THE ROLE OF NOS1AP, A SCHIZOPHRENIA SUSCEPTIBILITY GENE, IN THE

REGULATION OF DENDRITE BRANCHING, DENDRITIC SPINE FORMATION,

AND ACTIN DYNAMICS.

by

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Bonnie L. Firestein, Ph.D.

And approved by

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ABSTRACT OF THE DISSERTATION

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Proper communication between neurons is dependent upon the appropriate patterning of dendrites and the correct distribution and structure of spines. Schizophrenia is one of several neurodevelopmental disorders that are characterized by alterations in dendrite branching and spine density. NOS1AP is a protein encoded by a schizophrenia susceptibility gene, and its expression is upregulated in the dorsolateral prefrontal cortex of patients with schizophrenia. Previously, our laboratory showed that NOS1AP isoforms negatively regulate dendrite branching in cultured rat hippocampal neurons. Since dendrites and spines are influenced by changes in the cytoskeleton, we investigated whether the overexpression of NOS1AP isoforms in heterologous cells alters actin and microtubule organization. Overexpression of a long isoform of NOS1AP (NOS1AP-L) increases the presence of microtubule organizing centers, whereas overexpression of the short isoform of NOS1AP (NOS1AP-S) decreases microtubule organization. Furthermore, NOS1AP isoforms associate with F-actin in rat brain and can alter actin

organization in distinct ways. NOS1AP-S increases actin polymerization, and its overexpression in HEK293T cells decreases total Rac1 and cofilin protein expression. To elucidate the role of NOS1AP in spine formation and synaptic function, we overexpressed NOS1AP isoforms in cultured rat cortical neurons. Overexpression of NOS1AP-L increases the number of immature spines, whereas overexpression of NOS1AP-S increases the number of mature and immature spines. In addition, overexpression of NOS1AP-S increases the frequency of miniature excitatory postsynaptic currents (mEPSCs) but not the amplitude. Overexpression of NOS1AP-L decreases the amplitude of mEPSCs but not the frequency. To investigate whether NOS1AP-L can mediate changes to dendrite patterning *in vivo*, we overexpressed NOS1AP-L in neuronal progenitor cells of the embryonic rat neocortex and analyzed dendrite patterning three weeks later. Neurons overexpressing NOS1AP-L in layers II/III of the neocortex exhibit a reduction in dendrite length and number. Finally, to investigate the role that NOS1AP plays in human dendritic arbor development, human neurons were generated using induced pluripotent stem cell technology. Overexpression of either NOS1AP-L or NOS1AP-S in human neurons results in a decrease in dendrite branching. Interestingly, treatment of human neurons with D-serine results in a reduction in NOS1AP-L protein expression. Taken together, our data support a role for NOS1AP-L and NOS1AP-S in dendritogenesis and synaptic function.

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# Introduction

## Neural dendrite and spine development

Neurons are highly specialized eukaryotic cells of the brain that are responsible for transmitting information throughout the body by way of both chemical and electrical signals. Communication among neurons occurs through processes, called axons and dendrites, that extend from the cell body. Neurons transmit signals through axons and receive signals through dendrites. During development, neurons undergo morphological changes in both discrete and overlapping stages, consisting of immature neurite outgrowth, axon specification, dendrite extension and branching, spine formation and maturation, and finally, synapse formation (Figure 1). Synapses are sites of communication between neurons and consist of the presynaptic axon terminal, a small gap termed the synaptic cleft, and a small portion of membrane on the postsynaptic neuron. In the vertebrate central nervous system, there are two predominating types of synapses, axosomatic and axodendritic. Axosomatic synapses are synaptic contacts between the axon of the presynaptic neuron and the soma of the postsynaptic neuron, while axodendritc synapses occur between axons and dendrites. Spines are small protrusions that develop along the surface of dendrites, and their structure allows for neurons to communicate efficiently by forming axodendritic synapses. The stages of neuronal development are commonly studied *in vitro* using dissociated cortical or hippocampal cultures from embryonic mice or rats. Using this system, neuronal development is reasonably consistent from laboratory to laboratory, and neurons can be easily observed and manipulated.

Proper neuronal function and circuitry is dependent upon the appropriate patterning of dendrites as well as the appropriate number and structure of spines.

Dendrite and spine development are dynamic processes that are influenced by both intracellular factors, such as small GTPases (Tashiro et al., 2000; Negishi and Katoh, 2005), and extracellular factors, such as neurotrophins (Dijkhuizen and Ghosh, 2005; Orefice et al., 2013). Several neurodevelopmental and neuropsychiatric disorders are characterized by alterations in dendrite branching and spine density, resulting in the manifestation of the disease symptoms (Penzes et al., 2011; Kulkarni and Firestein, 2012). Large-scale genetic studies have identified many candidate genes that confer risk to these disorders, some of which have been linked to molecular pathways that influence dendrite and spine development. Understanding how these genes regulate dendritogenesis and spinogenesis will provide insight into the etiology of these disorders and potentially identify novel molecular targets for drug development.

## Cytoskeleton of neurons

The cytoskeleton of all eukaryotes consists of three main components: microtubules, intermediate filaments, and actin filaments. In neurons, the intermediate filaments are termed neurofilaments, and they provide structural strength and stabilize the cytoskeletal framework. Neurons undergo unique morphological changes during development due to the carefully orchestrated dynamics of actin and microtubules. Microtubules are polymers composed of heterodimers of α- and β-tubulin subunits. Axons are composed mainly of microtubules that elongate by the addition of tubulin heterodimers to their fast, growing ends, termed the "plus" ends. Within axons, microtubules are oriented with their "plus" ends distal to the cell body. Dendrites are composed of both microtubules and actin filaments. The microtubules within dendrites

exhibit mixed polarity, with some microtubules oriented with their "plus" towards the cells body and some away from the cell body. Actin filaments are polymers of globular actin (G-actin) and elongate by the addition of G-actin monomers to both ends of the filament to form filamentous actin (F-actin). The rapidly polymerizing end is termed the "plus" end or "barbed" end while the slow growing end is called the "minus" end or "pointed" end. Actin filaments are particularly concentrated in axonal growth cones, axonal nerve terminals, and dendritic spines. Both microtubules and actin filaments undergo periods of growth, stabilization, and disassembly. Microtubule- and actin-associated proteins can influence the rate at which microtubule and actin filaments polymerize or depolymerize.

Microtubules serve as the substrate for the transport of membrane-bound organelles and proteins necessary for neuronal growth and function. In addition, both microtubules and actin filaments play key roles in dendrite and spine development. The formation of a new neurite or dendrite branch occurs when actin filaments are locally destabilized, actin-rich protrusions termed filopodia extend, and microtubules invade the filopodia, providing stabilization of the structure (Georges et al., 2008). Spine formation also begins with filopodia formation at localized actin-rich sites along the dendrite; however, microtubules only transiently enter spines to influence spine development (Gu et al., 2008; Hu et al., 2008; Jaworski et al., 2009). Spine head enlargement is intimately tied to actin polymerization and the formation of highly branched actin filaments by the actin-related protein 2/3 (Arp2/3) complex (Hotulainen and Hoogenraad, 2010). Investigating how proteins can regulate cytoskeletal dynamics is crucial for shedding

light on how these key processes can be perturbed in the disease state resulting in abnormal neuronal morphology and function.

## NOS1AP in schizophrenia

An estimated 1.1 percent of the U.S. population age 18 and older in a given year is affected by schizophrenia, a severe and devastating neuropsychiatric disorder. Schizophrenia displays a complex pattern of inheritance, suggesting the involvement of multiple genetic factors in combination with environmental factors. The complexity of such a disorder results in patients with the manifestation of some symptoms, but not others, as well as varying degrees of symptom severity. The variable number of symptoms characterized in patients with schizophrenia includes positive symptoms (e.g. hallucinations, delusions, agitation, and disorganized thought), negative symptoms (e.g. a lack of motivation and interest, introversion, and low self-esteem leading to personal neglect), and cognitive impairments (e.g. attention deficit, problems with "working memory", and poor executive functioning). Currently available antipsychotic medications are most effective in treating the positive symptoms of the illness, while there is little to no improvement in the treatment of the negative or cognitive symptoms (Horacek et al., 2006). While it is important to treat the debilitating effects of the acute positive symptoms of the illness, it is necessary to find efficacious treatments for the negative and cognitive symptoms in order to see long-term improvement in the quality of life for individuals with schizophrenia.

More than 500 genes have been reported to contribute to the susceptibility of schizophrenia, including nitric oxide synthase 1 (*NOS1*), NOS1 adaptor protein

(*NOS1AP*), neuregulin-1 (*NRG1*), multiple N-methyl-D-aspartate (NMDA) receptor subunit genes, synapsin I, II, and III (*SYN1*, *SYN2*, *SYN3*), and Disrupted in Schizohrenia (*DISC1*) (e.g. (Millar et al., 2000; Stefansson et al., 2003; Baba et al., 2004; Brzustowicz et al., 2004; Chen et al., 2004; Tang et al., 2006). *NOS1AP* has been shown to be a promising candidate gene for schizophrenia susceptibility (Brzustowicz, 2008). Several independent studies have reported linkage of schizophrenia to chromosome 1q21-22, a locus that contains *NOS1AP* (Shaw et al., 1998; Brzustowicz et al., 2000; Brzustowicz et al., 2002; Rosa et al., 2002; Hwu et al., 2003). One study identified significant linkage disequilibrium between six single nucleotide polymorphisms (SNPs) within *NOS1AP* and schizophrenia in a group of 24 medium-sized Canadian families (Brzustowicz et al., 2004). In addition, an association study found one SNP within *NOS1AP* and haplotypes constructed from three SNPs within *NOS1AP* to be significantly associated with schizophrenia in the Chinese Han population (Zheng et al., 2005). More recently, eight SNPs within *NOS1AP* were found to be significantly associated with schizophrenia in a South American population isolate, two of which had been identified by Brzustowicz and colleagues, further strengthening a link between *NOS1AP* and schizophrenia (Kremeyer et al., 2008). None of the SNPs within NOS1AP that associate with schizophrenia alter the amino acid sequence of the protein, suggesting that they may instead play a role in altering gene expression levels. Indeed, NOS1AP expression, at both the mRNA and protein level, is upregulated in human postmortem samples from the dorsolateral prefrontal cortex (DLPFC), a region of the brain associated with cognitive function, of individuals with schizophrenia in an American population (Xu et al., 2005; Hadzimichalis et al., 2010). Furthermore, the increased NOS1AP mRNA expression

found among the American samples was significantly correlated to three SNPs identified in the Canadian population previously found to be associated with schizophrenia. Using a luciferase reporter assay in two human neural cell lines, a SNP identified in *NOS1AP* and shown to be associated with schizophrenia in the Canadian population was demonstrated to increase gene expression by enhancing transcription factor binding (Wratten et al., 2009). The DLPFC has been widely implicated in the pathophysiology of schizophrenia, showing perturbations at the anatomical, neuropathological, and neurochemical levels (Bunney and Bunney, 2000). The identification of *NOS1AP* as a schizophrenia susceptibility gene by linkage and association studies across different populations coupled with the finding of increased expression of *NOS1AP* in the DLPFC of individuals with schizophrenia make studies on NOS1AP function particularly attractive for identifying new therapeutic targets for the treatment of cognitive symptoms in schizophrenia.

## Use of iPSC technology to develop a human neuronal model system

Induced pluripotent stem cell technology involves the reprogramming of somatic cells to a pluripotent state by the overexpression of key transcription factors, such as OCT4, c-Myc, SOX2, and KLF4, which have been found to maintain pluripotency in embryonic stem cells (ESCs; (Takahashi and Yamanaka, 2006)). The expression of these transcription factors results in the activation of pluripotency genes and proliferative and metabolic pathways and the repression of differentiation and lineage-specific genes. Induced pluripotent stem cells can give rise to any cell type from the three germ layers. The study of neurodevelopmental disorders can particularly benefit from iPSC

technology due to the lack of or inadequacy of existing animal models and the difficulty of accessing human neural stem cells and neurons for *in vitro* studies. Despite the genetic similarities between humans and rodents, differences are seen in the downstream effects of genetic alterations. As a result, animal models do not always show the same disease symptoms as is observed in humans (Inoue and Yamanaka, 2011). The development of drugs relies on cell lines for proof-of-concept studies and toxicity screenings. Just as with animal models, rodent cell lines are not able to fully mimic human biological processes. One reason for this is that the type and/or distribution of ion channels and receptors on the surface of the cell may be different from those in human cells (Sabir et al., 2008). Immortalized human cells lines are not ideal for disease studies because often the disease-relevant cell type is not available and the immortalization process alters native cellular responses (Ebert and Svendsen, 2010). In fact, compounds that have shown efficacy in cell lines and animals do not show therapeutic effects in humans, and in the worst case scenario, have toxic effects in humans (Laustriat et al., 2010). Therefore, the use of human neural cell cultures can greatly complement the use of cell lines and animal models for disease studies. With iPSC technology, the study of the differentiation process of human neural stem cells to mature neurons in a more physiologically relevant manner is now possible.

## Thesis Overview

Many neurodevelopmental and neuropsychiatric disorders are associated with perturbations in dendrite branching, spine morphology and density, and connectivity. The focus of this thesis will be on elucidating the role of two isoforms of *NOS1AP*, a

schizophrenia susceptibility gene, in the regulation of dendrite and spine development, as well as synaptic function. Chapter 1 will focus on how NOS1AP regulates actin dynamics and key regulators of actin polymerization and depolymerization. In Chapter 2, work focuses on how NOS1AP influences dendrite branching when altered in an *in vivo* system, the developing rat brain, and spine formation and synaptic function using a rat cortical cell culture system. Finally, Chapter 3 will establish a human neuronal cell culture system and investigate the role of NOS1AP in human dendritogenesis.

**Stage 1**  **Stage 2**  **Stage 3**  **Stage 4**

dendritic outgrowth
and branching

spine and synapse
development

immature neurite
outgrowth

axon specification

axonal
growth
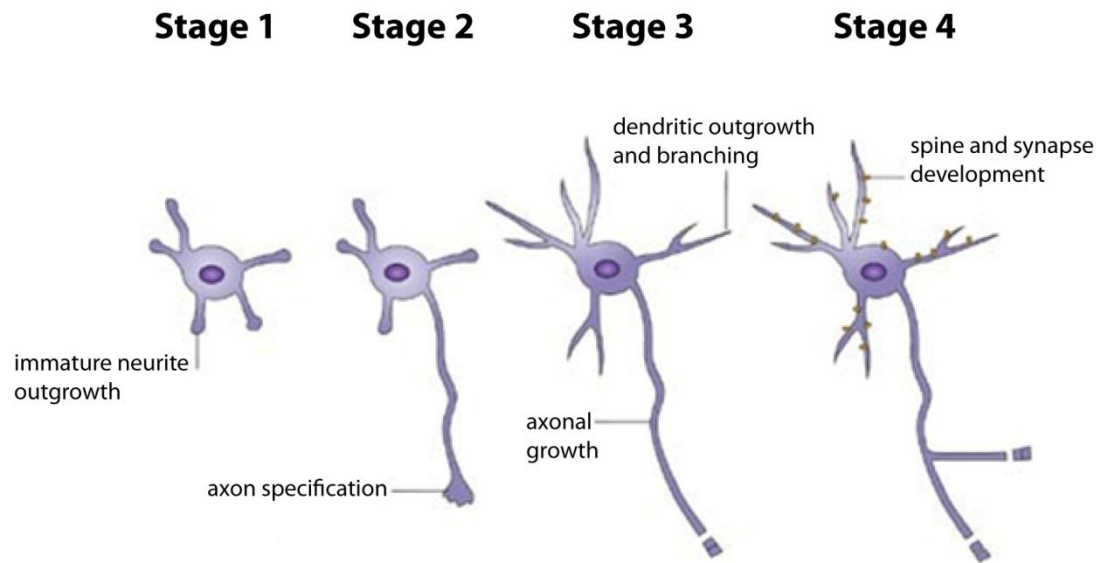
**Figure 1. Stages of neuronal development.** Schematic representation of the discrete and overlapping stages of development neurons undergo both *in vitro* and in vivo. Stage 1 represents the outgrowth of immature neurites from the cell body. At stage 2, one neurite grows at a faster rate than the other neurites and gets specified as the axon. The other neurites elongate and become mature dendrites during stage 3, followed by extensive branching. The last stage, stage 4, represents the formation of spines and synapses along the dendrites, which are necessary for synaptic neurotransmission. Figure adapted by author with permission from Macmillan Publishers Ltd: [Nature Reviews Neuroscience] Arimura N. and Kaibuchi K. Nat Rev Neurosci. 2007 Mar;8(3):194-205, copyright 2007.

# CHAPTER I:

Isoforms of NOS1AP regulate the actin cytoskeleton via

distinct mechanisms

## ABSTRACT

Proper dendrite and spine development is dependent on the tight regulation of actin dynamics. Our laboratory previously reported that two isoforms of NOS1AP, a long isoform (NOS1AP-L) and short isoform (NOS1AP-S), negatively regulate dendrite branching in rat hippocampal neurons. To elucidate the role of NOS1AP in the regulation of the cytoskeleton, we overexpressed a long (NOS1AP-L) or short (NOS1AP-S) isoform of NOS1AP in heterologous cells and investigated changes in actin and microtubule organization. Overexpression of NOS1AP-S increases the percentage of cells with irregular microtubules. In addition, both NOS1AP-L and NOS1AP-S alter actin organization, while only NOS1AP-L induces filopodia-like membrane protrusions. Overexpressing a mutant of NOS1AP-L lacking the PTB domain, the domain previously shown to influence Rac1 activation, blocks the induction of filopodia. We did not observe a change in activated Rac1 levels after overexpression of NOS1AP-S; however, we observed a significant decrease in total Rac1 protein levels. To further investigate how a reduction in Rac1 by NOS1AP-S overexpression can disrupt actin dynamics, we examined the activation state of cofilin, a downstream effector of Rac1. We observed a prominent decrease in cofilin total protein levels with NOS1AP-S overexpression, yet no change in the phosphorylated form of cofilin (P-cofilin). Overexpression of NOS1AP-L results in no change to either total cofilin levels or P-cofilin levels. Moreover, lysates from cultures expressing NOS1AP-S, but not NOS1AP-L, increase the rate of actin polymerization. Thus, our data suggest that NOS1AP-S, but not NOS1AP-L, acts to downregulate total Rac1 and the active, nonphosphorylated form of cofilin to promote actin reorganization.

## INTRODUCTION

Dynamic reorganization of the actin cytoskeleton is essential for numerous processes during neuronal development, such as dendritic growth, neuronal migration, and spine formation and maturation (Bellenchi et al., 2007; Georges et al., 2008; Jaworski et al., 2009). The Rho family of GTPases is composed of key intracellular regulators of dendritic development that act by influencing the actin cytoskeleton (Hall, 1994). GTPases are GTP-binding proteins that cycle between an active GTP-bound state and an inactive GDP-bound state. The three most commonly studied members of the Rho family of GTPases are Rac1, RhoA, and Cdc42. Activation of RhoA promotes the formation of actin stress fibers, and in neurons, this results in a reduction of dendrite growth (Hall, 1998). In contrast, both Rac1 and Cdc42 promote dendrite growth and branching, and in non-neuronal cells, induce lamellipodia and filopodia formation, respectively (Hall, 1998). When GTPases are in their active state, they transduce signals by binding to effector proteins, initiating a signaling cascade that directly influences actin dynamics (Figure I-1 A). A common signaling pathway among the three small GTPases involves the regulation of cofilin activity (Figure I-1 B). Cofilin is a member of the actin depolymerizing factor (ADF)/cofilin family of proteins that enhances the rate of actin filament turnover, both *in vivo* and *in vitro*, by severing and depolymerizing actin filaments (Carlier et al., 1997; Lappalainen and Drubin, 1997). The activity of cofilin is regulated by the phosphorylation of its Ser-3 residue, resulting in its inactivation (Moriyama et al., 1996). Activated Rho binds to and activates Rho-associated kinase (ROK) resulting in the phosphorylation of LIM-motif containing kinase (LIMK). Activated Rac and Cdc42 can also trigger LIMK activity by activating p21-activated

kinase (PAK), which then phosphorylates LIMK. Activated LIMK phosphorylates cofilin, which leads to the inactivation of its actin depolymerizing and severing activity. The inactivation of cofilin promotes increased stabilization of actin filaments and actin polymerization (Tybulewicz and Henderson, 2009).

Nitric oxide synthase 1 adaptor protein (NOS1AP) is an intracellular protein that influences dendrite branching, spine development, and neuronal migration (Carrel et al., 2009; Richier et al., 2010; Carrel et al., 2015). At least two isoforms of the NOS1AP protein have been identified (Jaffrey et al., 1998; Xu et al., 2005), with a third isoform recently reported by our group (Hadzimichalis et al., 2010). The longer isoform (NOS1AP-L), encoded by a ten exon mRNA transcript, consists of 501 amino acids and contains an amino-terminal phosphotyrosine-binding (PTB) domain and a carboxyl-terminal PDZ-binding motif, which stabilizes the interaction between NOS1 and NOS1AP (Li et al., 2015). The shorter isoform (NOS1AP-S), encoded by a transcript from the last two exons of the mRNA, consists of 211 amino acids and also contains the PDZ-binding motif at its carboxyl-terminus. The PTB domain of NOS1AP-L binds to Dexras1, synapsin, and Scribble (Fang et al., 2000; Jaffrey et al., 2002; Richier et al., 2010) and is responsible for the disruption of neuronal migration by NOS1AP-L during cortical development (Carrel et al., 2015). The PDZ-binding motif is important for stabilization of the binding of NOS1AP to neuronal nitric oxide synthase 1 (NOS1) (Jaffrey et al., 1998; Li et al., 2015), influencing NOS1 localization, and therefore, mediating nitric oxide (NO) signaling (Figure I-2).

Our laboratory previously reported that NOS1AP-L negatively regulates dendrite branching in cultures of primary rat hippocampal neurons at all time points examined but

that NOS1AP-S only transiently decreases dendrite number at an early time point in dendrite development (Carrel et al., 2009). NOS1AP-L binds to carboxypeptidase E (CPE) via a region not present in NOS1AP-S, mediating the effects of NOS1AP-L on dendrite branching (Carrel et al., 2009). CPE can exist in a soluble and a transmembrane form, and both have a C-terminal tail that can bind to dynactin. Dynactin is an adaptor protein that links CPE to microtubule motors, and this interaction is responsible for CPE-mediated transport of vesicles along microtubules (Cawley et al., 2012). The interaction of NOS1AP with CPE links NOS1AP to microtubule motors, which are important regulators of microtubule dynamics. In addition, one study found that overexpression of NOS1AP-L results in an increase in dendritic protrusions in rat hippocampal neurons and that the PTB domain is responsible for this effect (Richier et al., 2010). Although these studies have demonstrated that NOS1AP-L can influence both dendrite branching and spine formation in rat hippocampal neurons, it remains unclear whether NOS1AP isoforms do so by influencing actin or microtubule organization. Here, we investigate if overexpression of NOS1AP-L or NOS1AP-S in heterologous cells alters the cytoskeleton, with a focus on the actin cytoskeleton.

**A**
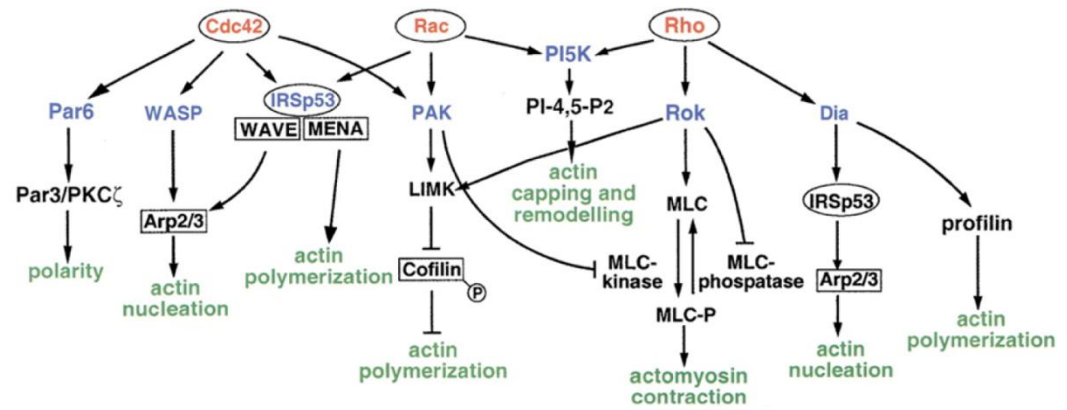


**B**
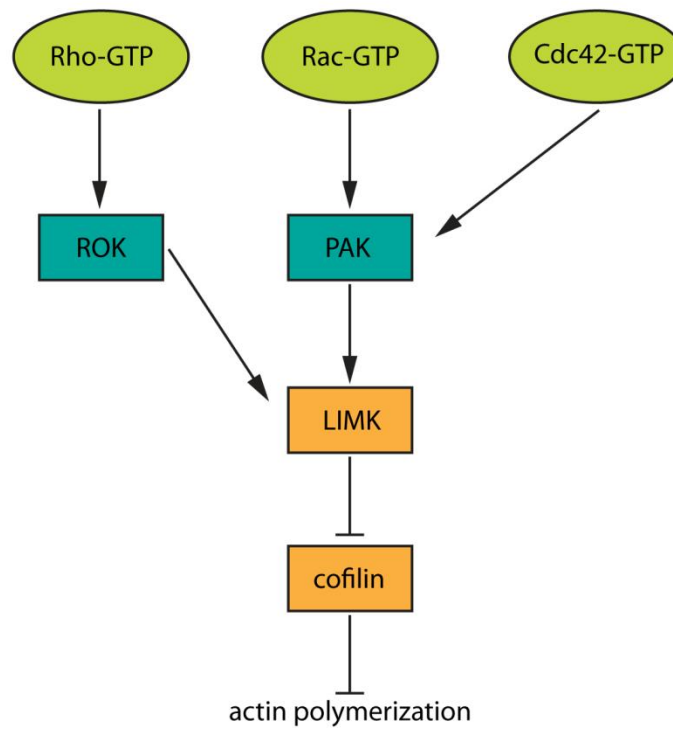
**Figure I-1. Downstream signaling pathways of the Rho family of GTPases. A,** Diagram of the effector proteins downstream of Cdc42, Rac, and Rho. The effector proteins mediate the numerous changes to the actin cytoskeleton that are initiated by the activated small GTPases. Diagram modified from Van Aelst and Symons, 2002. **B,** Diagram showing the cofilin signaling pathway, which is common to Cdc42, Rac, and Rho. GTP-bound Rho activates Rho-associated kinase (ROK) resulting in the phosphorylation of LIM-motif containing kinase (LIMK) by ROK. Activated Rac and Cdc42 can also activate LIMK by activating p21-activated kinase (PAK), which then phosphorylates LIMK. The activation of LIMK results in the phosphorylation of cofilin, inactivating its actin depolymerizing and severing activity and thereby promoting actin polymerization.

NOS1AP-L

Synapsin I, II, II
Dexras I
Scribble

nNOS

PTB
Domain

CPE-binding
Region

PDZ-binding
Motif

1    20                    180 181        307              489   501

NOS1AP-S

1                         199   211

PDZ-binding
Motif
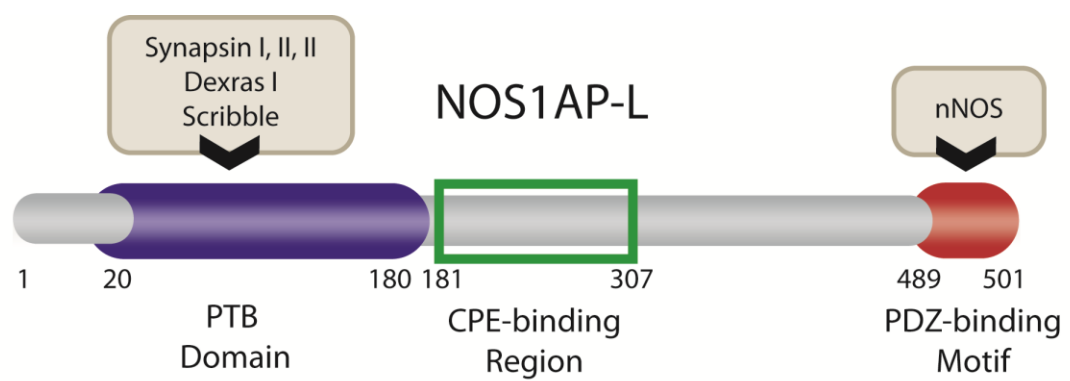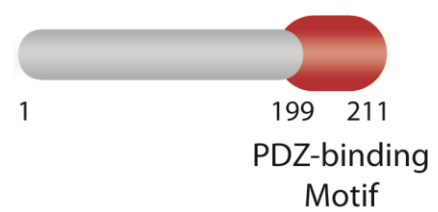
**Figure I-2. Domains of NOS1AP-Long and NOS1AP-Short Proteins.** The long isoform of NOS1AP (NOS1AP-L) produces a protein with an N-terminal phosphotyrosine binding domain (PTB; amino acids 1–180), a carboxypeptidase E (CPE)-binding region (amino acids 181–307), and a C-terminal PDZ-binding motif (amino acids 489–501). The short isoform of NOS1AP (NOS1AP-S) produces a protein with only the PDZ-binding motif. NOS1AP-L and NOS1AP-S have been identified as important adaptor proteins that can influence neuronal function and development. PDZ, postsynaptic density protein (PSD95), *Drosophilia* discs large tumor suppressor, and zonula occludens-1 protein domain. Figure adapted from Carrel et al., 2015.

# MATERIALS AND METHODS

## Statistics

All statistics were calculated using the Prism 5.0 software from GraphPad (La Jolla, CA). Tests used are noted in figure legends.

## Antibodies

Mouse monoclonal anti-acetylated tubulin and mouse monoclonal anti-actin from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal GAPDH antibody was from Millipore (Billerica, MA). Chicken and goat polyclonal green fluorescent protein (GFP) antibodies were from Rockland Immunochemicals (Limerick, PA). Mouse monoclonal Rac1 antibody was from Cytoskeleton, Inc (Denver, CO). Alexa Fluor® 647 phalloidin and chicken secondary antibody conjugated to Alexa-Fluor® 488 were from Life Technologies (Grand Island, NY). Mouse monoclonal cofilin antibody was from BD Biosciences (San Jose, CA) and rabbit monoclonal Phospho-cofilin (Ser3) antibody was from Cell Signaling Technology (Danvers, MA).

## DNA constructs

pCAG-GFP was obtained by subcloning EGFP from pEGFP-C1 (Clontech; Mountain View, CA) into a vector with CMV–actin–β-globin promoter (pCAG). cDNAs encoding long and short isoforms of human NOS1AP (NOS1AP-L and NOS1AP-S), NOS1AP-L-214-end (NOS1AP-L-ΔPTB), NOS1AP-L-1-487 (NOS1AP-L-ΔPDZ), and NOS1AP-L-181-307 (NOS1AP-M) were subcloned into pCAG-GFP as described previously (Carrel et al., 2009).

**Western Blotting of COS-7 cell lysates**

COS-7 cells were cultured in 60 mm dishes and transfected at 30-50% confluency with pCAG-GFP, pCAG-GFP-NOS1AP-L, or pCAG-GFP-NOS1AP-S using Lipofectamine 2000 following the manufacturer's protocol. Cells were collected two days after transfection and lysed, and expression of actin, GFP, and GAPDH was detected by immunoblotting after resolving proteins using SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes (Immobilon-P; Millipore). After blocking with 2% bovine serum albumin (BSA) in Tris-buffered saline (500 mM Tris, pH 7.4, 60 mM KCl, 2.8 M NaCl) with 1% Tween-20 (TBST), membranes were incubated with primary antibodies overnight at 4°C: 1:1000 for mouse anti-actin, 1:1000 for mouse anti-GAPDH, or 1:500 for goat anti-GFP. After washing, horseradish peroxidase-linked secondary antibody was applied at 1:5000 for one hour at RT. Immunoreactive bands were visualized using HyGlo quick spray (Denville Scientific; South Plainfield NJ) and quantified using Image Pro software (Media Cybernetics).

**Transfection of COS-7 cells and Immunocytochemistry for F-actin**

COS-7 cells were plated onto 0.1 mg/ml poly-d-lysine hydrobromide (Sigma-Aldrich) – coated coverslips at 10,550 cells/cm2 and transfected with pCAG-GFP, pCAG-NOS1AP-L, pCAG-NOS1AP-S, pCAG-NOS1AP-L-ΔPTB, pCAG-NOS1AP-L-ΔPDZ, or pCAG-NOS1AP-M using Lipofectamine 2000 (Life Technologies) following the manufacturer's protocol. Forty-eight hours after transfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 minutes and immunostained for GFP using chicken anti-GFP (1:500) and Alexa-Fluor® 488 anti-chicken (1:500) and for filamentous actin

(F-actin) using Alexa-Fluor® 647-Phalloidin, followed by nuclear staining with Hoechst dye. Coverslips were mounted onto glass slides using Fluoromount G (Southern Biotechnology; Birmingham, AL). For F-actin content analysis, cells were imaged at 600x with a fixed exposure time among experimental conditions using an Olympus Optical (Tokyo, Japan) IX50 microscope with a Cooke Sensicam CCD cooled camera, fluorescence imaging system, and ImagePro software (MediaCybernetics; Silver Spring, MD). Cells were traced with the experimenter blinded to the condition using ImageJ (NIH; Bethesda, MD) to quantify the mean fluorescence intensity of Alexa Fluor® 647-phalloidin staining.


**Transfection of COS-7 cells and Immunocytochemistry for Microtubules**

COS-7 cells were plated onto 0.1 mg/ml poly-d-lysine hydrobromide (Sigma-Aldrich) – coated coverslips at 10,550 cells/cm2 and transfected with pCAG-GFP, pCAG-NOS1AP-L, or pCAG-NOS1AP-S using Lipofectamine 2000 (Life Technologies) following the manufacturer's protocol. Forty-eight hours after transfection, cells were fixed with prewarmed PHEM buffer (60 mM PIPES, 21 mM HEPES, 10 mM EGTA, 2 mM MgCl2, 685 mM NaCl, pH7.5) containing 0.1% Triton-X-100 and 0.5% glutaraldehyde for 15 minutes. The fixation solution was removed and a 2 mg/mL sodium borohydride solution in 1x PBS was immediate added for 15 min followed by a wash with 1x PBS. Fixed cells were immunostained for GFP using chicken anti-GFP (1:500) and acetylated tubulin using mouse anti-acetylated tubulin (1:1000). Coverslips were mounted onto glass slides using Fluoromount G (Southern Biotechnology; Birmingham, AL). Cells were imaged at 600x using an Olympus Optical (Tokyo, Japan) IX50 microscope with a Cooke Sensicam

CCD cooled camera, fluorescence imaging system, and ImagePro software (MediaCybernetics; Silver Spring, MD). Cells were analyzed for presence of microtubule organizing center (MTOC) and microtubule organization with the experimenter blinded to the condition using ImageJ (NIH; Bethesda, MD).

**Rac Activation Assay and Western Blotting of HEK293T cell lysates**

Rac1 activation assay was performed with Rac1 Pull-down Activation Assay Biochem Kit using manufacturer's protocol (Cytoskeleton, Inc). HEK293T cells were transfected (30-50% confluency) with pCAG-GFP, pCAG-GFP-NOS1AP-L, or pCAG-GFP-NOS1AP-S using the calcium phosphate method (Kwon and Firestein, 2013), incubated overnight, and incubated in serum-free medium for an additional 24 h. Medium was changed to serum-containing medium for 10 min before scrape-harvesting protein. Cells were harvested in lysis buffer (50mM Tris pH 7.5, 10mM MgCl2, 0.5M NaCl, and 2% Igepal) supplemented with protease inhibitors (62 μg/ml Leupeptin, 62 Ég/ml Pepstatin A, 14 mg/ml Benzamidine and 12 mg/ml tosyl arginine methyl ester) and 1 mM sodium orthovanadate, pH 10. A positive control, GTPγS, was included, and the pull-down of activated Rac1 was performed using 20 μg PAK-PBD beads according to the manufacturer's protocol. Western blotting was performed for activated Rac1, total Rac1 using mouse anti-Rac1 (1:500), cofilin using mouse anti-cofilin (1:2500), phosphorylated-cofilin using rabbit anti-Phospho-cofilin (1:1000), and GAPDH using mouse anti-GAPDH (1:1000). Immunoreactive bands were quantified using Image Pro software.

*In Vitro* **Pyrene-Actin Polymerization Assays**

The rate of non-muscle actin polymerization in the presence of lysates from cultures overexpressing GFP, GFP-NOS1AP-L or GFP-NOS1AP-S was monitored according to the methods outlined in the Actin Polymerization Biochem Kit (Cytoskeleton, Inc). HEK293T cells were cultured in 10 cm dishes and transfected at 30-50% confluency with pCAG-GFP, pCAG-GFP-NOS1AP-L, or pCAG-GFP-NOS1AP-S using the calcium phosphate method. Forty-eight hours later, total protein was extracted in Buffer A (20 mM Tris-HCl, pH7.5, 20 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF)). Protein lysates were diluted to 1.5 mg/ml with Buffer A lacking Triton X-100 for final 0.1% [Triton X-100]. Pyrene-labeled rabbit muscle actin and human non-muscle actin (Cytoskeleton, Inc.) were mixed in a 1:10 ratio to monitor non-muscle actin polymerization. The 1:10 mixture of pyrene-muscle actin and unlabeled non-muscle actin was diluted to 0.45 mg/ml in G-buffer (5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, and 0.2 mM ATP). Pyrene muscle actin will not polymerize efficiently on its own at the concentration used in this assay, so the reaction is dependent on non-muscle actin polymerization for F-actin formation. In vitro polymerization assays (200 μl) were performed in black with clear bottom 96-well plates (Corning; Corning, NY). Duplicate or triplicate wells were assayed for G-buffer; pyrene-actin, lysis buffer (20 mM Tris-HCl, pH7.5, 20 mM NaCl, 0.1% Triton X-100, 1 mM PMSF); pyrene-actin, GFP; pyrene-actin, NOS1AP-L; and pyrene-actin, NOS1AP-S. Polymerization reactions were started 30 s prior to measurement by addition of 20 μl 10× actin polymerization buffer (Cytoskeleton, Inc.). The increase in pyrene fluorescence following polymerization was measured with CytoFluor Series 4000 fluorescence plate reader (Applied Biosystems,

Life Technologies): excitation, 360 ± 40 nm, emission, 460 ± 40 nm every 30 s. To quantify changes in polymerization rate, linear regression was performed using GraphPad, Prism (San Diego, CA) to calculate the Vmax for the growth phase of polymerization.

# RESULTS

**Overexpression of NOS1AP isoforms alters microtubule organization.**

We recently reported that both NOS1AP-L and NOS1AP-S can regulate dendrite branching distinctly during different time points of dendrite development (Carrel et al., 2009). Furthermore, NOS1AP-L exerts its effect on dendrite branching through a CPE-dependent pathway. To investigate whether NOS1AP can influence microtubule organization, we overexpressed NOS1AP-L or NOS1AP-S in COS-7 cells, a cell type in which changes in cytoskeletal organization can be more easily observed. Microtubule organization was monitored by immunofluorescence for acetylated tubulin. Control cells often exhibit a prominent microtubule-organizing center (MTOC) localized near the nucleus with microtubule bundles emanating from the MTOC in an astral fashion (Figure I-A). When NOS1AP-L is overexpressed in COS-7 cells, there is a lower percentage of cells without a prominent MTOC compared to control cells (Figure I-3 A,B). In contrast, a higher percentage of cells overexpressing NOS1AP-S do not exhibit a prominent MTOC compared to control cells (Figure I-3 A,B). Furthermore, overexpression of NOS1AP-S increases the percentage of cells with irregular microtubules, as observed by the circuitous or winding microtubule bundles (Figure I-3 A,C). Taken together, these data indicate that both NOS1AP-L and NOS1AP-S influence microtubule organization in distinct ways.

**A**

|  | Control | NOS1AP-L | NOS1AP-S |
| GFP | | | |
| Acetylated tubulin | | | |

**B**

% Cells without a prominent MTOC

**C**

% Cells with irregular MTs
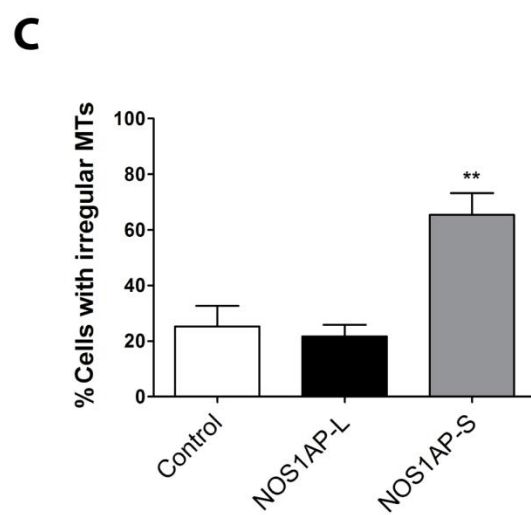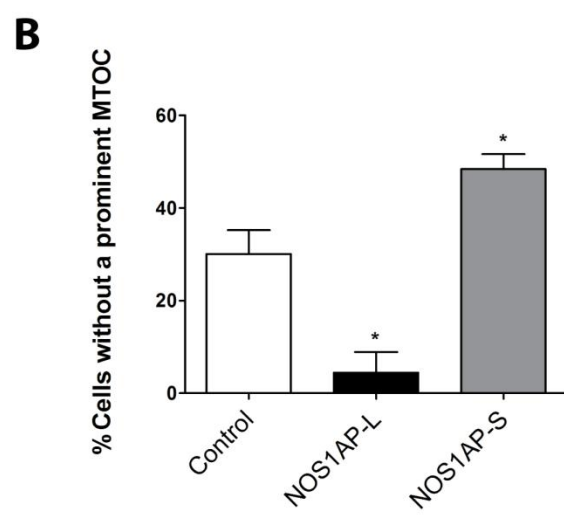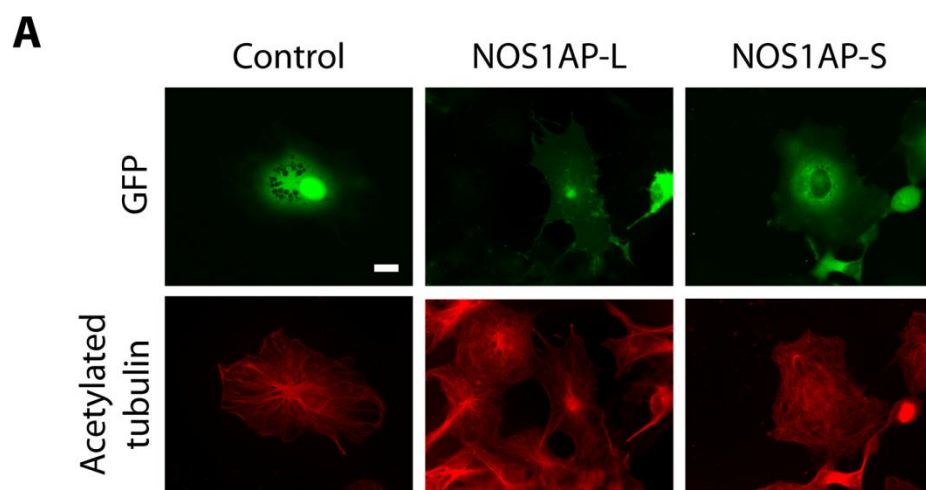
**Figure I-3. NOS1AP isoforms alter microtubule organization. A,** Representative images of anti-acetylated tubulin immunofluorescence of cells expressing GFP (Control), GFP-NOS1AP-L, or GFP-NOS1AP-S. **B,** Percentage of GFP-positive cells without a prominent microtubule organizing center (MTOC) 48 hours after transfection of COS-7 cells with plasmids encoding the indicated proteins. **C,** Percentage of GFP-positive cells with irregular microtubule organization 48 hours after transfection of COS-7 cells with plasmids encoding the indicated proteins. *$p < 0.05$ and **$p < 0.01$ versus GFP control. p values were determined by one-way ANOVA followed by Dunnett's Multiple Comparisons test. Error bars indicate ± s.e.m. n = 3 for each condition, representing 54 cells analyzed for GFP condition; 38 cells for NOS1AP-L; and 56 cells for NOS1AP-S. Scale bar = 10 μm.

**NOS1AP alters actin organization and cell morphology when overexpressed in COS-7 cells.**

We reported that NOS1AP-L and NOS1AP-S regulate dendrite branching (Carrel et al., 2009), and others reported that NOS1AP-L regulates dendritic spine development (Richier et al., 2010) in rat hippocampal neurons. To gain insight into how NOS1AP plays a role in these two cytoskeleton-based processes, we overexpressed NOS1AP-L or NOS1AP-S in COS-7 cells and analyzed actin expression 48 hours post-transfection. We found no difference in total actin protein when either isoform is overexpressed (Figure I-4 A,B). During new dendritic branch or spine formation in neurons, distinct types of reorganization of the actin cytoskeleton need to occur (Hotulainen and Hoogenraad, 2010). To investigate the role of NOS1AP isoforms in regulating actin organization, we characterized shape and measured F-actin content of cells overexpressing NOS1AP isoforms. Control cells exhibit typical fibroblast-like morphology (Fig. I-4 C), and the actin cytoskeleton is characterized by the presence of stress fibers and diffuse F-actin immunofluorescence, which we note as "actin organization." Expression of NOS1AP-L or NOS1AP-L-ΔPDZ, lacking the PDZ-binding motif, induces thin, long, and sometimes branched membrane protrusions, accompanied by a decrease in F-actin content (Fig. I-4 C,D), suggesting that the PDZ-binding motif is nonessential for this function of NOS1AP-L. Cells expressing NOS1AP-S or NOS1AP-L-ΔPTB, lacking the PTB domain, show normal shape, although the organization of actin is altered as shown by the decrease in F-actin staining (Fig. I-4 C,D). Expression of NOS1AP-M, the middle region in NOS1AP-L responsible for the effects of NOS1AP-L on dendrite branching (Carrel et al., 2009), has no effect on cell shape or actin organization. This region is responsible for

binding to carboxypeptidase E and is not present in NOS1AP-S, suggesting that NOS1AP-L regulates dendrite branching via a distinct domain and mechanism than it acts to regulate actin organization. Thus, the PTB domain is responsible for induction of filopodia-like membrane protrusions observed with NOS1AP-L overexpression, while an unknown shared region between NOS1AP-L and NOS1AP-S is responsible for the reduction in the diffuse F-actin staining. Our data suggest that NOS1AP-L and NOS1AP-S play roles in regulating actin organization via distinct mechanisms.

**A**



**B**



**C**



**D**
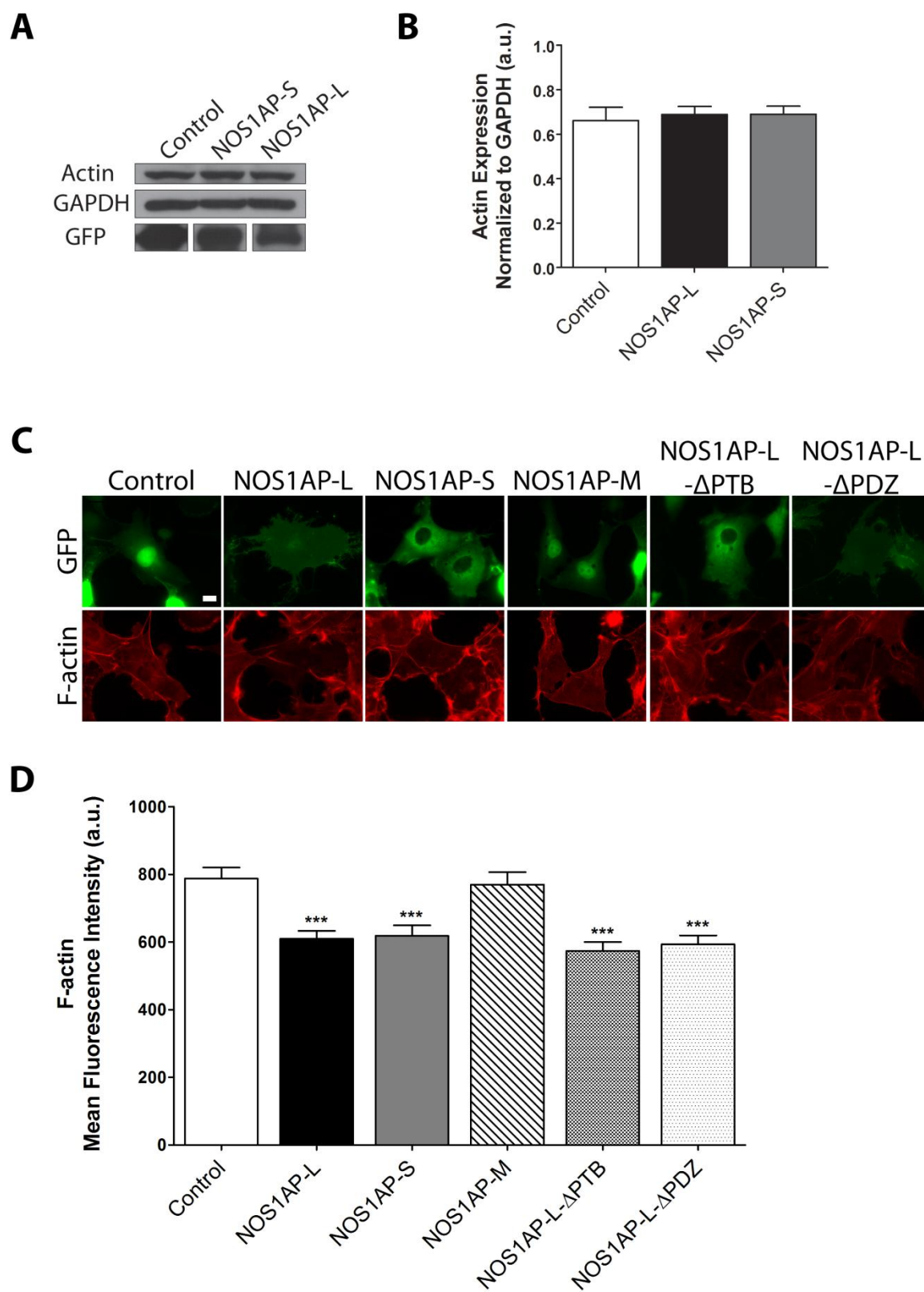
**Figure I-4. Expression of NOS1AP-L or NOS1AP-S in COS-7 cells decreases F-actin. A,** Extracts from cultures of transfected COS-7 cells expressing GFP (control), GFP-NOS1AP-L, or GFP-NOS1AP-S were resolved by SDS-PAGE and analyzed by Western blotting using antibodies that recognize actin or GAPDH. Representative blot is shown. **B,** Densitometry analysis of multiple blots represented in A. Error bars indicate ± s.e.m. n=6 for all conditions. a.u., arbitrary units. **C,** Representative images of Alexa Fluor® 647 phalloidin staining of cells expressing GFP (Control), GFP-NOS1AP-L, GFP-NOS1AP-S, GFP-NOS1AP-M, GFP-NOS1AP-L-ΔPTB, or GFP-NOS1AP-ΔPDZ. **D,** Intracellular F-actin content determined by Alexa Fluor® 647 phalloidin fluorescence intensity 48 hours after transfection of COS-7 cells with plasmids encoding the indicated proteins. ***$p < 0.001$ versus Control. p values were determined by one-way ANOVA followed by Dunnett's Multiple Comparisons test. Error bars indicate ± s.e.m. n = 36 cells, GFP; n = 36, NOS1AP-L; n = 36, NOS1AP-S; n = 36, NOS1AP-M; n = 34, NOS1AP-L-ΔPTB; n = 35, NOS1AP-ΔPDZ; from three experimental replicates. Scale bar = 10 μm.

**NOS1AP-S decreases total Rac1 protein expression.**

The Rho family of GTPases, including Rac1, are regulators of dendritic development by influencing the actin cytoskeleton (Nakayama et al., 2000; Tashiro et al., 2000; Negishi and Katoh, 2005; Zhang et al., 2005; Sekino et al., 2007). However, reorganization of the actin cytoskeleton may occur in a Rac1-independent manner (Papakonstanti and Stournaras, 2002). It has been reported that NOS1AP-L increases the activation of Rac1 (Richier et al., 2010). To investigate whether NOS1AP-S activates Rac1, we expressed NOS1AP-L or NOS1AP-S in HEK293T cells and measured the levels of GTP-bound Rac1 (Fig. I-5 A,B). We did not observe a change in activated Rac1 levels after NOS1AP-S overexpression and failed to observe consistent activation of Rac1 after NOS1AP-L overexpression (Fig. I-5 C). This may be due to variability in the responsiveness of the cells to Rac1 activation, although cells were subjected to the standard procedure for serum-starvation before examining activation of Rac1.

Activation of Rac1 is not the sole mechanism by which Rac1 may act to alter actin organization. Decreased total Rac1 levels, rather than amount of Rac1 activation, have been shown to inhibit the stabilization of actin-rich protrusions, affecting overall actin organization (Yip et al., 2007). As such, we examined whether overexpression of either NOS1AP isoform results in changes to overall Rac1 levels. Cells overexpressing NOS1AP-S, but not NOS1AP-L, demonstrate a decrease in total Rac1 protein (Fig. I-5 D). These data suggest that NOS1AP-L and NOS1AP-S regulate the Rac1 signaling pathway in distinct ways to influence the actin cytoskeleton.
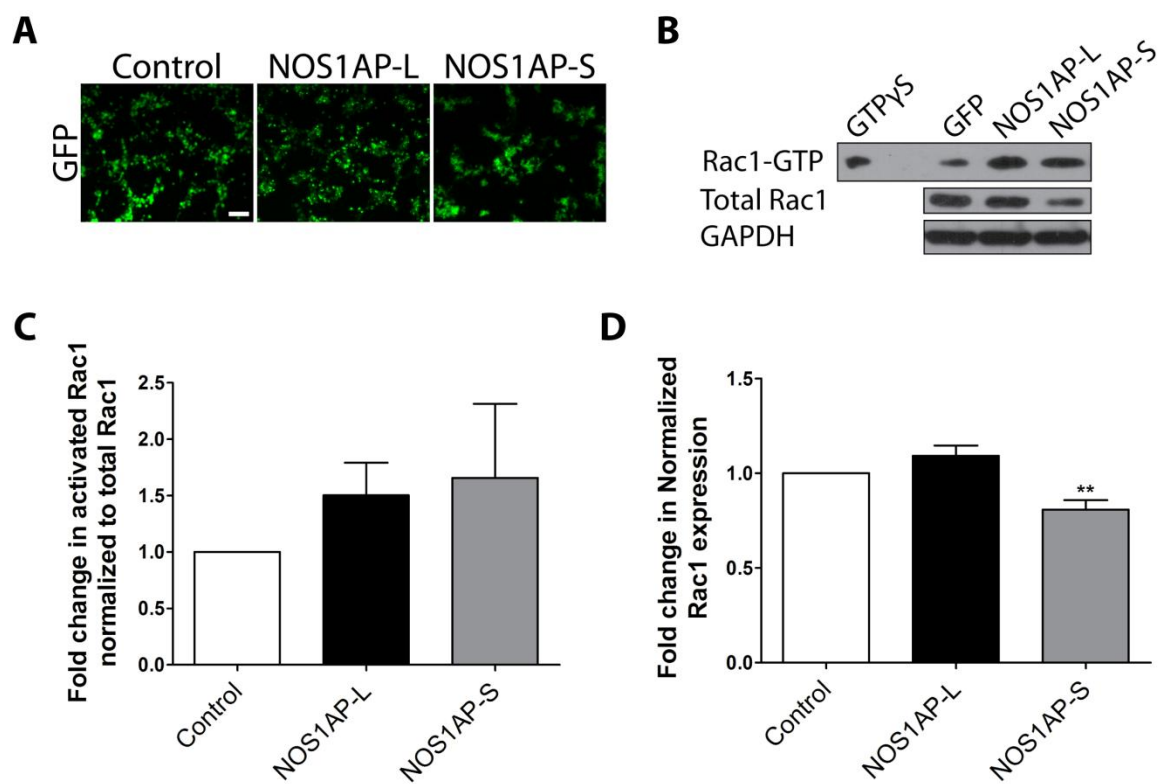
**Figure I-5. Expression of NOS1AP-S in HEK293T cells decreases total Rac1. A,** Representative images of HEK293T cells expressing GFP (control), GFP-NOS1AP-L, or GFP-NOS1AP-S. Scale bar = 100 μm. **B,** *Upper blot*, lysates from HEK293T cells overexpressing indicated proteins were incubated with PAK-PBD beads, and retained proteins were resolved by SDS-PAGE and analyzed by Western blotting using antibodies that recognize Rac1 to determine the amount of activated Rac1 (Rac1-GTP). GTPγS was included as a positive control. *Lower blots*, Lysates from HEK293T cells overexpressing indicated proteins were resolved by SDS-PAGE and analyzed by Western blotting using antibodies that recognize Rac1 and GAPDH to determine total Rac1 protein levels. Representative blot is shown. **C,** Relative quantification of Rac1-GTP normalized to total Rac1 protein from multiple blots represented in B. Error bars indicate ± s.e.m. n = 7 for all conditions. **D,** Relative quantification of total Rac1 protein normalized to control from multiple blots represented in B. Error bars indicate ± s.e.m. n = 9 for all conditions. All analyses were performed by first normalizing to GAPDH as an internal loading control and then comparing experimental condition to GFP control condition. **p< 0.01 versus control. p values were determined by one-way ANOVA followed by Dunnett's Multiple Comparisons test.

**NOS1AP-S decreases active cofilin and increases the proportion of inactive cofilin.**

To further investigate how a reduction in Rac1 levels, resulting from overexpression of NOS1AP-S, can disrupt actin dynamics, we assessed the activation state of cofilin, a common downstream effector of Rac1 and other Rho family GTPases. Cofilin is a member of the actin depolymerizing factor (ADF)/cofilin family of proteins and enhances the rate of actin filament turnover, both *in vivo* and *in vitro*, by severing and depolymerizing actin filaments (Carlier et al., 1997; Lappalainen and Drubin, 1997). The activity of cofilin is regulated by phosphorylation of its Ser-3 residue, resulting in its inactivation (Moriyama et al., 1996). When NOS1AP-S is overexpressed in HEK293T cells, a decrease in total cofilin protein levels results; however, there is no change in levels of the inactive, phosphorylated form of cofilin (P-cofilin) (Figure I-6 A-D). In contrast, overexpression of NOS1AP-L results in no change in total cofilin levels or P-cofilin levels (Figure I-6 A-D). To elucidate any changes in cofilin activity, we normalized P-cofilin levels to total cofilin, which allows for the analysis of the active, non-phosphorylated form of cofilin. We found that overexpression of NOS1AP-S decreases the levels of the active cofilin, resulting in an increase in the ratio of inactive cofilin to total cofilin (Figure I-6 E), a measure of cofilin activity standard in the literature. Taken together, our data suggest that NOS1AP-S, but not NOS1AP-L, acts to downregulate levels of total cofilin to promote actin reorganization.
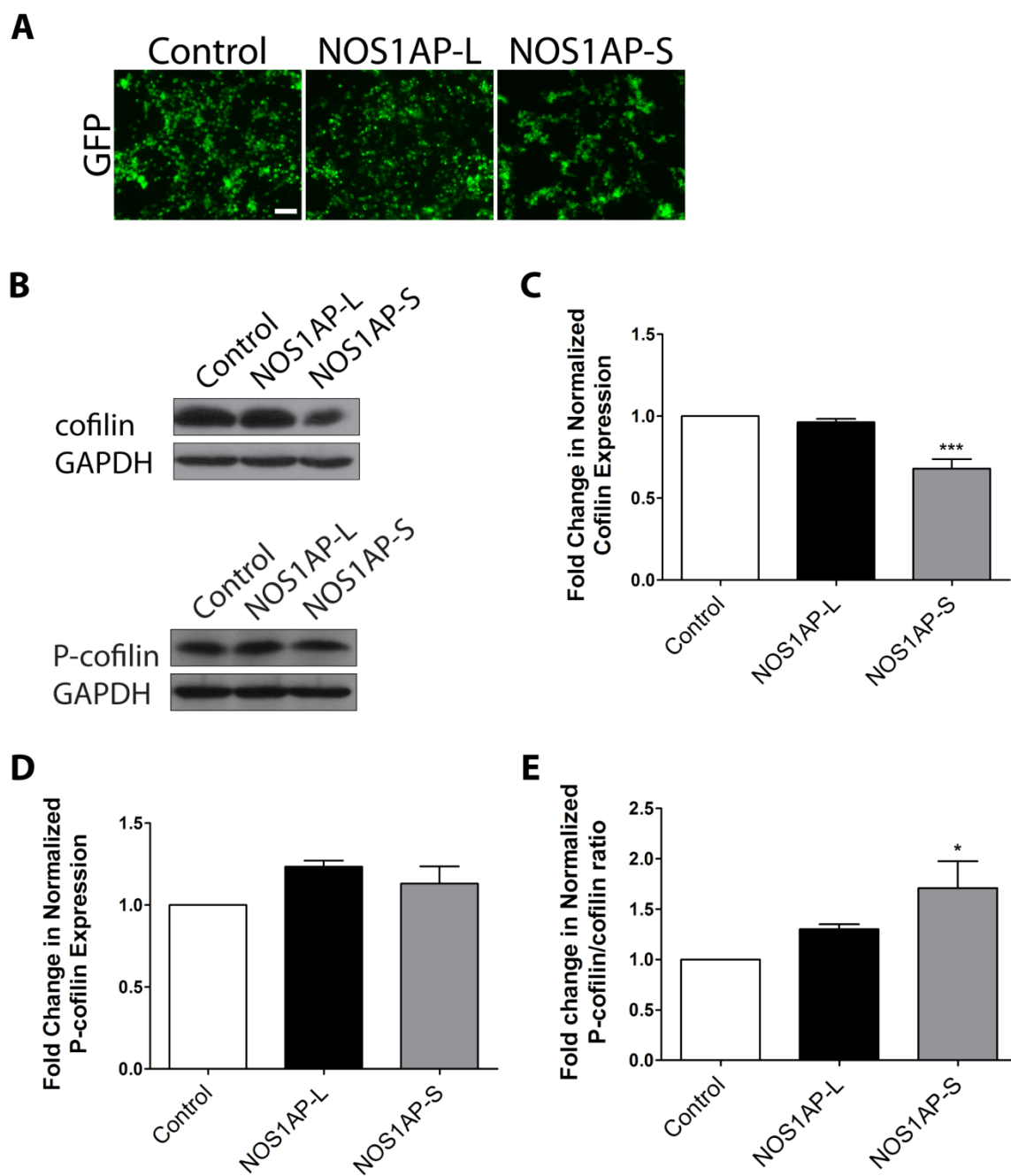
**Figure I-6. NOS1AP-S decreases active, non-phosphorylated cofilin protein expression in HEK293T cells. A,** Representative images of HEK293T cells expressing GFP (control), GFP-NOS1AP-L, or GFP-NOS1AP-S. Scale bar = 100 μm. **B,** Lysates from cultures of transfected HEK293T cells expressing GFP (control), GFP-NOS1AP-L, or GFP-NOS1AP-S were resolved by SDS-PAGE and analyzed by Western blotting using antibodies that recognize cofilin, phosphorylated cofilin (P-cofilin), and GAPDH. Representative blots are shown. **C,** Relative quantification of total cofilin normalized to control from multiple blots represented in B. Error bars indicate ± s.e.m. n = 4 for all conditions. **D,** Relative quantification of P-cofilin normalized to control from multiple blots represented in B. Error bars indicate ± s.e.m. n = 4 for all conditions. **E,** Relative quantification of normalized P-cofilin/cofilin ratio from multiple blots represented in B. Error bars indicate ± s.e.m. n = 4 for all conditions. All analyses were performed by first normalizing to GAPDH as an internal loading control and then comparing experimental condition to GFP control condition. *p< 0.05, **p< 0.01, and ***p< 0.001 versus control. p values were determined by one-way ANOVA followed by Dunnett's multiple comparisons test.

**NOS1AP isoforms influence actin dynamics.**

We have shown that expression of either NOS1AP isoform can alter F-actin content and that NOS1AP-S decreases expression of cofilin, a protein that directly regulates actin polymerization dynamics. To investigate whether NOS1AP isoforms can influence actin dynamics, as defined by the rate and amount of actin polymerization, we performed an *in vitro* actin polymerization assay. Recombinant NOS1AP-L or NOS1AP-S expression in *Escherichia coli* could not be achieved; therefore, HEK293T cell lysates from cultures expressing GFP, GFP-NOS1AP-L, or GFP-NOS1AP-S were used for actin polymerization assays (Figure I-7 A). The presence of NOS1AP-L or NOS1AP-S enhances polymerization of F-actin and results in an increased final amount of F-actin (Figure I-7 B). Using linear regression analysis, the maximum velocity, $V_{max}$, was calculated for the growth phase of actin polymerization (Figure I-7 C). Addition of extracts from cells expressing GFP has no effect on $V_{max}$ using buffer alone as a control (data not shown). Compared to actin polymerization in the presence of lysates from cultures expressing GFP, addition of lysates from cultures expressing GFP-NOS1AP-S, but not GFP-NOS1AP-L, increases the rate of actin polymerization (Figure I-7 D). Our data suggest that NOS1AP-L and NOS1AP-S regulate actin polymerization, a process necessary for spine formation and maturation, via distinct mechanisms.

**A**

Control    NOS1AP-L    NOS1AP-S

GFP

**B**



- buffer + actin
- GFP + actin
- NOS1AP-L + actin
- NOS1AP-S + actin

**C**



- GFP + actin
- NOS1AP-L + actin
- NOS1AP-S + actin

**D**
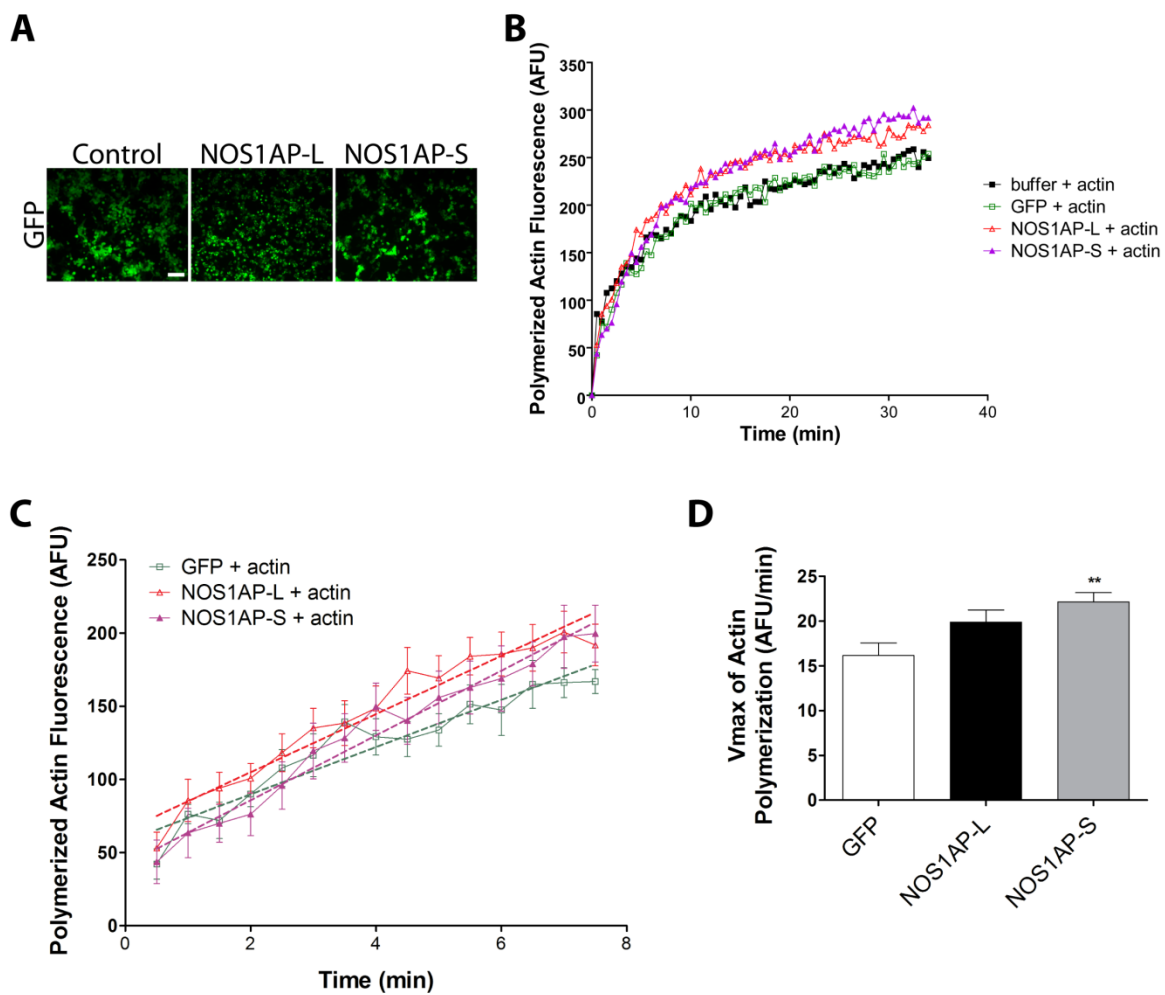
**Figure I-7. NOS1AP-L and NOS1AP-S promote actin polymerization. A,** Representative images of HEK293T cells expressing GFP (control), GFP-NOS1AP-L, or GFP-NOS1AP-S. Scale bar = 100 μm. **B,** Pyrene-actin polymerization assay was performed using a 1:10 mixture of pyrene-labeled muscle actin to unlabeled non-muscle actin. Polymerization of actin was initiated by the addition of polymerization buffer at time 0.5 min. The black trace depicts actin in the presence of lysis buffer alone, green trace depicts effect of adding HEK293T cell lysate from cultures expressing GFP, red trace depicts effect of adding HEK293T cell lysate from cultures expressing GFP-NOS1AP-L, and purple trace depicts effect of adding HEK293T cell lysate from cultures expressing GFP-NOS1AP-S. **C,** Effects of NOS1AP-L and NOS1AP-S on velocity during the growth phase of actin polymerization. Dotted lines are linear regression of polymerization curves in B from time 0.5 min to 7.5 min to determine $V_{max}$ values. **D,** $V_{max}$ data values for actin polymerization kinetics shown in C. Error bars indicate ± s.e.m. AFU, arbitrary fluorescence units. n = 10 polymerization reactions, buffer + actin; n = 9, GFP + actin; n = 13, NOS1AP-L + actin; n = 12, NOS1AP-S + actin. **p< 0.01 versus GFP + actin. p values were determined by one-way ANOVA followed by Dunnett's multiple comparisons test.

## DISCUSSION

In Chapter 1, we link NOS1AP to the regulation of the cytoskeleton, further implicating NOS1AP in the neurodevelopmental hypothesis of schizophrenia (Fatemi and Folsom, 2009; Andreasen, 2010). Regulation of the microtubule cytoskeleton is important for both dendrite and spine development. Here, we report that overexpression of NOS1AP-L increases the presence of a prominent MTOC when overexpressed in COS-7 cells. Centrosomes function as MTOCs during early neuronal development, while acentrosomal microtubule nucleation predominates later in development (Stiess et al., 2010). Studies in young neurons have demonstrated that centrosomes serve as a site of microtubule nucleation where they can then be released and transported into axons and dendrites (Yu et al., 1993; Ahmad and Baas, 1995; Ahmad et al., 1999). Furthermore, one study reports that the centrosome continues to play a critical role in dendrite development in more mature neurons by regulating dendrite patterning (Puram et al., 2011). The increased percentage of cells with a prominent MTOC caused by NOS1AP-L overexpression could have implications for proper regulation of dendrite development. Further investigation is needed to elucidate how NOS1AP-L may be regulating MTOC function. In contrast, overexpression of NOS1AP-S in COS-7 cells results in the disruption of microtubule organization, which may be mediated by the reduction in total Rac1 expression. When Rac1 activity is suppressed in 3T3 fibroblasts, microtubule dynamics are altered, resulting in the disruption of microtubule organization (Grigoriev et al., 2006). Furthermore, increasing Rac1 activity in neurons increases the number of dendrites (Threadgill et al., 1997). Our data presented in Carrel, et al., 2009 and in this

chapter suggest that NOS1AP-S may negatively regulate dendrite branching by reducing Rac1 protein expression.

Remodeling of the actin cytoskeleton is a common biological pathway shared among several risk factors for schizophrenia (Zhao et al., 2015). Here we report that both NOS1AP-L and NOS1AP-S induce remodeling of the actin cytoskeleton when overexpressed; however, their mechanisms of action are distinct. Specifically, NOS1AP-S, but not NOS1AP-L, exerts its effects on actin by increasing its polymerization rate. Our results demonstrate that a NOS1AP-L mutant lacking the PTB domain can reorganize the actin cytoskeleton, but cannot induce membrane protrusions as observed with overexpression of NOS1AP-L. These results suggest that the PTB domain of NOS1AP-L is responsible for the induction of filopodia-like membrane protrusions, and that there are multiple regulatory mechanisms by which NOS1AP-L can remodel the actin cytoskeleton. Additionally, a previous study reported that NOS1AP-L increases activation of the small GTPase Rac1 and that the PTB domain of NOS1AP-L is responsible for this activation (Richier et al., 2010); however we did not observe this result. In contrast, we observed a decrease in the protein levels of total Rac1 and cofilin, a downstream effector of Rac1, when NOS1AP-S, but not NOS1AP-L, is overexpressed. These results suggest that the remodeling of the actin cytoskeleton by NOS1AP-S may be mediated by the regulation of cofilin activity. Indeed, NOS1AP-S overexpression increases the ratio of inactive, phosphorylated cofilin (P-cofilin) to total cofilin, indicating that there is a decreased amount of the active, non-phosphorylated form of cofilin. The increased proportion of inactive cofilin observed with NOS1AP-S overexpression is in agreement with our finding that the rate of actin polymerization is

increased in the presence of lysates expressing NOS1AP-S. In summary, we demonstrate that the two NOS1AP isoforms act to regulate the actin cytoskeleton via distinct mechanisms.

# CHAPTER II:

The role of NOS1AP in the regulation of dendritogenesis

and synaptogenesis in rat cortical neurons.

## ABSTRACT

Schizophrenia is a neuropsychiatric disorder characterized by alterations in dendrite branching and spine density. Nitric oxide synthase 1 adaptor protein (NOS1AP), a risk gene for schizophrenia, encodes proteins that are upregulated in the dorsolateral prefrontal cortex of individuals with schizophrenia. To elucidate the role of NOS1AP in spine development *in vitro* and dendrite development *in vivo*, we overexpressed a long (NOS1AP-L) or short (NOS1AP-S) isoform of NOS1AP in neuronal progenitor cells of the embryonic rat neocortex or cultured rat cortical neurons and investigated changes in spine formation and dendrite branching. We observed that overexpression of NOS1AP-L in neurons in layers II/III of the neocortex results in a reduction in dendrite length and number. Overexpression of NOS1AP-L in cultured cortical neurons increases the number of immature spines and reduces the amplitude of miniature excitatory postsynaptic currents (mEPSCs). In contrast, overexpression of NOS1AP-S increases the number of mature and immature spines and frequency of mEPSCs, which may be attributed to a decrease in total Rac1 and cofilin expression. Our findings show that overexpression of NOS1AP-L or NOS1AP-S alters spine development, resulting in changes to synaptic function. However, the mechanisms by which these isoforms induce these changes are distinct. These results are important for understanding the cognitive deficits observed in schizophrenia.

## INTRODUCTION

Dendritic spines, which are enriched in actin, are the primary sites for excitatory synaptic input from presynaptic neurons. The typical morphology of a spine consists of a delta-shaped base, a narrow neck, followed by a bulbous head (Figure II-1 A). Changes in the structure or number of spines have implications for altered synaptic plasticity and function (Kasai et al., 2010). Dendritic spine abnormalities have been reported in several neuropsychiatric disorders, including schizophrenia (Glantz and Lewis, 2000; Penzes et al., 2011; Konopaske et al., 2014). In addition, genes linked to actin cytoskeleton remodeling are differentially expressed in schizophrenia (Matthews et al., 2012; Zhao et al., 2015). Further studies investigating the underlying cause of the spine pathology in schizophrenia are needed to better understand the etiology of the disorder.

The regulation of actin polymerization and depolymerization is important for new spine formation, spine maturation and spine stability, as well as spine remodeling and elimination. Spine development starts with the formation of an actin-rich protrusion followed by the formation of a mostly linearized actin network within the neck of the spine (Fig. II-1 B). In a mature state, the head of the spine is mushroom-shaped and contains a highly branched network of actin filaments. Three other main types of spine structures are filopodia-like spines, long-thin spines, and stubby spines, which are characterized by an absence of a spine neck (Bourne and Harris, 2008). Spine head growth is intimately connected with the regulation of actin dynamics. Cofilin, an actin severing protein, has been shown to be important for spine remodeling and synaptic plasticity. Activity-dependent spine growth is coupled to cofilin phosphorylation, which results in actin polymerization (Chen et al., 2007; Calabrese et al., 2014). In addition,

many other proteins act to alter the actin cytoskeleton, including the Arp2/3 complex and profilin. Profilin promotes actin filament assembly and the Arp2/3 complex promotes the growth of branched actin filaments, which is important for spine head enlargement (Pollard et al., 2000; Goley and Welch, 2006; Wegner et al., 2008).

Long-term potentiation (LTP) is the long-term strengthening of synapses following frequent stimulation. In hippocampal neurons, spine head enlargement promoted by LTP is accompanied by an increase in actin polymerization and F-actin content, which is dependent on the inactivation of cofilin (Yuste and Bonhoeffer, 2001; Fukazawa et al., 2003; Matsuzaki et al., 2004). Another important aspect of spine maturation is the enlargement of the postsynaptic density (PSD), a protein dense region located at the top of the spine head and directly across from the axon terminal of the presynaptic neuron. Mature spines contain more N-Methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the PSD compared to thinner, immature spines (Takumi et al., 1999; Matsuzaki et al., 2001). Furthermore, LTP results in the insertion and activation of glutamate receptors into the PSD (Liao et al., 1995; Petralia et al., 1999; Lu et al., 2001). In contrast, long-term depression (LTD), which results in a decrease in synaptic strength, induces actin depolymerization, exocytosis of glutamate receptors, and eventually, spine elimination (Beattie et al., 2000; Snyder et al., 2001; Nagerl et al., 2004; Okamoto et al., 2004; Zhou et al., 2004; Brown et al., 2005). Therefore, changes in the regulation of the actin cytoskeleton caused by overexpression of NOS1AP isoforms, as was shown in Chapter 1, can have implications for both early spine development and the synaptic changes that are necessary for learning and memory.

Altered connectivity in the brain can result from alterations in spine development and dendrite patterning. The complexity and pattern of a neuron's branches determine how many synaptic contacts it can make with the appropriate number and types of neurons. Several studies have demonstrated a reduction in the dendritic complexity of neurons in the prefrontal cortex of patients with schizophrenia (Selemon et al., 1995, 1998; Broadbelt et al., 2002; Black et al., 2004). This altered dendritic complexity is thought to underlie the disruption in normal cognitive function displayed by individuals with schizophrenia (Gur et al., 1999; Rusch et al., 2007). Although we have previously shown that NOS1AP isoforms can disrupt normal dendrite branching in cultured hippocampal neurons (Carrel et al., 2009), a demonstration of its *in vivo* relevance has not yet been shown. In the final section of this chapter, we investigate whether increased expression of NOS1AP-L can alter dendrite patterning *in vivo*.

**A**

postsynapse

glutamate receptors

adhesion molecules

PSD   EZ

spine head

spine neck

spine base

branched actin

lineair actin

microtubule

**B**

adhesion molecules

glutamate receptors

PSD

Myosin induced contractility

filopodia initiation

filopodia elongation

spine head formation

spine plasticity

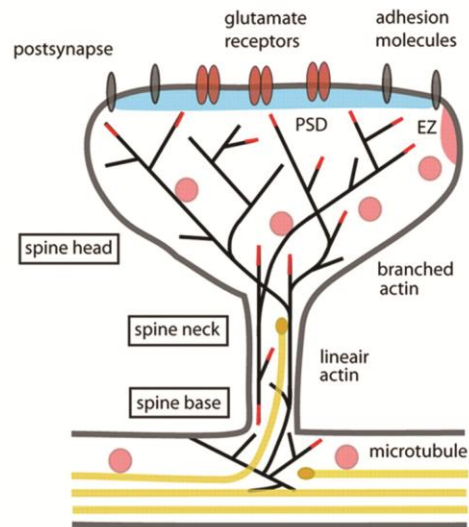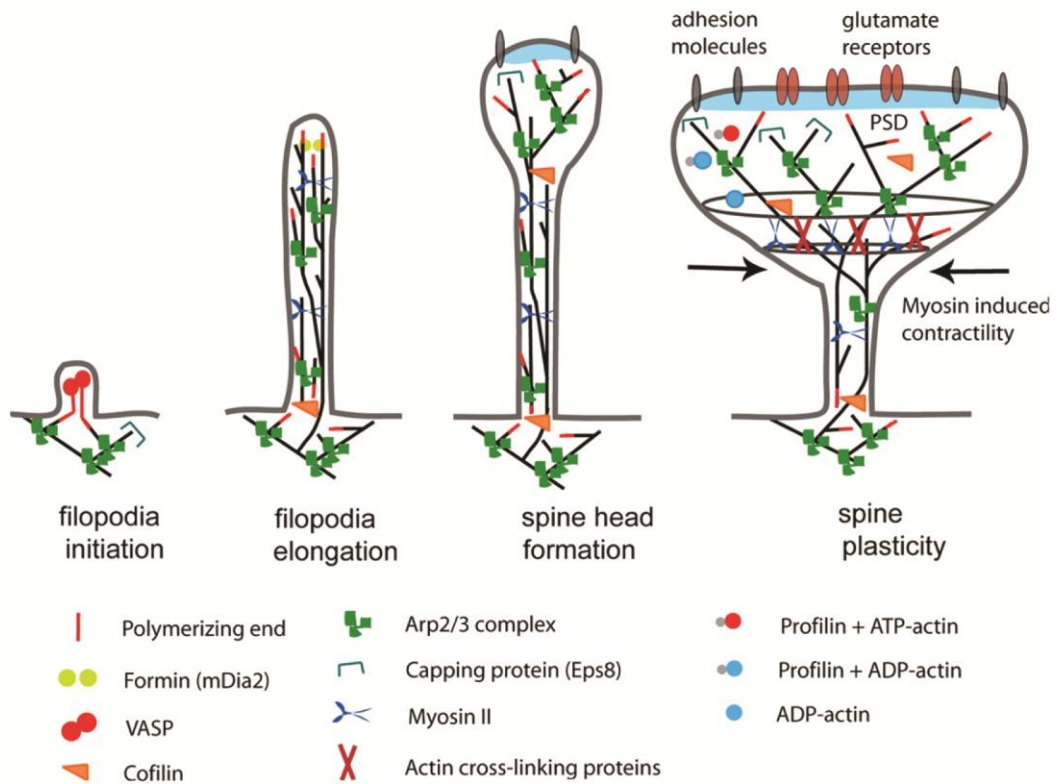| Polymerizing end | Arp2/3 complex | Profilin + ATP-actin |
| Formin (mDia2) | Capping protein (Eps8) | Profilin + ADP-actin |
| VASP | Myosin II | ADP-actin |
| Cofilin | Actin cross-linking proteins | |

**Figure II-1. Dendritic spine development. A,** Schematic of a mushroom-shaped spine illustrating the cytoskeletal organization of actin and microtubules in spines. The black lines represent actin in both branched and linear forms and the yellow lines represent microtubules. Spines possess a small portion of the membrane called the postsynaptic density (PSD; blue) that contains adhesion molecules (gray) and glutamate receptors (reddish brown). The endocytic zone (EZ) is located lateral of the PSD and recycling endosomes (pink) are throughout the spine and dendritic shaft. **B,** Schematic illustrating the stages of dendritic spine development. Present within the spine are many actin-binding proteins that can regulate the polymerization or depolymerization dynamics of actin filaments. The Arp2/3 complex promotes the growth of branched actin filaments. Cofilin is a severing protein that can promote the depolymerization of actin filaments. Profilin promotes actin filament assembly. This figure was adapted from Hotulainen and Hoogenraad, 2010. Originally published in the Journal of Cell Biology. doi:10.1083/jcb.201003008.

# MATERIALS AND METHODS

## Statistics

All statistics were calculated using the Prism 5.0 software from GraphPad (La Jolla, CA). Tests used are noted in figure legends.

## Antibodies

Rabbit polyclonal NOS1AP (sc-9138) and goat polyclonal DNase I antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal GAPDH antibody was from Millipore (Billerica, MA), mouse monoclonal anti-alpha-actinin-4 from Abcam (Cambridge, MA), and mouse monoclonal anti-actin from Sigma-Aldrich (St. Louis, MO). Chicken and goat polyclonal green fluorescent protein (GFP) antibodies were from Rockland Immunochemicals (Limerick, PA). Alexa Fluor® 647 phalloidin and chicken secondary antibody conjugated to Alexa-Fluor® 488 were from Life Technologies (Grand Island, NY). For *in vivo* studies, chicken polyclonal anti-red fluorescent protein (RFP) antibody was from AVES (Tigard, Oregon). Anti-chicken secondary antibody conjugated to Alexa-Fluor 555 was from Jackson ImmunoResearch (West Grove, Pennsylvania).

## DNA constructs

pCAG-GFP was obtained by subcloning EGFP from pEGFP-C1 (Clontech; Mountain View, CA) into a vector with CMV–actin–β-globin promoter (pCAG). cDNAs encoding long and short isoforms of human NOS1AP (NOS1AP-L and NOS1AP-S), NOS1AP-L-214-end (NOS1AP-L-ΔPTB), NOS1AP-L-1-487 (NOS1AP-L-ΔPDZ), and NOS1AP-L-

181-307 (NOS1AP-M) were subcloned into pCAG-GFP as described previously (Carrel et al., 2009). Complementary DNA encoding NOS1AP-L was subcloned in pCAG-IRES-TagRFP plasmid (pCIR, gift from Marie-Catherine Tiveron, Institut de Biologie du Développement de Marseille).

**Cell Fractionation and Western Blotting of COS-7 cell lysates**

COS-7 cells were cultured in 60 mm dishes and transfected at 30-50% confluency with pCAG-GFP, pCAG-GFP-NOS1AP-L, or pCAG-GFP-NOS1AP-S using Lipofectamine 2000 following the manufacturer's protocol. Cells were collected two days after transfection and lysed in F-actin stabilization/lysis buffer (50 mM PIPES pH 6.9, 50 mM KCl, 5 mM MgCl2, 5 mM EGTA, 5% (v/v) glycerol, 0.1% nonidet P40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% 2-mercaptoethanol, and 1 mM ATP). Lysates were incubated at 37°C for 10 minutes followed by centrifugation at 350 x $g$ at room temperature for 5 minutes to pellet unbroken cells. High speed centrifugation of the lysates was performed at 100,000 x $g$, 37°C for 1 hour to pellet F-actin with G-actin remianing in the supernatant. F-actin depolymerization buffer (8M urea, 15mM β-mercaptoethanol and 10 mM Tris pH 8.0) was added to each pellet and incubated on ice for 1 hour. The fractions were then separated by SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes (Immobilon-P; Millipore). After blocking with 2% bovine serum albumin (BSA) in Tris-buffered saline (500 mM Tris, pH 7.4, 60 mM KCl, 2.8 M NaCl) with 1% Tween-20 (TBST), membranes were incubated with goat anti-GFP (1:500) overnight at 4°C. After washing, horseradish peroxidase-linked secondary antibody was applied at 1:5000 for one hour at RT. Immunoreactive bands were visualized using

HyGlo quick spray (Denville Scientific; South Plainfield NJ) and quantified using Image Pro software (Media Cybernetics).

**F-actin Immunoprecipitation**

Adult rat brain tissue was homogenized and cells lysed in F-actin stabilization/lysis buffer (50 mM PIPES pH 6.9, 50 mM KCl, 5 mM MgCl2, 5 mM EGTA, 5% (v/v) glycerol, 0.1% nonidet P40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% 2-mercaptoethanol, and 1 mM ATP) supplemented with 1 Complete, Mini, EDTA-free protease inhibitor tablet (Roche; Indianapolis, IN). Extracted proteins were diluted two-fold and 500 µl volumes were incubated with 5 µg of biotin labeled-phalloidin (Sigma-Aldrich) or without (negative control) followed by precipitation of the captured complexes with 20 µl streptavidin-linked magnetic beads (Dynabeads® M-280 Streptavidin, Life Technologies). Beads were washed three times with phosphate-buffered saline, pH 7.4, with the samples being used for biochemical analysis after the third wash. Precipitated fractions were subjected to SDS-PAGE and sequentially immunoblotted to detect NOS1AP, alpha-actinin-4, Dnase I, and actin using the following dilutions: rabbit anti-NOS1AP (1:250), mouse anti-alpha-actinin-4 (1:500), goat anti-DNase I (1:250), and mouse anti-actin (1:500).

**Primary cortical neuron culture and spine analysis**

Neuronal cultures were plated from cortices of rat embryos at 18 days gestation on glass coverslips (12 mm diameter; 53,000 cells/cm$^2$), as previously reported (Carrel et al., 2009). At day *in vitro* (DIV) 14, cultures were transfected with indicated constructs using

calcium phosphate method (Kwon and Firestein, 2013). Neurons were fixed at DIV 17 and immunostained for GFP. Images of dendritic segments were taken with a high numerical aperture objective lens (40x C. Apochromat, N.A. 1.2) on a laser scanning confocal microscope, LSM510 META (Carl Zeiss Microscopy; Thornwood, NY). X-Y and Z-resolution was set as 0.1 μm-0.1 μm and 0.3 μm, respectively, to define dendritic spines. Spines along dendritic segments were counted and classified starting from 20 μm to 50 μm from the soma. Spines were classified as immature or mature based on morphology (Galvez and Greenough, 2005). Spine densities and types were manually counted from at least 12 neurons for each experimental condition, and analysis was performed with the experimenter blinded to the condition.

**Electrophysiology**

Whole cell patch-clamp recordings were made on the soma of cortical neurons. For recordings, cells were bathed in artificial cerebrospinal fluid containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.4 adjusted with NaOH; 290-310 mOsmol). Recording electrodes (3 −5 MΩ) contained a $K^+$-based internal solution composed of (in mM): 126 K-gluconate, 4 KCl, 10 HEPES, 4 ATP-Mg, 0.3 GTP-$Na_2$, 10 phosphocreatine, and 10 QX-314 bromide (pH 7.2; 280 −300 mOsmol). To record miniature excitatory postsynaptic currents (mEPSCs), we blocked action potentials with 1μM tetrodotoxin (Tocris, R & D Systems; Minneapolis, MN). The membrane potential was held at -70mV throughout all experiments. Data were amplified and filtered at 2 kHz by a patch-clamp amplifier (Multiclamp 700B), digitalized (DIGIDATA

1440A), stored, and analyzed by pCLAMP (Molecular Devices; Union City, CA). Data were discarded when the input resistance changed >20% during recording.

**Ethics approval statement**

This study was carried out in accordance with the recommendations of the National Institute of Health's Guide for the Care and Use of Laboratory Animals (DHHS Publication No. [NIH] 85-23 and all subsequent revisions thereof) and to the Public Health Service Policy on Humane Care and Use of Laboratory Animals followed by Rutgers Institutional Animal Care and Use Committee. The protocol was approved by the Rutgers Institutional Animal Care and Use Committee.

*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 for 3 weeks after electroporation (until P14).

All animals used for *in vivo* studies 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**

Postnatal day (P) 14 rat brains (P14) were dissected and fixed by transcardial perfusion of 4% paraformaldehyde (PFA) in phosphate buffered saline (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 30 μm and 80 μm (P14 brains) using a cryostat. For analysis of neuronal morphology at P14, 40- and 50-μm-thick z-stacks were acquired on a Zeiss LSM 510 confocal laser-scanning microscope (Zeiss) using a 20× NA .5 objective, and z-series were projected to two-dimensional representations. For analysis of neuronal morphology at P14, neurites were traced and quantified with NeuronJ software (http://rsb.info.nih.gov/ij/) (Meijering et al., 2004). Total neurite length and number of branches for each individual neuron were measured with the experimenter blinded to the condition.

## RESULTS

**NOS1AP isoforms associate with the cytoskeleton.**

Overexpression of either NOS1AP isoform in COS-7 cells can alter the cytoskeleton (Chapter 1). To determine if NOS1AP-L or NOS1AP-S associates with the cytoskeleton, we performed a high-speed centrifugation assay to isolate the cytoskeletal fraction. COS-7 cells were transfected with cDNA encoding GFP, GFP-NOS1AP-L or GFP-NOS1AP-S, and after 48 hours of expression, cells were lysed in a buffer that stabilizes and maintains F-actin and microtubules. The cell lysates were subjected to high-speed centrifugation to pellet F-actin and microtubules, and the supernatant and pellet fractions were analyzed by SDS-PAGE and Western blotting. Results demonstrate that both GFP-NOS1AP-L and GFP-NOS1AP-S are enriched in the cytoskeletal fraction compared to the GFP control (Fig. II-2 A).

Given that overexpression of NOS1AP isoforms can regulate actin organization (Chapter 1), we next investigated whether NOS1AP-L or NOS1AP-S associates with F-actin in rat brain. Previous studies demonstrated that biotinylated-phalloidin specifically precipitates F-actin (Fulga et al., 2007; Clarke and Mearow, 2013). Tissue extract was incubated with or without (negative control) biotinylated-phalloidin, followed by precipitation of captured complexes with streptavidin-linked magnetic beads. Precipitated fractions were subjected to Western blotting to detect F-actin. Pull-down of F-actin (Fig. II-2 B) captured NOS1AP-S and NOS1AP-L lacking posttranslational modifications (~55 kDa). Pull-down of F-actin also captured alpha-actinin-4, a known F-actin binding partner (Maruyama and Ebashi, 1965; Drabikowski et al., 1968). DNAse I, which preferentially binds to G-actin (Schafer et al., 1975), was not detected in precipitated

fractions (negative control), as well as a third isoform of NOS1AP identified previously by our laboratory termed NOS1AP-S' (Hadzimichalis et al., 2010). These data further strengthen a possible role for NOS1AP in the regulation actin dynamics in rat neurons.
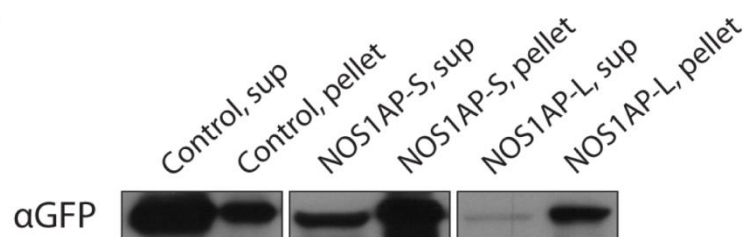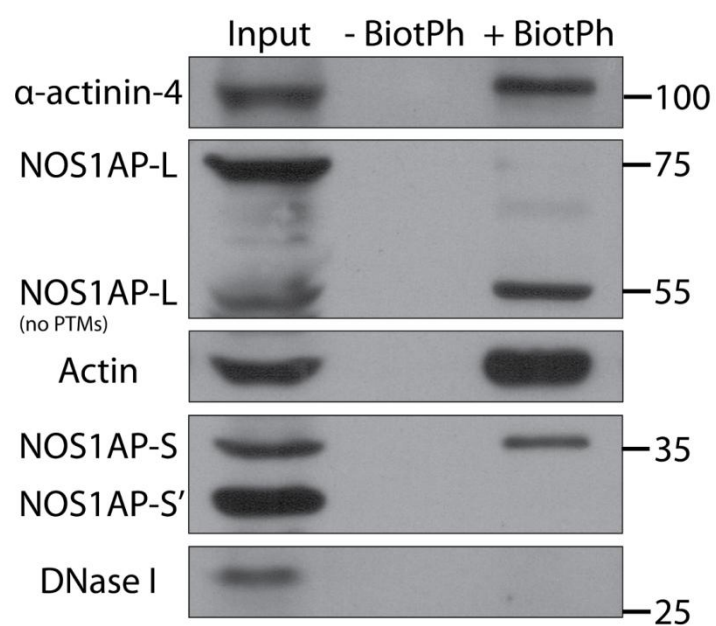
**Figure II-2. NOS1AP-L and NOS1AP-S associate with F-actin in rat brain. A,** Extracts from cultures of COS-7 cells overexpressing GFP-NOS1AP-L, GFP-NOS1AP-S, or GFP (control) were used to separate the G-actin fraction (sup) and the F-actin fraction (pellet) by high-speed centrifugation and analyzed by SDS-PAGE and Western blotting using antibodies that recognize GFP. Representative blot is shown. **B,** Representative blot showing immunoprecipitation of phalloidin-bound F-actin and NOS1AP from adult rat brain lysate. Tissue was homogenized and cells were lysed in F-actin stabilization buffer. Samples were incubated with or without (negative control) biotinylated-phalloidin followed by precipitation of the captured complexes with streptavidin-linked magnetic beads. Precipitated fractions were then subjected to SDS-PAGE and sequentially immunoblotted to detect NOS1AP, alpha-actinin-4, Dnase I, and actin. Pulldown of F-actin also captures alpha-actinin-4 (positive control). BiotPh, biotin–phalloidin; PTMs, posttranslational modifications.

**NOS1AP influences spine formation and maturation in rat cortical neurons.**

We have previously reported that the mRNA and protein levels of NOS1AP isoforms are increased in postmortem samples from the DLPFC of subjects with schizophrenia (Xu et al., 2005; Hadzimichalis et al., 2010). Furthermore, the expression of NOS1AP-L and NOS1AP-S protein increases during E15 to P14 in rat forebrain, developmental time periods linked to both dendrite branching and spine formation (Carrel et al., 2009). Our data now link NOS1AP-L and NOS1AP-S to actin dynamics, which are important for regulating spine formation and maturation. Thus, we investigated the role of NOS1AP-L and NOS1AP-S in the formation and maturation of dendritic spines in cultured rat embryonic cortical neurons. Extensive spine formation and maturation occurs from DIV 14 to DIV 21; therefore, we transfected neurons at DIV 14 and performed spine analysis at DIV 17. Cortical neurons overexpressing either isoform of NOS1AP or a NOS1AP-L mutant lacking the PDZ-binding motif (NOS1AP-L-ΔPDZ) display an increased number of spines, although the increase was not as dramatic with NOS1AP-S compared to NOS1AP-L or NOS1AP-L-ΔPDZ overexpression (Fig. II-3 A,B).

Furthermore, dendritic spines were classified as immature or mature, based on morphology (Galvez and Greenough, 2005). Overexpression of NOS1AP-L in cortical neurons resulted in an increase in thinner dendritic spines, indicative of immature spines, compared to control neurons (Fig. II-4 A,B). In contrast, overexpression of NOS1AP-S resulted in an increase in both immature and mature neurons (Fig. II-4 A-C). Interestingly, expression of a mutant of NOS1AP-L lacking the PTB domain (NOS1AP-L-ΔPTB) eliminated the effects on spine number (Fig. II-3 A,B), suggesting that the PTB domain is responsible for the formation of new, immature spines promoted by

overexpression of NOS1AP-L. Expression of NOS1AP-L-ΔPTB resulted in a similar increase in the number of mature spines as was observed with NOS1AP-S overexpression (Fig. II-4 A-C). Neurons overexpressing NOS1AP-M, the middle region in NOS1AP-L responsible for the effects of NOS1AP-L on dendrite branching (Carrel et al., 2009), show no changes to total spine number and spine morphology (Figs. II-3, II-4). Conversely, neurons overexpressing NOS1AP-L-ΔPDZ display increased total spine number, number of immature spines, and number of mature spines (Figs. II-3, II-4), suggesting that the interaction between NOS1 and NOS1AP may play a role in regulating spine number and maturation. Taken together, our data suggest that increased expression of NOS1AP-L and NOS1AP-S have distinct, yet dramatic, effects on spine formation and maturation.
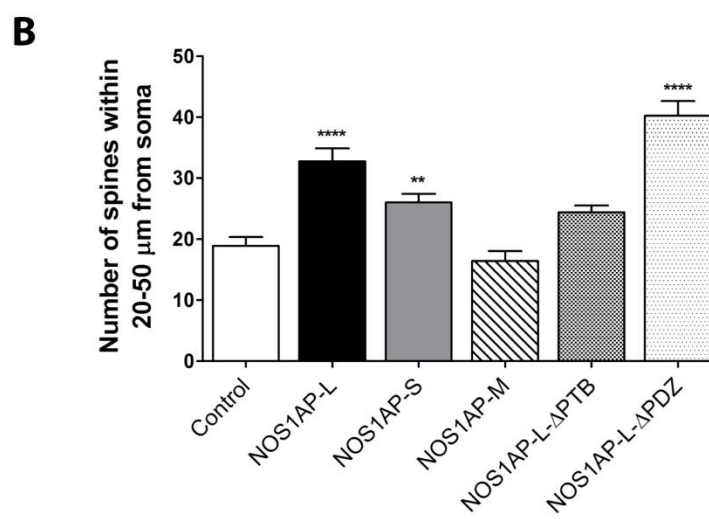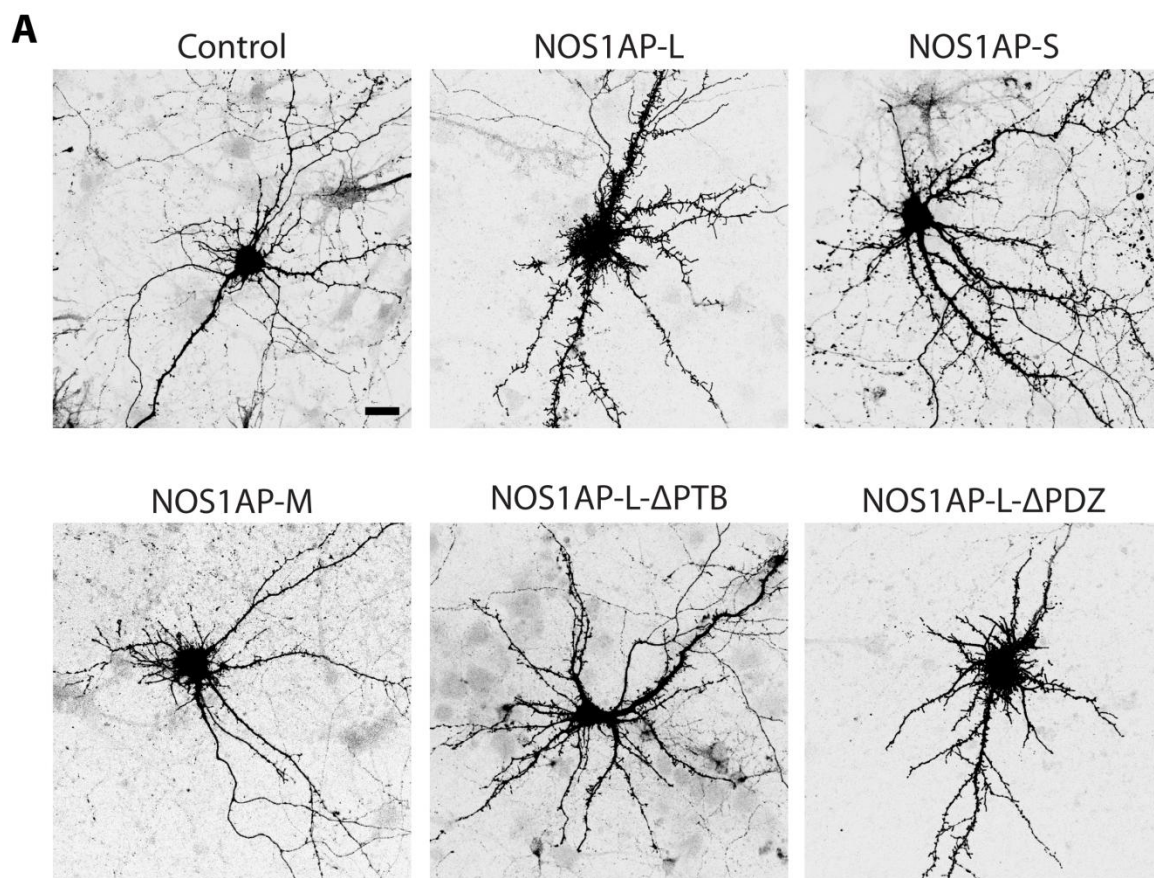
**A**



Control   NOS1AP-L   NOS1AP-S

NOS1AP-M   NOS1AP-L-ΔPTB   NOS1AP-L-ΔPDZ

**B**

**Figure II-3. Overexpression of NOS1AP-L and NOS1AP-S increase spine number in rat cortical neurons. A,** Representative inverted GFP images of rat cortical neurons (DIV17) transfected with pCAG-GFP (control), pCAG-GFP-NOS1AP-L, pCAG-GFP-NOS1AP-S, pCAG-GFP-NOS1AP-M, pCAG-GFP-NOS1AP-L-ΔPTB, or pCAG-GFP-NOS1AP-ΔPDZ. Scale bar = 20 μm. **B,** Number of spines per 30 μm segment in cultured neurons expressing indicated proteins. **p< 0.01 and ****p< 0.0001 versus GFP. p values were determined by one-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate ± s.e.m. n = 50 dendrites, GFP; n = 41, NOS1AP-L; n = 50, NOS1AP-S; n = 37, NOS1AP-M; n = 55, NOS1AP-L-ΔPTB; n = 35, NOS1AP-ΔPDZ. Dendrites were analyzed from 12-20 neurons per condition with 2-3 dendrites/neuron.

**A**

Control



NOS1AP-L



NOS1AP-S



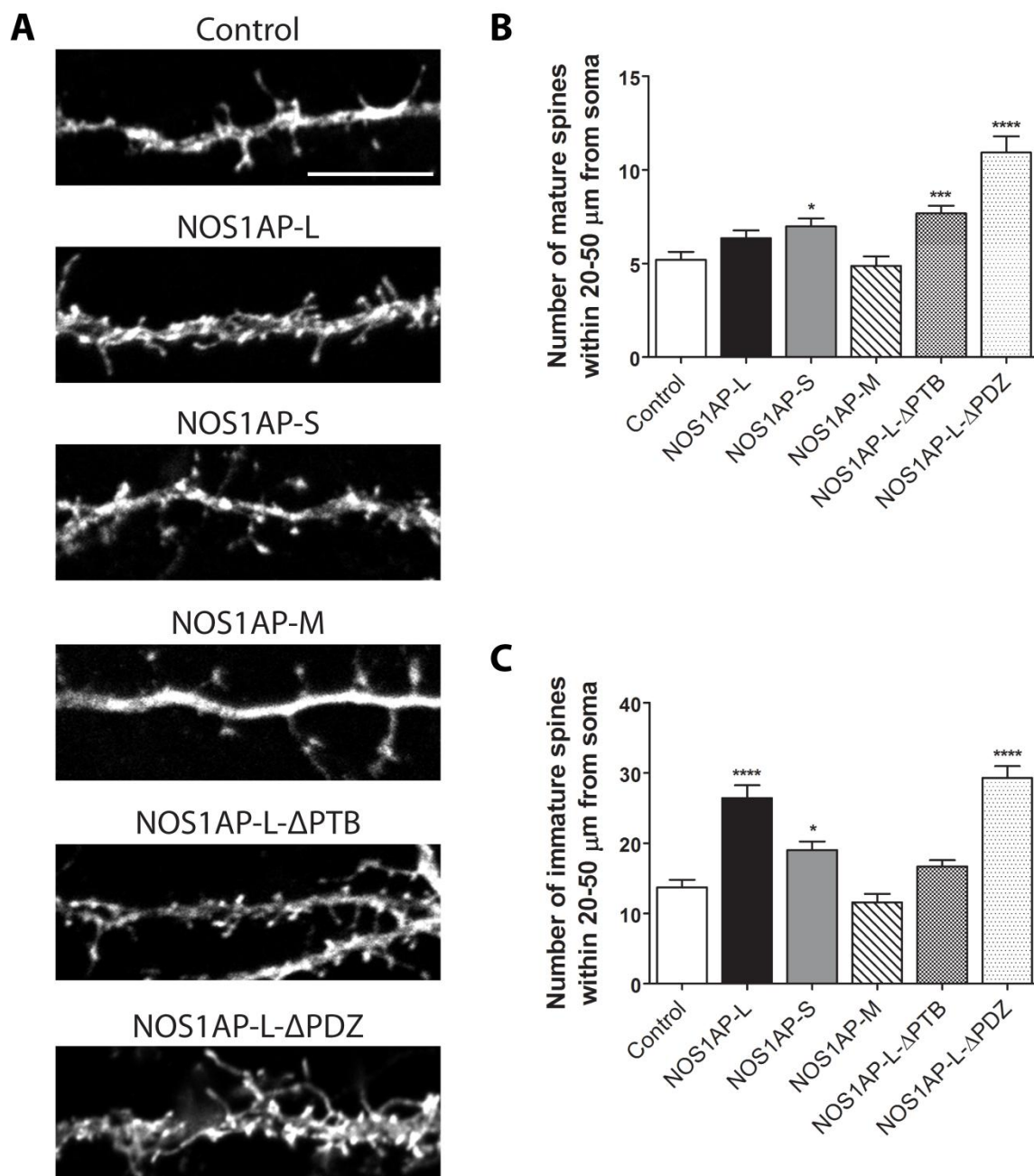NOS1AP-M



NOS1AP-L-ΔPTB



NOS1AP-L-ΔPDZ



**B**



**C**

**Figure II-4. NOS1AP-L and NOS1AP-S influence spine morphology. A,** Representative images of dendrite segments from rat cortical neurons (DIV17) transfected with pCAG-GFP (control), pCAG-GFP-NOS1AP-L, pCAG-GFP-NOS1AP-S, pCAG-GFP-NOS1AP-M, pCAG-GFP-NOS1AP-L-ΔPTB, or pCAG-GFP-NOS1AP-ΔPDZ. Scale bar = 10 μm. **B,** Number of mature spines per 30 μm segment in cultured neurons expressing indicated proteins. *p< 0.05; ***p< 0.001 and ****p< 0.0001 versus GFP. p values were determined by one-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate ± s.e.m. **C,** Number of immature spines per 30 μm segment in cultured neurons expressing indicated proteins. *p< 0.05 and ****p< 0.0001 versus Control. p values were determined by one-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate ± s.e.m. n = 50 dendrites, GFP; n = 41, NOS1AP-L; n = 50, NOS1AP-S; n = 37, NOS1AP-M; n = 55, NOS1AP-L-ΔPTB; n = 35, NOS1AP-ΔPDZ. Dendrites were analyzed from 12-20 neurons per condition with 2-3 dendrites/neuron.

**NOS1AP isoforms alter synaptic properties in rat cortical neurons.**

Changes in spine number and morphology promoted by overexpression of NOS1AP-L or NOS1AP-S could lead to distinct functional alterations in synaptic transmission. Thus, we performed whole-cell patch-clamp recordings of miniature excitatory postsynaptic currents (mEPSCs) in cultured rat embryonic cortical neurons. In correspondence with our spine studies, we transfected neurons at DIV 14 with cDNA encoding NOS1AP isoforms or NOS1AP-L mutants and recorded mEPSCs at DIV 17 (Fig. II-5 A). Neurons overexpressing NOS1AP-L show no change in the frequency of mEPSCs but exhibit a significant decrease in the amplitude of mEPSCs (Fig. II-5 B-C). This change in amplitude may result from a decrease in the amount of transmitter contained in presynaptic vesicles or a change in the function or number of postsynaptic receptors (Turrigiano and Nelson, 2004). Since transfection efficiency is <10% and we do not observe two or more transfected neurons making synaptic contacts with each other, changes in mEPSCs are most likely due to changes in postsynaptic strength. Therefore, our results suggest that overexpression of NOS1AP-L decreases mEPSC amplitude by altering the composition of the postsynaptic density. When either NOS1AP-L-ΔPTB or NOS1AP-L-ΔPDZ is overexpressed, the decrease in mEPSC amplitude is lost (Fig. II-5 C), suggesting that both the PTB domain and the PDZ-binding motif play roles in altering synaptic properties. Interestingly, neurons overexpressing NOS1AP-S or NOS1AP-L-ΔPTB demonstrate increased mEPSC frequency with no change in amplitude (Fig. II-5 B-C). These results suggest that the postsynaptic mechanism for increase in mEPSC frequency is an increase in the number of functional synapses. Overexpression of NOS1AP-L-ΔPDZ also results in an increase in mEPSC frequency (Fig. II-5 B), similar

to that seen with NOS1AP-S overexpression, indicating that the PDZ-binding motif is not responsible for the effect. The observed increase in mEPSC frequency correlates with our findings of increased number of mature spines resulting from NOS1AP-S overexpression. Taken together, our results suggest that NOS1AP-L overexpression decreases synaptic strength and NOS1AP-S overexpression increases synaptic strength.
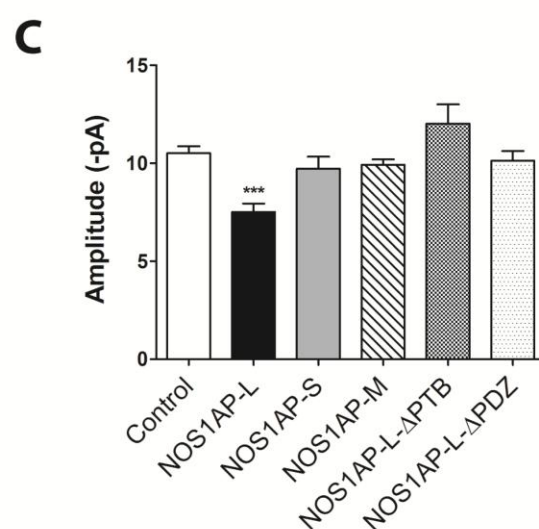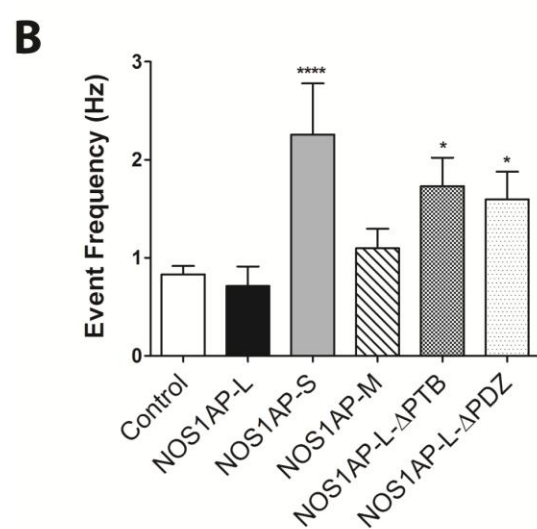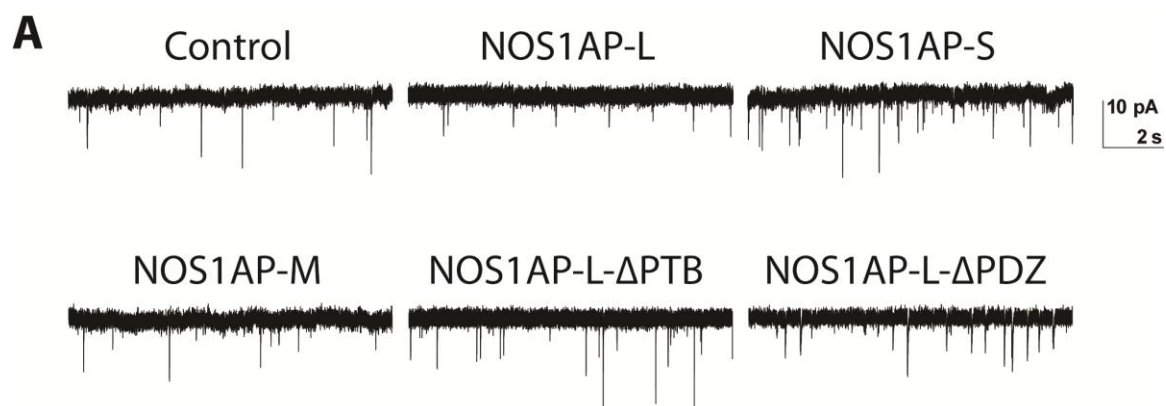
**Figure II-5. NOS1AP-S overexpression increases synaptic strength, whereas NOS1AP-L overexpression decreases synaptic strength. A,** Representative tracings of miniature excitatory postsynaptic currents (mEPSCs) from rat cortical neurons (DIV17) transfected with pCAG-GFP (control), pCAG-GFP-NOS1AP-L, pCAG-GFP-NOS1AP-S, pCAG-GFP-NOS1AP-M, pCAG-GFP-NOS1AP-L-ΔPTB, or pCAG-GFP-NOS1AP-ΔPDZ. **B,** Average frequency of mEPSCs in cultured neurons expressing indicated proteins. *C,* Average amplitude of mEPSCs in cultured neurons expressing indicated proteins. Error bars indicate s.e.m. n = 62 cells, Control; n = 14, NOS1AP-L; n = 19, NOS1AP-S; n = 15, NOS1AP-M; n = 15, NOS1AP-L-ΔPTB; n = 22, NOS1AP-L-ΔPDZ. *$p < 0.05$; ***$p < 0.001$ and ****$p < 0.0001$ versus Control. p values were determined by one-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate ± s.e.m. This experiment was performed in collaboration with Przemyslaw Swiatkowski.

**NOS1AP-L negatively regulates dendrite patterning of cortical neurons *in vivo*.**

We previously reported that overexpression of NOS1AP-L in cultured rat cortical neurons during both early dendrite formation and later during active dendrite branching results in a decrease in dendrite number (Carrel et al., 2009). As *in vitro* experiments cannot fully replicate cellular conditions within an organism, *in vivo* experiments can offer a more relevant and conclusive insight into the role of NOS1AP-L in dendrite development. To investigate the role of NOS1AP in dendritogenesis, we electroporated neuronal precursors with cDNA encoding NOS1AP-L and RFP or RFP alone (as a control) into the lateral ventricular wall at embryonic day (E) 16 and analyzed dendrite patterning of transfected neurons three weeks later at postnatal day (P) 14 (Fig. II-6 A). Neurons in layers II/III of the neocortex overexpressing NOS1AP-L exhibited a reduction in total dendrite length and number compared to control neurons (Fig. II-6 B-D). This finding correlates with our previous *in vitro* data and further supports the concept that increased expression of NOS1AP perturbs normal dendritogenesis.

**A**  TagRFP  NOS1AP

**B**

TagRFP

NOS1AP

**C**

**D**
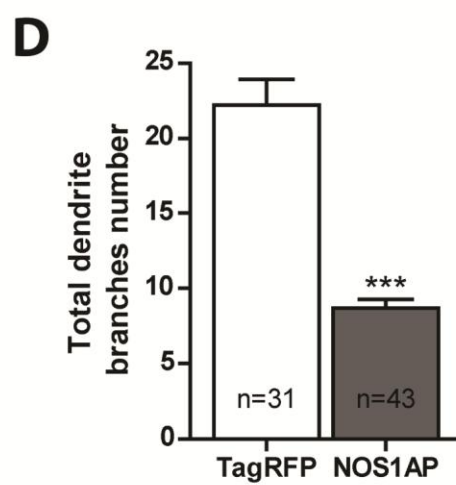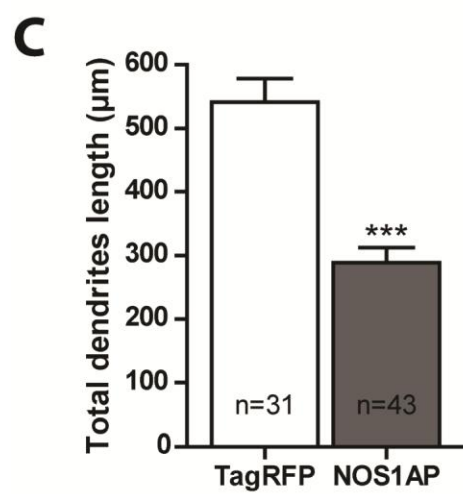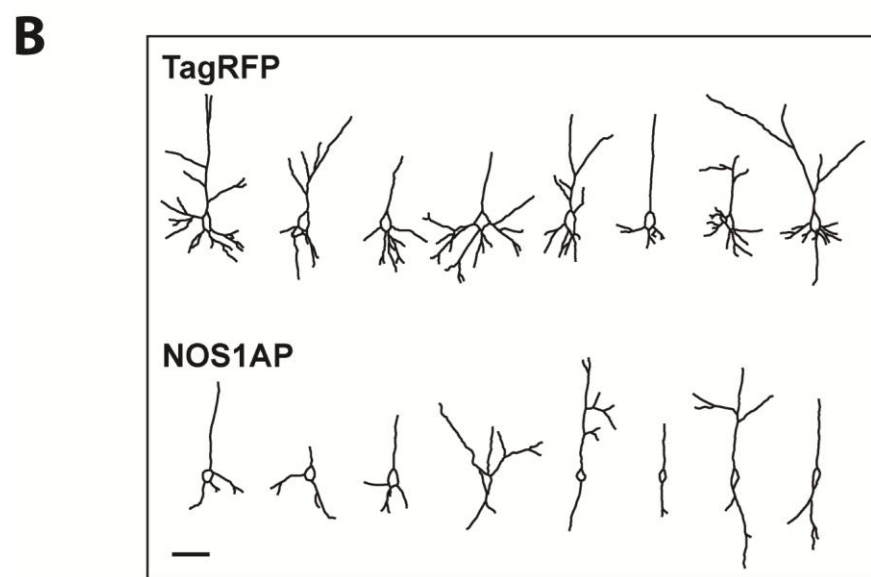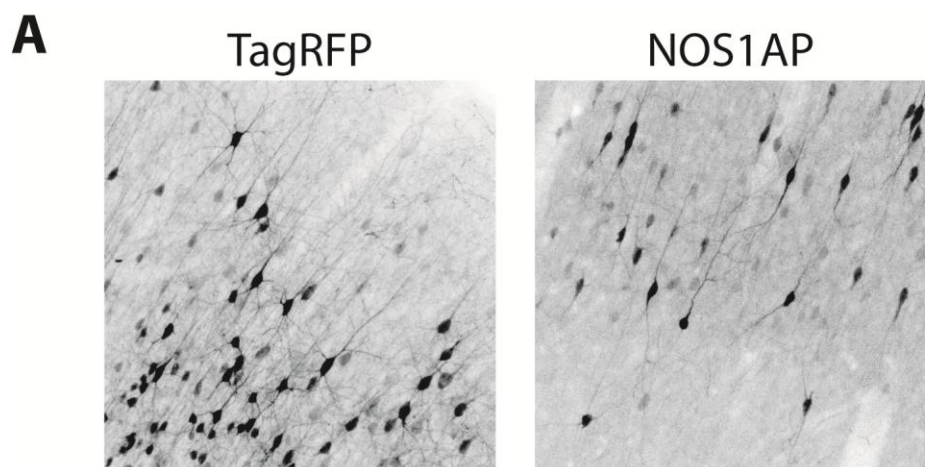
**Figure II-6. Overexpression of NOS1AP-L decreases total dendrite length and number *in vivo*. A,** Representative inverted RFP images of layer II/III cortical neurons overexpressing TagRFP (control) or RFP and NOS1AP (long isoform, NOS1AP-L). **B,** Representative NeuronJ tracing of transfected cells in layers II/III for each condition. **C,** Quantification of the total dendritic length of layer II/III neurons overexpressing indicated proteins**. D,** Quantification of the total number of dendrite branches of layer II/III neurons overexpressing indicated proteins. TagRFP, red fluorescent protein TagRFP. Statistical significance was calculated using Student's t test. Error bars indicate ± s.e.m. ***$p < .001$ versus control. *n* values refer to the number of analyzed cells from five different brains per condition. This figure was modified from Carrel et al., 2015.

## DISCUSSION

Numerous studies support the concept that schizophrenia is a disorder of altered connectivity (Narr and Leaver, 2015; Zhou et al., 2015), resulting in the impairment of cognitive, social, and behavioral functions. Connectivity in the brain can be disrupted by the dysregulation of dendritogenesis, spinogenesis, or synaptogenesis. Individuals with schizophrenia display reduced dendritic length and number in layer 3 and reduced dendritic number and field size in layer 5 of the prefrontal cortex and reduced density of dendritic spines (Moyer et al., 2014). In the present chapter, we report that overexpression of either NOS1AP-S or NOS1AP-L alters the number and morphology of spines in rat cortical neurons. Specifically, overexpression of NOS1AP-S increases the number of mature spines, which may be attributed to the downregulation of total Rac1 and active cofilin (reported in Chapter 1). Both Rac1 and cofilin, a downstream effector of Rac1, have been shown to play important roles in dendritic spine development and maintenance. One study reported that a reduction in Rac1 activity results in an increase in thin, longer filopodia-like protrusions (Tashiro and Yuste, 2004). Another group reported that the conditional deletion of Rac1 in mice results in increased thin and stubby spine formation (Golden et al., 2013). Furthermore, neurons lacking cofilin and neurons with suppressed cofilin activation exhibit a mature spine phenotype (Rust et al., 2010; Pontrello et al., 2012).

Here, we report that changes to Rac1 and cofilin expression results from the overexpression of NOS1AP-S and that NOS1AP-S overexpression increases both immature and mature spines in rat cortical neurons. In contrast, overexpression of NOS1AP-L increases the number of immature spines. When the PTB domain of

NOS1AP-L is deleted, this effect is lost, further suggesting that the PTB domain, which has been shown to increase Rac activity (Richier et al., 2010), is important for the induction of new membrane protrusions. We also found that overexpression of NOS1AP-L-ΔPDZ, which results in a less stable interaction between NOS1 and NOS1AP (Jaffrey et al., 1998; Li et al., 2015), increases total spine number, number of immature spines, and number of mature spines, suggesting that the interaction between NOS1 and NOS1AP may play a role in regulating both spine number and maturation.

To investigate if the changes in spine number and morphology caused by the overexpression of NOS1AP isoforms results in changes to synaptic strength, we recorded mEPSCs in neurons overexpressing NOS1AP isoforms. We show that an increased number of mature spines by NOS1AP-S overexpression correlates with increased mEPSC frequency, suggesting an increase in the number of functional synapses. Additionally, overexpression of NOS1AP-L leads to reduced amplitude of mEPSCs, suggestive of a reduction in the function or number of postsynaptic glutamate receptors (Turrigiano and Nelson, 2004). This is consistent with our findings that overexpression of NOS1AP-L increases the number of immature spines and can remodel the actin cytoskeleton, a process that regulates the endocytosis of glutamate receptors in spines. Interestingly, overexpression of NOS1AP-L-ΔPDZ does not reduce the amplitude of mEPSCs. These results suggest that NOS1AP-L may regulate NO signaling through its interaction with NOS1, resulting in the nitrosylation of NMDA receptors, thereby inhibiting the activity of the receptor, leading to reduced amplitude of mEPSCs. The perturbations in spine morphology resulting from increased NOS1AP isoform expression may have negative

consequences for spine development and spine remodeling necessary for synaptic plasticity (Fig. II-7).

We have previously shown that NOS1AP-L can negatively regulate dendrite branching in cultured hippocampal neurons (Carrel et al., 2009). Furthermore, knock-down of NOS1AP-L in cultured hippocampal neurons increases dendrite branching (Carrel et al., 2009), indicating that the effect of NOS1AP-L on dendrite branching is not an artifact of overexpression. However, it is unknown whether NOS1AP-L can mediate changes to dendrite patterning *in vivo*. To investigate this, we overexpressed NOS1AP-L in neuronal progenitor cells of the embryonic rat neocortex using *in utero* electroporation and analyzed dendrite patterning of transfected neurons three weeks later. Overexpression of NOS1AP-L inhibits normal dendritogenesis with neurons displaying a reduction in total dendrite length and in the number of branches. *In vitro*, we reported the effect of NOS1AP-L on dendrite branching to be mediated through its interaction with CPE. CPE can bind to dynactin (Park et al., 2008), linking NOS1AP to microtubule motors and regulation of the microtubule cytoskeleton. Indeed, CPE knockout mice exhibit increased dendritic patterning in layer V of the cortex and abnormalities in dendritic pruning (Woronowicz et al., 2010). Our results show that NOS1AP-L may be contributing to the underlying pathophysiology of schizophrenia through its regulation of dendritogenesis.

Overexpression of
NOS1AP-L

Overexpression of
NOS1AP-S

Key:
AMPAR
NMDAR
mGluR

Reorganization of actin cytoskeleton
Increased number of immature spines
Altered composition of postsynaptc density
Decreased amplitude of mEPSCs

Reorganization of actin cytoskeleton
Decreased Rac1 and cofilin expression
Increased proportion of inactive cofilin
Increased rate of actin polymerization
Increased number of mature spines
Increased frequency of mEPSCs

**Figure II-7. Model of the actions of NOS1AP-L and NOS1AP-S on the actin cytoskeleton, dendritic spine number and maturity, and synaptic strength.** Overexpression of NOS1AP isoforms result in distinct changes to neurons. A hypothetical model of action for NOS1AP-L and NOS1AP-S is shown. Future studies will determine the effects of overexpression of NOS1AP-L and NOS1AP-S on NMDA and AMPA receptor localization.

## ACKNOWLEDGEMENTS

I would like to thank the following people for their contributions to the work presented in this chapter of my thesis: Przemyslaw Swiatkowski for his contriubution to Fig. II-5 by performing the recordings and analysis of mEPSCs; Damien Carrel for his contribution to Fig. II-6 by performing the *in utero* electroporation of the animals and imaging of brain sections. Results in Figure II-6 were published in Carrel et al., 2015.

# CHAPTER III:

The characterization and use of hiPSC-derived neural progenitor cells and neurons to investigate the role of NOS1AP in human dendritogenesis

## ABSTRACT

Abnormal dendritic arbor development has been implicated in a number of neurodevelopmental disorders, such as autism and Rett syndrome, and the neuropsychiatric disorder schizophrenia. Postmortem brain samples from subjects with schizophrenia show elevated levels of NOS1AP in the dorsolateral prefrontal cortex, a region of the brain associated with cognitive function. Furthermore, we previously reported that NOS1AP negatively regulates dendrite branching in rat hippocampal neurons. For patients with a *NOS1AP* allele associated with schizophrenia, the cognitive deficits could be linked to neurodevelopmental defects. To investigate the role that NOS1AP plays in human dendritic arbor development, we adapted methods to generate human neural progenitor cells and neurons using induced pluripotent stem cell (iPSC) technology. We found that increased protein levels of NOS1AP decrease dendrite branching in human neurons at the developmental time point when both primary and secondary branching actively occurs. Next, we tested whether pharmacological agents can decrease the expression of NOS1AP isoforms. Treatment of human iPSC-derived neurons with D-Serine results in a reduction in NOS1AP-L protein expression. Lastly, we generated neurons from iPSC lines that were derived from the blood of subjects with schizophrenia containing the associated allele within the *NOS1AP* gene and iPSC lines from control subjects and began studies to investigate NOS1AP expression in these iPSC lines. In summary, we demonstrate how an *in vitro* model of human neuronal development can help in understanding the etiology of schizophrenia and can also be used as a platform to screen drugs for preventative or adult therapies.

# INTRODUCTION

Induced pluripotent stem cell technology involves the reprogramming of somatic cells to a pluripotent state by the overexpression of key transcription factors, such as OCT4, c-Myc, SOX2, and KLF4, which have been found to maintain pluripotency in embryonic stem cells (ESCs) (Takahashi and Yamanaka, 2006). Induced pluripotent stem cells can give rise to any cell type from the three germ layers. The study of neurodevelopmental disorders can particularly benefit from iPSC technology due to the lack of or inadequacy of existing animal models and the difficulty of accessing human neural stem cells and neurons for *in vitro* studies. The disease phenotype cannot always be recapitulated in an animal, especially for neuropsychiatric disorders, such as schizophrenia, which involve higher order cognitive function. In addition, despite the genetic similarities between humans and rodents, differences are seen in the downstream effects of genetic alterations. As a result, animal models do not always display the disease symptoms that are observed in humans (Inoue and Yamanaka, 2011). The development of drugs relies on cell lines for proof-of-concept studies and toxicity screenings. Just as with animal models, rodent cell lines are not able to fully mimic human biological processes. One reason for this is that the type and/or distribution of ion channels and receptors on the surface of the cell may be different from those in human cells (Sabir et al., 2008). Immortalized human cells lines are also not ideal for disease studies because often the disease-relevant cell type is not available and the immortalization process alters native cellular responses (Ebert and Svendsen, 2010). In fact, compounds that have shown efficacy in cell lines and animals have not shown therapeutic effects in humans, and in the worst case scenario, have shown toxic effects in humans (Laustriat et al.,

2010). Therefore, the use of human neural cell cultures can greatly complement the use of cell lines and animal models for disease studies. With the availability of iPSCs, the study of the differentiation process of human neural stem cells to mature neurons in a more physiologically relevant manner is now possible.

In this chapter, we demonstrate how iPSC technology can be used to ectopically express a gene that has been linked to a neuropsychiatric disorder in the disease-relevant cell type, neurons. For multi-genic based diseases such as schizophrenia, this type of disease modeling aids in the understanding of how each gene contributes to the disease phenotype. Postmortem studies from patients with a number of cognitive and neurological disorders show that neurons from these patients have improper neuronal morphology, such as a reduction in dendrite number or branching (Zoghbi, 2003; Kulkarni and Firestein, 2012). Specifically, it has been shown that in patients with schizophrenia, the dendritic arbor of layers III and V pyramidal neurons in the prefrontal cortex is less complex than those arbors in control patients (Broadbelt et al., 2002; Black et al., 2004). A number of reports indicate that many of the genes that have been linked to neurological disorders are involved in determining dendrite morphology (e.g. (Ma et al., 2011; Lepagnol-Bestel et al., 2013; Pathania et al., 2014; Watanabe et al., 2014). Specifically, our group has tested the functional role of increased NOS1AP expression both *in vitro* and *in vivo* in the rat model system. These studies indicate that NOS1AP plays a major role in the regulation of dendrite branching, and perturbations in its expression result in altered dendrite patterning (Carrel et al., 2009; Carrel et al., 2015). Furthermore, NOS1AP has been linked to the NMDA receptor pathway through its interaction with NOS1 (Jaffrey et al., 1998). An ExF motif-bearing region present in both

long, NOS1AP-L, and short, NOS1AP-S, isoforms is required for the binding to NOS1, and this interaction is further stabilized by the C-terminal PDZ-binding motif of NOS1AP isoforms (Li et al., 2015). Through this interaction, NOS1AP can influence NOS1 localization and influence downstream NOS1 signaling pathways (Jaffrey et al., 1998; Li et al., 2013; Li et al., 2015) that may have an effect on the regulation of dendrite branching.

The use of human neural cell cultures can greatly complement the use of cell lines and animal models for disease studies as despite the genetic similarities between humans and rodents, differences are seen in the downstream effects of genetic alterations (Inoue and Yamanaka, 2011). To investigate the role that NOS1AP plays in human dendritic arbor development, we used human induced pluripotent stem cell (iPSC) technology to generate human neural progenitor cells (NPCs) and neurons. Furthermore, we begin to use this human neuronal cell culture system to test whether pharmacological agents can reduce NOS1AP expression to find compounds that can restore normal dendritogenesis in human neurons with increased expression of NOS1AP.

# MATERIALS AND METHODS

## Statistics

All statistics were calculated using the Prism 5.0 software from GraphPad (La Jolla, CA). Tests used are noted in figure legends.

## Antibodies

Chicken polyclonal green fluorescent protein (GFP) antibody was purchased from Rockland Immunochemicals. Mouse monoclonal GAPDH, mouse monoclonal OCT4, mouse monoclonal Nanog, rabbit polyclonal SOX2, mouse monoclonal Tra-1-60, and rabbit polyclonal synaptophysin antibodies were from Milipore. Chicken polyclonal microtubule associated protein 2 (MAP2), mouse monoclonal PAX6, mouse monoclonal MusashiI, and rabbit polyclonal Tbr2 antibodies were from Abcam. Rabbit polyclonal NOS1AP (sc-9138) antibody was from Santa Cruz Biotechnology. Rabbit polyclonal vesicular glutamate transporter 1 (VGLUT1) antibody was from Synaptic Systems.

## DNA constructs

pCAG-GFP was obtained by subcloning EGFP from pEGFP-C1 (Clontech; Mountain View, CA) into a vector with CMV–actin–β-globin promoter (pCAG). cDNAs encoding long and short isoforms of human NOS1AP (NOS1AP-L and NOS1AP-S), NOS1AP-L-214-end (NOS1AP-L-ΔPTB), and NOS1AP-L-181-307 (NOS1AP-M) were subcloned into pCAG-GFP as described previously (Carrel et al., 2009). cDNA encoding NOS1AP-L-1-487 (NOS1AP-L-ΔPDZ) and NOS1AP-L were subcloned into 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), respectively.

**Human induced pluripotent stem cell (hiPSC) Derivation**

Human foreskin fibroblasts (HFFs) were infected with retroviruses expressing OCT4, SOX2, KLF4, and c-MYC. Five days after transduction, cells were plated onto Matrigel (BD Biosciences)-coated 10 cm dishes in mTESR (StemCell Technologies). Media change was performed daily with fresh mTESR, and the cells were grown for 23 days. hiPSC colonies were picked manually and plated onto Matrigel-coated 6-well plates in mTESR. hiPSCs were passaged after disociation using Dispase (BD Biosciences) and a cell scraper and replating into mTESR.

The NIMH Stem Cell Center, together with RUCDR Infinite Biologics, generated patient derived iPSCs from Cryopreserved Lymphocytes (CPLs) through Sendai viral vectors expressing OCT4, Klf4, c-Myc, and SOX2. Human iPSCs were not used for experiments until passage 13, when the Sendai virus can no longer be detected.

**hiPSC differentiation to Neural Progenitor Cells (NPCs) and neurons**

hiPSCs were grown in mTeSR/Neurobasal (Invitrogen) media supplemented with 500 ng/ml Noggin. After six days, the medium was changed to Neurobasal medium supplemented with 500 ng/ml Noggin. On day 10, the cells were dissociated and plated in Neurobasal medium onto 20 µg/ml laminin (Sigma)-coated dishes. Eight days later, the medium was changed to DMEM/F12 with Glutamax (Invitrogen)/Neurobasal supplemented with 20ng/ml Fibroblast Growth Factor (FGF; Peprotech), 0.5x N2

supplement (Invitrogen), and 0.5x B27 (-) Vitamin A supplement (Invitrogen), which we refer to as Neural Progenitor Medium (NPM). NPCs were grown and passaged in NPM onto ¼ diluted Matrigel-coated plates. For differentiation into neurons, NPCs were plated in NPM onto a ¼ diluted Matrigel-coated 10 cm dish. One day after plating, the medium was changed to Neurobasal medium supplemented with 1x B27 (-) Vitamin A, and 10ng/ml of Brain Derived Neurotrophic Factor (BDNF; Peprotech), which we refer to as Neural Differentiation Medium (NDM). Four days later, the cells were dissociated using Accutase (Stem Cell Technologies) and plated in NDM onto substrates that were coated with 10 µg/ml poly-D-lysine (PDL; Sigma) for 2 hours followed by 10 µg/ml laminin for 2 hours.

Patient derived iPSCs were differentiated into NPCs using Gibco® PSC Neural Induction Medium (Thermo Fisher Scientific) following the manufacturer's protocol. NPCs were differentiated into neurons as described above, except NPCs were plated on undiluted Matrigel-coated dishes to begin differentiation.

**Neuronal culture and drug administration**

hiPSC-derived neurons grown for 4 days post-differentiation (DD 4) were plated at 210,000 cells/cm$^2$ (158,000 cells/cm$^2$ for neurons differentiated from NPCs generated using Gibco® PSC Neural Induction Medium) onto PDL/laminin-coated substrates. Substrates were coated with 10 µg/ml PDL for 2 hours, followed by a 2 hour coating with 10 µg/ml laminin. Medium changes were performed three times per week with NDM supplemented with 1 µg/ml laminin. Flupenazine, clozapine, haloperidol, D-serine, and GLYX-13 (all from Sigma) were dissolved in sterile dimethyl sulfoxide (DMSO). The

hiPSC-derived neurons were treated on DD 19 by adding drugs or vehicle to the medium. Antipsychotic concentrations were chosen based on the therapeutic plasma level concentrations, with 10- to 30-fold higher levels in brain tissue (Zhang et al., 2007).

**Immunocytochemistry**

Cells were fixed for 15 min in 4% paraformaldehyde (PFA) in 1 x phosphate-buffered saline (PBS) for neuronal marker staining or for 15 min in methanol at -20°C for 15 min stem cell marker staining.  Cells were permeabilized and blocked in 2% normal goat serum and 0.1% Triton-X-100 in 1 x PBS at room temperature for 1 hour. The cells were incubated with primary antibodies for 2 hours, washed twice with 1 x PBS, and then incubated with secondary antibodies for 1hour.

**Western Blot**

Twelve micrograms of protein from cell lysate homogenized in TEE buffer (25 mM Tris-HCl, pH 7.4, 1mM EDTA, 1mM EGTA) were loaded and resolved on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to Immobilon P membrane (Millipore) in transfer buffer lacking SDS.  The blot was probed with a rabbit antibody to NOS1AP (Santa Cruz, #R-300) and visualized using ECL Plus (Amerisham Biosciences) with a secondary antibody coupled to horseradish peroxidase. For GAPDH normalization, the blot was probed with a mouse antibody to GAPDH (Millipore).

**Electrophysiology**

DD 28 neurons were patch-clamped in the whole cell configuration using thick walled glass pipettes with a resistance of 4-6 mega Ohms. To activate voltage-gated ion channels, neurons were voltage-clamped at -70mV, with a jump to -40 mV, and returned to -80 mV. Additional jumps were made at 10 mV increments, with the final jump to +10 mV. In current clamp mode, a depolarizing current injection was applied to activate voltage-gated sodium channels to generate action potential activity. Internal solution contained (in mM) 125 K-gluconate, 20 KCl, 10 Na2-phosphocreatine, 4 MgATP, 0.3 GTP, 10 HEPES, 5 EGTA, and was adjusted to pH 7.2 using KOH. The external recording solution contained (in mM)  125 NaCl, 2.5 KCl, 1 MgCl2, 2 CaCl2, 25 glucose, 1.25 NaH2PO4, 0.4 ascorbic acid, 3 myo-inositol,  2 sodium pyruvate and 25 NaHCO3, and was adjusted to pH 7.4 by bubbling with carbogen (95% O2, 5% CO2).

**Transfection and Cell Imaging for Dendrite Branching Analysis**

At DD19, human neurons were transfected with pCAG-GFP, pCAG-GFP-NOS1AP-L, or pCAG-GFP-NOS1AP-S using Lipofectamine 2000 (Invitrogen).  Forty eight hours after transfection, cells were fixed with 4% paraformaldehyde in PBS for 15 min and immunostained for GFP and MAP2.  For dendrite branching analysis, Bonfire analysis was performed (Langhammer et al., 2010). Neurons were imaged at 200x using an Olympus Optical (Tokyo, Japan) IX50 microscope with a Cooke Sensicam CCD cooled camera, fluorescence imaging system, and ImagePro software (MediaCybernetics, Silver Spring, MD).  Neurons were traced with the experimenter blinded to the condition using NeuronJ (NIH, Bethesda, MD) and NeuronStudio (Mt. Sinai Medical School, NYC, NY).

Data were analyzed using MatLab (MathWorks, Natick, MA). Sholl analyses were performed at 6 μm intervals, and dendrites were counted if >3 μm.

**Transfection of hiPSC-derived NPCs and Immunocytochemistry for F-actin**

NPCs were plated in NPM onto ¼ diluted Matrigel-coated coverslips at 21,000 cells/cm2 and transfected with pCAG-GFP, pCAG-NOS1AP-L, pCAG-NOS1AP-S, pCAG-NOS1AP-L-ΔPTB, pCIG-NOS1AP-L-ΔPDZ, or pCAG-NOS1AP-M using Lipofectamine 2000 (Life Technologies) following the manufacturer's protocol. Forty-eight hours after transfection, cells were fixed with 4% paraformaldehyde in 1 x PBS for 15 minutes and immunostained for GFP using chicken anti-GFP (1:500) and Alexa-Fluor® 488 anti-chicken (1:500) and for filamentous actin (F-actin) using Alexa-Fluor® 647-Phalloidin, followed by nuclear staining with Hoechst dye. Coverslips were mounted onto glass slides using Fluoromount G (Southern Biotechnology; Birmingham, AL). Images of NPCs were taken with a high numerical aperture objective lens (63x C. Apochromat, N.A. 1.2) on a laser scanning confocal microscope, LSM510 META (Carl Zeiss Microscopy; Thornwood, NY).

## RESULTS

**Generation and characterization of human iPSCs and NPCs.**

NOS1AP is expressed at higher levels in the DLPFC of subjects with schizophrenia and may play a role in glutamatergic neurotransmission. Therefore, to assess the role of NOS1AP in human neuronal development, we used somatic cell reprogramming to generate human induced pluripotent stem cells (hiPSCs) and differentiated the iPSCs into neuronal progenitor cells (NPCs) using conditions that favor the generation of forebrain NPCs. The hiPSCs generated were positive for the pluripotency markers OCT4, Tra-1-60, Nanog, and SOX2 (Figure III-1 A). The NPCs were faintly positive for the early neural progenitor cell markers MusashiI and PAX6 and strongly positive for NPC marker SOX2 and the cortical progenitor marker Tbr2 (Figure III-1 B).
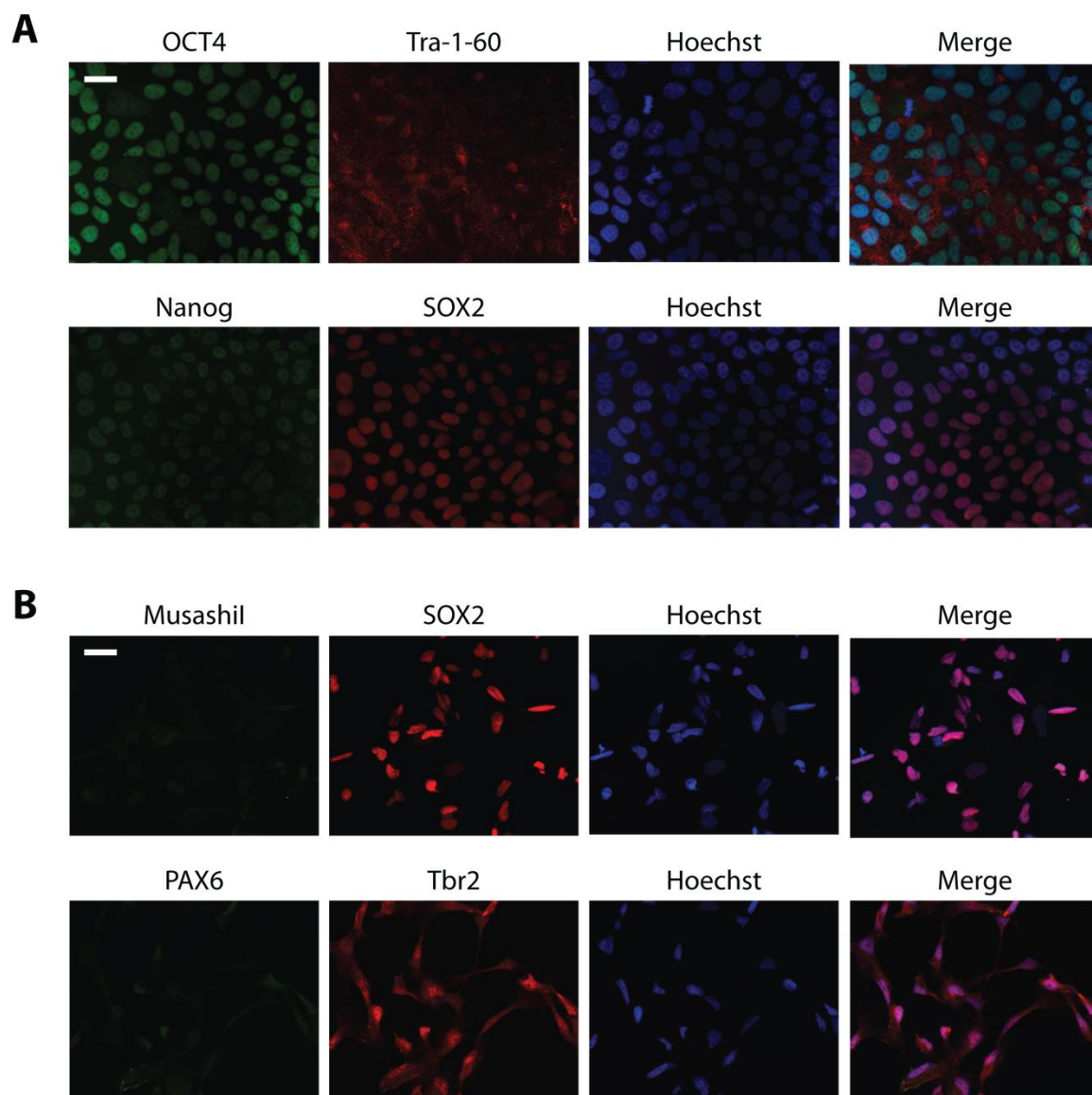
**A**

| OCT4 | Tra-1-60 | Hoechst | Merge |

| Nanog | SOX2 | Hoechst | Merge |

**B**

| Musashil | SOX2 | Hoechst | Merge |

| PAX6 | Tbr2 | Hoechst | Merge |

**Figure III-1. Characterization of hiPSCs and hiPSC-derived NPCs. A,** hiPSCs express pluripotency markers OCT4, Tra-1-60, Nanog, and SOX2. **B,** hiPSC-derived NPCs express NPC markers MusashiI, SOX2, PAX6 and the cortical progenitor marker, Tbr2. Scale bar = 20 μm.

**Generation and characterization of human iPSC-derived neurons.**

We generated a mostly glutamatergic population of neurons by withdrawing FGF and adding BDNF to the medium. We found that starting the differentiation procedure with the NPCs grown on dishes coated with Matrigel optimizes cell survival. We observed optimal attachment of the differentiating NPCs when replating onto PDL-laminin coated substrates after allowing the NPCs to differentiate on the Matrigel-coated dishes for four days. Concentrations of PDL higher than 10 µg/ml are toxic to the cells and result in a large amount of cell death. The human neuronal cultures are immunoreactive for the neuronal markers, microtubule-associated protein 2 (MAP2) and synaptophysin, and the glutamatergic marker, vesicular glutamate transporter 1 (VGLUT1) at days post-differentiation (DD) 14. Figure III-2 A). The hiPSC-derived neurons show normal sodium and potassium currents when voltage-clamped and normal induced action potentials when current-clamped (Figure III-2 B).

To assess maturation of the human neurons in our culture system, we quantified the number of MAP2-positive cells in the population of cells over two different time points during differentiation (Figure III-3 A,B). The percentage of MAP2-positive cells significantly increases from 58.5% to 94% from DD 14 to DD 42. The increase in MAP2-positive cells in our neuronal cultures over time is indicative of maturation of the neurons.
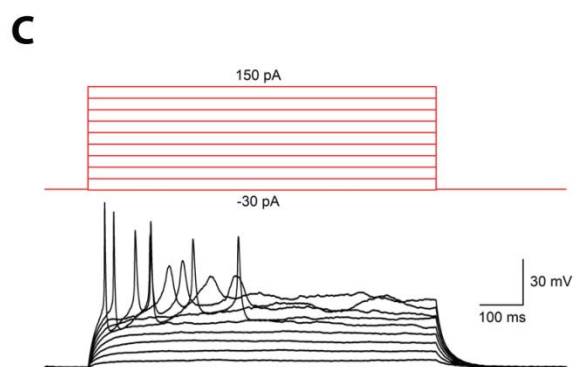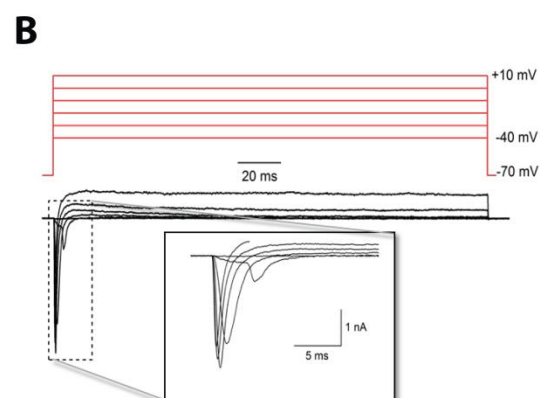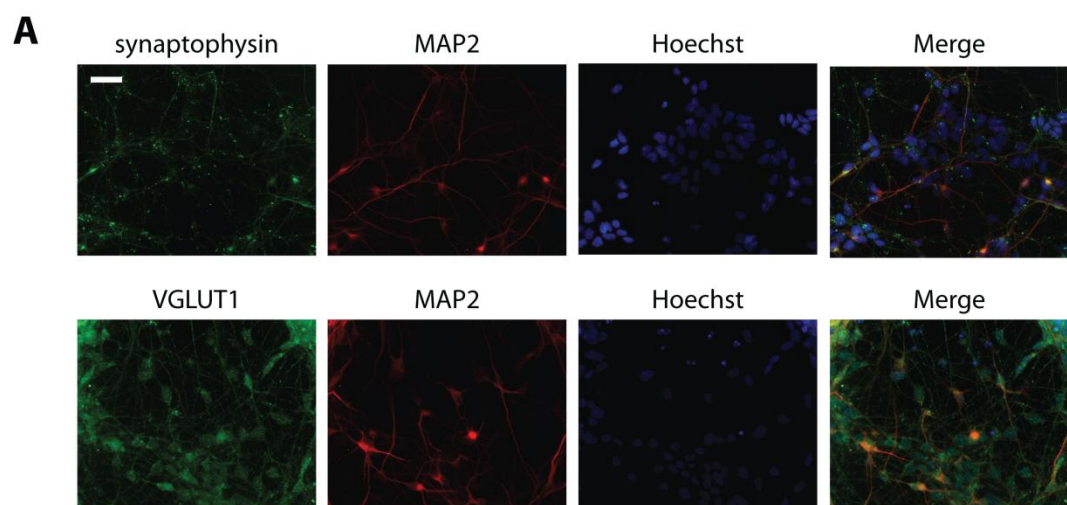
**A**

| synaptophysin | MAP2 | Hoechst | Merge |
|---|---|---|---|

| VGLUT1 | MAP2 | Hoechst | Merge |
|---|---|---|---|

**B**

+10 mV

−40 mV

−70 mV

20 ms

1 nA

5 ms

**C**

150 pA

−30 pA

30 mV

100 ms

**Figure III-2. Characterization of hiPSC-derived neurons. A,** hiPSC-derived neurons express neuronal markers MAP2, synaptophysin, and VGLUT1 at days post-differentiation (DD) 14. Scale bar = 20 μm. **B,** hiPSC-derived neurons show normal sodium and potassium currents when voltage-clamped on DD 28. **C,** hiPSC-derived neurons show normal induced action potentials when current-clamped on DD 28. Electrophysiology experiment was performed in collaboration with Steven Clarke in Dr. Kenneth Paradiso's laboratory.
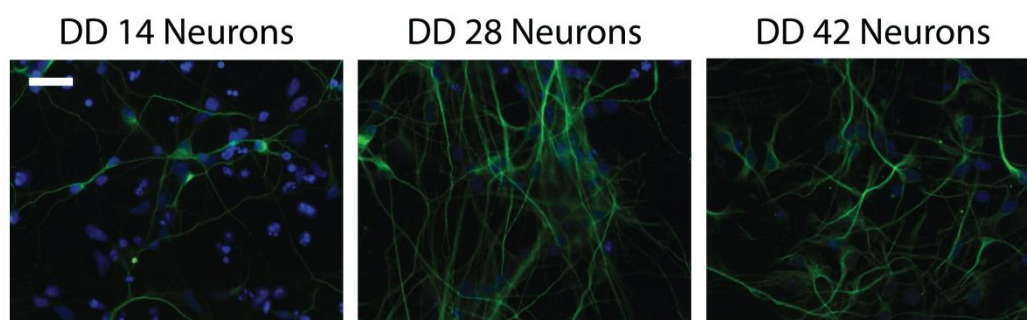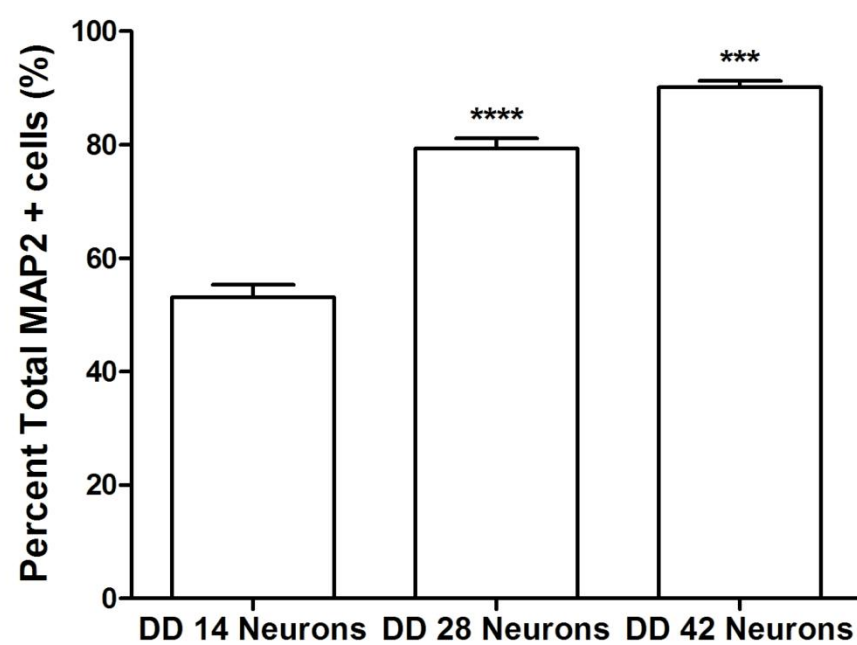
**A**



DD 14 Neurons   DD 28 Neurons   DD 42 Neurons

**B**

**Figure III-3. hiPSC-derived Neurons mature *in vitro*. A,** MAP2 immunoreactivity increases from days post-differentiation (DD) 14 to DD 42. **B,** Percentage of MAP2-immunopositive cells per 400x magnification field for neurons on DD 14, DD 28, and DD 42. Scale bar = 20 μm. ***p< 0.001 and ****p< 0.0001 versus neurons on DD 14. p values were determined by one-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate ± s.e.m. n = 59 neurons, DD 14; n = 56, DD 28, n = 56, DD 42.

**NOS1AP protein expression increases during hiPSC-derived neuronal maturation.**

Before the effects of exogenous NOS1AP expression can be tested in hiPSC-derived neurons, it would be informative to determine at what stage in development NOS1AP expression occurs and at what levels. To investigate this, we isolated protein from cell lysates from hiPSCs, hiPSC-derived NPCs, hiPSC-derived neurons on DD 14, and hiPSC-derived neurons on DD 28 and performed Western blot analysis to detect NOS1AP protein expression (Figure III-4 A). Interestingly, NOS1AP-L protein levels stay relatively consistently throughout neural differentiation. NOS1AP-L expression trends toward a decrease from the iPSC state to the NPC state, although this did not reach statistical significance. NOS1AP-S protein expression significantly increases from both the iPSC state and the NPC state to neurons on DD 28. NOS1AP isoforms have a similar protein expression profile in human neurogenesis compared to the expression profile of NOS1AP isoforms in the developing rat forebrain (Carrel et al., 2009).

To investigate how NOS1AP affects the arborization of developing human neurons, we determined the developmental time period when these neurons undergo active dendritogenesis. To identify this time window, we transfected differentiating NPCs at different time points with a plasmid that allows for GFP expression to monitor changes in cellular morphology (Figure III-4 B). The cells undergo neurite extension from DD 1 to DD 9, with axon specification occurring on DD 12. Secondary dendrite extension begins on approximately DD 17, with active primary and secondary branching occurring by DD 21. Our *in vitro* human neuronal culture system exhibits the hallmark stages of dendritogenesis (neurite extension, axon specification, axon and dendrite elongation, and active dendritic branching), which have been observed in mouse and rat model systems.
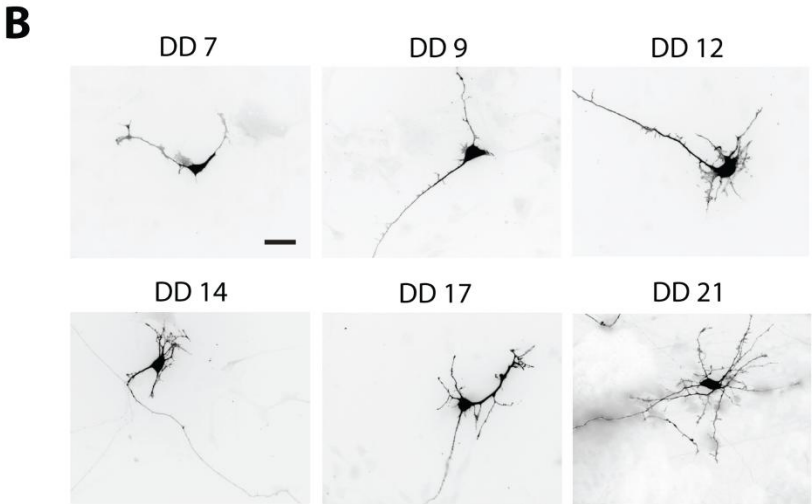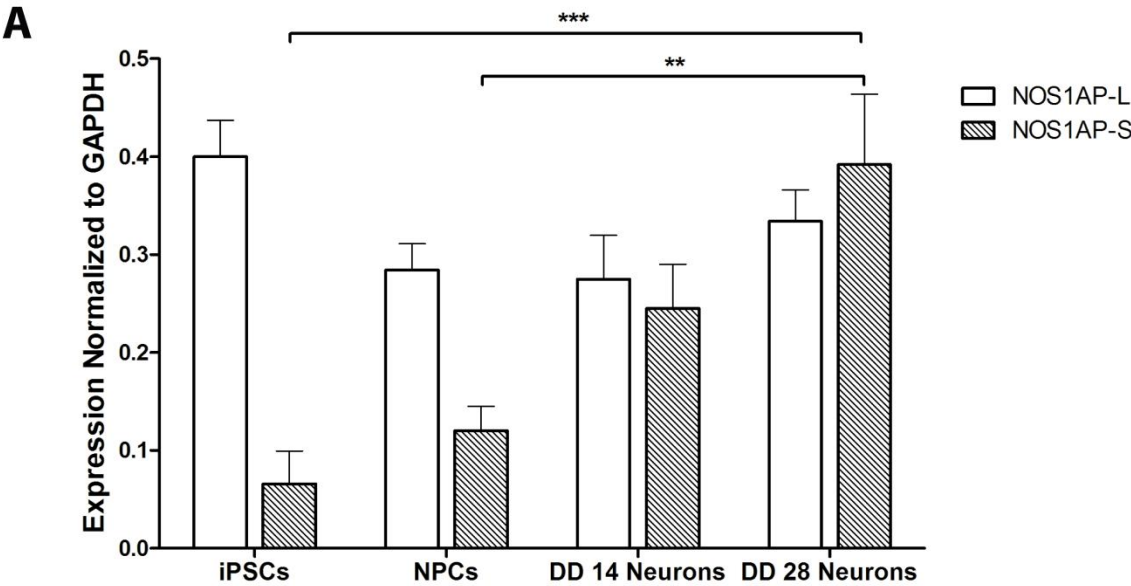
**A**



**B**

**Figure III-4. NOS1AP protein levels alter during neurogenesis. A,** Densitometry analysis of cell lysates from various hiPSC-derived cell types via immunoblotting for two isoforms of NOS1AP, long (L) and short (S). NOS1AP-S increases during neural differentiation, whereas NOS1AP-L expression stays relatively the same. **p< 0.01 and ***p< 0.001 versus neurons on DD 14. p values were determined by one-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate s.e.m. n = 6 for each cell type. **B,** Pattern of neurite outgrowth monitored through transfection of differentiatiing hiPSC-derived NPCs with peGFP. Active primary and secondary branching occurs at DD21 in developing hiPSC-derived neurons. Scale bar = 20 μm.

**Overexpression of NOS1AP isoforms decreases dendrite branching in hiPSC-derived neurons.**

To investigate the role that NOS1AP isoforms play in dendrite branching, we transfected human neurons with constructs encoding GFP or GFP fusions of either the short or long isoform of NOS1AP on DD 19. After 48 hours of expression, the cells were fixed and immunostained for GFP and MAP2 (Figure III-5 A). For dendrite branching analysis, only GFP- and MAP2-positive cells were included, and neurons were traced and analyzed using an automated Sholl analysis (Langhammer et al., 2010) with the experimenter blinded to the condition. In contrast to our findings in rat hippocampal cultures where overexpression of NOS1AP-L decreases dendrite branching between DIV 1-12 and overexpression of NOS1AP-S only has an effect during DIV 5-7 (Carrel et al., 2009), overexpression of either NOS1AP-L or NOS1AP-S significantly decreases dendrite branching in human neurons during the time point when active branching is occurring (Figure III-5 B). These data demonstrate that both NOS1AP-L and NOS1AP-S decrease dendrite branching, and when expression is upregulated too early in development, may result in a reduction in dendritic complexity.

**A**



Control    NOS1AP-L    NOS1AP-S

**B**



| | p > 0.05 |
| | p < 0.05 |
| | p < 0.01 |
| | p < 0.001 |
| | p < 0.0001 |

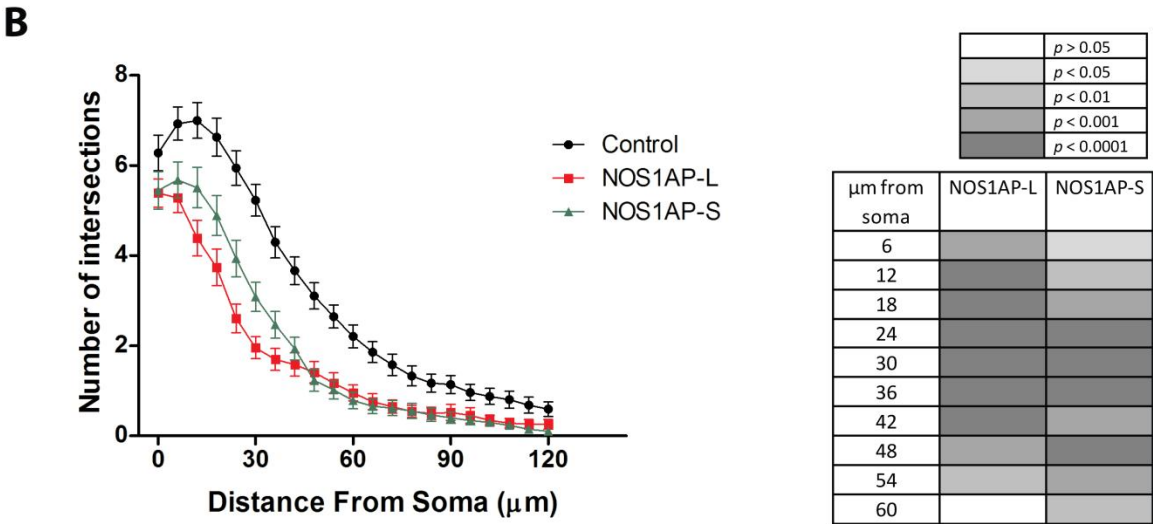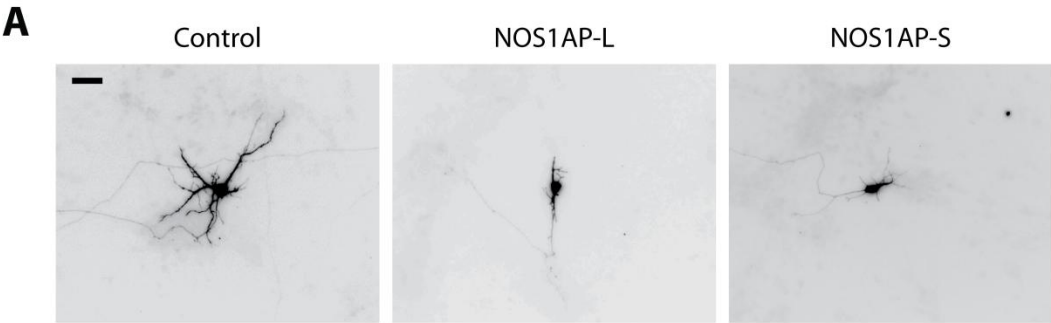| µm from soma | NOS1AP-L | NOS1AP-S |
|---|---|---|
| 6 | | |
| 12 | | |
| 18 | | |
| 24 | | |
| 30 | | |
| 36 | | |
| 42 | | |
| 48 | | |
| 54 | | |
| 60 | | |

**Figure III-5. Overexpression of NOS1AP isoforms decreases dendrite branching in hiPSC-derived neurons. A,** Inverted GFP images of representative hiPSC-derived neurons transfected on DD 19 with constructs encoding GFP (control), GFP-NOS1AP-L, or GFP-NOS1AP-S, and fixed and immunostained for GFP and MAP2 for dendrite counting on DD 21. Scale bar = 20 μm. **B,** Proximal Sholl analysis within the first 120 μm from the soma. Two-way ANOVA followed by Bonferroni multiple comparisons test was performed to compare NOS1AP-L or NOS1AP-S versus control. p values are shown in table. Error bars indicate s.e.m. n = 57 neurons, Control; n = 46, NOS1AP-L; n= 47, NOS1AP-S. Neurons are from three independent cultures for GFP and NOS1AP-L conditions and two independent cultures for NOS1AP-S condition.

**Overexpression of NOS1AP isoforms decreases secondary and higher order dendrite number.**

To better understand how NOS1AP-L and NOS1AP-S overexpression alters dendrite patterning of human neurons, we investigated changes in dendrite length and number when isoforms are overexpressed. Overexpression of either isoform of NOS1AP has no effect on the number of primary dendrites (Figure III-6 A). In contrast, overexpression of NOS1AP isoforms decreases the number of secondary and higher order dendrites compared to control neurons. We did not observe a change in the length of primary, secondary, or higher order dendrites (Figure III-6 B). Our results indicate that overexpression of NOS1AP isoforms decreases higher order dendrites.
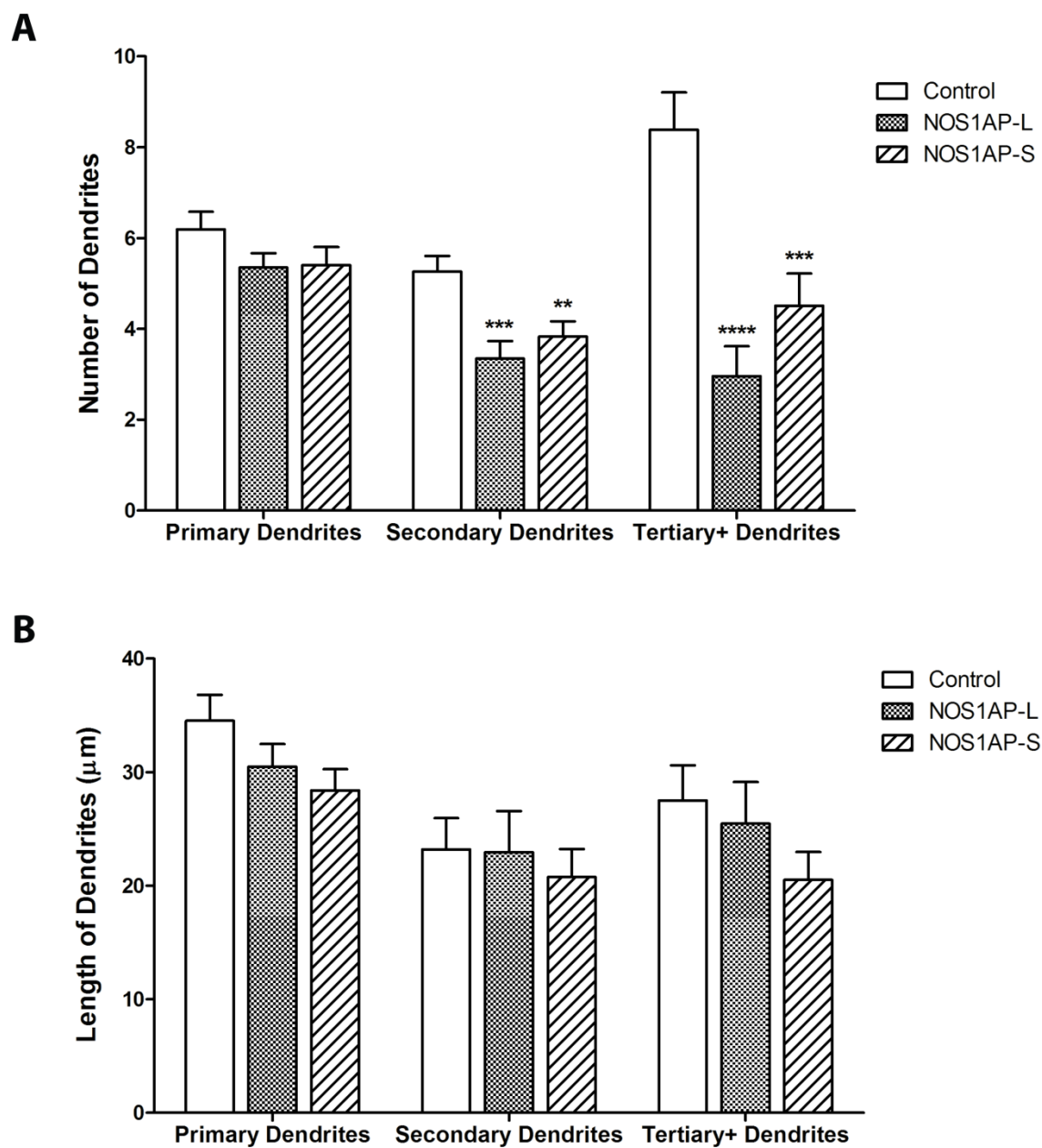
**Figure III-6. Overexpression of NOS1AP isoforms decrease secondary and higher order dendrite number. A,** Quantification of average number of primary, secondary, and higher order (tertiary+) dendrite branches from hiPSC-derived neurons (on DD 21) overexpressing GFP (control), NOS1AP-L, or NOS1AP-S. $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$ versus control. p values were determined by one-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate $\pm$ s.e.m. n = 57 neurons, Control; n = 46, NOS1AP-L, n = 47, NOS1AP-S. **B,** Quantification of average length of primary, secondary, and higher order (tertiary+) branches from DD 21 hiPSC-derived neurons overexpressing GFP (control), NOS1AP-L, or NOS1AP-S. Error bars indicate $\pm$ s.e.m. n = 57 neurons, Control primary length; n = 54 neurons, Control secondary length; n = 51 neurons, Control tertiary+ length; n = 46, NOS1AP-L primary length; n = 37, NOS1AP-L secondary length; n = 21, NOS1AP-L tertiary+ length; n = 47, NOS1AP-S primary length; n = 44, NOS1AP-S secondary length; n = 34, NOS1AP-S tertiary+ length. Neurons are from three independent cultures for GFP and NOS1AP-L conditions and two independent cultures for NOS1AP-S condition.

**Overexpression of NOS1AP isoforms in human NPCs trends towards altering F-actin organization.**

Given that NOS1AP isoforms can alter dendrite branching in human neurons, we investigated whether overexpression of NOS1AP isoforms can influence actin organization in human neural cells. We overexpressed NOS1AP isoforms in human NPCs, as actin filament organization can be more easily observed in this cell type as compared to neurons (Figure III-7 A). Overexpression of NOS1AP-L in human NPCs trends toward a decrease in F-actin organization, whereas overexpression of NOS1AP-S trends toward an increase in F-actin organization. An additional experimental replicate is needed to confirm these findings since the experiment was performed on two independent cultures.

**A**



**B**
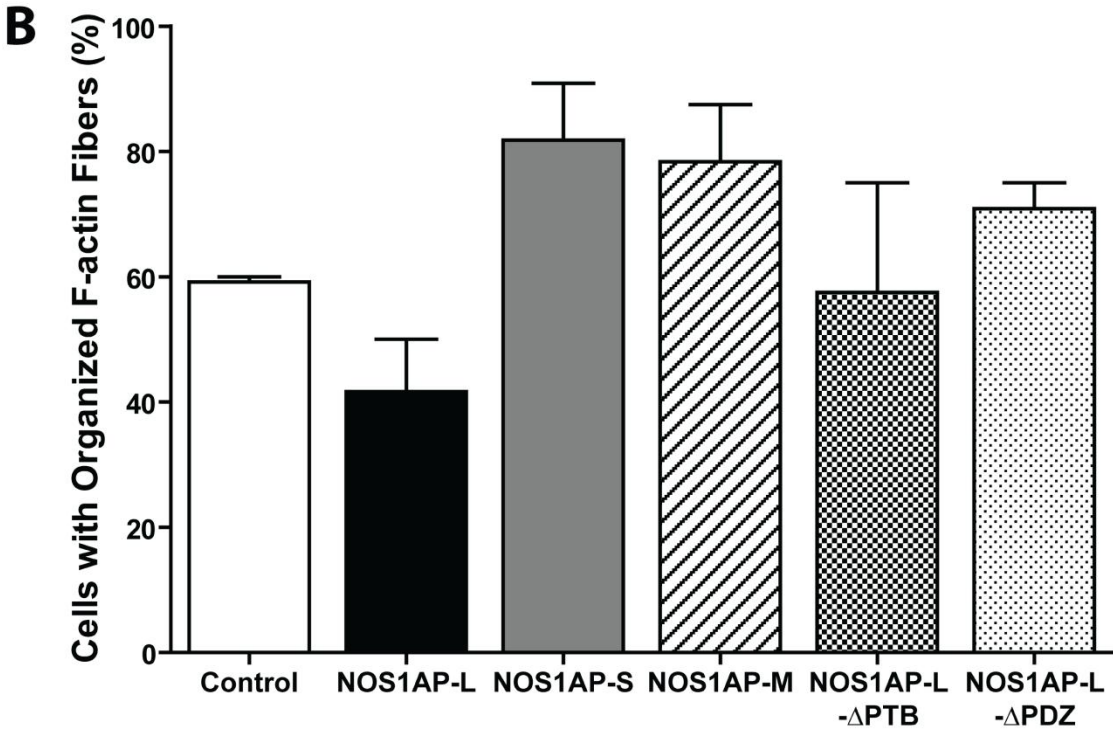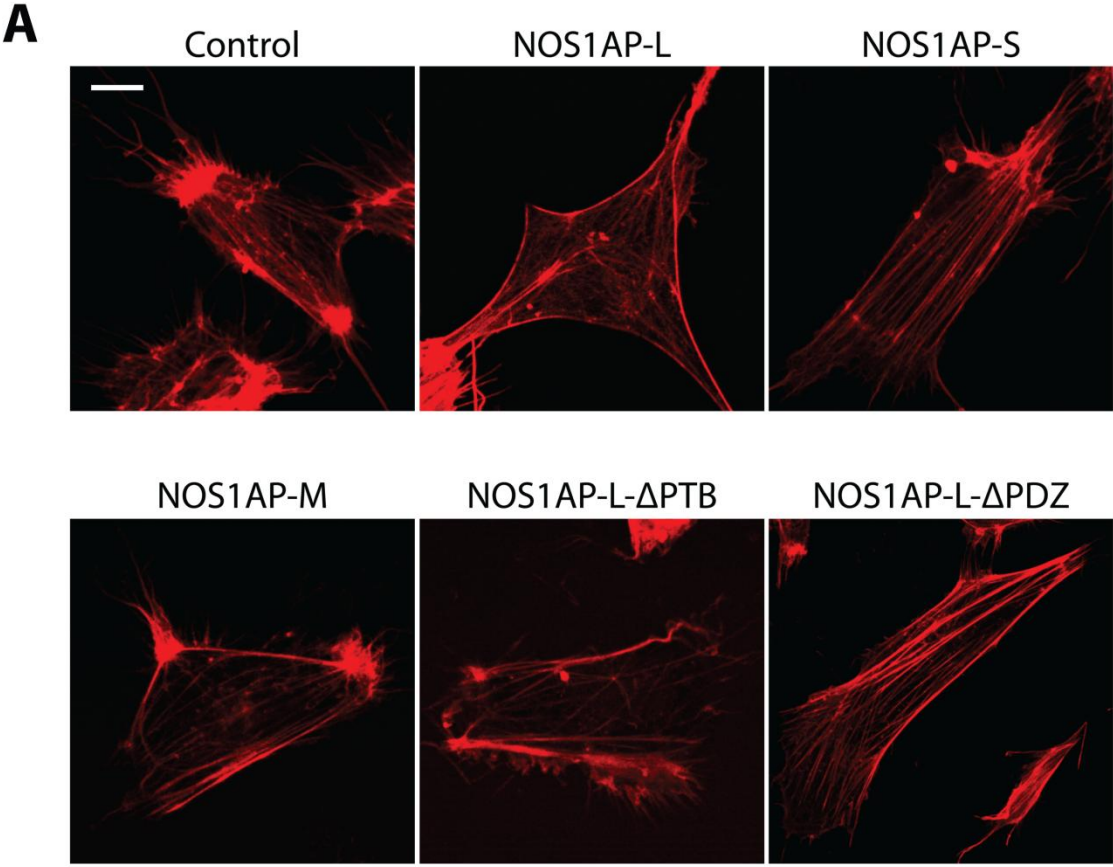
**Figure III-7. Overexpression of NOS1AP-L in hiPSC-derived NPCs trends towards inducing F-actin disorganization. A,** NSCs were transfected with cDNA encoding GFP, NOS1AP-L, or NOS1AP-S and fixed and immunostained for GFP and phalloidin to observe F-actin organization 48 hours later. Representative phalloidin images are shown. Scale bar = 10 µm. **B,** Percentage of GFP + cells with organized F-actin fibers. n = 2 cultures for each condition, with 22 cells analyzed for GFP condition; 19 cells for NOS1AP-L; and 22 cells for NOS1AP-S.

**NMDAR agonist treatment trends toward decreasing NOS1AP protein levels.**

The ability of NOS1AP isoforms to compete with PSD-95 for binding to NOS1 suggests that NOS1AP isoforms may sequester NOS1 away from NMDA receptors (Jaffrey et al., 1998). The decoupling of NOS1 and NMDA receptors prevents the activation of NOS1, and therefore, may disrupt NMDA receptor-dependent dendrite arborization. To determine whether endogenous NOS1AP protein expression in human neurons can be reduced after treatment with pharmacological agents, we treated hiPSC-derived neurons on DD 19 with three commonly prescribed antipsychotic agents, fluphenazine, clozapine, and haloperidol, and two NMDA receptor agonists, D-serine and GLYX-13. Given the therapeutic plasma levels of fluphenazine (0.4 ng/ml), clozapine (100-400 ng/ml), and haloperidol (5-14 ng/ml), with 10- to 30-fold higher levels in brain tissue, neurons were treated with vehicle (DMSO), 12 ng/ml fluphenazine, 3 ug/ml clozapine, or 250 ng/ml haloperidol for 48 hours, from DD 19-21. D-serine is a potent agonist of the glycine co-agonist site of NMDA receptors, whereas GLYX-13 is a partial agonist of the glycine site. To maximally activate NMDAR glycine binding sites, neurons were treated for 48 hours, from DD 19-21, with 10 µM D-serine or 1 µM GLYX-13, concentrations that have been shown to simultaneously enhance long-term potentiation (LTP) and suppress long-term depression (LTD) (Zhang et al., 2008). Protein extracts from neurons treated for 48 hours with either an antipsychotic or a NMDAR agonist were subjected to Western blot analysis to analyze NOS1AP isoform protein expression (Figure III-8 A). Treatment with antipsychotics has no effect on NOS1AP protein expression (Figure III-8 B). Interestingly, treatment with D-serine or GLYX-13 trends toward a decrease in

expression of both NOS1AP isoforms, yet only treatment with D-serine significantly decreases NOS1AP-L protein expression (Figure III-8 B).

**A**
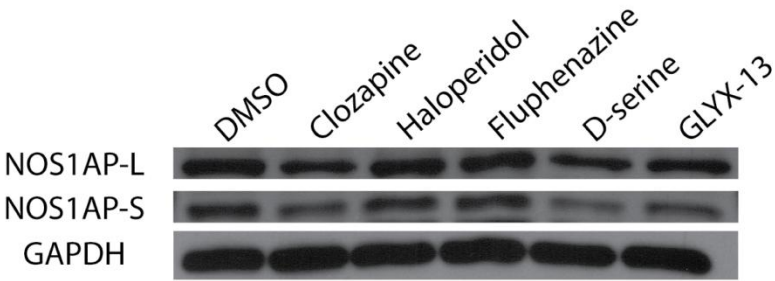


**B**
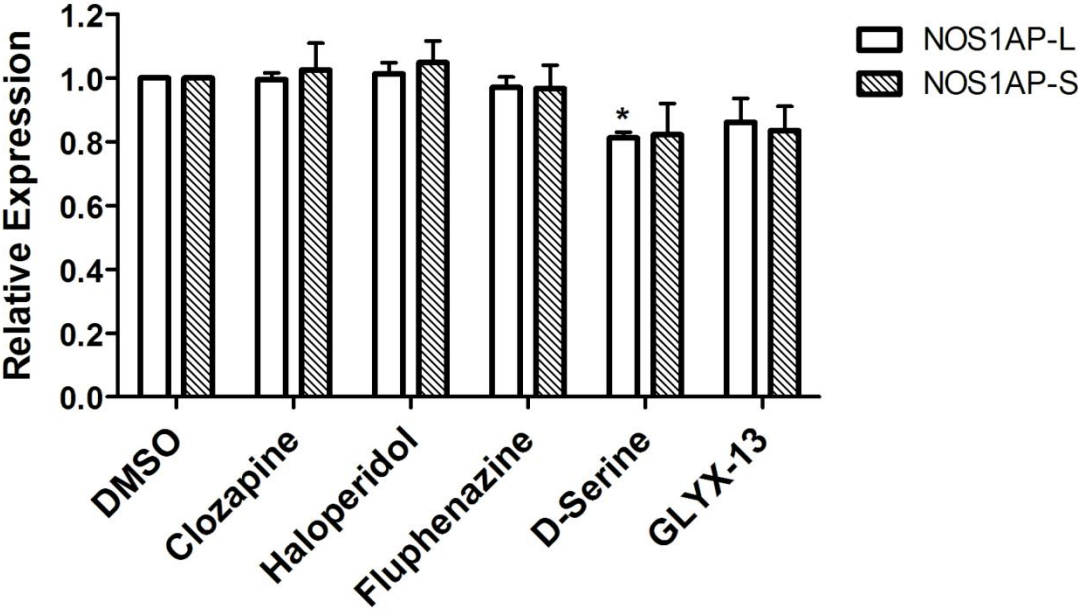
**Figure III-8. NMDAR agonists trends toward decreasing NOS1AP protein levels. A,**
hiPSC-derived neurons were treated with antipsychotics or NMDA receptor agonists on
DD 19 for 48 hours. Extracts from cultures were analyzed by SDS-PAGE and Western
blotting using antibodies that recognize both isoforms of NOS1AP or GAPDH.
Representative blot is shown. **B,** Densitometry analysis of NOS1AP normalized to
GAPDH expression. Error bars indicate ± s.e.m. n = 6, DMSO; n = 5, clozapine, n = 6,
Haloperidol; n = 6, Fluphenazine; n = 6, D-Serine; n = 6, GLYX-13 from three
experimental replicates. All analyses were performed by first normalizing to GAPDH as
an internal loading control and then comparing experimental condition to DMSO. *$p <$
$0.05$ versus DMSO. p values were determined by one-way ANOVA followed by
Bonferroni multiple comparisons test.

**NOS1AP protein expression in SCZ patient-derived human neurons.**

To complement overexpression studies, iPSC lines derived from the blood of subjects with schizophrenia (SCZ) containing the associated allele within the *NOS1AP* gene and iPSC lines from control subjects were differentiated into neurons. Protein extracts from iPSCs, NPCs, neurons on DD 14, and neurons on DD 28 derived from SCZ and control subjects were subjected to Western blot analysis to analyze NOS1AP protein expression. Neurons (DD28) derived from subjects with schizophrenia trend toward increased protein levels of NOS1AP-L and NOS1AP-S compared to neurons derived from control subjects (Figure III-9). Additional experimental replicates and affected subjects and control subjects are needed to verify these preliminary findings.
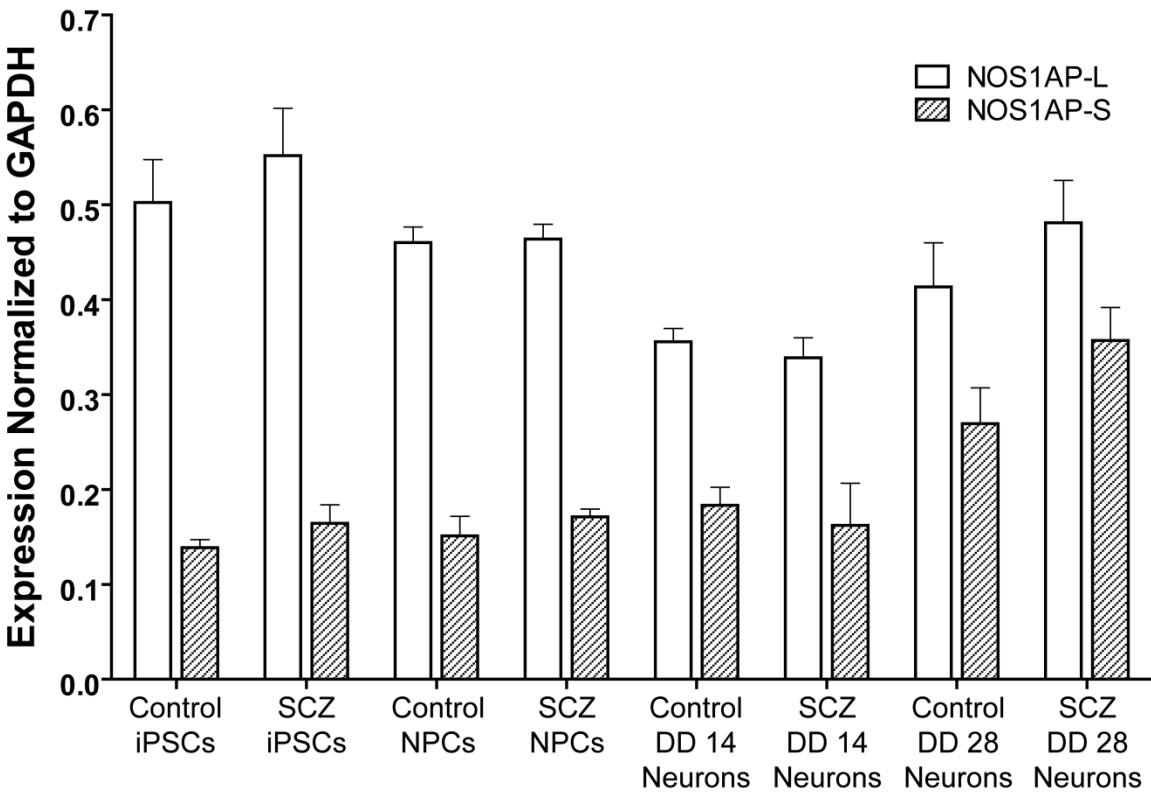
**Figure III-9. NOS1AP protein expression in SCZ patient-derived human neurons.**

Densitometry analysis of cell lysates from various human induced pluripotent stem cell (iPSC)-derived cell types via immunoblotting for NOS1AP-L and NOS1AP-S. Preliminary data show that neurons (DD28) derived from patients with schizophrenia trend toward increased NOS1AP protein levels compared to control neurons. iPSC lines were generated from the blood of patients with schizophrenia carrying the associated allele within *NOS1AP* and unaffected subjects as controls. SCZ, schizophrenia affected. n = 2 for all conditions representing results from 2 SCZ subjects and 2 control subjects.

## DISCUSSION

A subset of patients with schizophrenia display single nucleotide polymorphisms (SNPs) within *NOS1AP* that have been found to be significantly associated with schizophrenia (Brzustowicz et al., 2004; Zheng et al., 2005; Kremeyer et al., 2008). Furthermore, a published study identified one SNP in *NOS1AP* that increases gene expression by enhancing transcription factor binding (Wratten et al., 2009). Coupling these data with the finding of an increase in both mRNA and protein expression of NOS1AP isoforms in postmortem brain samples of subjects with schizophrenia (Xu et al., 2005; Hadzimichalis et al., 2010) points to the study of functional implications of increased levels of NOS1AP in a human neuronal culture system.

In this chapter, we characterize a human neuronal cell culture system that we use to investigate the role of NOS1AP isoforms in human neuronal development. We found that NOS1AP-S protein levels increase along neural development from NPCs to neurons on DD 28. However, NOS1AP-L expression maintains relatively constant expression throughout development. Our results are consistent with NOS1AP expression in the developing rat brain, with NOS1AP-S expression increasing dramatically during development from E15 to P14 and NOS1AP-L expression increasing only moderately (Carrel et al., 2009). To investigate the role of NOS1AP-L and NOS1AP-S in the regulation of dendrite branching of human neurons, we overexpressed two isoforms of NOS1AP at the time point when active primary and secondary branching occurs in our cell culture system. Overexpression of either NOS1AP-L or NOS1AP-S dramatically decreases the dendritic complexity of the developing human neuron. Our results indicate that both isoforms of NOS1AP can influence the dendritic architecture of human

forebrain glutamatergic neurons and that altered expression of NOS1AP may contribute to the dendritic defects observed in the prefrontal cortex of patients with schizophrenia.

An *in vitro* model of human neuronal development affords the ability to screen drugs for their potential therapeutic or harmful effects on human neurodevelopment. To identify a drug that would rescue the dendrite branching phenotype seen with overexpression of NOS1AP isoforms, we tested two NMDA receptor agonists, D-serine and GLYX-13. The NMDA receptor plays an important role in dendritogenesis. Specifically, studies have shown that antagonism of the NMDA receptor results in reduced dendritic arborization of Purkinje cells and spinal motor neurons (Kalb, 1994; Vogel and Prittie, 1995). Interestingly, after treatment with D-Serine we observe a significant decrease in NOS1AP-L protein expression and a trend towards a decrease in NOS1AP-S protein expression in hiPSC-derived neurons. Treatment with GLYX-13 results in a trend towards a decrease in NOS1AP isoform expression in hiPSC-derived neurons, but results did not reach significance. NMDA receptor agonists D-serine and GLYX-13 have been shown to be cognitive enhancers in learning and memory models (Kantrowitz et al.; Bado et al., 2011; Burgdorf et al., 2011). Further studies are needed to determine whether NMDA receptor glycine site agonists can prevent the reduction in dendritic complexity caused by increased levels of NOS1AP by reducing NOS1AP protein levels to control levels. Our studies demonstrate how a human neuronal cell culture system can be used as a platform to screen drugs to be used for the treatment of specific schizophrenia genotypes.

Lastly, we generated human neurons using iPSC technology from subjects with schizophrenia that have the associated *NOS1AP* allele and control subjects. Preliminary

results indicate that protein expression of NOS1AP isoforms may be increased in mature neurons compared to control neurons of the same maturation state. This system could be used to identify biomarkers for schizophrenia for a subset of patients, such as increased NOS1AP protein levels. In summary, these studies demonstrate how hiPSC technology can circumvent the difficulty of accessing human neural stem cells and neurons for large-scale *in vitro* studies.

## ACKNOWLEDGEMENTS

I would like to thank Steven Clarke in Dr. Kenneth Paradiso's laboratory for his contribution to Fig. III-2 by performing the electrophysiology recordings.

# Summary and Future Directions

Individuals with schizophrenia display a variable number of symptoms that fall into three main categories: positive, negative, and cognitive. Currently available antipsychotic medications largely target the dopamine D2 receptor and are most effective in treating the positive symptoms of the illness, while there is little to no improvement in negative or cognitive symptoms (Horacek et al., 2006; Keefe et al., 2007; Davidson et al., 2009; Lally and MacCabe, 2015). As such, investigations into other molecular targets or cellular processes that are altered in these individuals could identify new therapeutic strategies. Increased NOS1AP-L and NOS1AP-S protein expression in the DLPFC of individuals with schizophrenia coupled with alterations in neurodevelopmental processes regulated by NOS1AP isoforms suggest that NOS1AP isoforms play a role in cognitive function. To support this notion, it has been reported that healthy individuals carrying a schizophrenia-associated allele within *NOS1AP* show significantly greater activation of the DLPFC during a task of working memory (Brzustowicz, 2008). These findings suggest that identifying potential pathways and molecular targets affected by NOS1AP isoforms may prove important for understanding the cognitive deficits observed in schizophrenia and can guide future therapeutic studies.

Improper regulation of the actin cytoskeleton can result in the disruption of several key neurodevelopmental processes. Changes in normal expression of proteins involved in early migration of neurons, axonal and dendritic outgrowth, and synaptogenesis have been observed in postmortem brain tissues from individuals with schizophrenia (Fatemi and Folsom, 2009), including a study by our group showing that expression of three isoforms of NOS1AP is increased in the DLPFC of individuals with schizophrenia (Hadzimichalis et al., 2010). Two of these isoforms, NOS1AP-L and

NOS1AP-S, have been reported to influence dendrite branching in cultured rat hippocampal neurons (Carrel et al., 2009). In chapter 1, we provided insight into how NOS1AP isoforms can alter the microtubule actin cytoskeleton to disrupt normal neuronal development. Our results suggest that overexpression of NOS1AP-L may play a role in the stabilization of microtubules, whereas overexpression of NOS1AP-S disrupts microtubule organization. With regards to the actin cytoskeleton, both isoforms of NOS1AP can influence actin dynamics, although they act via distinct mechanisms. Overexpression of NOS1AP-L induces filopodia formation in heterologous cells. On the other hand, overexpression of NOS1AP-S results in no obvious change in cell shape, yet it significantly increases the rate of actin polymerization. The increased rate of actin polymerization may be the result of the observed decrease in total Rac1 and cofilin protein levels after NOS1AP-S overexpression. Future studies are needed to determine if either isoform of NOS1AP can directly bind to F-actin and if their association with F-actin is required to influence actin dynamics.

As spine formation and maturation are intimately tied to the regulation of the actin cytoskeleton, we next investigated the role of NOS1AP isoforms in spinogenesis. In chapter 2, we report that both NOS1AP-L and NOS1AP-S associate with F-actin in rat brain and can influence spine formation and maturation. Overexpression of NOS1AP-L significantly increases the number of immature spines in cultured rat cortical neurons. In contrast, overexpression of NOS1AP-S significantly increases the number of both immature and mature spines. To investigate whether these morphological changes in spines result in functional changes, we recorded mEPSCs from cortical neurons overexpressing NOS1AP-L or NOS1AP-S. Cortical neurons overexpressing NOS1AP-L

exhibit a significant reduction in the amplitude of mEPSCs, suggesting a reduction in the number or function of glutamate receptors in the postsynaptic density. This finding is consistent with the observation that NOS1AP-L overexpression results in an increase in immature spines, i.e. spines that may not have functional synapses. Cortical neurons overexpressing NOS1AP-S exhibit a significant increase in the frequency of mEPSCs, suggesting an increase in the number of functional synapses. The increase in mEPSC frequency correlates with the observed increase in the number of mature spines with NOS1AP-S overexpression. Taken together, our results demonstrate that NOS1AP-L overexpression decreases synaptic strength, whereas NOS1AP-S overexpression increases synaptic strength. Future studies are needed to determine if NOS1AP isoforms alter the number of or the function of AMPA and NMDA receptors at the synapse. Lastly, in chapter 2, we show that overexpression of NOS1AP-L in neural progenitor cells *in vivo* can disrupt normal dendrite development. Cortical neurons overexpressing NOS1AP-L exhibit a reduction in dendrite length and number. These results further strengthen the notion that NOS1AP-L plays a major role in dendrite development.

In the final chapter, we demonstrate the use of human induced pluripotent technology to establish a human neural cell culture system. We adapted methods to generate a population of human glutamatergic neurons derived from forebrain-specific neural progenitor cells. Using this cell culture system, we show that overexpression of NOS1AP-L or NOS1AP-S in developing human neurons during the time period of active dendrite branching results in the reduction of secondary and higher order dendrite number. These results differ from our findings in rat neurons and demonstrate the importance of a human neuronal model system. We then tested antipsychotic drugs and

NMDA receptor agonists for their potential to reduce NOS1AP expression in human neurons. Only treatment with the NMDA receptor agonists trended toward a reduction in NOS1AP protein expression. Indeed, treatment with D-Serine significantly reduced NOS1AP-L protein expression in hiPSC-derived neurons. Future studies are needed to determine if treatment with NMDA receptor agonists can rescue normal dendrite branching in human neurons with increased protein levels of NOS1AP. NMDA receptor agonists may be a potential novel treatment for patients with schizophrenia associated with specific risk alleles at SNPs in the *NOS1AP* gene.

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