonparametric ANOVA followed by Dunn's analysis comparing cotransfected groups with GFP.

The effects of NOS1AP isoforms on reduction of dendrite branching in earlier cultures

The time points examined (DIV 10–12) for the effects of NOS1AP were chosen because it is during this time period that active branching occurs (Charych et al., 2006). To assess whether NOS1AP affects the initiation or maintenance of dendrites, the effects of NOS1AP overexpression were examined in younger hippocampal neuron cultures. We constructed GFP-NOS1AP-L, GFP-NOS1AP-S, and GFP-NOS1AP-M plasmids by cloning the NOS1AP-L, NOS1AP-S, and NOS1AP-M into the eGFP-C1 vector. To explore whether the initial branching of dendrites were influenced by NOS1AP, freshly dissected hippocampi were dissociated and transfected with these plasmids using the Amaxa Nucleofector kit for rat neurons (VPG-1003). Neurons were fixed on DIV 5 and dendrite branching was examined. GFP-NOS1AP-L and GFP-NOS1AP-M reduced branching while GFP-NOS1AP-S had no effect (Fig. 5*A*). To further examine the roles played by NOS1AP in dendrite branching during neuron development, cultured hippocampal neurons were transfected on DIV 2, DIV 5, and DIV 7 with GFP, GFP-NOS1AP-L, GFP-

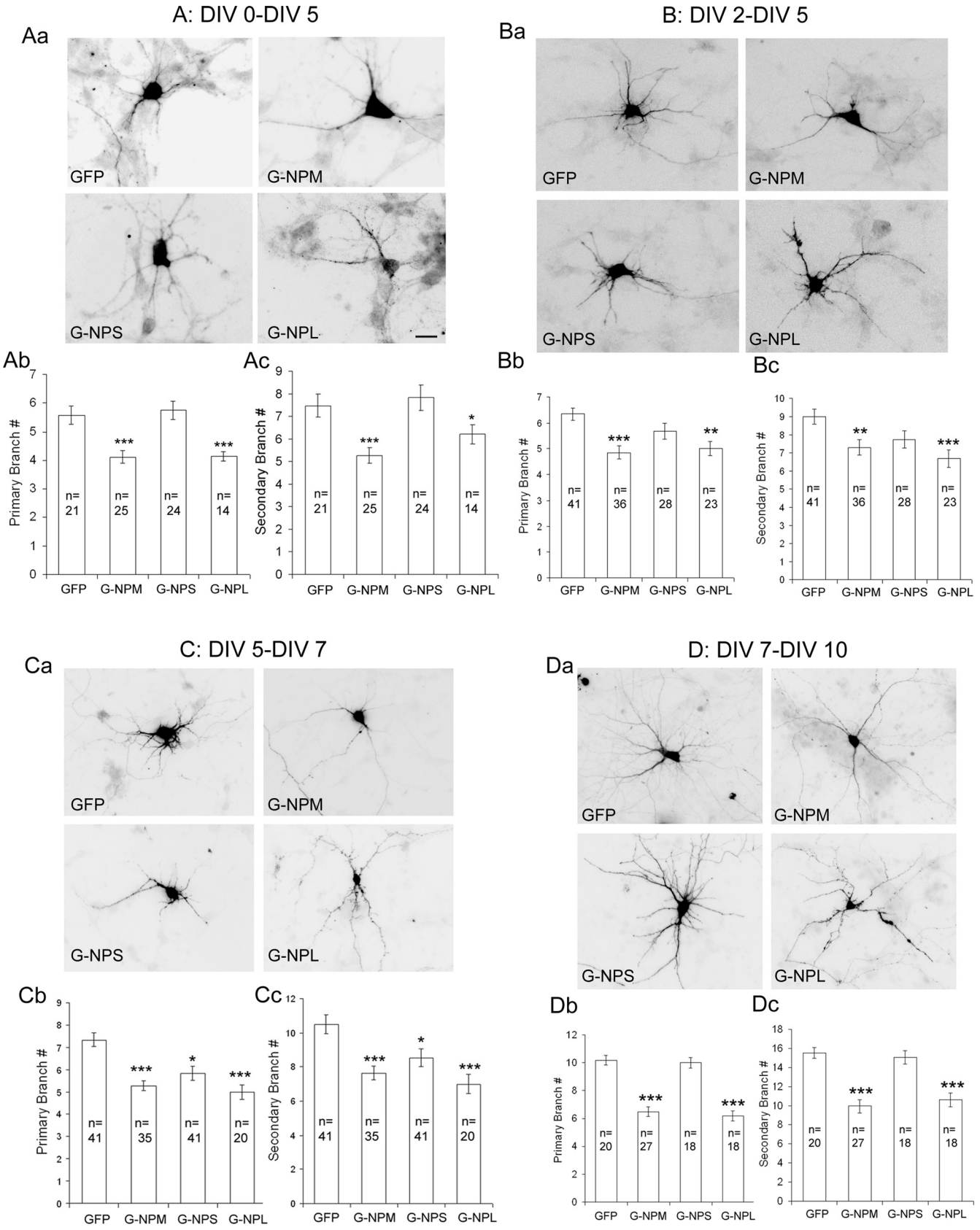

Figure 5. NOS1AP alters dendrite number in young hippocampal neuron cultures. Cells were transfected with cDNA encoding GFP, GFP-NOS1AP-L (G-NPL), GFP-NOS1AP-S (G-NPS), or GFP-NOS1AP-M (G-CPM) at the indicated dates (***A***, DIV 0; ***B***, DIV 2; ***C***, DIV 5; ***D***, DIV 7). Neurons were fixed and immunostained for dendrite counting at DIV 5 (***A***), DIV 5 (***B***), DIV 7 (***C***), or DIV 10 (***D***). ***Aa***, ***Ba***, ***Ca***, ***Da***, GFP images of representative neurons. ***Ab***, ***Bb***, ***Cb***, ***Db***, ***Ac***, ***Bc***, ***Cc***, ***Dc***, Average number of primary and secondary dendrites in transfected neurons. Scale bar, 10 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by nonparametric ANOVA followed by Dunn's analysis comparing G-NPL, G-NPS, and G-NPM groups with GFP.

NOS1AP-S, and GFP-NOS1AP-M and fixed on DIV 5, DIV 7, and DIV 10, respectively. Examination of dendrite morphology revealed a similar level of reduction resulting from overexpression of GFP-NOS1AP-L and GFP-NOS1AP-M (Fig. 5*B–D*). These results indicate that NOS1AP-L can alter the dendritic development of hippocampal neurons at almost any early stage. On the other hand, overexpression of GFP-NOS1AP-S reduced branching when its expression was elevated from DIV 5 to DIV 7 (Fig. 5*B*), suggesting a possible role of NOS1AP-S in earlier stages of neuronal development. We replotted these data as a time course and confirmed that overexpression of NOS1AP-S only affects early dendrite development while NOS1AP-L affects all stages (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Thus, the actions of NOS1AP-L on neurons at different developmental stages suggests that NOS1AP-L alters both dendrite initiation and maintenance, while the limited effect of NOS1AP-short at a single developmental time point suggests that NOS1AP-S may only influence a specific stage of dendrite outgrowth and branching.

Coexpression of NOS1AP-S with NOS1AP-L does not alter the effects of NOS1AP-L

In contrast to NOS1AP-L, overexpression of NOS1AP-S had no direct effect on dendrite branching at most time points studied. Given that NOS1AP-L and NOS1AP-S share the same PDZ-binding domain, they could compete with each other for binding to the same substrates. In this way NOS1AP-S might be able to regulate the actions of NOS1AP-L. To test this, hippocampal neurons were cotransfected with constructs for NOS1AP-L and GFP-NOS1AP-S. The expression of NOS1AP-L was monitored with the goat-anti-NOS1AP antibody, which recognizes the N terminus of NOS1AP-L. Dendrite number of cotransfected neurons was assessed. Overexpression of GFP-NOS1AP-S did not change the effects of NOS1AP-L (Fig. 6), suggesting that NOS1AP-S may not play a direct regulative role in the actions of NOS1AP-L on dendrite branching.

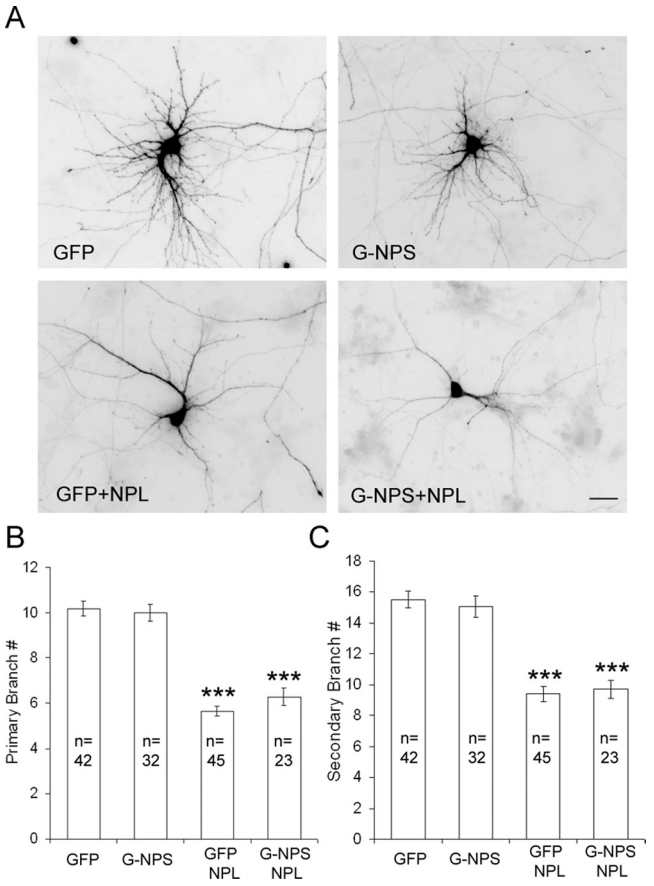

Figure 6. GFP-NOS1AP-S does not block the effects of NOS1AP-L on dendrite number. Hippocampal neurons were transfected on DIV 10 with cDNAs encoding GFP, GFP-NOS1AP-S (G-NPS), GFP plus NOS1AP-L (NPL), or GFP-NOS1AP-S plus NOS1AP-L. Neurons were fixed and immunostained for dendrite counting at DIV 12. ***A***, GFP images of representative neurons. Scale bar, 10 μm. ***B***, ***C***, Average number of primary and secondary dendrites in transfected neurons. ***$p < 0.001$, by nonparametric ANOVA followed by Dunn's analysis comparing cotransfected groups with GFP.

Identification of NOS1AP-L binding partners

In previous studies, a small number of binding proteins have been identified for the two distinct domains of NOS1AP (Fang et al., 2000; Jaffrey et al., 2002). However, our mutation studies indicate that NOS1AP-M, amino acids 181–307, is essential for NOS1AP-L-promoted decreases in dendrite number. No interacting proteins have yet been identified for this region. Therefore, we performed a yeast two-hybrid screen of a rat brain cDNA library to identify possible binding partners for NOS1AP-M, which served as bait for the screen. Several putative binding partners were identified. Out of the 19 positive clones identified, carboxypeptidase E (CPE, also termed carboxypeptidase H) represented 11.

To confirm the binding of NOS1AP and CPE in the brain, we performed a series of biochemical experiments. As shown in Figure 7, *A* and *C*, CPE and NOS1AP coimmunoprecipitate from adult rat brain lysate, verifying that the two proteins exist in a complex in the brain. Moreover, glutathione-*S*-transferase (GST) fusion protein affinity chromatography studies show that CPE binds to NOS1AP-M, compared with minimum binding by GST and GST-NOS1AP-S, confirming that the middle region of NOS1AP is responsible for CPE binding (Fig. 7*B*). These results, together with our yeast two-hybrid screen, suggest that NOS1AP-L directly binds to CPE via amino acids 181–307.

Carboxypeptidase E is a component of large dense-core vesicles in splenic nerve and of vas deferens (Schwarzenbrunner et al., 1990), PC12 cells (Laslop and Tschernitz, 1992), β-cell lines (Fiedorek and Parkinson, 1992), cultured neurons (Park et al., 2008), and Neuro2A cells (Normant and Loh, 1998). Interestingly, there are no published data on the subcellular localization of CPE in forebrain neurons. Thus, we prepared synaptosomes from adult rat cortex and separated membrane, cytosolic, and vesicular components using a glycerol gradient. As seen in Figure 7*D*, although some CPE is found in the large dense-core vesicle fraction, as evidenced by the presence of secretogranin II, the majority of CPE comigrates with syntaxin, a plasma membrane marker. Interestingly, NOS1AP is localized to all fractions (Fig. 7*D*). In contrast, nNOS is concentrated at the top of the gradient, which represents a soluble or cytosolic fraction. In addition, immunostaining of cultured hippocampal neurons shows that NOS1AP does not colocalize with secretogranin II (data not shown) and that CPE is evenly distributed throughout the dendrite (Fig. 8*A*). Together, our data suggest that a majority of CPE and NOS1AP complexes most likely do not occur at large core vesicles and that nNOS is not a component of this complex.

CPE mediates NOS1AP-promoted reduction in dendrite branching

To determine whether regulation of dendritic branching by NOS1AP-L is CPE-dependent, we constructed a CPE shRNA

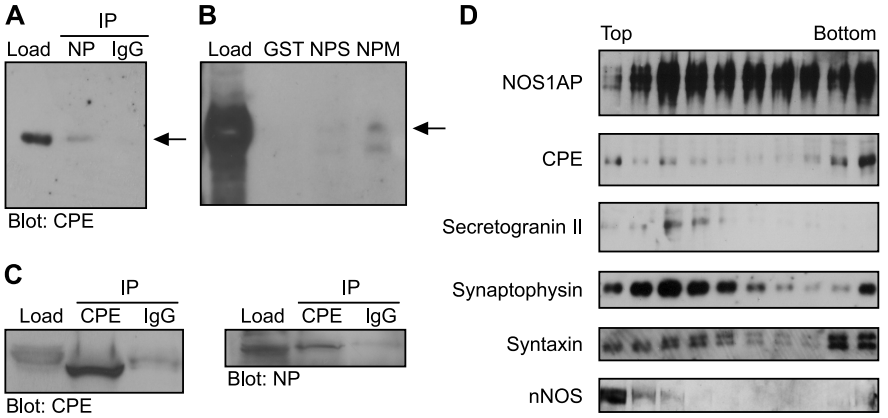

Figure 7. CPE binds to NOS1AP-M. ***A***, Immunoprecipitated proteins from rat brain by anti-NOS1AP or IgG were resolved using SDS-PAGE. Western blots were probed with an antibody to CPE. CPE coimmunoprecipitates with NOS1AP. ***B***, Lysates of rat brains were applied to GST fusion protein affinity columns. CPE binds to GST-NOS1AP-M (NPM) compared with minimal binding to GST and GST-NOS1AP-S (NPS). ***C***, Immunoprecipitated proteins from rat brain by anti-CPE or IgG were resolved using SDS-PAGE. Western blots were probed with antibodies to CPE and NOS1AP. NOS1AP coimmunoprecipitates with CPE. In all cases, a representative Western blot of three individual experiments is shown. ***D***, Equal volumes of fractions 1–10 from the top of a 5–25% glycerol velocity gradient of a synaptosome preparation analyzed by Western blotting. NOS1AP and CPE cofractionate predominantly with the synaptic plasma membrane marker syntaxin at the bottom of the gradient. A smaller amount of NOS1AP and CPE cofractionate with synaptophysin and secretogranin II in synaptic and large dense-core vesicles, respectively. nNOS is present in the luminal top fraction. Loads for coimmunoprecipitations represent 4% of total protein.

(pS-CPE) to knock down CPE protein levels. pS-CPE and an unrelated shRNA (negative-control, pS-NC) were subcloned into the pSUPER-GFP RNAi vector. Hippocampal neurons were transfected on DIV 10 with pS-CPE and pS-NC. Cells were fixed 48 h later and stained for CPE. By comparing the intensity of CPE immunostaining in neurons transfected with pS-CPE and pS-NC (Fig. 8*A*), we found that CPE shRNA attenuated CPE protein expression by ~40% (Fig. 8*B*). Coexpression of pS-CPE with NOS1AP-L attenuated the effects of NOS1AP-L in reducing primary and secondary dendrite numbers (Fig. 8*C–E*), suggesting that NOS1AP acts via a CPE-dependent pathway to regulate dendrite number. Furthermore, coexpression of pS-CPE with NOS1AP-M completely abolished the effects of NOS1AP-M (data not shown).

Discussion

NOS1AP-L was originally identified as a binding protein for nNOS (Jaffrey et al., 1998). It reduces the interaction of nNOS with PSD-95, an important scaffolding protein localized at the postsynaptic density of excitatory synapses. The PDZ-binding domain on the C terminus of NOS1AP-L competes with PSD-95 for nNOS binding. Further studies revealed that the PTB domain, located close to the N terminus of NOS1AP-L, binds to Dexras1 and synapsin (Fang et al., 2000; Jaffrey et al., 2002). Dexras1 is a member of the Ras small G-protein family. It is coupled to nNOS by binding to NOS1AP-L, and this proximity enables nNOS to elicit Dexras1 *S*-nitrosylation and activation, which then leads to iron uptake (Cheah et al., 2006). Our studies now establish that, in addition to these roles, NOS1AP-L regulates dendrite morphology in cultured hippocampal neurons, presenting an important function for NOS1AP in development and plasticity. Moreover, the possible involvement of NOS1AP with the pathophysiology seen in schizophrenia casts new insight into the mechanisms of this illness.

NOS1AP, schizophrenia, and dendrite morphology

During the past few years, comprehensive studies have revealed overwhelming evidence for a connection between schizophrenia and abnormal dendrite morphology. For example, postmortem examination of patients with schizophrenia has demonstrated that there are reductions in spine density, dendritic length, and complexity in the basilar dendrites of pyramidal cells in the prefrontal cortex (Glantz and Lewis, 2000; Kalus et al., 2000; Broadbelt et al., 2002; Kalus et al., 2002; Black et al., 2004). Moreover, examination of pyramidal dendrites in layers II/III in prefrontal cortex of rhesus macaques that underwent amphetamine sensitization, which elicited aberrant schizophrenia-like behaviors, demonstrated reduced overall dendritic branching and decreased peak spine density (Selemon et al., 2007). However, the molecular mechanisms underlying dendrite irregularities in schizophrenia have not been established.

Recent genetic studies revealed that NOS1AP is associated with schizophrenia (Rosa et al., 2002; Brzustowicz et al., 2004; Zheng et al., 2005; Kremeyer et al., 2009; Wratten et al., 2009). Moreover, the expression of NOS1AP is distinctively altered in postmortem brain tissue from patients with bipolar disorder or schizophrenia (Xu et al., 2005). NOS1AP-L mRNA expression is correlated with antipsychotic treatment history, with significantly reduced expression in patients of a given diagnosis who were treated with antipsychotics compared with untreated patients (Xu et al., 2005). NOS1AP-S mRNA did not demonstrate any changes in expression level based on medication treatment history, but was significantly increased in patients with schizophrenia and bipolar disorder compared with psychiatrically normal controls (Xu et al., 2005). The association of *NOS1AP* with schizophrenia and bipolar disorder implicates NOS1AP in the etiology of these psychiatric diseases. Moreover, our current study showing that NOS1AP-L reduces dendrite branching suggests a likely mechanism for NOS1AP-L to be involved in the pathology of schizophrenia and bipolar disorders.

We have used cultures of primary hippocampal neurons for our studies since they are more homogenous in their dendritic morphology than are cortical neurons. Even more importantly, the hippocampus has been implicated in the etiology of schizophrenia. Several research approaches, such as magnetic resonance imaging (MRI), proton magnetic resonance imaging, positron emission tomography (PET), neuropsychological, neurochemical, and neuropathological studies, as well as consideration of animal models, provide evidence for hippocampal involvement in schizophrenia (for review, see Harrison, 2004). In particular, a reduction of hippocampal size (Lawrie and Abukmeil, 1998; Nelson et al., 1998; Wright et al., 2000) and altered hippocampal shape (Csernansky et al., 2002) have been observed. Interestingly, this modification as well as several others found in schizophrenia have been observed in prodromal and first episode subjects supporting a causal role of hippocampal involvement in the disease (Harrison, 2004). At the cellular level, the most consistent change observed is an abnormal synaptic connectivity, supported by altered protein and mRNA levels of the presynaptic proteins synaptophysin and the synaptosomal associated protein SNAP-25 (Rehn and Rees, 2005). Thus, the studies presented here are relevant to the changes found in schizophrenia.

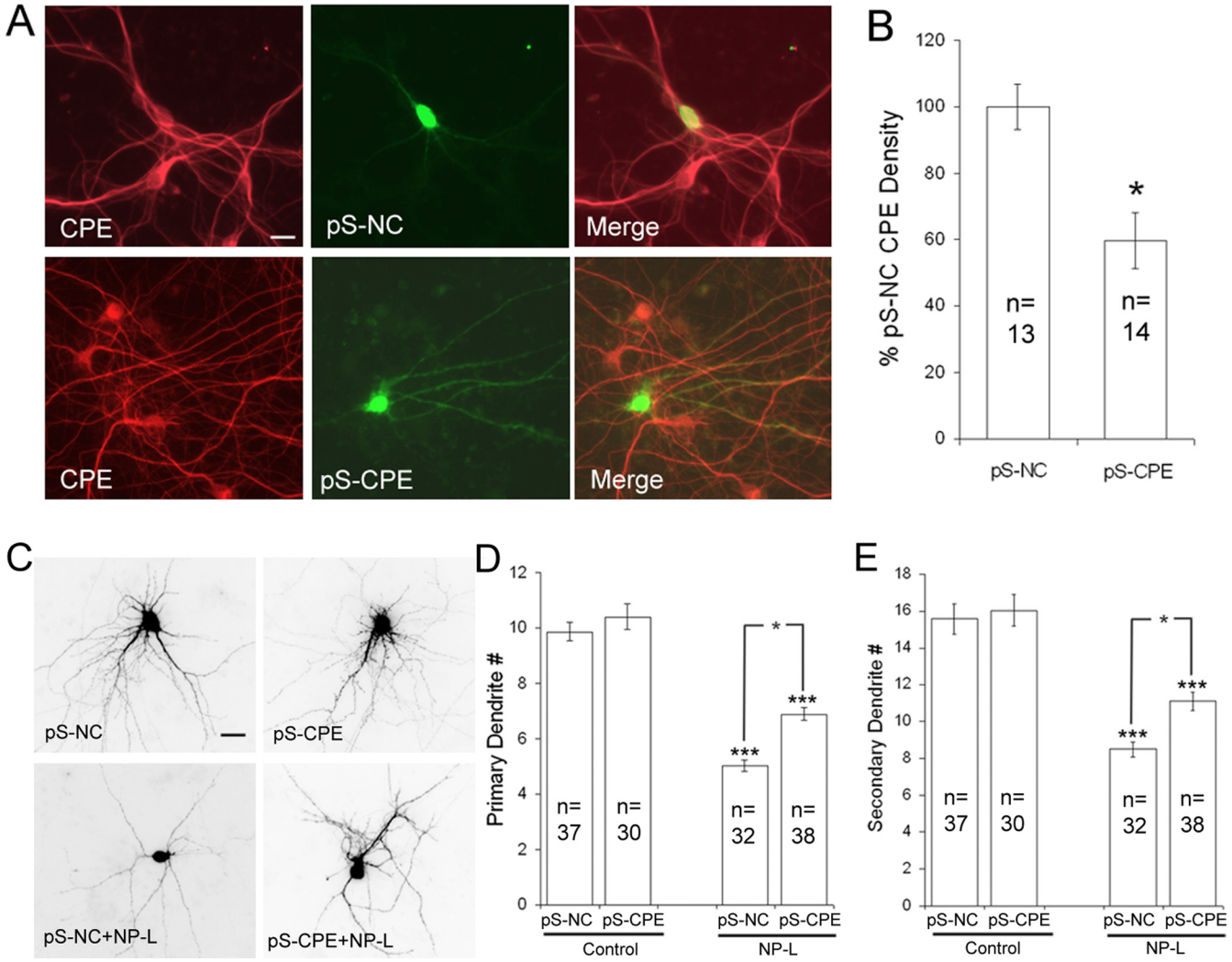

Figure 8. Knocking down CPE reduces the effects of NOS1AP-L. Hippocampal neurons were transfected on DIV 10 with pS-CPE and pS-NC. Cells were fixed 48 h later and immunostained for CPE. ***A***, Illustration of representative neurons transfected with pS-CPE or pS-NC and immunostained for CPE. Scale bar, 10 μm. ***B***, The intensity of CPE fluorescence was compared. *$p < 0.05$, by Student's *t* test. ***C***, GFP images of representative neurons transfected with pS-NC, pS-NC plus NOS1AP-L (NP-L), pS-CPE, or pS-CPE plus NP-L. ***D***, ***E***, Average number of primary and secondary dendrites in transfected neurons. *$p < 0.05$, by nonparametric ANOVA followed by Dunn's analysis comparing pS-NC plus NP-L with pS-CPE plus NP-L. ***$p < 0.001$, by nonparametric ANOVA followed by Dunn's analysis comparing cotransfected groups with pS-NC dendrite numbers.

NOS1AP isoforms and stages of dendrite development

We have used dissociated rat hippocampal neurons in culture to assess the roles of NOS1AP-L and NOS1AP-S on the establishment of mature dendrites and their branches. In our cultures, primary dendrites are extended from the cell body from DIV 1 until at least DIV 10 (stages 2–4) (Dotti et al., 1988). Active dendritic branching begins at approximately DIV 6 until approximately DIV 12 (stage 4) (Dotti et al., 1988; Akum et al., 2004). From DIV 12 until ~3 weeks in culture, a portion of the primary and secondary dendrites are pruned until the process of spine formation and maturation occurs (stage 5) (Dotti et al., 1988). We overexpressed the two isoforms of NOS1AP during these different developmental time points, and interestingly, we found that overexpression of NOS1AP-L reduced all phases of development, while overexpression of NOS1AP-S only reduced dendrite number from DIV 5–7. This suggests that NOS1AP-L may perform a long-term role in the initiation, growth, and maintenance of dendrites whereas NOS1AP-S plays a short-term role in regulating dendrite branching. Furthermore, the binding of CPE to the middle region of NOS1AP-L suggests that CPE also may play an important role in initiation, growth, and maintenance of dendrites. Thus, the effect of antipsychotics on NOS1AP-L may have relevance to maintaining mature dendrites as a treatment for psychotic disorders.

Possible roles played by nNOS and NOS1AP-S

Our current data support a regulatory role played by nNOS in mediating the effects of overexpression of NOS1AP-L on dendrite morphology. When nNOS was blocked by L-NAME, the reduction elicited by NOS1AP-L on dendrite branching was attenuated (Fig. 3). Moreover, the deletion of the PDZ-binding domain, which is responsible for NOS1AP/nNOS interactions, also results in a marginal decrease in these effects by NOS1AP-L (Fig. 4). These data suggest that while nNOS is not playing a central role in mediating the actions of NOS1AP, its presence enhances the reduction of dendrite branching elicited by NOS1AP-L.

Why does the inhibition of nNOS partially reverse the effect of NOS1AP-L on dendrite number, and why do overexpression of NOS1AP-L-ΔPDZ and NOS1AP-181-ΔPDZ have less of an effect

on dendrite numbers than does the middle region of NOS1AP, amino acids 181–307? Based on our data, we believe that NOS1 plays a modulatory role in NOS1AP-promoted decreases in dendrite number. Although not previously reported, one possibility is that NO produced by activation of NOS1 may result in nitrosylation of NOS1AP. *S*-Nitrosylation can be elicited nonenzymatically on cysteine residues. More likely, binding partners to NOS1AP-L may be nitrosylated. In fact, it has been reported that Dexras1, which binds to the PTB domain of NOS1AP, is nitrosylated (Cheah et al., 2006). The nitrosylation of NOS1AP-L or of NOS1AP-L binding partners could change the three-dimensional structure of NOS1AP-L, exposing the middle region and increasing the affinity of NOS1AP-L for other proteins, such as CPE. Thus, when only amino acids 181–307 are present, they can interact with CPE since there is no other portion of NOS1AP-L to keep this region hidden in the three dimensional structure. When NOS1 is inhibited, NOS1AP-L or its partners cannot be nitrosylated and cannot change NOS1AP-L conformation to allow maximal interaction with CPE. Thus, when NOS1AP-L-ΔPDZ and NOS1AP-181-ΔPDZ are expressed, there may be protein folding that weakens the interaction of NOS1AP with binding partners, and this folding can no longer be changed by nitrosylation.

While overexpression of NOS1AP-L dramatically reduces dendrite branching, overexpression of NOS1AP-S, which is an alternatively expressed form of NOS1AP, had no effect during most time periods studied (Figs. 1, 5). NOS1AP-S shares an identical C-terminal PDZ-binding domain with NOS1AP-L, which is responsible for the interaction of NOS1AP with nNOS. Thus, it is natural to hypothesize that NOS1AP-S can compete with NOS1AP-L for nNOS binding, and the reduction of nNOS binding to NOS1AP-L in turn reduces the effects of NOS1AP-L. Our experiments, however, failed to validate this hypothesis for this function of NOS1AP. The coexpression of GFP-NOS1AP-S and NOS1AP-L in cultured hippocampal neurons demonstrated a similar reduction in dendrite numbers and branching as that of coexpression of GFP and NOS1AP-L (Fig. 6). It should be noted that these results do not mean that NOS1AP-S does not play a role in modulating other functions of NOS1AP-L.

The involvement of CPE in actions of NOS1AP-L on dendrite morphology

Our yeast two-hybrid screening studies using NOS1AP-181–307 (NOS1AP-M) as bait and subsequent RNA interference experiments revealed that CPE is a likely candidate as a NOS1AP-L binding partner and mediator of NOS1AP-L's regulation of dendrite morphology (Figs. 7, 8). CPE is a member of the carboxypeptidase family that processes various proteins in the CNS (Fricker, 1988; Fricker and Leiter, 1999). It has also been shown to work as a sorting receptor for prohormones (Cool and Loh, 1998). A mutation in CPE in mice results in a reduction of CPE activity, lower expression, and early-onset obesity (Naggert et al., 1995). Further studies are still needed to address the underlying mechanisms for mediation of NOS1AP-L function by CPE.

An important but striking finding of our work is that the majority of CPE is not localized to large dense-core vesicles but is evenly distributed throughout dendrites. This is consistent with previously published data in sections of rat brain where strong immunoreactivity for endogenous CPE was detected in the cell bodies and dendrites of cortex, striatum, and hippocampus (Billova et al., 2007). In addition, two soluble forms of CPE have been identified with different N termini (Parkinson, 1990), and ~70% of CPE in the pituitary gland is soluble and 30% is membrane bound (Parkinson, 1992). These data suggest that CPE serves a function other than that of sorting and secretion of target substrates, such as brain-derived neurotrophic factor (BDNF) (Lou et al., 2005), which has been implicated in the regulation of dendrite development and maintenance (McAllister et al., 1995, 1997; Yacoubian and Lo, 2000; Horch and Katz, 2002; Horch, 2004). Interestingly, knockdown of CPE in our culture system has no effect on baseline dendritic growth parameters. Thus, NOS1AP actions on dendrite number most likely are not mediated via BDNF.

The cytoplasmic tail of CPE interacts with microtubule-based motors, such as dynactin and kinesin (Park et al., 2008). Although this interaction was identified for transmembrane CPE, the soluble forms of CPE share a common C-terminal sequence with that of the transmembrane CPE (Parkinson, 1990), suggesting that these forms can also interact with microtubule-based motors. Since motors, such as dynactin and kinesin, play a role in dendrite patterning (Homma et al., 2003; Satoh et al., 2008), NOS1AP may regulate the interaction between CPE and these motors to regulate dendrite patterning. Future studies will address this possibility.

If CPE is crucial to NOS1AP-L action on dendrites, then why is there no change when CPE is knocked down? Studies using CPE knock-out mice provide insight into this question. For example, these knock-out mice show normal CA3 pyramidal neuron number and morphology at 3 weeks of age (Woronowicz et al., 2008). In striking contrast, there is marked degeneration of these CA3 neurons, which normally express high levels of CPE, at 4 weeks of age and older (Woronowicz et al., 2008). Furthermore, lack of active CPE is also thought to exacerbate ischemic brain injury (Zhou et al., 2004). Thus, CPE itself may not normally serve to regulate neuronal development, but coupled with other signaling molecules, such as NOS1AP-L, may be important for maintenance of proper dendrite morphology or for normal brain function.

In summary, our studies not only reveal novel functions for NOS1AP and CPE but also provide a possible molecular mechanism to further explain the dendritic abnormalities in schizophrenia.

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