THE NITRIC OXIDE SYNTHASE ADAPTOR PROTEIN (NOS1AP) ASSOCIATES WITH SCRIBBLE AND REGULATES DENDRITIC SPINE DEVELOPMENT

by

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DEDICATION PAGE

I need to dedicate this work to those of you who have shown me such incredible never-ending support throughout this process. You know who you are so I don’t feel the need to mention names. As unpredictable as life can be, I really look upon you all for guidance and wisdom and you always deliver! Thank-you.

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ABSTRACT

In a targeted proteomic screen to identify polarity protein complexes, a number of Scribble (Scrib) -associating proteins were identified; of particular interest was the Nitric Oxide Synthase 1 Adaptor Protein (NOS1AP). NOS1AP contains an N-terminal phosphotyrosine binding (PTB) domain and a C-terminal PSD-95/Dlg homology/ZO-1 (PDZ) binding motif that associates with neuronal NOS (NOS1). We show that the PTB domain of NOS1AP associates with the fourth PDZ domain of Scrib. We identified NOS1AP binding partners including three key regulators of dendritic spine formation, β-Pix, Git1, and PAK, which require Scrib to associate with NOS1AP. Overexpression of NOS1AP in cultured hippocampal neurons increases dendritic protrusions, a process dependent on the PTB domain. The increase in dendritic protrusions can be blocked by the co-expression of a dominant negative Rac construct. NOS1AP, and the PTB domain of NOS1AP influence Rac activity. Together these data suggest that Scrib and NOS1AP function as important scaffolding proteins in the mammalian synapse and that NOS1AP functions in the dendritic spine by influencing Rho GTPase activity.

LIST OF ABBREVIATIONS AND SYMBOLS USED

μm	micrometer
β-Pix	β-p21-activated kinase interacting exchange factor
AMOT	Angiomotin
AMPAR	α-amino-3-hydroxyl-5-methyl-4-isoxazote-propionic acid receptor
APC	Adenomatous polyposis coli
aPKC	atypical Protein Kinase C
Arp2/3	Actin-related protein 2 and 3
Asp	Asparagine
$CaPO_4$	Calcium phosphate
CAZ	Cytoskeletal matrix at the active zone
Cdc42	Cell division cycle 42
CM	Conditioned medium
CNS	Central nervous system
COOL	cloned out of library
CP	Capping Protein
CPE	Carboxypeptidase E
Crb	Crumbs
CRIB	cdc42/Rac interactive binding
CRMP2	Collapsin response mediator protein 2
DH	Dbl homology
DISC	Disrupted in Schizophrenia
DIV	Days *in vitro*
Dlg	Disc-Large
DMEM	Dubelco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E19	Embryonic Day 19
EDTA	Ethylenediaminetetraacetic acid
FGF	Fibroblast Growth Factor
Erc	Extracellular signal-related kinase
ERC1	ELKS/RAB6-interacting/CAST family member1
F-actin	Filamentous actin
FBS	Fetal Bovine Serum
FMR-1	Fragile-X mental retardation
G-actin	monomeric or soluble actin
GAP	GTPase activating protein
GBD	Git binding domain
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green Fluorescent Protein
Git1	G-protein coupled receptor kinase interacting protein1

GSKβ	Glycogen synthase kinase 3
GST	Glutathione-S-Transferase
GTP	Guanosine triphosphate
GUK	Guanylate kinase
Gukh	Guanylate kinase-holder
HBSS	Hanks Balanced Salt Solution
HeBS	HEPES Buffered Saline
HEK	Human Embryonic Kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPV	Human Papillomavirus
hScrib	mammalian homologue of Scrib
IP	Immunoprecipitate
IRS	Insulin Receptor Substrate
Lgl	Lethal Giant Larvae
LNX	Ligand of Numb Protein X
LPP	Lipoma Preferred Partner
LQTS	Long QT syndrome
LRR	Leucine Rich Repeat
LTP	Long-term potentiation
MAGUK	membrane-associated guanylate kinase
MAP	Microtubule associated protein
MARK2	Microtubule affinity regulating kinase -2
MDCK	Madin-Darby canine kidney
MEM	Minimum Essential Medium
$MgCl_2$	Magnesium Sulphate
MLC	Myosin-II regulatory light chain
MR	Mental Retardation
NaCl	Sodium Chloride
NAK	Numb Associated Kinase
NGF	Nerve Growth Factor
NMDAR	N-methyl-D-aspartate Receptor
NMJ	Neuromuscular Junction
nNOS	neuronal Nitric Oxide Synthase
NO	Nitric Oxide
CAPON	Carboxyl-terminal PDZ ligand of nNOS
NOS1AP	Nitric Oxide Synthase 1 Adaptor Protein
NOS1APc	Nitric Oxide Synthase 1 Adaptor Protein c (CAPONLong)
NP40	Nonident-P 40
nWASP	neuronal Wiskott-Aldrich syndrome protein
PAK	p21-activated kinase
PALS	protein associated with LIN7
PAR	Partitioning Defective
Patj	Pals-associated tight junction protein
PBD	PAK binding domain
PBS	Phosphate Buffered Saline
PDZ	PSD-95/ZO-1/Dlg

PEI	Polyethyleneimine
PFA	Paraformaldehyde
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-Bisphosphate
PKC	Protein kinase C
PMSF	Phenylmethylsuphonyl Fluoride
PNS	Peripheral nervous system
Pro	Proline
PSD	Post-synaptic density
PTB	Phosphotyrosine binding
pY	phosphorylated tyrosine
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras-related homologue member A
RP	Reserve Pool
RRP	Readily Releasable pool
SAP97	synapse-associated protein 97
Scrib	Scribble
SDS	Sodium dodecyl sulphate
Sdt	Stardust
Ser	Serine
SFM	Serum Free Medium
SH3	Src-homology3
STEF	STF and TIAM1-like exchange factor
Thr	Threonine
TIAM1	T-lymphoma and metastasis protein
TJ	Tight Junction
Val	Valine
WB	Western Blot
WD-40	Tryptophan-aspartic acid 40
X	Any amino Acid
ZO	Zonula Occludens

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CHAPTER 1 INTRODUCTION

1.1 Components of a Neuron

The neuron is the fundamental unit of the nervous system. Neurons are formed from progenitor cells and upon terminal differentiation, mature and develop a complex polarized morphology. Neurons typically contain a cell body, one long extension designated as the axon, and multiple small extensions known as dendrites (Arimura and Kaibuchi, 2007). Connections between the axon of one cell and its target cell occurs at a unique structure known as the synapse (Arimura and Kaibuchi, 2007).

1.1.1 Axon

Each neuron extends one axon from the cell body. Axons are typically long processes and are homogeneous in width (Arimura and Kaibuchi, 2007). The axon propagates signals that have been integrated in the cell body. This signal is then sent along the axon to the synapse (Arimura and Kaibuchi, 2007). The signal is then propagated across the synaptic cleft to activate receptors on the post-synaptic region allowing for conduction or continuation of the signal (Arimura and Kaibuchi, 2007).

1.1.2 Synapse

The synapse consists of a presynaptic region where the axon connects with a target dendrite and postsynaptic region where excitatory synaptic inputs are received by dendritic spines on the target cell (Fig. 1.1) (Dillon and Goda, 2005). The presynaptic region contains many neurotransmitter-filled synaptic vesicles (Wiggin et al., 2005). There are two main locations within the presynaptic compartment that contain synaptic vesicles, the readily releasable pool (RRP) and the reserve pool (RP) (Dillon and Goda, 2005; Wiggin et al., 2005). The RRP contains a synaptic vesicle pool docked and primed for release at the active zone (Dillon and Goda, 2005). The active zone is the point of contact between the membrane and synaptic vesicles containing neurotransmitters ready for release (Rosenmund et al., 2003). The RP consists of vesicles in the presynaptic

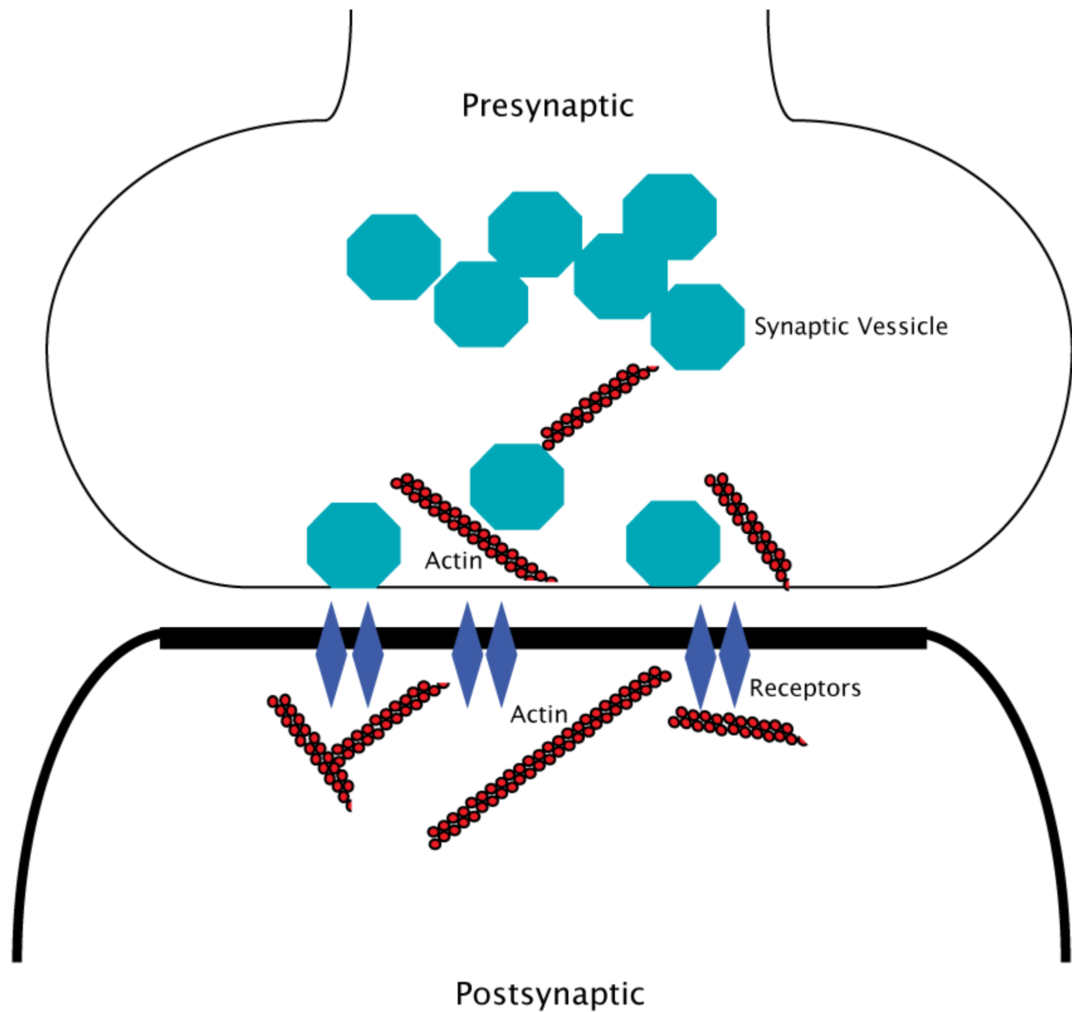


Figure 1.1 Model of the synapse and related components.
The axon presynaptic terminal has a a reserve vesicle pool and a readily releasable vesicle pool ready to be released in the active zone. The dendritic spine contains the receptors ready to receive neurotransmitters from the presynaptic side. Note the actin cytoskeleton at both the pre- and post- synaptic terminal, regulating vesicle release and in the structure of the postsynaptic compartment. Modified from Dillon and Goda, 2005.

compartment that have to translocate the active zone before being released (Dillon and Goda, 2005). Once in an active zone, a synaptic vesicle can fuse with the membrane and send neurotransmitters to the postsynaptic compartment (Kandel et al., 2000). From the postsynaptic compartment, a chemical signal is converted to an electrical signal and propagated along a dendrite towards the cell body (Kandel et al., 2000).

1.1.3 Dendrites

Each neuron typically has several dendrites that extend out from the cell body (Arimura and Kaibuchi, 2007). Dendrites are commonly shorter than axons and start thicker at the base of the cell body and thin out distally (Arimura and Kaibuchi, 2007). Dendrites have small protrusions called dendritic spines. These can be small finger-like projections or mushroom shaped structures known as spine-heads found along the dendrite (Sekino et al., 2007). These are enriched with receptors that bind neurotransmitters (Arimura and Kaibuchi, 2007). At the leading edge of a dendritic spine is the postsynaptic density (PSD), an electron dense region composed of an actin rich structural element (Sekino et al., 2007). In addition to neurotransmitter receptors that receive signal input from the presynaptic compartment, dendritic spines contain an array of postsynaptic proteins responsible for binding actin, scaffolding proteins, and signaling molecules (Nimchinsky et al., 2002; Sekino et al., 2007).

Development of a functional polarized phenotype in neurons is dependent on actin dynamics. Evidence for this comes from studies using actin depolymerizing drugs that, when applied to a developing neuron, cause altered neuronal phenotypes such as multiple axon formation (Bradke and Dotti, 1999). In the synapse actin plays a structural role in formation of the pre- and post- synaptic compartments (Fig. 1.1) (Dillon and Goda, 2005). In addition to its structural role, actin also regulates the function of the synapse (Dillon and Goda, 2005). For example, presynaptically actin plays a role in synaptic vesicle dynamics, primarily in the RP where synaptic vesicles are stored before release (Dillon and Goda, 2005). Since much of the work in this thesis focuses on actin dynamics, an introduction to actin is necessary.

1.1.4 (a) Key Regulators of Neuronal Development – Actin

Actin is a major structural protein found within a cell that is important for both maintaining cell shape and allowing cellular migration (Dillon and Goda, 2005). Actin can be found in two different forms, Filamentous (F-actin) and monomeric or soluble actin (G-actin) (Dillon and Goda, 2005). F-actin is comprised of molecules of G-actin noncovalently bound to one another forming the filaments (Dillon and Goda, 2005). F-actin is lengthened at the fast growing or barbed end through the addition of G-actin and undergoes catastrophe at the other end known as the pointed end (Welch and Mullins, 2002; Dillon and Goda, 2005). Here, the molecules of filamentous actin are removed and returned to the G-actin pool (Welch and Mullins, 2002; Dillon and Goda, 2005). Thus, G-actin is recycled from the pointed end to the barbed end (Dillon and Goda, 2005). This process maintains the supply of soluble actin for the continuous development of F-actin (Dillon and Goda, 2005).

The addition and removal of actin is regulated by a number of effector proteins such as neuronal Wiskott-Aldrich syndrome protein (nWASP), cofilin, actin-related protein 2/3 (Arp2/3), Gelsolin, and Lim kinase (Dillon and Goda, 2005). These proteins serve various functions in the life cycle of filamentous actin such as capping, actin nucleation, and catastrophe (Dillon and Goda, 2005). Actin filaments can be capped at either end by F-actin Capping Protein (CP) or Gelsolin (Machesky and Insall, 1999; Dent and Gertler, 2003; Dillon and Goda, 2005; Cooper and Sept, 2008). Here proteins are added to either end preventing the addition of new G-actin or removal of actin (Machesky and Insall, 1999; Dent and Gertler, 2003; Dillon and Goda, 2005; Cooper and Sept, 2008). For example, CP and Gelsolin are responsible for binding to actin filament barbed ends to block the extension of actin filaments (Machesky and Insall, 1999; Cooper and Sept, 2008). New actin filaments can grow out from already formed filaments. For this to occur, new actin must be nucleated (Dillon and Goda, 2005). In the case of actin nucleation, proteins such as Arp2/3 are responsible for initiation of new filaments at nucleation sites (Dent and Gertler, 2003; Dillon and Goda, 2005). Finally, actin filaments can be destroyed via a process known as catastrophe (Dent and Gertler, 2003; Dillon and Goda, 2005). Filament catastrophe is initiated by another set of proteins, one important

member being cofilin (Dent and Gertler, 2003; Dillon and Goda, 2005), Cofilin binds either G- or F- actin in a phospho-dependent manner (Arber et al., 1998; Bamburg, 1999; Edwards et al., 1999; Stanyon and Bernard, 1999; Maciver and Hussey, 2002; Bokoch, 2003). Once bound, cofilin serves two separate functions (Maciver, 1998; Rosenblatt and Mitchison, 1998; Maciver and Hussey, 2002). The first function for cofilin is to sever actin filaments and the second is to increase the rate of G-actin removal from the pointed end of actin filaments (Maciver, 1998; Rosenblatt and Mitchison, 1998; Maciver and Hussey, 2002). This leads to overall actin filament catastrophe (Maciver, 1998; Rosenblatt and Mitchison, 1998; Maciver and Hussey, 2002).

1.1.4 (b) Key Regulators of Neuronal Development – Rho GTPases

Proteins that regulate actin filament development and catastrophe are themselves regulated by small GTPases known as Rho GTPases. (Dillon and Goda, 2005). Rho GTPases are a subgroup of the small GTPase superfamily (Luo, 2000). The three most common Rho GTPases affecting the actin cytoskeleton are Ras homologue member A (RhoA), Ras-related C3 botulinum toxin substrate (Rac), and cell division cycle 42 (cdc42) (Luo, 2000). These Rho GTPases function as molecular switches (Luo, 2000). They can be found in either an active GTP bound state or an inactive GDP bound state (Fig 1.2) (Luo, 2000). Evidence for the involvement of activated Rho GTPases in regulating the actin cytoskeleton stems from overexpression studies. Overexpression of Rac in cells results in lamellipodia formation or membrane ruffling (Ridley et al., 1992; Luo, 2000). Overexpression of cdc42 leads to filopodia formation or finger-like projections (Nobes and Hall, 1995; Luo, 2000). Overexpression of Rho leads to a loss of stress fibers (Ridley and Hall, 1992; Luo, 2000). When activated, these Rho GTPases target downstream, actin effectors.

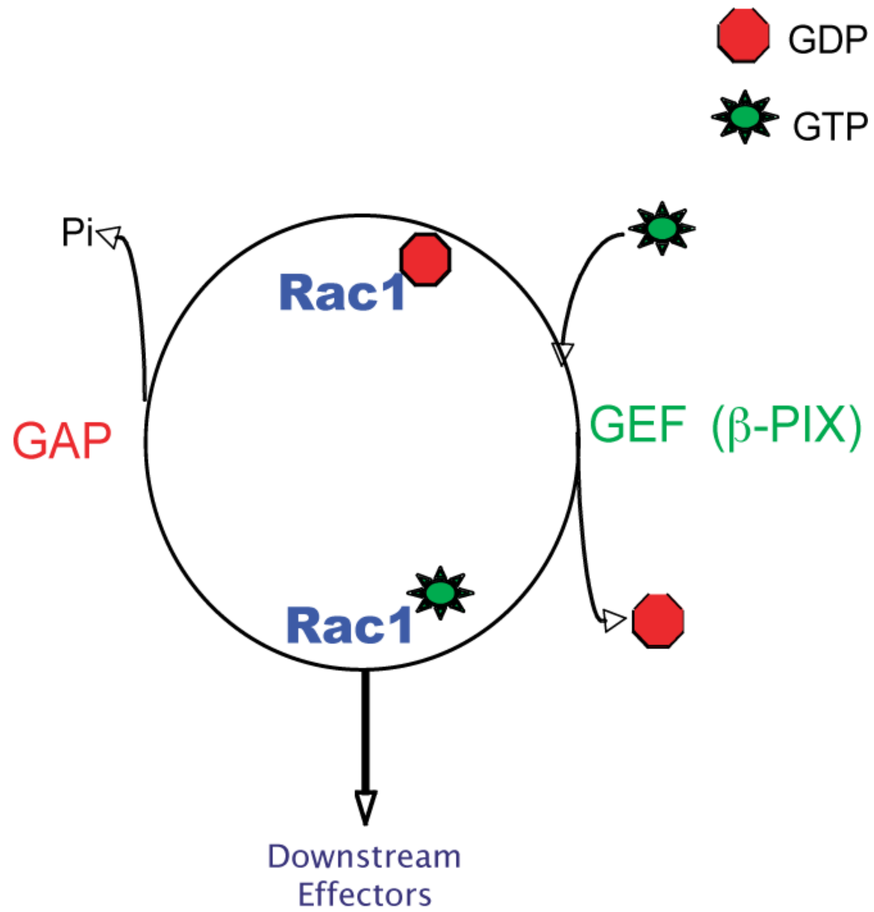


Figure 1.2 Model of RhoGTPase activation as modelled by Rac activation. Rho-GTPases are activated in a GTP bound state by Guanine Nucleotide Exhange Factors (GEFs) and inactivated by GTP hydrolysis by GTP activating protiens (GAPs), Adopted from Etienne-Manneville and Hall, 2002.

Rho GTPases target various downstream effectors to elicit responses in actin binding proteins. For example, activated Rac targets synthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2), which in turn removes the capping protein, Gelsolin, from the barbed ends of actin filaments allowing filament polymerization (Machesky and Insall, 1999). The protein n-WASP binds and is activated by the Rho GTPase cdc42.
The association between cdc42 and nWASP functions as a switch to enable n-WASP association with the actin nucleating protein Arp2/3 leading to the development of actin filaments (Luo, 2000). In an example of actin catastrophe, active Rac binds p21-activated kinase (PAK) leading to kinase activation of PAK (Bamburg, 1999; Edwards et al., 1999; Stanyon and Bernard, 1999; Bokoch, 2003). Active PAK in turn activates Lim kinase by a phospho-dependent mechanism (Bamburg, 1999; Edwards et al., 1999; Stanyon and Bernard, 1999; Bokoch, 2003). Active Lim kinase then phosphorylates cofilin (Arber et al., 1998; Bamburg, 1999; Edwards et al., 1999; Stanyon and Bernard, 1999; Bokoch, 2003). Phospho-cofilin can no longer bind actin, thus loses its ability to cause actin catastrophe (Arber et al., 1998; Rosenblatt and Mitchison, 1998; Bamburg, 1999; Maciver and Hussey, 2002). In the nervous system, Rho GTPases play a role in regulating actin dynamics in axon and dendrite formation and in synaptic function through the regulation of many different actin regulatory proteins (Van Aelst and D'Souza-Schorey, 1997; Dent and Gertler, 2003; Dillon and Goda, 2005). Thus, understanding actin regulation is critical to understanding neuronal polarity. Much of this work on actin and actin regulatory proteins has been characterized using primary dissociated hippocampal neurons as a model system.

1.2 Dissociated Hippocampal Neurons and Neuron Development

One of the best characterized models for neuronal development and polarization is the rat dissociated hippocampal neuron culture (Arimura and Kaibuchi, 2007). This model system was first characterized by Banker, Dotti, and Goslin in the 1980’s (Dotti and Banker, 1987; Dotti et al., 1988; Goslin and Banker, 1989; Arimura and Kaibuchi, 2007). In studying these neurons, Banker and colleagues noted five distinct morphological stages of development (Fig. 1.3) (Dotti et al., 1988; Arimura and Kaibuchi, 2007).

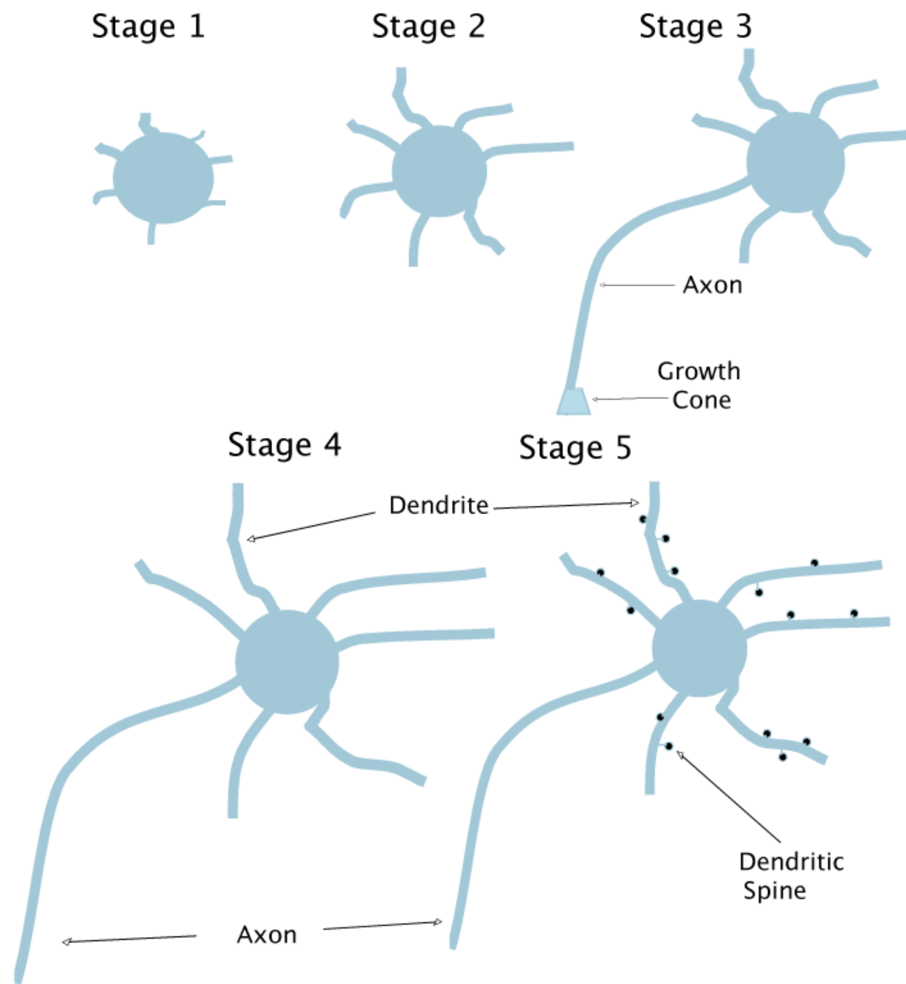


Figure 1.3 Model of the five stages of cultured hippocampal neuron development as proposed by Banker and Colleagues.Modified from Dotti et al, 1988 and Arimura and Kaibuchi, 2007.

The first stage occurs immediately after isolation, dissociation and plating of E18 rat hippocampal neurons (Dotti et al., 1988; Arimura and Kaibuchi, 2007). Upon plating, individual cells extend membrane ruffles known as lamellipodia around the cell body (Dotti et al., 1988; Arimura and Kaibuchi, 2007). Stage two occurs a few hours later, here small or "minor" processes extend from the cell body (Dotti et al., 1988; Arimura and Kaibuchi, 2007). In the next stage, stage 3, one of the minor processes rapidly extends out, becoming an axon, a process occurring within 12 hours to two days of plating (Dotti et al., 1988; Arimura and Kaibuchi, 2007). In stage 4, the other minor processes then go through growth and retraction stages for another few days before extending out to become dendrites (Dotti et al., 1988; Arimura and Kaibuchi, 2007). Finally, stage 5 occurs after seven days and involves maturation of dendrites including formation of dendritic spines (Dotti et al., 1988; Arimura and Kaibuchi, 2007) (Fig. 1.3). Interestingly, only one axon develops during neuronal differentiation, which raises an important question, how/why does one process become an axon while the others remain dendrites? Understanding this may provide insight into mechanisms for functional regeneration following nerve or spinal cord injury where axons have been severed.

Axon outgrowth and neurite outgrowth in general are thought to involve the tight regulation of positive and negative feedback loops controlling actin dynamics and microtubules, another major structural protein found in cells (Andersen and Bi, 2000; Arimura and Kaibuchi, 2007). This feedback is thought to be balanced during stage 2 development where all neurites are growing equally, however, during axon outgrowth (in stage 3), it is thought that a net positive feedback loop initiates the newly forming axon and a net negative feedback loop slows the growth of the remaining neurites with a net result of a single axon forming (Andersen and Bi, 2000; Arimura and Kaibuchi, 2007). In order for an axon to elongate, four major cellular events must occur (Arimura and Kaibuchi, 2007). The four steps involve (i) increases in plasma membrane, (ii) receptor activation, (iii) the formation of actin filaments and (iv) microtubules (Arimura and Kaibuchi, 2007). It is now thought that subsets of proteins are important for formation of the different components including axon, dendrites, and synapses of the developing

neuron. This thesis will focus on the development of neuron polarity and the role of polarity proteins in neuron development.

1.3 Protein Communication: Protein domains

In recent years it has become more evident that the development of a differentiated neuron is controlled in part by a set of conserved polarity protein complexes. These polarity proteins function by regulating actin dynamics (Wiggin et al., 2005). Many of these polarity proteins associate with one another to modify intracellular signaling cascades regulating Rho GTPase activity and thus affecting actin dynamics. Successful protein – protein communication is essential for regulating these cellular events.

1.3.1 Protein Domains

It is now clear that proteins interact with one another through a vast number of different mechanisms. Proteins are composed of a primary amino acid structure, which take on secondary structures such as β-folds and/or α-helices. Several of these secondary structures combine to allow a protein to take on a unique three-dimensional structure. It is evident now that even within a protein there are unique regions which form discrete modular domains (Pawson and Nash, 2003). Proteins are composed of several of these modular three dimensional structures giving rise to protein structural diversity (Pawson and Nash, 2003). Specifically, these modular domains allow for specificity in binding to targets, commonly a recognition sequence or motif on another protein (Pawson and Nash, 2003). Protein domains are typically 35 to 150 amino acid sequences that recognize short (four to ten) amino acid motifs in a target protein (Pawson et al., 2002). Domains can function as independent units within a protein. Indeed protein domains, when removed from the parental protein, maintain their structure, function, and binding affinities (Pawson and Scott, 1997; Howard et al., 2003; Pawson and Nash, 2003). These modular structures or protein domains are classified by their specificity for a binding site, structure, and sequence similarity (Margolis et al., 1999). The majority of the work in this

thesis will focus on proteins that contain two major protein-binding domains, the phosphotyrosine binding (PTB) domain and PSD-95/Dlg homology/ZO-1 (PDZ) domain.

1.3.2 Phosphotyrosine Binding (PTB) Domains

The PTB domain was first identified in the adaptor protein Shc (Margolis et al., 1999). Shortly following identification of the PTB domain in Shc, a PTB domain was also identified in the Insulin Receptor Substrate (IRS) protein (Margolis et al., 1999). Interestingly, the PTB domains of these two proteins show similar structural folds, yet differ in binding specificity (Margolis et al., 1999). Thus, PTB domains are commonly considered Shc-like or IRS-like. PTB domains were identified as binding primarily to recognition sequences surrounding phosphotyrosine (pY) residues, these PTB domains commonly recognize Asp-Pro-X-pTyr motifs, where X is any amino acid (Pawson and Scott, 1997; Margolis et al., 1999; Pawson and Nash, 2000; Yaffe, 2002). PTB domain-containing proteins gain specificity by the subset of amino acids (usually five to eight) found N-terminally to the core pY site on their target binding protein (Pawson and Scott, 1997). Interestingly, some PTB domains also associate with recognition sequences involving the same consensus sequence independently of tyrosine phosphorylation (Margolis et al., 1999). For example, the PTB domain of the cell fate protein, Numb, recognizes a non-phosphorylated tyrosine binding site on the E3 ubiquitin ligase, Ligand of Numb Protein X (LNX), which targets Numb for degradation (Dho et al., 1998; Yaich et al., 1998; Nie et al., 2002; Yaffe, 2002; Schlessinger and Lemmon, 2003). Furthermore, the PTB domains of proteins X11 and FE65 also recognize non-phosphorylated tyrosine sites (Pawson and Scott, 1997; Schlessinger and Lemmon, 2003). Recent reports show that some PTB domains recognize binding sites that lack tyrosines altogether (Yaffe, 2002). For example, PTB domain of Numb is bound by Numb-associated kinase (NAK) in an amino acid sequence motif independent of tyrosines (Yaffe, 2002). Finally, PTB domains, such as the Shc PTB domain, can bind to phospholipids in a mechanism independent of protein recognition sites (Yaffe, 2002). The ability of PTB domains to recognize and bind multiple different recognitions sites illustrates the modular, dynamic, and diverse nature of these interaction domains.

1.3.3 PDZ Domains

Another common protein domain is the PDZ domain. PDZ domains are typically 90 amino acids long and contain six anti-parallel β-strands and two α–helices (Sheng and Sala, 2001). PDZ domains typically associate with target proteins in one of two ways. They either recognize a short peptide sequence immediately preceding a free carboxylate group at the C-terminus of their target, this sequence is commonly X-thr/Ser-X-Val-COO^-, where X is any amino acid (Pawson and Scott, 1997; Pawson and Nash, 2000, 2003; Kim and Sheng, 2004). The C-terminus of the target peptide fits into a binding pocket between the last β-strands and the first α–helix (Doyle et al., 1996; Sheng and Sala, 2001). In the other mechanism of binding, PDZ domains associate with other PDZ domains (Pawson and Nash, 2000, 2003). This typically involves a β-finger fold that can insert into the PDZ binding pocket (Sheng and Sala, 2001). For example, the postsynaptic density protein (PSD), PSD-95, interacts with neuronal Nitric Oxide Synthase (nNOS) through a PDZ-PDZ domain interaction and acts to localize nNOS to NMDA receptors located at the PSD (Christopherson et al., 1999). Finally, recent data suggests that PDZ domains can also recognize lipids (Feng and Zhang, 2009), again suggesting the highly dynamic nature of these protein domains.

Many proteins are not limited to one modular protein domain, but contain several domains that allow these proteins to form multiple interactions, to amplify signals by binding multiples of the same target, or have different binding specificities for different targets (Pawson and Scott, 1997; Pawson and Nash, 2000). For example many PDZ domain containing proteins contain other signaling domains including Src-Homology 3 (SH3), PTB domains or catalytic domains in addition to a PDZ domain (Pawson and Scott, 1997). Some PDZ domain containing proteins, such as GRIP, contain as many as 7 tandem PDZ domains, making these proteins great adaptors for signaling purposes (Kim and Sheng, 2004). The idea that protein domains can function together has been adapted more recently to connect different signaling pathways to rewire cellular signaling (Howard et al., 2003). By selectively generating constructs from proteins within specific signaling pathways, these pathways can be reengineered for novel function. For example turning a prosurvival pathway into an apoptotic response, may provide a mechanism to

overcome diseases such as cancer (Howard et al., 2003). Furthermore, these modular domains are key components to polarity protein networks that allow for proper formation and function of polarized cell and cell structures such as neurons and associated synapses.

1.4 POLARITY PROTEINS

1.4.1 Identification of Cellular Polarity

Proteins responsible for cell polarity developed from initial studies by Ken Kemphues (Cornell University) and colleagues who focused on *Caenorhabditis elegans* embryo development (Kemphues et al., 1988; Kemphues, 2000; Macara, 2004b). In a screen looking for defects in the development of a single cell embryo, they found that once fertilized, the one cell embryo undergoes a characteristic stage of development. This is characterized by the development of the mitotic spindle with one spindle pole moving in close opposition to the cortical membrane relative to the other mitotic spindle, while the other pole remains in place (Kemphues et al., 1988; Kemphues, 2000; Goldstein and Macara, 2007). Upon division, this creates two cells, with the more anterior cell being larger in size relative to the posterior cells (Kemphues et al., 1988; Kemphues, 2000). The mitotic spindle of the more anterior cell then rotates 90 degrees relative to the posterior cell (Kemphues et al., 1988; Kemphues, 2000). This process leads to cellular diversity and contributes to multicellular organisms (Kemphues et al., 1988; Kemphues, 2000). The screen Kemphues used was designed to identify defects in this characteristic division paradigm (Kemphues et al., 1988; Kemphues, 2000). They identified six genes they called *Partition defective* or *PAR* genes in addition to *protein kinase C3 (pkc3; atypical Protein Kinase C - aPKC)* (Kemphues et al., 1988; Kemphues, 2000; Macara, 2004b; Goldstein and Macara, 2007). The proteins were initially identified in *C.elegans* have been shown to function in many species and cell types including *Drosophila*, *Saccharomyces cerevisiae*, and mammalian systems. Since the discovery of the initial PAR genes, three major polarity complexes have been identified and the best-characterized cell system to date is the epithelial cell.

1.4.2 Three major protein complexes

Subsequent studies, mainly focused in epithelial cells, have identified three major conserved protein complexes responsible for formation of cellular polarity (Fig. 1.4). The first complex involves Scribble (Scrib)/Discs Large (Dlg)/Lethal Giant Larvae (Lgl), the second involves PAR3/PAR6/atypical Protein Kinase C (aPKC), and the third involves Crumbs (Crb)/Stardust (Sdt)/Pals-associated tight-junction protein (Patj)) (Roh and Margolis, 2003; Macara, 2004b). These three complexes localize to specific regions of epithelial cells and appear to act in concert to regulate cellular polarity. For example, the Scrib/Dlg(or SAP97 in mammalian systems)/Lgl complex localizes more to the basolateral surface (Bilder et al., 2003) or below the TJ on the lateral membrane. The PAR3/PAR6/aPKC complex localizes to the tight junctions (TJ) of mammalian epithelial cells, each member is required to be in a heterotrimeric complex in order for this localization to occur (Roh and Margolis, 2003; Macara, 2004b). In addition, the Crb/Std/Patj complex also localizes to the apical region in mammalian epithelia (Roh and Margolis, 2003; Macara, 2004b). Some studies suggest that the Par3/Par6/aPKC complex signals through the Crb/Std/Patj complex to define apical polarity (Roh and Margolis, 2003). Further, it is thought that the Scrib/Dlg/Lgl complex antagonizes the Crb/Std/Patj complex after initiation by the PAR3/PAR6/aPKC complex in a feedback loop, this allows tight regulation of epithelial polarity (Bilder et al., 2003; Roh and Margolis, 2003; Tanentzapf and Tepass, 2003; Macara, 2004b). Interestingly, members from the three major polarity complexes interact directly (Macara, 2004b). Par6 binds directly to Lgl (Betschinger et al., 2003; Plant et al., 2003; Yamanaka et al., 2003) and the Std mammalian homologue, protein associated with LIN7 (PALS) (Hurd et al., 2003). Together, these studies suggest that although three general polarity complexes have been identified, they nonetheless communicate with and regulate the function of one another.

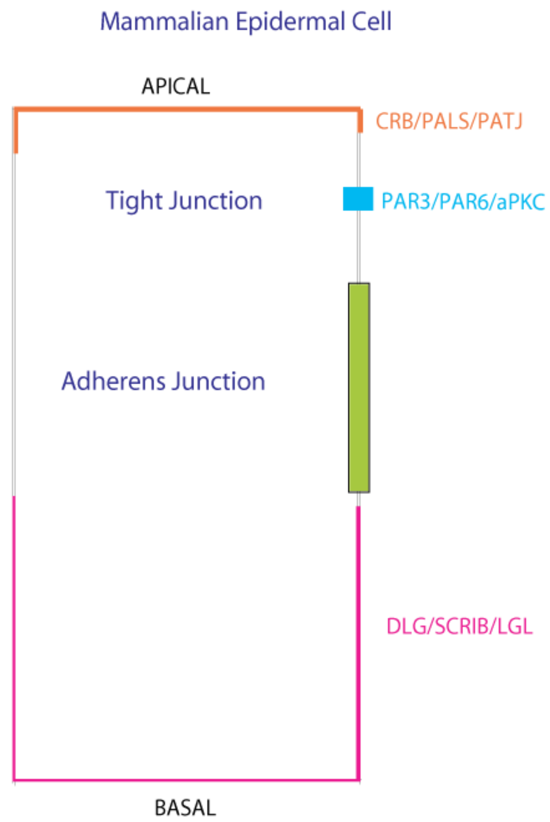


Figure 1.4 Model of the three major polarity complexes in epidermal cells. Modified from Macara, 2004.

1.5 Polarity Proteins in Epithelial Polarity and Neuron Development

Polarity proteins have been shown to regulate many aspects of both epithelial and neuronal polarity (Macara, 2004b). I will discuss evidence for their roles in epithelial polarity, axon specification, dendrite development and dendritic spine development. Here I will focus on two of the three polarity complexes, PAR3/PAR6/aPKC and Scrib/Lgl/Dlg. Finally, much of the work in this thesis will focus on the role of Scrib in cellular polarity, so a brief introduction to the Scrib polarity complex and the role of Scrib in epithelial polarity and in tumor suppression is necessary. For the purposes of this section, I will use dendritic spines and synapses interchangeably.

1.5.1 (a) PAR3/PAR6/aPKC discovery and epithelial polarity

Much of how the PAR complex regulates cellular polarity came from studies in epithelial cells (Etienne-Manneville and Hall, 2003b). Epithelial cells provide a nice model system to study cellular polarity since they have defined regions upon their terminal differentiation. In particular, canine epithelial cells known as Madin-Darby canine kidney (MDCK) cells have been used extensively to study models of epithelial cell polarity. The formation of MDCK polarity is well established (Bacallao et al., 1989). When in a cultured monolayer, MDCK cells form tight junctions (TJs) that serve as important landmarks separating apical and basal regions and allow for cell polarization (Etienne-Manneville and Hall, 2003b; Sabatini, 2005). Basolaterally, subapical to the TJ is the Adherens Junction (AJ) (Colman, 1999). AJs act as a link connecting two neighboring cells and supply structural support to the cell (Colman, 1999) (Fig. 1.4). In fully polarized MDCK cells a well-defined apical cilia develops (Roth et al., 1988; Bacallao et al., 1989). These characteristics of MDCK cells have been used extensively to study polarity complexes responsible for regulating this polarity.

Since the discovery of the *PAR* genes, they have been shown to play major roles in regulating epithelial and neuronal polarity (Macara, 2004a; Chen and Macara, 2005). Some of the initial studies involving the *PAR* genes have outlined a signaling pathway

involving PAR3, PAR6, aPKC and cdc42 (Macara, 2004b). The first PDZ domain of PAR3 associates with the PDZ domain found in PAR6 (Etienne-Manneville and Hall, 2003b; Macara, 2004b). The Phox and Bem 1 (PB1) domain of PAR6 in turn associates directly with the PB1 domain of aPKC (Etienne-Manneville and Hall, 2003b; Macara, 2004b). PAR3 and PAR6 are both substrates for aPKC (Macara, 2004b). Further, PAR6, is a downstream effector of cdc42 (Macara, 2004b). The question still remains as to how cdc42 and aPKC act to regulate PAR6 (Etienne-Manneville and Hall, 2003b). It has been suggested that cdc42 enhances the association between PAR3 and PAR6 (Etienne-Manneville and Hall, 2003b) by binding to the cdc42/rac interactive binding (CRIB) domain of PAR6 (Mertens et al., 2006). This interaction is thought to release a blocking function of PAR6 on aPKC activity, leading to aPKC activation (Mertens et al., 2006). Nonetheless, these proteins function together and regulate cellular polarity.

1.5.1 (b) PAR3/PAR6/aPKC in Tight Junctions

Interestingly, the PAR3/PAR6/aPKC complex has been implicated in TJ formation in a mechanism dependent on Rac activation through the Rho GEF protein T-lymphoma and metastasis 1 protein (TIAM1) (Mertens et al., 2006). Normally, TIAM1 and PAR3 both localize to TJs and function together for appropriate development of epithelial polarity (Chen and Macara, 2005; Mertens et al., 2005; Mertens et al., 2006). Consistent with this it has been shown that when correctly localized to TJs, TIAM can activate both Rac and aPKC an important event for polarization (Mertens et al., 2006). Indeed, TIAM1 deficient cells fail to form mature TJs, while PAR3 deficient cells have improper TJ formation and increased global Rac activation (Mertens et al., 2006). Interestingly, PAR3 overexpression mislocalizes PAR3 away from TJs, decreases cell-cell junctions, and again results in a global activation of Rac (Mertens et al., 2006). This mislocalization of PAR3 and lack of cell junction formation can be rescued by overexpression of the TIAM1 binding domain of PAR3 alone (Mertens et al., 2006). The current thought is that PAR3 recruits TIAM1 and associated activated Rac to TJs, which in turn recruits and activates PAR6/aPKC (Chen and Macara, 2005; Mertens et al., 2005; Mertens et al.,

2006). When PAR3 is mislocalized or removed, it results in global activation of Rac since TIAM1 is no longer localized to TJs (Mertens et al., 2006).

1.5.2 PAR3/PAR6/aPKC in axon and process outgrowth

In addition to epithelial TJ formation, the PAR3/TIAM1 association is important in axonal specification, however, the mechanism remains slightly different (Mertens et al., 2006). Specifically, the PAR complex (PAR3/PAR6/aPKC) has also been implicated in the development of the axon. Support for this comes from a number of studies. Using the dissociated hippocampal neuron paradigm studies revealed that PAR3/PAR6 and aPKC are all localized to the tips of immature neurites (Shi et al., 2003; Arimura and Kaibuchi, 2007). Late in stage 2, however, when the axon grows out rapidly from the cell body, the PAR3/PAR6/aPKC complex assembles to one process, the presumptive axon (Shi et al., 2003; Arimura and Kaibuchi, 2007). By stage 3 this complex is enriched in the tip of the newly formed axon (Shi et al., 2003; Arimura and Kaibuchi, 2007). Using both N- and C-terminal deletion mutants of PAR3, this study was able to show that the N-terminal region of PAR3 is critical for subcellular localization and polarity of PAR3 (Shi et al., 2003). Since all three members of the PAR complex are critical for cellular polarity, the next question to be addresses involved what was acting upstream to regulate this complex (Shi et al., 2003) and the result was phosphatidylinositol 3-kinase (PI3K). It was shown that Akt, a product of PI3K, was located at the tips of axons (Shi et al., 2003). Finally, similar to the PAR complex, by inhibiting PI3K activity, axon formation was blocked and PAR3 was no longer transported to the neurites (Shi et al., 2003). Therefore, both the PAR complex in conjunction with PI3K is required for proper neuronal polarization.

How does the PAR complex regulate this axonal specification? In one model TIAM1 is thought to function with PAR3 to regulate Rac activity leading to local Rho GTPase activation favouring axogenesis (Mertens et al., 2006). Evidence for this came from the localization of TIAM1 or a protein related to TIAM1 (STEF for STF and TIAM1-like exchange factor) at the tip of a developing axon during stage 3 (Arimura and Kaibuchi, 2007). Loss of TIAM1 protein leads to a decrease in axons, while overexpression of

TIAM1 results in multiple axons forming (Mertens et al., 2006). Interestingly, unlike epithelial cells, it appears that cdc42 acts upstream of TIAM1 (Nishimura et al., 2005; Mertens et al., 2006; Arimura and Kaibuchi, 2007). Specifically, it is thought that cdc42, through another GTPase, Rap1B, acts upstream of the PAR complex to recruit both the PAR complex and TIAM1 to the tip of the developing axon (Schwamborn and Puschel, 2004; Nishimura et al., 2005; Mertens et al., 2006; Arimura and Kaibuchi, 2007). Studies have shown that PAR6 colocalizes with this complex; however, an underlying mechanism behind PAR6 association has not been determined in the developing axon model (Nishimura et al., 2005; Arimura and Kaibuchi, 2007).

In another model of the PAR complex in axogenesis involves PAR3 acting upstream of and negatively regulating glycogen synthase kinase 3 (GSK3β) (Etienne-Manneville and Hall, 2003a). GSK3β was first identified as an important component in the migration of astrocytes in a PAR3 dependent manner by regulating microtubule dynamics and was later shown to play a role in axogenesis (Etienne-Manneville and Hall, 2003a; Arimura and Kaibuchi, 2007). In the presence of PAR3, GSK3β undergoes phosphorylation and subsequent inactivation (Etienne-Manneville and Hall, 2003a). The precise mechanism leading to this phosphorylation state is unknown, however, it is thought that aPKC or cdc42 are important for the phosphorylation of GSK3β (Etienne-Manneville and Hall, 2003a). Importantly, the serine/threonine kinase activity of GSK3β becomes activated in an unphosphorylated state. Unphosphorylated GSK3β is known to phosphorylate Adenomatous polyposis coli (APC), a protein involved in stabilization and growth of microtubules (Rubinfeld et al., 1996; Bienz, 2002). Once phophorylated, APC loses its ability to stabilize microtubules (Dikovskaya et al., 2001) and causes a loss in neuronal polarity (Shi et al., 2004). A relationship between PAR3, APC and GSK3β as an important signaling complex necessary for axon development comes from studies showing that axon formation requires APC for correct localization PAR3 to axons (Shi et al., 2004). Further, inhibiting GSK3β results in a mislocalization of PAR3 (Shi et al., 2004). Thus, GSK3β and APC are important for correct PAR3 localization in developing axons (Shi et al., 2004). These two studies indicate that in addition to the PAR proteins, GSK3β and APC are also important regulators of axogenesis.

In another pathway, the serine/threonine kinase microtubule affinity-regulating kinase 2 (MARK2)/PAR1 was shown to have an increased ratio of the phosphorylated form to unphosphorylated form at the tips of developing axons in stage 3 development (Chen et al., 2006). Interestingly, phosphorylation and subsequent inactivation of MARK2 occurs through aPKC (Hurd et al., 2003). Overexpression of MARK2 results in a loss of axon formation and knockdown of MARK2 leads to the formation of numerous axons (Chen et al., 2006). MARK2 knockdown also leads to a decrease in the phosphorylation of the microtubule-associated protein Tau (Chen et al., 2006). It is thought that Tau phosphorylated by MARK2, leads to disassembly of microtubules (Drewes et al., 1997). Together this leads to the working hypothesis that aPKC phosphorylation and inhibition of MARK2 results in decreased Tau phosphorylation (Chen et al., 2006). Decreased phospho-Tau causes increased microtubule assembly and thus axon formation. Interestingly, expression of all three members of the PAR complex – PAR3/PAR6/aPKC rescues the effects and allows correct polarization to occur in half of the MARK2 overexpressing cells. This indicates that axonal differentiation and neuronal polarization is a highly dynamic process that is dependent on the tight regulation of many different cellular factors.

1.5.3 PAR3/PAR6/aPKC in dendritic spines

In addition to axon outgrowth, members of the PAR3/PAR6/aPKC pathway have also been implicated in dendritic spine formation. Here, PAR3 and TIAM1 are required for maturation of dendritic spines by regulating of Rac activity (Mertens et al., 2006; Zhang and Macara, 2006). PAR3 recruits TIAM1 to dendritic spines allowing for the localized activation of Rac in spine heads resulting in spine maturation (Zhang and Macara, 2006). Indeed, loss of PAR3 results in mislocalized TIAM1 leading to the formation of multiple filopodial-like structures reminiscent of globally activated Rac (Zhang and Macara, 2006). Here, PAR3 is thought to be required to recruit TIAM1 into spine heads, thus localizing active Rac in spines to increase the maturation of the spine (Zhang and Macara, 2006). Interestingly, this role for PAR3 is independent of the association of PAR6/aPKC (Zhang and Macara, 2006), although, aPKC has been shown to be important

for regulating actin dynamics leading to changes in spine morphology (Mertens et al., 2006). This led to the theory that the aPKC binding protein PAR6 may affect spine development (Zhang and Macara, 2008). Consistent with this, knockdown of PAR6 decreases spine density, which is only rescued by a PAR6 construct containing an aPKC binding region (Zhang and Macara, 2008). This same study showed that the effects of PAR6 on spine density were not through Rac or cdc42, but rather through a RhoA dependent mechanism (Zhang and Macara, 2008). Here, PAR6 recruits p190RhoGAP by an unknown mechanism to locally influence Rho signaling ultimately leading to changes in spine morphology (Zhang and Macara, 2008). Nonetheless, together these studies illustrate the important role polarity complexes play in regulating cellular structures such as axon and dendritic spine formation. Increasingly there is evidence for the role of the PAR complex in neuronal polarity. More recently, the Scrib complex has been shown to play an ever-increasing role in neuronal polarity.

1.5.4 The Scrib Complex

Proteins within the Scrib complex, Dlg, Lgl, and Scrib, are all known tumor suppressors and serve as important membrane localization proteins in different cell types (Bilder et al., 2000; Bilder, 2004). Perhaps the best-characterized member of the Scrib complex is Dlg. Dlg, also known as synapse-associated protein 97 (SAP97), is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins (Bilder, 2004). Dlg is a multi-domain scaffolding protein that contains three PDZ domains, an SH3 domain, and a guanylate kinase (GUK)-like domain (Bilder, 2004). Dlg is a scaffolding protein and has been shown to bind the glutamate receptor 1 subunit of the α-amino-3-hydroxyl-5-methyl-4-isoxazote-propionic acid (AMPA) receptors and plays an important role in regulating the synaptic dynamics (Feng and Zhang, 2009).

Lgl is a WD40 repeat containing protein (Wodarz, 2000), acts as a substrate for atypical protein kinase C (lambda and iota) and binds directly to PAR6 (Plant and Fawcett et al., 2003; Yamanaka et al., 2003). In the developing nervous system, Lgl1 plays an important role in neuronal differentiation (Vasioukhin, 2006), since loss of Lgl1 leads to the

inability of neuronal progenitor cells to exit the cell cycle (Vasioukhin, 2006). These cells lacking Lgl develop neuroepithelial-derived tumors (Vasioukhin, 2006). In Drosophila, Lgl has been shown to form a stable complex with fragile X mental retardation protein (Fmrp1) and modulates the size of the neuromuscular junction in Drosophila (Zarnescu et al., 2005). However, the focus of this thesis is related to Scrib.

Scrib is a Leucine Rich Repeat (LRR) and PDZ (LAP) family protein that acts as a scaffold with 16 N-terminal LRRs and four PDZ domains (Bilder and Perrimon, 2000). In epithelial cells, Scrib is restricted to basolateral surfaces (Bilder, 2004) and it is thought that the LRR region of Scrib is required to maintain Scrib at these surfaces (Navarro et al., 2005); although some evidence points to either the LRR or PDZ domains of Scrib being sufficient to maintain Scrib at the plasma membrane (Kallay et al., 2006). The LRR domain of Scrib is responsible for binding Lgl and the PDZ domains are responsible for binding many proteins including the membrane associated protein Vangl2 (Kallay et al., 2006). Scrib is also found at cell-cell junctions with other known junction proteins, for example two of the PDZ domains of Scrib bind the C-terminus of Zonula Occludens (ZO)-2, a cell-cell junction protein (Metais et al., 2005) and Scrib can associate with the cell adhesions protein Lipoma Preferred Partner (LPP) (Petit et al., 2005). In different regions of the murine eye Scrib co-localizes with Dlg, ZO-l, E- and N-Cadherin, specifically at adherins junctions (Nguyen et al., 2005). All of these studies point to a role for Scrib in epithelial polarity by acting as a scaffold to restrict proteins to distinct areas within the cell.

1.5.5 Scrib in Tumorigenesis

Loss in cellular polarity is present in late stage tumor development (Wodarz and Nathke, 2007). During epithelial to mesenchymal transition (EMT), where cells switch from an epithelial phenotype a less differentiated or mesenchymal phenotype, cells lose their apical-basal polarity (Wodarz and Nathke, 2007). There has been great interest over the past few years to better understand the role of polarity proteins in tumor progression (Wodarz and Nathke, 2007). Scrib being both a polarity protein and a tumor suppressor

has attracted much recent attention. In mammary epithelial tissue, loss of Scrib, results in a loss of cell polarity, an inhibition of apoptosis, and eventual tumor progression *in vivo* (Zhan et al., 2008). Incorrect localization and decreased expression of Scrib is thought to play a role in some murine and human mammary tumors (Zhan et al., 2008). Interestingly, one of the products of the human papillomavirus (HPV) E6 virus, the virus common to most cervical cancers, is the E6 ubiquitin ligase, which binds directly to Scrib and targets Scrib for degradation (Takizawa et al., 2006). Loss of Scrib is thought to contribute to the loss of the differentiated epithelial phenotype, a hallmark of an early cancerous cell (Takizawa et al., 2006).

Scrib may be involved in tumor suppression by altering the adhesion and migration of epithelial cells in at least two distinct pathways, one that involves Rac and the other that involves E-cadherin. Scrib is thought to act through two proteins β P-21 activated kinase (PAK) interacting exchange factor (β-Pix) and G-protein coupled receptor kinase interacting protein (Git1) (Zhan et al., 2008). Specifically, β-Pix is a Rac GEF (Manser et al., 1998) and Git1 is a scaffolding protein that directly associates with β-Pix (Zhao et al., 2000; Zhang et al., 2005; Frank and Hansen, 2008). Acting through β-Pix and Git1, Scrib influences Rac activity leading to changes in cellular migration (Zhan et al., 2008). Scrib reduction in MCF10A cells, a cancer cell line, show a decrease in lamellipodia as well as decreased expression and localization of the Rho GTPase proteins, cdc42 and Rac, at the leading edge (Dow et al., 2007). In some cases the presence of Scrib does not affect Rho GTPase activity (Qin et al., 2005), suggesting this may be a cell type specific response. For example, Scrib is important for cell adhesion in MDCK epithelial cells and loss of Scrib in these cells leads to an increase in cell migration and lack of directional movement (Qin et al., 2005), two features associated with tumorigenesis. Interestingly, knockdown of Scrib in MDCK cells does not effect β-Pix activation of Rac, however, cell adhesion through E-cadherin is affected (Qin et al., 2005). This phenotype can be rescued by co-expression with E-cadherin fused to α-catenin (Qin et al., 2005). Furthermore, Scrib requires E-cadherin for localization to cell-cell junctions (Navarro et al., 2005). Thus, Scrib is important for restricting and defining cellular polarity, an event that may be disrupted in certain tumors. It is critical to establish the involvement of

proteins like Scrib to better understand cellular polarity in all mammalian systems from epithelial cells to neurons.

1.5.6 (a) Presynaptic Scrib

Studies in both *Drosophila* and mammalian systems have implicated Scrib as an important protein in the development of polarized cellular structures, while more recent studies have implicated Scrib in the presynaptic compartment. For example, the *Circletail* mouse model, which produces a truncated form of Scrib missing the last two PDZ domains, shows developmental defects in neural tube formation (Murdoch et al., 2003). In *Drosophila,* Scrib has been implicated at the synapse, particularly the neuromuscular junction (NMJ) and is localized to the synapse by Guanylate Kinase - holder (GUKh), forming a ternary complex along with Dlg (Mathew et al., 2002). *Scrib* mutant *Drosophila* show a decreased number of active zones, impaired vesicle dynamics, decreased facilitation and increased synaptic depression, all suggesting that Scrib plays a role in synaptic plasticity (Roche et al., 2002). More recent work suggests that Scrib plays a major role in the presynaptic compartment of mammalian neurons. Both Scrib and binding partner, β-Pix, show partial co-localization to the presynaptic region (Audebert et al., 2004). Furthermore, Scrib has been shown to regulate presynaptic vesicle clustering (Sun et al., 2009). In this model, Scrib acts downstream of β-catenin to induce vesicle clustering (Sun et al., 2009). Thus, new evidence exists for Scrib being an important component of presynaptic vesicles.

1.5.6 (b) Scrib in postsynaptic function with Pix/Git/PAK proteins

In mammalian neurons, Scrib, Git1, and β-Pix can be found postsynaptically (Wiggin et al., 2005) suggesting that these proteins may be important in dendritic spine development. Previous studies have illustrated a functional connection between Scrib and β-Pix (Audebert et al., 2004; Osmani et al., 2006; Nola et al., 2008). β-Pix acts as a GEF for Rac and induces membrane ruffling in cultured cells (Manser et al., 1998) and locally activates Rac in dendritic spines (Zhang et al., 2005). β-Pix has three known isoforms

p50Cool, p85Cool (β-Pix), and α-Pix (Bagrodia et al., 1998; Manser et al., 1998; Frank and Hansen, 2008). β-Pix (also known as cloned out of library, COOL) interacts directly with PAK, a cdc42/rac effector molecule (Bagrodia et al., 1998; Frank and Hansen, 2008). β-Pix contains an SH3 domain that interacts directly with a proline-rich region of PAK (Bagrodia et al., 1998; Manser et al., 1998) as well as Dbl homology (DH) and Pleckstrin homology (PH) domains (Bagrodia et al., 1998) and a Git-binding domain (GBD) (Frank and Hansen, 2008). β-Pix binds to Rac1 through the GBD (Shin et al., 2006) and further acts to recruit PAK to cdc-42 and Rac1 positive focal adhesions (Manser et al., 1998). Specifically, β-Pix binds directly to the PDZ domains of Scrib and coupled together, these proteins have been shown to affect Rho GTPase activity (Audebert et al., 2004). Some evidence for this comes from one study where both Scrib and β-Pix were important for regulating cdc42 activity and localization (Osmani et al., 2006). Scrib along with β-Pix are partially responsible for cellular polarity during migration of astrocytes, Scrib co-localizes with β-Pix at the leading edge and both Scrib and functional GEF activity are required for β-Pix mediated cellular polarity (Osmani et al., 2006). Dominant negative constructs of either Scrib or β-Pix inhibit the exocytosis of calcium affecting cellular polarization states (Audebert et al., 2004). Thus, together Scrib and β-Pix regulate calcium efflux to effect cell polarization.

Scrib has been shown to co-immunoprecipitate with β-Pix, Git1 and PAK in MCF-10.2A cells, the interaction between Scrib and PAK is dependent on β-Pix (Nola et al., 2008). The complex involving Scrib, β-Pix, and PAK localizes to the leading edge of epithelial cells and is thought to be involved in polarity (Nola et al., 2008). It appears that Git1 is responsible for increasing the number and maturation of synaptic spines, while β-Pix through activation of Rac is responsible for increasing dendritic protrusions (Zhang et al., 2003). When activated, PAK is found on synapses and plays a role in the formation of dendritic spines (Zhang et al., 2005). In dendritic spines, one major downstream effector for PAK is mysosin II regulatory light chain (MLC) (Zhang et al., 2005). Both PAK and MLC function downstream of Git1/β-Pix in influencing dendritic spine morphology (Zhang et al., 2005). Furthermore, in endothelial cells PAK acts as an extracellular

signal-regulated kinase (Erk) effector in order to phosphorylate and activate MLC (Stockton et al., 2007). The effect of PAK on Erk is dependent on the presence of a PAK, β-Pix , and Git1 complex (Stockton et al., 2007). Many of these Scrib-associating proteins are important in dendritic spine maturation, although it remains unclear how these proteins may all function together in synapse.

1.5.8 Rho GTPases and Neuron Polarity

Multiple lines of evidence implicate a close connection between known polarity proteins and Rho GTPase signaling. As mentioned, Scrib and Rac/cdc42 are functionally linked to and promote changes in cell morphology (Dow et al., 2007). Cdc42 has been implicated as a critical regulator of TJ formation through the Patj complex (Wells et al., 2006). In this model, Rich1 acts as a GAP for cdc42 and associates with angiomotin (AMOT) to target Patj to TJs allowing proper TJ formation (Wells et al., 2006). In its activated form, cdc42 also interacts with PAR6 by binding to the PAR6 cdc42/Rac interactive binding (CRIB) domain and influences the entire PAR3/PAR6/aPKC complex activation (Joberty et al., 2000). Cdc42 is also an effector for the Arp2/3 binding protein nWASP (Fawcett and Pawson, 2000; Ramesh and Geha, 2009). In this model nWASP binds to activated cdc42 through a GTPase binding domain to lead to actin filament formation (Fawcett and Pawson, 2000; Ramesh and Geha, 2009).

Rac is an effector for p-21 activated kinase (PAK) proteins (Bokoch, 2003; Kreis and Barnier, 2009). PAK proteins contain an N-terminal CRIB domain as well as a Pak Binding Domain (PBD), which enhances affinity for and binds Rac and cdc42 (Bokoch, 2003; Kreis and Barnier, 2009). PAK proteins have a C-terminal catalytic domain that acts as a serine/threonine kinase (Bokoch, 2003). As mentioned, once activated, PAK proteins are then thought to target Lim kinase which in turn phosphorylates cofilin, inhibiting the ability of cofilin to bind and break down actin filaments (Edwards et al., 1999; Bokoch, 2003). These examples help illustrate the critical role that play in regulating neuronal polarity by targeting downstream polarity proteins.

Most of the proteins implicated in axon and spine development converge on regulating the actin cytoskeleton through the Rho GTPases. Therefore, it is not surprising that Rac and cdc42 play a major role in initial neurite outgrowth (Etienne-Manneville and Hall, 2002). Rac has been implicated in the formation and maintenance of dendritic spines in hippocampal neurons (Nakayama et al., 2000). Indeed, decreasing activated Rac results in a loss of dendritic spines, this is similar to constitutively active Rac, except that constitutively active Rac also increases filopodial-like process outgrowth structures (Nakayama et al., 2000). Furthermore, several Rho GEF's (for example, β-Pix) have been reported to localize both pre- and post-synaptically (Luo, 2000). Further, studies have shown that mouse mutants expressing active Rac in Purkinje cells have increased numbers of smaller spines (Luo et al., 1996). These studies suggest that Rac and cdc42 play largely a structural role in epithelial and neuron cells by targeting downstream components to cause changes in actin dynamics.

1.5.9 Actin Dynamics, Development, and Neurological Disease Progression

A number of studies have implicated the Rho GTPases in several disorders of learning and memory dysfunction; implicating actin dynamics as playing a role in diseased states (Dillon and Goda, 2005). Therefore, understanding actin dynamics and actin regulatory proteins is critical to studying human diseased states that include, but are not limited to mental retardation (MR), schizophrenia, and bipolar disorder as well as normal learning and memory. For example, there are several links between MR and genetic mutations in Rho GTPase related genes that suggests that actin cytoskeleton regulation is critical for normal function (Dillon and Goda, 2005). For example, human genetic diseases involve mutations in PAK3, α-Pix, Lim kinase, and Fragile-X-mental retardation (FMR)-1 (Dillon and Goda, 2005). The latter involve binding partners for PAK protein (Edwards et al., 1999; Bokoch, 2003) and all involve proteins that regulate or are regulated by Rho GTPases (Dillon and Goda, 2005). Patients with these forms of MR show alterations in spine morphology including increases in immature spine numbers (Dillon and Goda, 2005). These genes and resultant condition are not necessarily mutually exclusive events.

For example, in the case of FMR1-related mental retardation, which as mentioned before, results in the loss of a critical synaptic protein, Fmrp (Wiggin et al., 2005), some of the behavioral symptoms of this form of mental retardation can be partially rescued by expression of a catalytically deficient form of PAK (Hayashi et al., 2007). Kinase deficient PAK also rescues some of the decreased long-term potentiation (LTP) or changes in synaptic strength that occurs in FMR-l deficient mice (Hayashi et al., 2007).

Current theories suggest that actin plays a dynamic role in learning and memory (Dillon and Goda, 2005). Learning and memory are thought to be the result of synaptic plasticity. Current models of synaptic plasticity suggest that actin is involved in changes in both spine shape and in the movement of presynaptic components (Dillon and Goda, 2005). This idea was first shown in 1998 in an experiment using GFP-actin transfected cells to look at changes in actin dynamics that resulted in changes in dendritic spine shape (Fischer et al., 1998). These changes in spine shape were dependent on the presence of a presynaptic terminal (Fischer et al., 1998). Later studies have shown that motility of dendritic spines is controlled in part by different glutamate receptors, such as α-amino-3-hydroxyl-5-methyl-4-isoxazote-propionic acid (AMPA) glutamate receptors (AMPAR) and N-methyl-D-aspartate (NMDA) Receptors (NMDAR) (Dillon and Goda, 2005). In both cases, stimulation of these receptors results in stabilization of spines and decreased motility (Dillon and Goda, 2005). It does appear, however, that this phenomenon is dependent on the extent to which these receptors are stimulated since some studies have shown that receptor stimulation leads to increased F-actin, while other have shown that receptor stimulation results in a collapse of spines (Dillon and Goda, 2005). Presynaptic actin also changes as a result of postsynaptic plasticity, studies have shown that presynaptic actin increases and shifts towards the postsynaptic compartment (Dillon and Goda, 2005). Models of LTP, long term changes in synaptic dynamics, illustrate that LTP involves an increase in F-actin and subsequent decrease in G-actin (Dillon and Goda, 2005). Studies have shown that when induced, LTP involves an enlargement of the spine head that can last for several weeks (Dillon and Goda, 2005). Interestingly, some current models suggest that LTP is a result of regulation of Lim kinase and ADF/cofilin (Dillon and Goda, 2005), downstream targets of PAK (Edwards et al., 1999; Bokoch, 2003). For

example, dominant negative PAK3 expression in the forebrain of mice results in decreased number, but increased size of synaptic spines and presynaptic terminals in cortical neurons, increased LTP, but decreases in hippocampal-related memory (Hayashi et al., 2004; Dillon and Goda, 2005). In addition, Lim kinase, a target of PAK, deficient mice show deficiencies in memory tasks (Dillon and Goda, 2005).

1.6 Nitric Oxide Synthase1 Adaptor Protein

A recent mass spectrometry screen using Scrib as bait identified a novel interaction to the protein carboxyl-terminal PDZ ligand of neuronal Nitric Oxide Synthase (nNOS) (CAPON) or Nitric Oxide Synthase1 Adaptor Protein (NOS1AP), herein NOS1AP (Richier et al., submitted manuscript). This forms the basis for my thesis work. It is therefore, important to introduce NOS1AP.

1.6.1 Domain Architecture and Signaling

NOS1AP is a protein that contains an amino-terminal phosphotyrosine binding (PTB) domain and a carboxyl-terminal PDZ binding motif (Jaffrey et al., 1998). NOS1AP was first identified as a novel nNOS-binding partner, whereby the C-terminal PDZ binding motif of NOS1AP was shown to associate with the PDZ domain of nNOS (Jaffrey et al., 1998). This study proposed that NOS1AP acts to compete with PSD-95 for nNOS and may act to sequester nNOS away from NMDAR (Jaffrey et al., 1998). Two separate ternary complexes and mechanisms of action have been proposed through NOS1AP's association with nNOS. The first involves an association between the dexamethasone induced Ras family member, Dexras1, and the PTB domain of NOS1AP (Kemppainen and Behrend, 1998; Fang et al., 2000). NO activates Dexras through S-nitrosylation converting Dexras from an inactive GDP-bound to an active GTP-bound state (Fang et al., 2000; Jaffrey et al., 2002a). In this model, Dexras1 is activated in the GTP bound state by NMDA-Receptor mediated NO production (Fang et al., 2000). The activity of Dexras1 is increased in the presence of nNOS and this nNOS dependent activation is further increased in the presence of NOS1AP (Fang et al., 2000). The PTB domain of

NOS1AP has also been shown to bind to the synaptic vesicle associate proteins, synapsin I, II, and III, again forming a ternary complex with nNOS (Jaffrey et al., 2002b). Synapsin 1 is a synaptic vesicle membrane binding protein and an actin associating protein involved in synaptic vesicle dynamics (Hirokawa et al., 1989) and is localized to the presynaptic terminals of cultured hippocampal neurons (Fletcher et al., 1991). Synapsin I and II double knockout mice show altered distributions of NOS1AP and nNOS, suggesting that synapsins localize NOS1AP and nNOS to certain subcellular compartments (Jaffrey et al., 2002b). A recent study has shown that NOS1AP is involved in dendritic patterning early in development of cultured hippocampal neurons (Carrel et al., 2009). Primarily, NOS1AP overexpression in hippocampal neurons leads to a decrease in the number of dendrites independently of either the PTB or PDZ domains and the region required for this phenotype binds to the protein modifier, carboxypeptidase E (CPE) (Carrel et al., 2009).

1.6.2 nNOS signaling

NOS1AP was first identified as an nNOS binding partner (Jaffrey et al., 1998) and has subsequently been shown to play a role in at least two nNOS-ternary complexes (Fang et al., 2000; Jaffrey et al., 2002b). The PDZ domain of nNOS (NOS1) binds to the C-terminal region of PDS-95 (Brenman et al., 1996). PDS-95 also associates with NMDAR (Christopherson et al., 1999). Together NMDAR, PDS-95, and nNOS form a ternary complex (Christopherson et al., 1999). NOS enzymes are involved in NO production from L-arginine (Moncada and Higgs, 1993; Nathan and Xie, 1994). NO is a modulator of synaptic function and regulates spine development (Brenman and Bredt, 1997; Nikonenko et al., 2008). Studies have illustrated a possible link between NMDAR, PSD-95, nNOS and NO production (Nikonenko et al., 2008). Studies have shown that PSD-95 regulates the complexity of the presynaptic terminal and this is dependent on nNOS and NO (Nikonenko et al., 2008). Further, PSD-95 and NO production has recently been shown to have effects on the morphology of the synapse, both pre- and post-synaptically (Nikonenko et al., 2008). Finally, nNOS is thought to play a role in neurotransmission

and excitotoxicity (Kim and Sheng, 2004), although the underlying mechanism is not known.

1.6.3 NOS1AP mRNA and Protein Expression

NOS1AP RNA is enriched in many regions of the brain including the cortex, cerebellum, hippocampus, striatum, and olfactory bulbs (Jaffrey et al., 1998), making it an ideal candidate for brain development. Northern blotting and immunostaining have also revealed that NOS1AP is expressed in other regions throughout the body including spinal cord, skeletal muscle, heart, and gut (Segalat et al., 2005; Chang et al., 2008). Developmental expression of NOS1AP mRNA in the spinal cord shows that through embryonic day 14 to 18 rats have low level expression, which then peaks at postnatal day 1 and returns to lower levels after 12 weeks (Li et al., 2008). Thus, NOS1AP is found in many different organs throughout the body and shows developmental expression patterns in the spinal cord.

1.6.4 NOS1AP levels affected by injury

NOS1AP mRNA and protein levels are affected by injury. Consistent with this idea, following injury to the rat spinal cord, NOS1AP mRNA and protein levels increase between two and eight hours following injury, then decrease to normal levels two weeks post-injury (Cheng et al., 2008). Following injury, immunofluorescence studies revealed that NOS1AP localizes to nNOS positive neurons and oligodendrocytes with maximal NOS1AP mRNA expression within the white matter as well as the ventral and dorsal horns (Cheng et al., 2008). In other injury models, similar changes in NOS1AP expression have been observed (Chen et al., 2008; Shen et al., 2008)). For example, following sciatic nerve injury models, mRNA levels for both NOS1AP and Dexras increase (Shen et al., 2008). Following injury levels of NOS1AP and Dexras return to basal levels (Shen et al., 2008). Thus, NOS1AP may play a major role in nerve plasticity in the period of time immediately following after spinal cord injury.

1.6.5 NOS1AP and Disease

NOS1AP has recently been implicated in several disorders including cardiovascular disease, schizophrenia and bipolar disorder. Linkage studies suggest that NOS1AP is a candidate in two diseases, long-QT syndrome (Arking et al., 2006; Aarnoudse et al., 2007; Post et al., 2007) and schizophrenia (Brzustowicz et al., 2004; Zheng et al., 2005). In cardiovascular disease research, whole genome studies have linked NOS1AP to congenital long- or short-QT syndrome, whereby patients suffer life-threatening cardiac anomalies (Arking et al., 2006; Aarnoudse et al., 2007; Post et al., 2007). In heart tissue, NOS1AP has been shown to bind NOS1 and inhibit L-type calcium channels, which increases the rate of repolarization (Chang et al., 2008) a system that may be affected in congenital QT syndrome.

Interestingly, Linkage disequilibrium studies have also shown that the *NOS1AP* gene confers susceptibility to schizophrenia (Brzustowicz et al., 2004; Zheng et al., 2005). Many of the regions linked to schizophrenia are non-coding regions, suggesting they may play a role in gene transcription (Wratten et al., 2009). This comes from studies on a Chinese Han population (Zheng et al., 2005), a Canadian population (Brzustowicz et al., 2004), a Columbian population (Miranda et al., 2006), and a South American Population (Kremeyer et al., 2009). One study looked as several single nucleotide polymorphisms (SNPs) and showed that one in particular may play a role in altering binding of transcription factors, thus increasing gene expression (Wratten et al., 2009). One UK study failed to find a link between *NOS1AP* and schizophrenia, but attributed these findings to genetic heterogeneity or sample design (Puri et al., 2006). In addition, another Chinese Han population study also failed to find an association between *NOS1AP* and schizophrenia (Fang et al., 2008). Additional studies have revealed that susceptibility might be due to a novel isoform of NOS1AP that consists of the two terminal exons of *NOS1AP* and can be found in the human dorsolateral prefrontal cortex (Xu et al., 2005). This study revealed that there was increased expression of this novel isoform of NOS1AP in patents with both schizophrenia and bipolar disorder (Xu et al., 2005). Since NOS1AP associates with nNOS and recruits nNOS from PDS-95 at NMDA receptors (Jaffrey et al., 1998) and NMDA receptors are thought to be involved in schizophrenia (Harrison

and Weinberger, 2005), evidence supports a potentially strong role for NOS1AP in schizophrenia and bipolar disorder (Xu et al., 2005).

1.7 RATIONALE

Clearly, evidence indicates that Scrib and NOS1AP play important roles in the CNS. The precise mechanisms behind their function remain unknown. Here we have identified a novel interaction between a known polarity protein, Scrib, and NOS1AP. We show that together these proteins couple with other proteins, namely β-Pix, Git1 and PAK and that this complex is involved in influencing Rho GTPase activity.

1.8 HYPOTHESIS

In this thesis I hypothesized that NOS1AP, through its association with Scrib, can bind the β-Pix, Git1, Pak complex and this complex influences dendritic spine development through the Rho GTPase, Rac1.

1.9 OBJECTIVES

1. Determine the nature of the association between NOS1AP and Scrib.

2. Determine the nature of the association between NOS1AP and the β-Pix, Git1, PAK complex.

3. Determine the effects of NOS1AP overexpression in mature hippocampal neurons.

4. Determine if NOS1AP can influence the Rho GTPase, Rac1.

CHAPTER 2 METHODS

2.1 Reagents

All reagents were purchased from Sigma Aldrich unless otherwise noted.

2.2 Constructs

The full length YFPNOS1AP was created with 5' primer AAT GAC ATT CTA GAA TTC AGC CGA and 3' primer TTT GTC GAC CCA CTA CAC GGC GAT CTC. The YFPNOS1APPTB construct was made from 5' TTT CCG AAT TCC ATC CCC AGC AAA ACC AND 3' primer CTT AAG ATC TTA CAG TAA GGG. The YFPNOS1APdelPTB was created using 5' primer GTC GAG ATG GTG CCA GCA TAT CTT CAG and 3' primer TTT GTC GAC CCA CTA CAC GGC GAT CTC. The Flag Git1 was cloned using 5' primer TTT GCG AAT TCG ATG TCC CGA AAG GGG and 3' primer TTT AGA GTC GAC TCA CTG CTT CTT CTC. The YFPScrib and myc Scrib were cloned using 5' primer TTT GCG AAT TCA ATG CTC AAG TGC ATC and 3' primer TTT AGA GTC GAC TGG TAC CCT CTA GGA GGG CAC AGG GCC CAG. The mycD.N.RacN17T came from (Lin et al., 2000). The FlagPix was cloned using 5' primer TTT GCC GCG ATT TCA ATG ACC GAC ATT AGC and 3' primer TTT GTC GAC TGG TTA TAG ATT GGT CTC ATC. For the FlagPixAAA mutant, the same 5' primer for FlagPix was used, however, the 3' primer was TTT GTC GAC TGG TTA CGC CGC CGC CTC ATC CCA GGC.

2.3 Antibodies

The NOS1AP rabbit polyclonal antibody was raised against a GST-fusion protein encompassing the C-terminus of rat NOS1AP. The following primers were used to amplify the C-terminal region before it was inserted in frame into the pGEXT3 vector, 5'primer TTT CCG AAT TCC ATG GGC TCC CAG, 3'primer TTT CGA GTC GAC CTA CAC GGC GAT CTC. The Scrib rabbit polyclonal antibody was generated against a GST fusion of the C-terminal region of Scrib 5'primer TTT CCA GGA ATT CCC CTT

CTG GGC AGG CCC TCA CCC GGC, 3'primer TTT AGA GTC GAC TGG TAC CCT CTA GGA GGG CAC AGG GCC CAG. The NOS1APc (NOS1APLong) rabbit polyclonal antibody was generated against the sequence CAFPLLDPPPPITRKRT. The N-terminal cysteine residue was used to directly conjugate the peptide to KLH for immunization using the Sulfo-Link Kit (Pierce). The PAK1 polyclonal antibody was purchased from Cell Signaling (#2602) used at 1:1000 for western blot (WB). Beta-Pix (611648) and nNOS (610309) antibodies were from BD Transduction Laboratories, both used at 1:1000 for WB. The myc (sc-40) and Git1 (sc-13961) antibodies were from Santa Cruz Biotechnology; used at 1:500 and 1:1000 for WB, respectively (Santa Cruz, CA). The GFP polyclonal antibody was purchased from ABCAM (Ab290), and used at 1:5000 for immunoprecipitation (IP) and 1:10,000 for WB. The Rac1 monoclonal antibody (Part No. 240106, Cell Biolabs, Cat# STA-401) and used at 1:1000 for WB.

2.3 Cell culture and Transfections

Human Embryonic Kidney (HEK) 293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Wisent Cat# 319-005-CL) with sodium pyruvate, 10% heat inactivated fetal bovine serum (FBS) (Wisent; Cat# 090150, Lot# 112622), 2mM L-glutamine (Wisent; Cat# 609-065-EL), 100 U/ml penicillin and 100 mg/ml streptomycin (Wisent; Cat# 450-201-EL) at 37 °C, 5% CO_2. For transfection, cells were plated to reach a density of between 50 % to 70 % confluency. For 10 cm plates, cells were transfected by mixing 25 μl poly(ethyleneimine) (PEI) transfection reagent (2 mg/ml Sigma) with 5 μg of cDNA in 750 μl serum free media (SFM), vortexed, left for 5 minutes, then added dropwise to cells in serum-containing media (Ehrhardt et al., 2006).

2.4 Immunoprecipitation

Mouse brains were homogenized using a dounce homogenizer in an NP40 lysis buffer containing 20mM tris pH 8.0, 37.5mM NaCl, 10% glycerol, 1%NP40 with 1mM phenylmethylsulphonylfluoride (PMSF), 10 μg/ml aprotinin (Sigma A1153), 10 μg/ml leupeptin (Sigma M92884), and 1 mM benzamidine (sigma) at a 1/10 (weight/volume).

Following homogenization, clarified lysate was generated by spinning samples three times at 13,000 rpm for 15 minutes at 4 °C. For whole cell lysate, 30 μl was removed and added to a 2XSDS sample buffer containing 250mM Tris pH 6.8, 4% sodium dodecylsulphate (SDS), 20% glycerol, 0.01% bromophenol blue, 0.4M Dithiothreitol (DTT). The remaining cleared lysate (1ml, approximately 4 mg total protein) was incubated with an appropriate antibody. This was then left to incubate overnight at 4 °C followed by a one-hour incubation with 100 μl of 10 % slurry of protein A sepharose (GE Healthcare) or 10 μl of 100 % slurry of protein G sepharose (Sigma) under constant agitation. The beads were then washed by spinning the mixture at 3000 rpm for 1 minute. The resulting supernatant was removed and 1 ml of fresh NP40 lysis buffer was added. This was repeated three times. Following the final wash, the sample was spun, the supernatant removed, and 30 μl of 2X SDS sample buffer was added. Samples were then eluted by boiling at 95 ºC for five minutes prior to loading and running through SDS-PAGE gels.

In addition to rodent brain lysate, we also immunoprecipitated from transfected cell lines. Here, cultured HEK 293T cells in 10 cm dishes were washed twice in 10 ml cold phosphate buffered saline (PBS) (Wisent; Cat # 311-010-CL). Cells were then suspended in 1 ml NP40 lysis buffer (see above) containing 1mM phenylmethylsulphonylfluoride (PMSF), 10 μg/ml aprotinin (Sigma A1153), 10 μg/ml leupeptin (Sigma M92884), and 1 mM benzamidine (sigma). Cells were incubated with the 1 ml lysis buffer on ice for 10 minutes, then scraped, collected, and transferred to eppindorf tubes. The 1 ml lysed cell suspension was then spun at 13,000 rpm for 15 minutes at 4 °C. Thirty microliters of the cleared lysate (supernatant) was removed and added to 30 μl 2X SDS sample buffer to generate whole cell lysate. The remaining lysate was then incubated with appropriate antibodies overnight at 4 °C with constant agitation. The following day, lysate was further incubated with 100 μl of a 10% slurry of protein A sepharose (GE Healthcare) at 4 °C with constant agitation. The beads were then washed as described above and run on a 7.5% SDS-PAGE and transferred onto PVDF membrane for western blot analysis.

2.5 HIPPOCAMPAL NEURON CULTURE AND TRANSFECTION

Hippocampal neurons were isolated from E19 rat embryos in a protocol modified from Krueger et al (2003). The hippocampi were dissected from four to six E19 rat embryos, then incubated with 1 ml 0.03% trypsin (Sigma Cat # T99355) at 37 °C, 5% CO_2 and then washed three times with 1 ml Hanks Balanced Salt Solution (HBSS; Invitrogen Cat # 14175) (Brewer et al., 1993; Krueger et al., 2003). After the final removal of HBSS, 2 ml of fresh HBSS was added to the hippocampi, followed by careful mechanical trituration to break-up and dissociate the hippocampi. Cells were counted by the addition of 10 µl Trypan Blue (FloLabs Cat# 16-910-49) with 90 µl of the cell/HBSS suspension. The mixture (10 µl) was added to the haemocytometer containing a coverslip, four chambers were counted to give an average, which was used to calculate the total number of cells. Dissociated cells were plated at a density of 40,000 cells per 35 mm dish. The 35 mm dishes (BD falcon) contained five 12 mm German glass cover slips (Cedar lane; Cat#72196-12) pre-coated in 0.05% (w/v) poly-L-lysine (Peptides International, Louisville, KY Cat# OKK3056). Cells were suspended in 1.5 ml of media containing 2% B27 Neurobasal medium (Maintenance media; Invitrogen, Carlsbad, CA) supplemented with 2mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Wisent). At day seven, cells were supplemented with an additional 500 µl of the maintenance media. Cells were then maintained at 37 °C, 5% CO_2, for 13 days in vitro (DIV) and then transfected using a $CaPO_4$ method (modified from (Jiang et al., 2004)) with cDNA constructs outlined. For $CaPO_4$ transfection, 13 days following plating, conditioned maintenance media (CM) was removed from cells and replaced with 1 ml of 37 °C high glucose Minimal Essential Medium (MEM) (Invitrogen) with 5% B27 (Invitrogen). DNA mix was then prepared by mixing 24 µg of a particular cDNA with 10 µl 2.5M $CaCl_2$, and sterile water (Sigma, W 3500) to bring up to a total volume of 200 µl. This mixture was then added dropwise to 200 µl 2XHeBS (274 mM NaCl, 10mM KCl, 1.4 mM Na_2HPO_4, 15 mM D-glucose, 42 mM HEPES (free acid); pH 7.1.). This mixture was vortexed after each drop was added. The combined mixture was allowed to incubate at room temperature for 20 minutes. The DNA/HeBS mixture was then added dropwise to cells, which were then incubated at 37 °C, 5% CO_2 for three hours. Cells were then

washed twice with 1 ml 2X HBSO/2 (144 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES; pH 6.7) and once with CM supplemented with 1/4th volume maintenance medium. Supplemented CM was then replaced (1.5 ml) on the cells overnight. Cells were fixed 24 hours post-transfection as described below.

2.6 IMMUNOCYTOCHEMISTRY

The dissociated hippocampal neurons grown as mentioned above were washed once with 1 ml 1XPBS, then fixed with 1 ml PBS containing 4% paraformaldehyde (PFA) with 4% sucrose for 10 minutes at room temperature. The PFA solution was removed and cells were washed twice with 1 ml PBS. Cells were then permeabilized by addition of 1 ml 0.1% Triton X-100 in PBS for 30 minutes. The Triton X-100 solution was removed and cells were then blocked in 1 ml 5% goat serum (Sigma Cat # G9023) in PBS for 20 minutes. This was followed by overnight incubation at 4 °C in 1 ml myc antibody (1:500) suspended in a mixture containing 2% goat serum in PBS. Cells were then washed two times in 1 ml PBS. Cover slips were then incubated for 1 hour at room temperature in 1 ml secondary antibody. Here, Goat anti-mouse alexa 594 (Invitrogen; Cat # A11005) was diluted to 1:2000 in 2% goat serum. Cells were then washed two times in 1 ml PBS. BisBenzamide (Hoescht – 33258; Sigma Cat # B1155) was used as a nuclear stain at 1:10,000 in PBS. Here cells were incubated for 10 minutes (1 ml) at room temperature. Cells were then washed three times in 1 ml PBS. Cells were then washed once by dipping in sterile double deionized water and then mounted in 20 μl fluoromount mounting medium (Sigma Cat# F4680) on glass slides (VWR) (three coverslips per slide).

2.7 IMAGE QUANTIFICATION

Cells were imaged on an inverted Leica CTR6000 with a Hamamatsu camera using a 63X oil objective. Three experiments were conducted for each treatment (transfection), which included a minimum total of 17 neurons. Three different neurites were used for each neuron counted. For each neurite a 50 μm region was selected and the number of processes extending out within this region were counted. Any process extending at least

0.1 μm from the neurite was counted as a dendritic protrusion. Neurons were counted by the initial experimenter and an individual blinded to the study. Both data sets were pooled and a t-test was used to assess significance between the different treatments. For YFP, YFPNOS1AP, and YFPNOS1APPTB transfected neurons, data was represented as percentage from YFP control values. For data involving the D.N.Rac constructs, a ONE-WAY ANOVA was used to assess significance and a Bonferroni post-test was used to assess significance between groups.

2.8 RAC ACTIVATION ASSAY

The Rac activation assay protocol was followed from Cell Biolabs, San Diego, CA (Cat#STA-401). HEK293T cells were transfected with constructs encoding either YFP, YFPNOS1AP, or YFPNOS1APPTB. Following transfection, cells were left for approximately 18 hours, then incubated in serum free media for an additional 24 hours. The serum free media was then changed to media containing 10% FBS for 10 minutes. Cells were then treated according to manufacturers protocol. Cells were washed twice in 10 ml ice cold PBS, then lysed in 1 ml lysis buffer (25mM HEPES, pH 7.5, 150 mM NaCl, 1%NP-40, 10mM MgCl2, 1 mM EDTA, 2% glycerol) containing 1mM PMSF, 10 μg/ml aprotinin (Sigma A1153), 10 μg/ml leupeptin (Sigma M92884), and 1 mM benzamidine (sigma) (see above protocol for cell lysis). Cell were then scraped off the plate and placed into eppindorf tubes. Cells were spun at 13,000 rpm for 10 minutes at 4°C. Supernatant (lysate) was then removed for the rac activation experiment. For each experiment, an extra YFP-transfected plate was used for a positive control. For the positive control, the 1ml cell lysate was mixed with 20 μl 0.5M EDTA, pH 8.0, followed by the addition of 10 μl GTPγS. This reaction was incubated at 30°C for 30 minutes in a water bath with agitation. After the 30-minute incubation, 65 μl 1M $MgCl_2$ was added to stop the reaction. Next, all lysate from the various transfected cells (including the positive control) was incubated with 40 μl of the resuspended GST-PBD beads for one to one and a half hours at 4°C with constant agitation. Cells were then washed three times with 500 μl lysis buffer by spinning for 1 minute at 3000rpm between washes. Then 30 μl 2X SB

was added to the GST-PBD pellet. Samples were then boiled at 95 ºC for five minutes, then run on a 12% SDS-PAGE gel prior to being transferred onto PVDF membrane for western blot analysis and probed with Rac antibody (supplied; 1:1000) and YFP (ABCAM; 1:5000).

CHAPTER 3 RESULTS

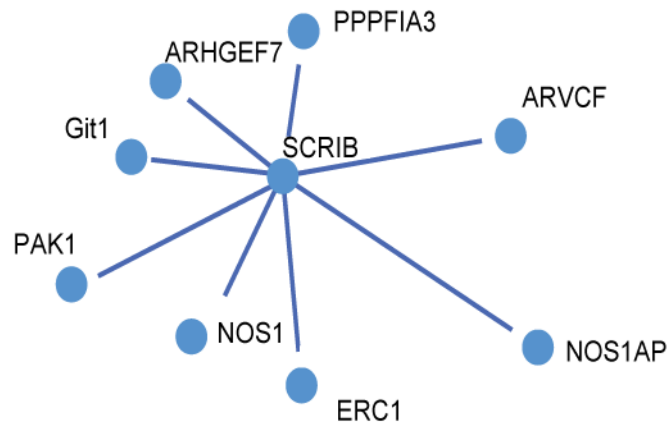
The work in this thesis stems from a larger mass spectrometry/proteomic screen conducted by Dr. Fawcett. Figure 3.1 shows a schematic of selected proteins identified in the proteomic screen. Important Scrib and NOS1AP associating proteins are identified, including Git1, β-Pix, and PAK.

3.1 Scrib and NOS1AP interactions

3.1.1 Scrib Associates with NOS1AP

One of the more interesting proteins identified in the mass spectrometry screen using Scrib as bait was the nitric oxide synthase adaptor protein NOS1AP (Fig. 3.1). NOS1AP has been linked as a susceptibility protein for schizophrenia (Brzustowicz et al., 2004; Brzustowicz, 2008; Kremeyer et al., 2009), and more recently to long QT syndrome (LQTS) (Arking et al., 2006; Arking et al., 2009), a congenital heart condition. NOS1AP contains an amino terminal PTB domain important for its association with Dexras (Fang et al., 2000), a Ras homologue, and the synaptic vesicle associated protein synapsin1 (Jaffrey et al., 2002b). NOS1AP also contains a carboxyl-terminal PDZ binding motif, responsible for the interaction with the PDZ domain of nNOS (Jaffrey et al., 1998). To confirm the interaction between NOS1AP and Scrib, an antibody to the C-terminal region of NOS1AP was generated and we used this antibody to precipitate endogenous NOS1AP. Figure 3.2A confirms that endogenous NOS1AP was able to precipitate endogenous Scrib. In our NOS1AP IP's we noted a slower migrating band of 100kDa, we mapped this protein to the same locus as NOS1AP and were able to show this is a novel splice variant of NOS1AP (NOS1APc or CAPONLong) (Fig. 3.2A-C). We then cloned this novel isoform. NOS1APc contains all the same elements as NOS1AP, but has a novel C-terminal region that destroys the C-terminal PDZ binding motif. We next tested whether NOS1APc could associate with Scrib. For this we precipitated Scrib and its associating proteins from rodent brain lysate and using a NOS1APc antibody were able to show that NOS1APc could associate with Scrib (Fig. 3.2 A).

A



B

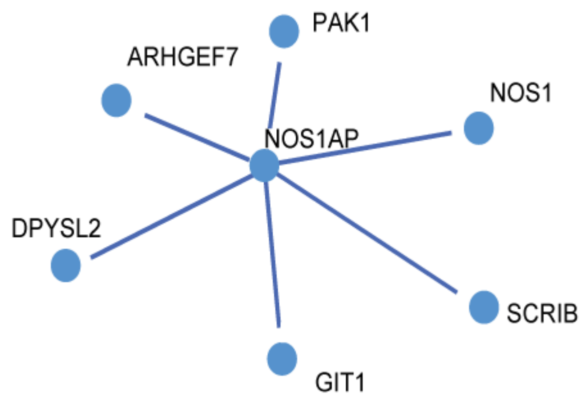


Figure 3.1 Original Mass spectrometry/proteomic screen to identify Scrb and NOS1AP associating proteins (A) Selected Scrib associated proteins identified in a Mass Spectrometry screen. (B) Selected NOS1APassociated proteins identified in the Mass Spectrometry Screen.

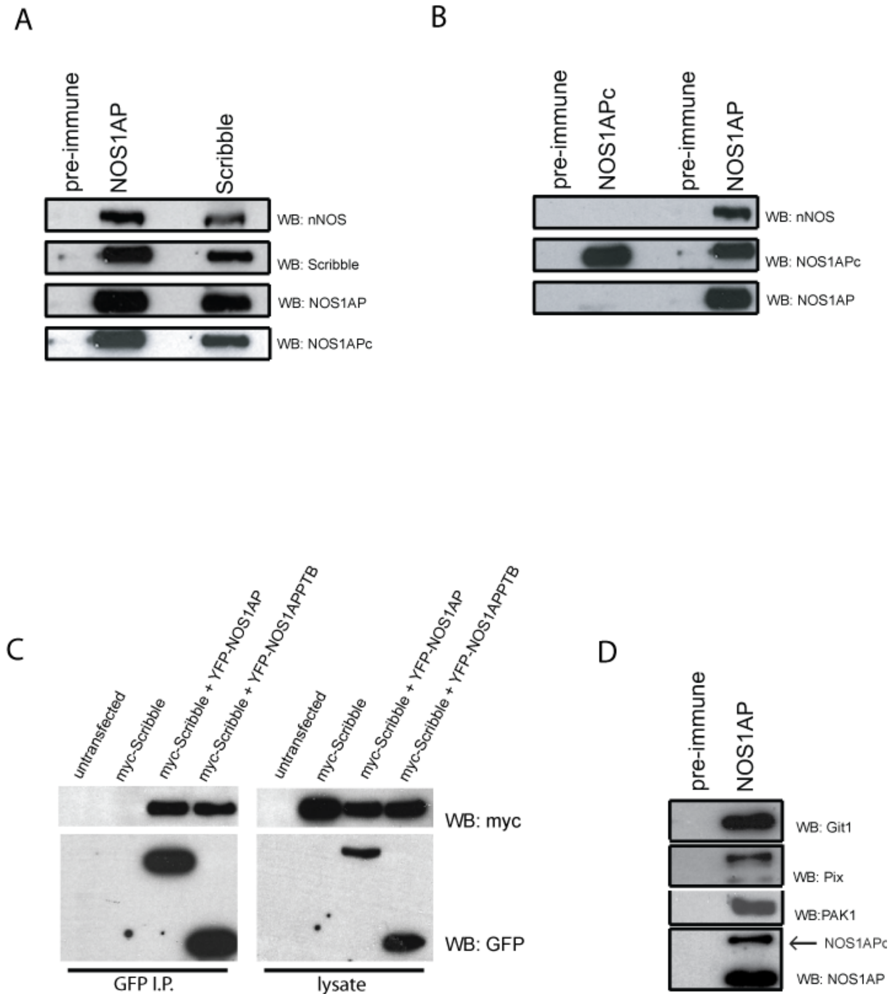


Figure 3.2 (A) Mouse brain lysate was immunoprecipitated (IP'd) with the antibodies indicated. The resulting blot was probed with nNOS, Scrib, or NOS1AP. (B) Mouse brain lysate was IP'd with the antibodies indicated. The resulting blot was probed with nNOS, NOS1AP, NOS1APc (CAPONLong). (C) cDNA constructs were transfected into 293T cells as indicated. The lysates were IP'd with anti-GFP and probed with anti-myc or anti-GFP as indicated. (D) Mouse brain lysate was IP'd with the antibodies indicated. The resulting blot was probed with Git1, β−Pix, PAK or NOS1AP (Lower panel). Note a slower mobility band detected with our NOS1AP antibody (NOS1APc).

3.1.2 Scrib Associates with nNOS

Since NOS1AP is known to associate with nNOS, we wanted to test whether Scrib could also associate with nNOS. For this we used an antibody raised against Scrib and we were able to show an association between Scrib and nNOS (Fig. 3.2A), suggesting that Scrib may play a role in previously identified NOS1AP – nNOS complexes. We next tested whether NOS1APc could associate with nNOS. For this we immunoprecipitated NOS1APc from rodent brain lysate and could not detect an association with nNOS (Fig. 3.2B). This suggests that NOS1AP and NOS1APc may be involved in different complexes.

3.1.3 Scrib Associates with the PTB domain of NOS1AP

Since we were able to show endogenous Scrib and NOS1AP can form a complex, we next wanted to investigate the precise nature of the interaction. First we confirmed that exogenously expressed Scrib and NOS1AP could co-precipitate with each other (Fig. 3.2C). Next deletion mutants of both NOS1AP and Scrib were generated by Dr. Fawcett. Since Scrib contains four PDZ domains (Humbert et al., 2003) and NOS1AP contains a C-terminal PDZ binding motif, necessary for the interaction with nNOS (Jaffrey et al., 1998), we wondered whether this C-terminal region in NOS1AP is required for its interaction with Scrib. Since a construct of NOS1APc, that destroys the C-terminal PDZ binding motif, still interacts with Scrib, we ruled out the possibility that the C-terminal PDZ binding motif of NOS1AP was important for the interaction with Scrib. Therefore we focused on the other important signaling domain found in NOS1AP, the PTB domain. Previously, PTB domains have been shown to associate with PDZ domains (Nie et al., 2004) giving precedence to the idea that the PTB domain of NOS1AP may be important for association with Scrib. To test this a construct containing only the PTB domain fused in frame with YFP was co-transfected along with myc-tagged Scrib into HEK 293T cells. Using GFP antibody we precipitated the PTB domain of NOS1AP and showed that myc-Scrib co-precipitated (Fig. 3.2C). Therefore, the PTB domain is sufficient to associate with Scrib. Others in the lab were able to show that the PTB domain associated with the fourth PDZ domain of Scrib.

3.2 A NOS1AP Complex

3.2.1 NOS1AP Associates with Git, β-Pix, Pak

The initial proteomic screen identified a number of proteins that precipitated with NOS1AP, these included the neuronal isoform of nitric oxide synathase (nNOS), β-Pix, Git1, and PAK3 (Fig. 3.1B). Using an antibody raised against NOS1AP, we confirmed that a number of these proteins were specifically precipitated with NOS1AP (Fig. 3.2D). These proteins included β-Pix, Git1, and PAK1. Although the original screen identified PAK3 peptides, using PAK1 specific antibodies, we also determined that PAK1 could precipitate with NOS1AP (Fig. 3.1B and 3.2D), suggesting that more than one PAK protein can associate with NOS1AP. Previous work has identified β-Pix, Git1, and PAK as associating proteins (Bagrodia et al., 1998; Manser et al., 1998; Premont et al., 2004; Za et al., 2006), together these data suggest that NOS1AP forms a complex with β-Pix, Git1, and PAK, yet the underlying mechanism of this interaction and morphological consequence remain to be determined. Further, a number of these proteins, previously shown to associate with Scrib (Audebert et al., 2004; Nola et al., 2008), including, β-Pix, Git1, and PAK were also identified in the Scrib proteomic screen (Fig. 3.1A).

3.2.2 NOS1AP Associates with β-Pix, Git1, Pak through Scrib

Since NOS1AP precipitated β-Pix and Git1, and β-Pix has been shown to bind directly to Scrib (Audebert et al., 2004), we next wanted to test whether Scrib was an important scaffold to bridge an interaction between NOS1AP on the one hand and β-Pix and Git1 on the other. To test this possibility, we co-transfected β-Pix and NOS1AP in the presence or absence of Scrib. Only in the presence of Scrib were we able to see an association between β-Pix and NOS1AP (Fig. 3.3A and B). Since β-Pix has been shown to bind Scrib directly through a PDZ binding motif (Zeniou-Meyer et al., 2005) we mutated the last three amino acids of β-Pix to alanines to test the effects of destroying this PDZ domain recognition sequence. Interestingly, the β-PixAAA mutant was not able to associate with Scrib, confirming previous reports; although, we still detected an interaction between NOS1AP and Scrib in the same assay (Fig. 3.3C and D) further

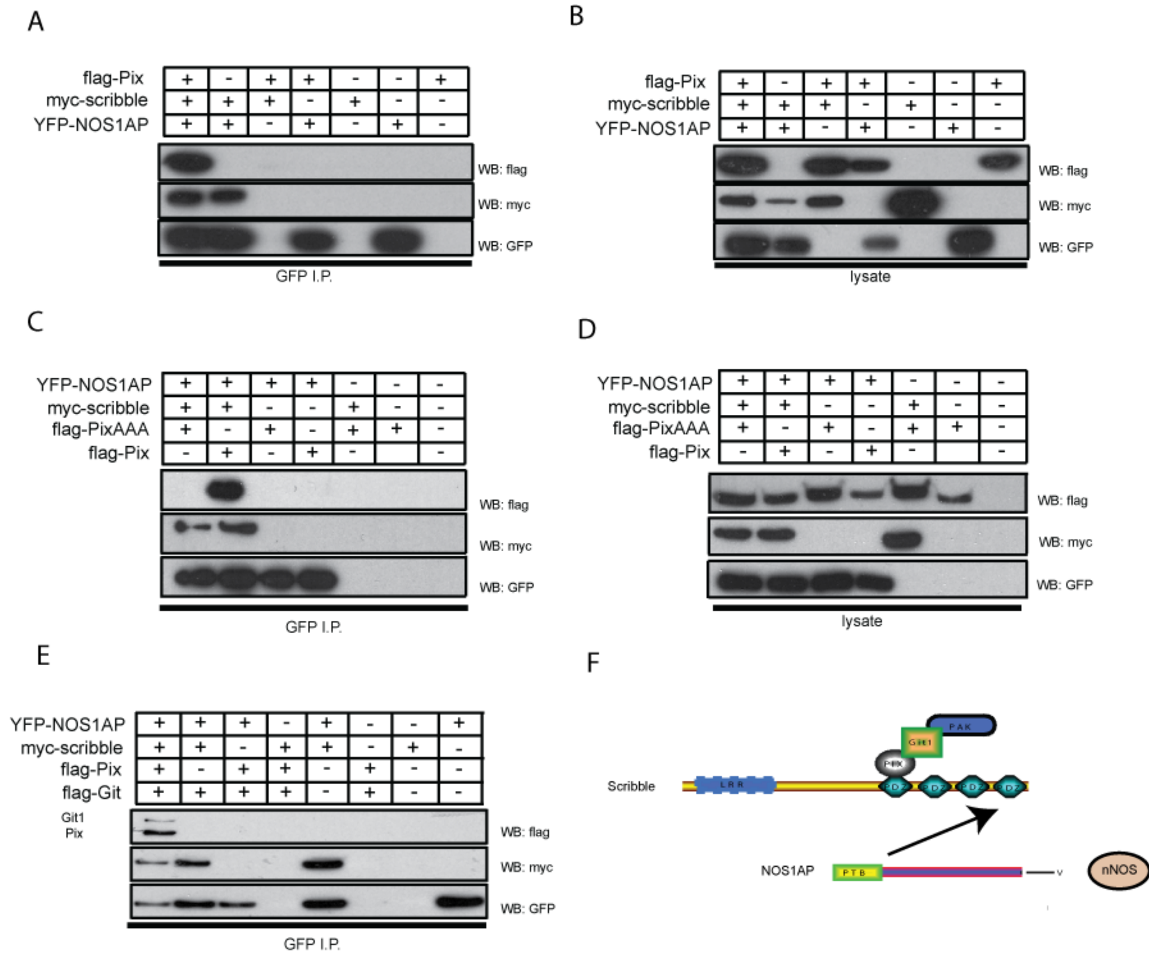


Figure 3.3 NOS1AP interacts with β-Pix through an interaction with Scribble. (A, B) HEK 293T cells were transfected with cDNA constructs as indicated. The resulting lysates were immunoprecipitated (IP'd) with GFP antibody (A) and probed as indicated. (B) Lysate controls. (C, D) 293T cells were transfected with cDNA constructs indicated. The resulting lysates were IP'd with GFP antibody (C) and probed as indicated. (D) Lysate controls. (E) 293T cells were transfected with cDNA constructs as indicated. The resulting lysates were IP'd with GFP antibody (E) and probed as indicated. (F) Summary of the interactions outlined in (A-E).

confirming the role of Scrib as an important scaffolding protein connecting NOS1AP with β-Pix. Finally, to determine whether Git1 associates with NOS1AP, through both Scrib and β-Pix, we tested whether Git1 could precipitate with NOS1AP and Scrib in the absence of β-Pix. As expected, Git1 co-immunoprecipitated with NOS1AP only in the presence of both Scrib and β-Pix (Fig. 3.3E). Together these data suggest that Scrib functions to bridge an interaction between NOS1AP and the β-Pix, Git1, Pak complex (Fig. 3.3F).

3.3 NOS1AP and the PTB domain of NOS1AP localize to the membrane

We next wanted to determine a role for NOS1AP in a cell system. For this we initially transfected HEK 293T cells with yellow fluorescent protein (YFP) or constructs with YFP fused in frame with NOS1AP, the PTB domain of NOS1AP alone (NOS1APPTB) or all of NOS1AP except the PTB domain (NOS1APdelPTB). We note membrane localization in cells expressing YFP-NOS1AP or YFP-NOS1APPTB (Fig. 3.4A and B). While cells expressing the YFP-NOS1APdelPTB construct revealed a cytosolic localization similar to YFP (Figure 3.4C and D). Further examination of cells expressing YFPNOS1AP or the PTB domain alone revealed cells extending processes compared to cells expressing YFP or the YFPNOS1APdelPTB construct (Figure 3.4A and B; see arrows). Taken together these data suggest that the PTB domain of NOS1AP is important for localizing NOS1AP to membranes and stimulating process outgrowth.

3.4 NOS1AP and Scrib in dendrites

3.4.1 A role for a NOS1AP-Scrib complex in dendritic spines

We have identified an important scaffolding complex for NOS1AP that includes Scrib β-Pix, Git1, and Pak proteins. In neurons, β-Pix, Git1 and Pak proteins have been shown to play a significant role postsynaptically by regulating dendritic spine development (Wiggin et al., 2005; Zhang et al., 2005). In addition, nNOS, a protein identified as a Scrib (Fig. 3.2A) and NOS1AP associating protein (Jaffrey et al., 1998), has previously

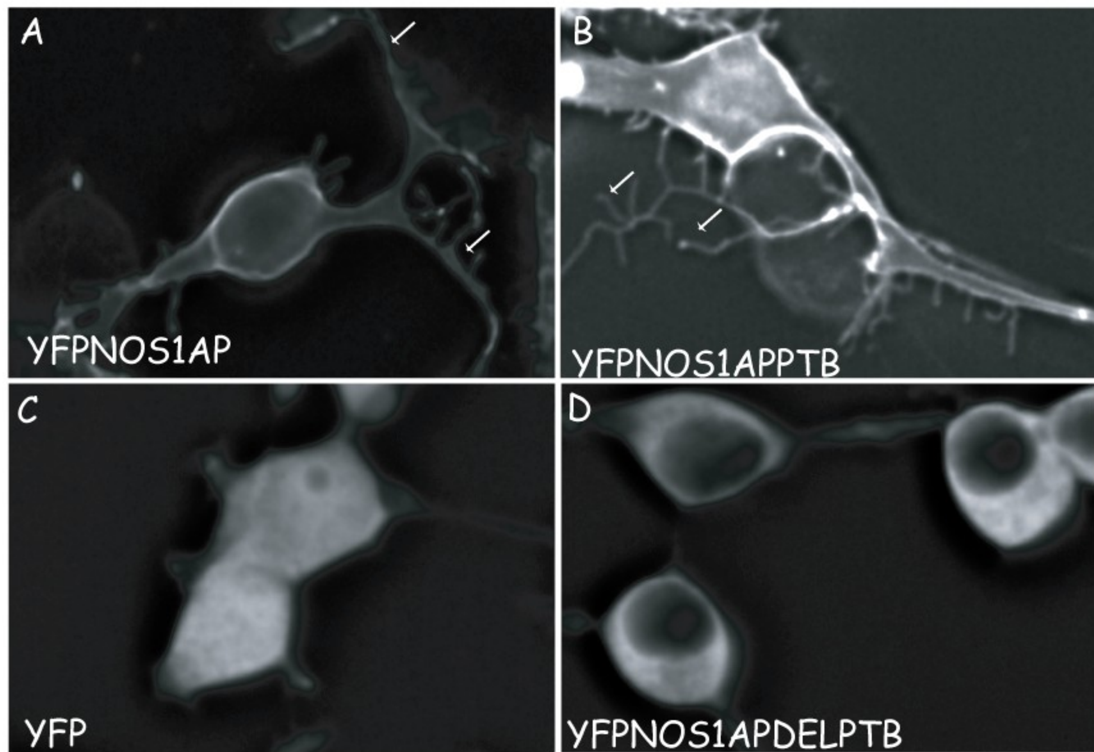


Figure 3.4 NOS1AP and the PTB domain of NOS1AP localize to the membrane. HEK 293T cells were transfected with cDNA constructs encoding (A) YFPNOS1AP, (B) YFPNOS1APPTB, (C) YFP, or (D) YFPNOS1APdelPTB (NOS1AP mutant devoid of PTB domain).

been shown to localize in the postsynaptic region (Christopherson et al., 1999). Further, Scrib, in multiple organisms, has been localized to the postsynaptic region (Roche et al., 2002; Audebert et al., 2004). Since these data implicate a potential role for NOS1AP and Scrib in the postsynaptic region, we tested whether NOS1AP and Scrib could localize to dendrites. For this purpose we co-transfected 13DIV hippocampal neurons with YFP-NOS1AP and myc-Scrib. We showed a clear colocalization in dendritic shafts (Fig. 3.5A-C arrowheads), and in dendritic spine, and spine heads (Fig. 3.5A-C arrows) that is not present in the YFP and myc-Scrib control. Taken together, these data suggest that NOS1AP and Scrib proteins can localize postsynaptically in dendrites.

3.4.2 NOS1AP PTB domain co-localizes with Scrib in Dendritic spines

Since the PTB domain of NOS1AP is sufficient to bind Scrib, we wanted to test whether the PTB domain could co-localize with Scrib in dendritic spines in a similar manner to full length NOS1AP. For this we co-transfected dissociated hippocampal neurons that had been cultured for 13 DIV with YFP-PTB and myc-Scrib. The YFP-PTB co-localized with myc-Scrib along dendritic shafts, along dendritic protrusions, and in dendritic spines (Figure 3.5D-F arrows). This suggests that the PTB domain is partially responsible for associating with Scrib and perhaps plays a role in the recruitment of Scrib to the dendritic spines.

3.4.3 NOS1AP increases dendritic protrusions

Since NOS1AP localized in dendrites, and we have linked NOS1AP with proteins important for regulating dendritic morphology namely the RhoGEF β-Pix and its associated proteins, Git1 and Pak through Scrib, and NOS1AP induces a profound outgrowth phenotype in 293T cells, we next wanted to investigate whether NOS1AP had an effect on dendritic spine morphology. For this purpose we overexpressed either YFP or YFP-NOS1AP in dissociated hippocampal neurons that had been cultured for 13 DIV (Fig. 3.6A and B). We counted the number of dendritic protrusions twenty-four hours post-transfection and noted 25.3 +/- 0.6 processes/50 μm segment in YFP-transfected

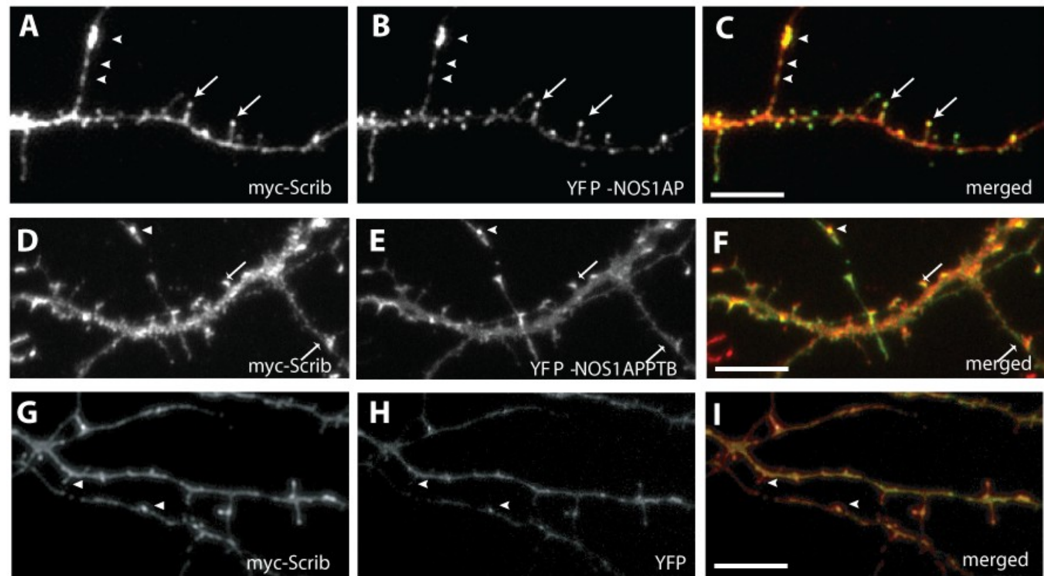


Figure 3.5 NOS1AP and Scrib colocalize in dendrites. Fluorescent image of a dissociated hippocampal neuron cultured for 13 days, then transfected with a constructs encoding (A) myc-Scrib (B) YFP-NOS1AP and (C) merged. Note the colocalization of Scrib and NOS1AP in dendritic shafts (arrowhead A, B, and C) and in dendritic spines (A,B, and C arrows).Cultured neurons were also transfected with constructs encoding (D) myc-Scrib, (E) YFPNOS1APPTB and (C) merged. Note the colocalization of Scrib and NOS1AP-PTB domain in dendritic shafts (arrowhead D, E, and F) and in dendritic spines (D, E, and F arrows). As a control, cultured neurons were transfected with constructs encoding (G) myc-Scrib, (H) YFP and (I) merged. Note the lack of co-localization at punctate structures (arrowhead D, E, and F). Scale Bar = 10 μm.

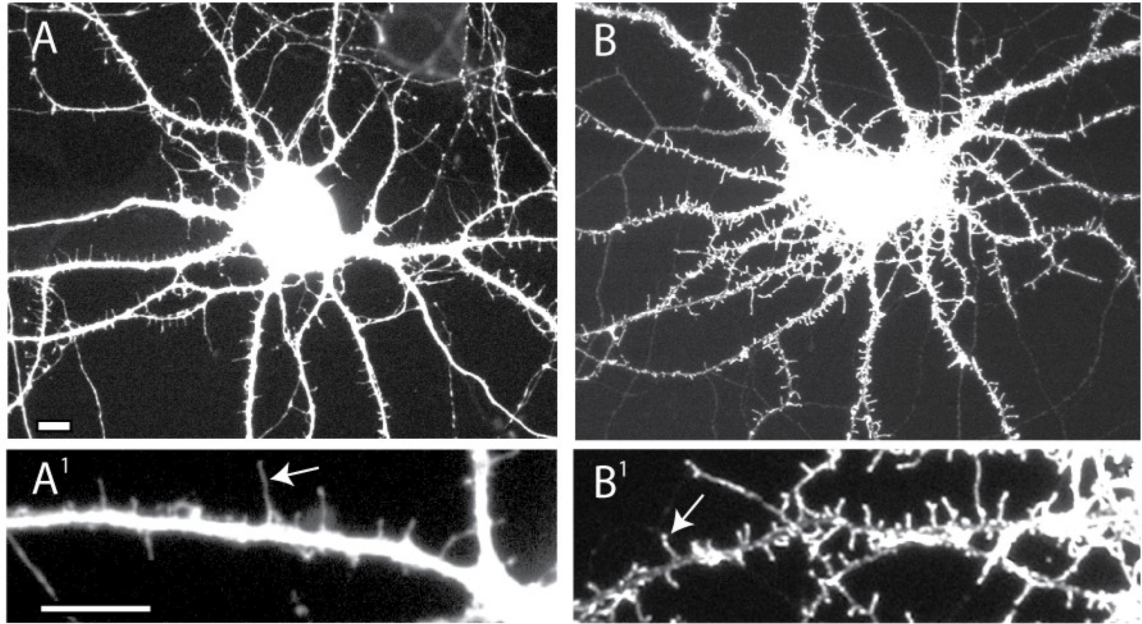


Figure 3.6 NOS1AP induces process outgrowth in hippocampal neurons. Primary hippocampal neurons transfected at 13 DIV with either (A) YFP or (B) YFP-NOS1AP were imaged 24 hours post transfection. (A^1-B^1) Enlarged regions from (A) and (B) showing individual dendrites and dendritic spines (arrows) fom the (A^1) YFP or (B^1) YFP-NOS1AP transfected neurons. Scale 10 μm.

neurons and 36.0 +/- 0.8 processes/50 μm in YFPNOS1AP-transfected neurons ($p<0.0001$). This represents a 30% increase in the number of dendritic protrusions in the YFP-NOS1AP-transfected neurons. (Fig. 3.6 and 3.7; Table 1).

3.4.4 NOS1AP increases dendritic protrusions through its PTB domain

Since the PTB domain showed a similar localization and effect in 293T cells to full length NOS1AP, we next wanted to determine the effects of overexpressing the PTB domain in neurons. For this we transfected dissociated hippocampal neurons with a construct encoding YFP-NOS1APPTB or YFP control alone. We counted 38.7 +/- 1.6 processes/50 μm in YFPNOS1APPTB expressing neuron, an increase of 35% compared to YFP control ($p<0.0001$) (Fig. 3.7; Table 1). This result suggests that the PTB domain is sufficient to elicit a response in neurons.

3.5 NOS1AP AND RAC ACTIVATION

3.5.1 NOS1AP activates the small Rho GTPase Rac

Previous work has shown that β-Pix is a RacGEF and along with Git1 and Pak, regulates the small Rho GTPases, Rac and cdc42 (Manser et al., 1998; Bokoch, 2003; Zhang et al., 2005; Kreis et al., 2007). As well, others have shown that β-Pix, Git1 and PAK function to regulate dendritic spine morphology through the Rho GTPase Rac (Zhang et al., 2003; Zhang et al., 2005). Since NOS1AP associates with β-Pix, Git1 and PAK through Scrib, we wondered whether NOS1AP could activate the Rho family GTPases, specifically Rac. To test this possibility we overexpressed NOS1AP in HEK293T cells, and investigated its effect on the level of GTP-bound Rac. In YFP-NOS1AP expressing cells we saw a 3-fold increase in Rac activity compared to cells expressing YFP alone (Fig. 3.8 A and B).

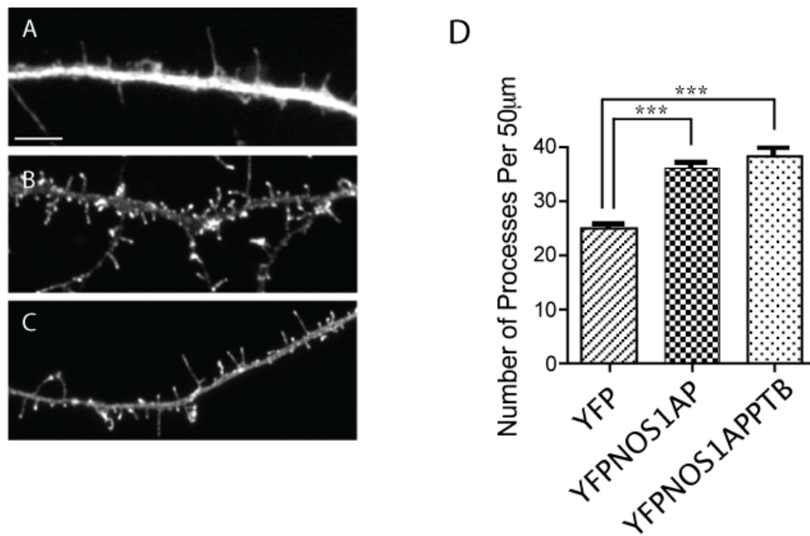


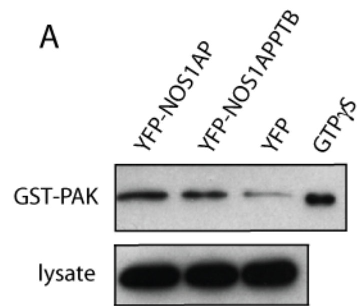
FIgure 3.7 NOS1AP increases processes. Epifluorescent image of hipporamapal neurons cultured for 13 days transfected with (A) YFP (B) YFPNOS1AP or (C) YFPNOS1APPTB. (D) Quantification of process number in YFP vs. YFPNOS1AP vs YFPNOS1APPTB. *** = $p<0.0001$; Scale Bar = 5 µm.

Table 1 Quantification of the number of processes/50 μm for neurons expressing constructs below including % increase from YFP control and p-values. Note: *** p-value <0.0001

	Processes/50 μm	Percent of YFP (%)	P-value from YFP
YFP	25.3 +/- 0.6	100 +/- 2.4	
YFPNOS1AP	36.0 +/- 0.8	143 +/- 3.2	***
YFPNOS1APPTB	38.7 +/- 1.4	153 +/- 5.5	***

Table 2 Quantification of the number of processes/50 μm for neurons expressing constructs below. Note: *** p-value <0.001; ** p-value <0.01; N.S. (not significant)

	Processes/50 μm	YFP	YFPNOS1AP	YFPNOS1AP +D.N.Rac
YFP	25.3 +/- 0.6			
YFPNOS1AP	36.0 +/- 0.8	***		
YFPNOS1AP + D.N.Rac	27.8 +/- 0.7	N/S	***	
YFP + D.N.Rac	29.6 +/- 1.0	**	***	N/S



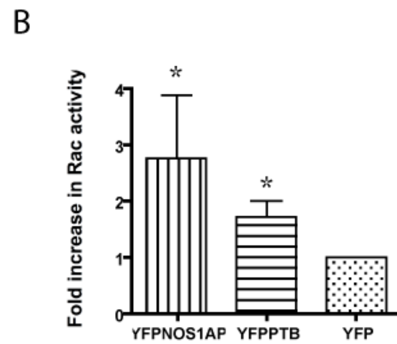


FIgure 3.8 YFPNOS1AP and YFPNOS1APPTB activate Rac. HEK 293T cells were transfected with cDNA encoding YFPNOS1AP, YFPNOS1APPTB (YFPPTB) or YFP control and Rac activation was measured (B) Quantification of activated Rac. * = $p<0.05$

3.5.2 The PTB domain is sufficient to activate Rac

We have shown that the PTB domain of NOS1AP is sufficient to induce process outgrowth in cultured neurons and to associate with Scrib. Further, Scrib is necessary to connect NOS1AP with the RhoGEF protein β-Pix, we next tested whether the PTB domain of NOS1AP alone can activate Rac. For this we overexpressed the PTB domain alone in HEK293T cells, and investigated its effect on the level of GTP-bound Rac. Expression of the PTB domain showed 2-fold Rac activation above YFP control (Fig. 3.8A and B), suggesting that the PTB domain plays an important role in regulating Rho GTPase activity. Together, these data suggest that the PTB domain of NOS1AP is sufficient to increase Rac activity.

3.5.3 D.N.Rac attenuates NOS1AP-induced dendritic protrusions

We suspected that Rac may act downstream of NOS1AP, to confirm this we co-expressed a dominant negative version of Rac (D.N. Rac) along with NOS1AP. We used a D.N. version of Rac in which asparagine 17 was mutated to threonine (N17T), preventing GTP from binding and activating Rac. We co-transfected dissociated hippocampal neurons that had been cultured for 13 DIV with 1: YFP-NOS1AP and myc-tagged D.N.Rac (YFPNOS1AP/D.N.Rac); 2: YFP-NOS1AP and myc vector (YFPNOS1AP) and two controls 3: YFP and myc vector (YFP); 4: YFP and myc-tagged D.N.Rac (YFP/D.N.Rac). We then counted the number of dendritic protrusions per 50 μm segment of neurite. Our quantification revealed 27.8 +/- 0.7 processes/50 μm for YFPNOS1AP/D.N.Rac, 36.0 +/- 0.8 processes/50 μm for YFPNOS1AP, 25.3 +/- 0.6 processes/50 μm for YFP, and 29.6 +/- 1.0 processes/50 μm for YFP/D.N.Rac (Fig. 3.9; Table 2). Co-expression of YFP-NOS1AP and D.N.Rac reduces process outgrowth by 33% returning the number of processes to levels seen in YFP overexpressed neurons. Thus, D.N.Rac blocks the phenotypic effects of NOS1AP overexpression. We note, when we compare the two controls (YFP with YFP/D.N.Rac) there is a small but statistically significant increase with YFP/D.N.Rac ($p<0.01$). However, comparing YFP/D.N.Rac with YFPNOS1AP/D.N.Rac, there is no statistically significant difference again supporting the notion that D.N.Rac blocks NOS1AP dependent process outgrowth.

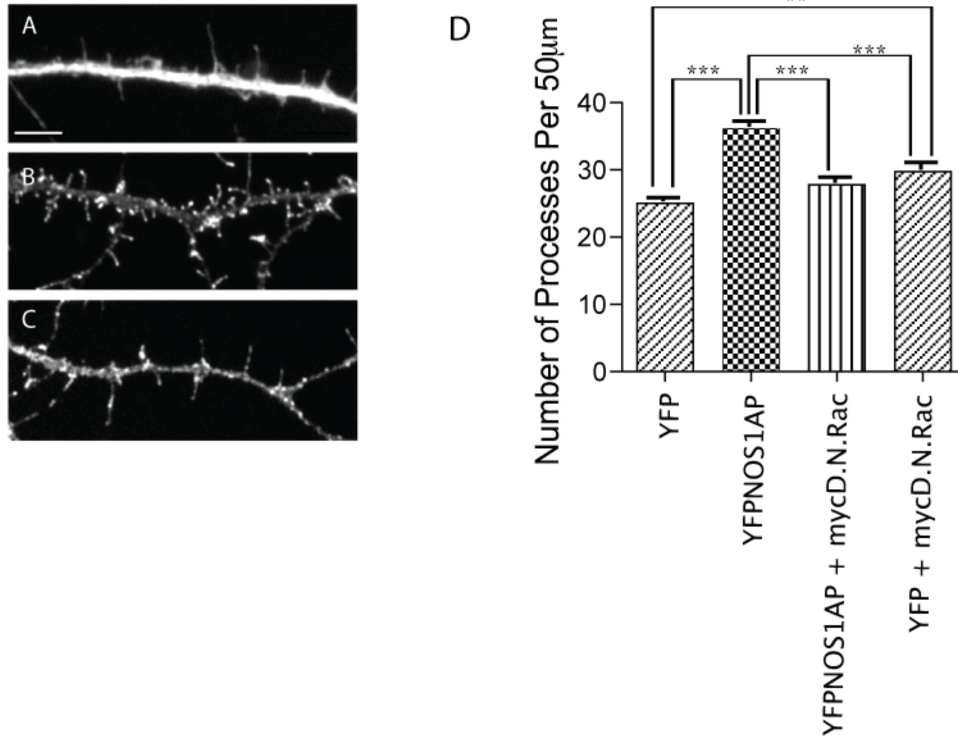


Figure 3.9 NOS1AP increases spine numbers and activates Rac. Epifluorescent image of a hippocampal neuron cultured for 13 days transfected with (A) YFP (B) YFPNOS1AP or (C) YFPNOS1AP plus D.N.Rac. (D) Quantification of process number for YFP, YFP-NOS1AP, YFPNOS1AP + D.N.Rac and YFP + D.N.Rac. *** = $p<0.001$. ** = $p<0.01$; Scale Bar = 5 μm.

CHAPTER 4 DISCUSSION

4.1 Scrib Associates with The PTB domain of NOS1AP

Our study indicates that NOS1AP influences Rac activation through a Scrib complex to regulate synapse formation and function. We identified a unique interaction between the NOS adaptor protein NOS1AP and the polarity protein Scrib through a targeted proteomic screen. Scrib contains a number of signaling domains including LRR and four PDZ domains, and we show that the fourth PDZ domain of Scrib interacts with the PTB domain of NOS1AP. However, we note that the PTB domain of NOS1AP was both sufficient and necessary to precipitate Scrib protein implicating its importance for the NOS1AP-Scrib interaction (Fig. 3.2). Previously, the PTB domains of the p72 and p66 isoforms of the cell fate determinant protein NUMB, have been reported to be necessary for an interaction with the first PDZ domain of the E3 ligase protein LNX (Nie et al., 2004), supporting the notion that this type of interaction does occur. Interestingly, the PTB domain of NOS1AP is most similar to the PTB domain of mouse Numb (Jaffrey et al., 2002b) suggesting that the PTB domain of NOS1AP may have a similar fold. The association of the NUMB PTB domain with the PDZ domain of LNX is dependent on an 11 amino-acid insertion between the α-helix A2 and β-strand B2 (Nie et al., 2004); however alignments between the NOS1AP and NUMB PTB domains fail to show homology in this 11-amino acid region (data not shown). Nonetheless, the unique interaction identified here suggests that PTB-PDZ domain interactions may be more common than previously supposed, especially in the context of the polarity network.

4.2 NOS1AP signaling in the synapse

4.2.1 Scrib and NOS1AP pre- and post- synaptically

In our proteomic screen we identified β-Pix, Git1 and Pak as associating proteins with both Scrib, supporting previous studies (Audebert et al., 2004; Nola et al., 2008), and with NOS1AP. Further, we show that Scrib functions as a bridge between NOS1AP and the β-Pix, Git1 and PAK complex. This is consistent with a role for Scrib as a multi-

faceted scaffolding protein. Many PDZ containing proteins function to scaffold multiple protein complexes (Tonikian et al., 2008). PDZ proteins are especially important to scaffold signaling complexes involved in the development and function of the synapse (Feng and Zhang, 2009). In addition to PDZ domain containing proteins, recent evidence suggests that LRR containing proteins also function as important regulators of synaptic function (Ko and Kim, 2007; Linhoff et al., 2009; Woo et al., 2009). Here, we suggest that Scrib is an organizing protein that functions to localize NOS1AP, β-Pix, Git1 and PAK pre- and post- synpatically (Fig 4.1).

Consistent with a role for Scrib as a major presynaptic scaffold, a number of proteins identified in our screen function in the cytoskeletal matrix at the active zone (CAZ) (Schoch and Gundelfinger, 2006; Richier et al., submitted manuscript). These include, in addition to NOS1AP, β-Pix and Git1, liprin-alpha and ERC1 (Rab6IP). Liprin-alpha functions in the development and maturation of the presynaptic zone (Zhen and Jin, 1999; Dai et al., 2006), while NOS1AP has been shown to bind to synapsin1 (Jaffrey et al., 2002b), an important phospho-protein involved in linking synaptic vesicles and the actin cytoskeleton to regulate vesicle dynamics (Bloom et al., 2003). Git1 localizes to presynaptic in active zones (Ko et al., 2003) and associates with CAZ-associated protein, piccolo (Kim et al., 2003). In addition, endogenous NOS1AP and Scrib co-localize with the pre-synaptic marker synaptophysin (Richier et al., submitted manuscript) further supporting our idea that NOS1AP localizes to the pre-synaptic compartment.

Many of the Scrib and NOS1AP associating proteins also function postsynaptically and are particularly important in spine morphology and maturation. For example, liprin-alpha localizes postsynaptically and functions with Git1 (Ko et al., 2003) in the trafficking of AMPA receptors (Wyszynski et al., 2002; Ko et al., 2003). Git1 can form a complex with Ephrin B and Grb4, an interaction that is important for spine maturation (Segura et al., 2007). β-Pix, Git1 and PAK function together to regulate dendritic spine morphology through MLC (Zhang et al., 2003; Zhang et al., 2005). Consistent with the idea that Scrib and NOS1AP associate with proteins important in dendritic spine maturation, we find that both NOS1AP and Scrib colocalize in the postsynaptic regions (Fig. 3.5).

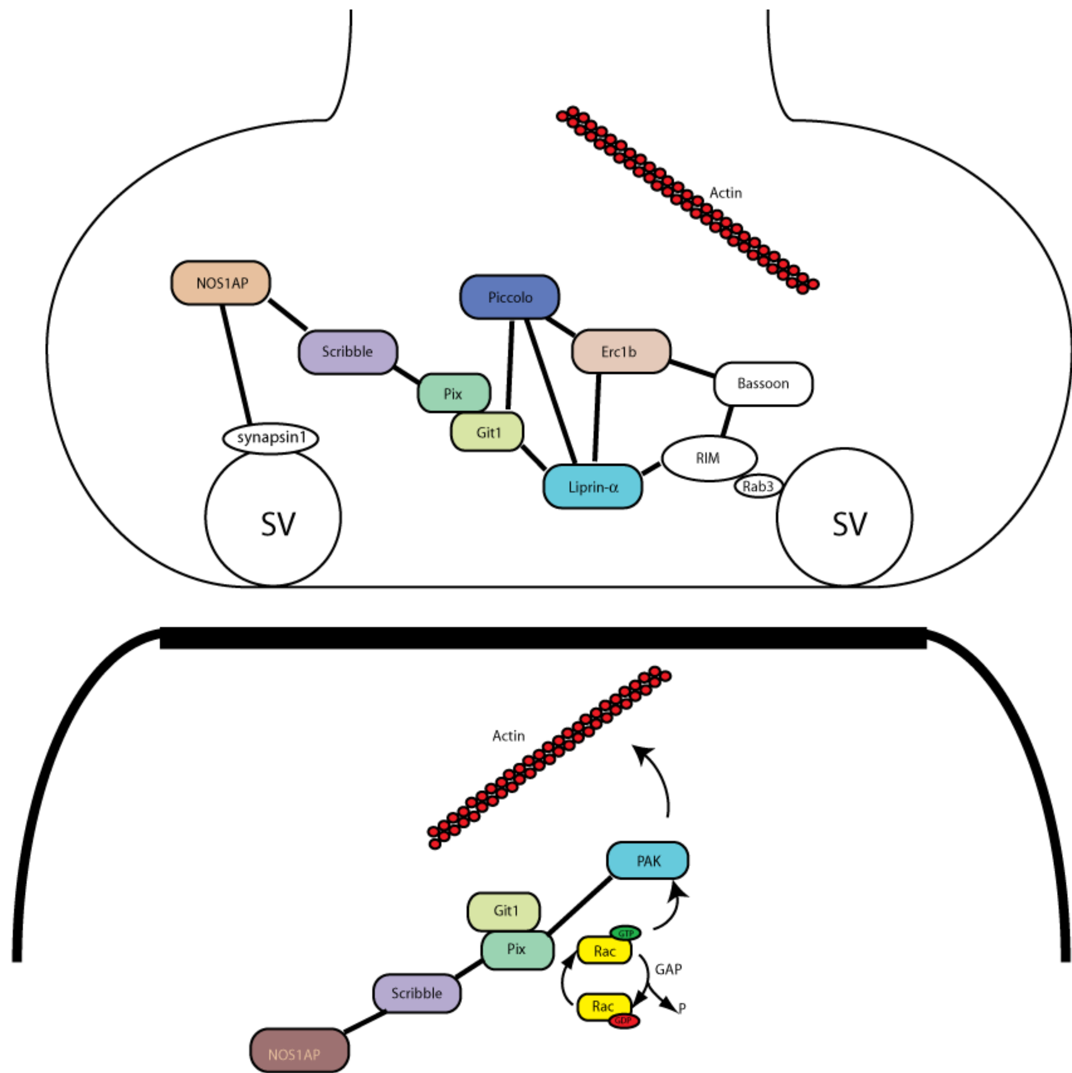


Figure 4.1 Model of the role NOS1AP and scribble play in the presynaptic region (upper) and postsynaptic region (lower). From Richier et al (submitted manuscript)

4.2.2 NOS1AP and Scrib, β-Pix, Git, PAK

Functionally, many of the proteins identified in our proteomic screen as Scrib and NOS1AP-associating proteins have been implicated in dendritic spine morphology. Here we show that overexpression of NOS1AP increases spine number, a role dependent on the PTB domain of NOS1AP. Recent evidence increasingly suggests that polarity proteins function in regulating spine morphology by regulating Rho GTPase signaling. For example, PAR3 functions through the Rac GEF, TIAM1, to regulate the maturation of dendritic spines (Zhang and Macara, 2006), while PAR6 and aPKC function through a Rho dependent mechanisms to regulate spine morphology independently of PAR3 (Zhang and Macara, 2008). In our study, we show that overexpression of NOS1AP or its PTB domain in non-neuronal cells influences Rac activity and that D.N.Rac can attenuate NOS1AP-induced dendritic protrusions (Fig. 3.8 and 3.9). This raises the question of how might NOS1AP influence Rac activity to affect dendritic spine development? One possibility is that the NOS1AP-Scrib association may affect nitric oxide signaling to regulate spine development, since NO signaling is known to modify spine morphology (Nikonenko et al., 2008; Steinert et al., 2008). However, this possibility seems unlikely since the NOS1AP PTB domain, which is not coupled to NOS, is the critical region in NOS1AP affecting spine development. Rather we speculate that NOS1AP induces or stabilizes a Scrib, β-Pix, Git1, PAK complex leading to an increase in Rac activity (Fig. 4.2). This is consistent with previous studies showing that a complex of Scrib, β-Pix, Git1 PAK can be regulated leading to an increase in Rac activity (Zhan et al., 2008) and that the Rac GEF activity of β-Pix is dependent on the oligomerization state of β-Pix (Schlenker and Rittinger, 2009). Finally, we cannot rule out that NOS1AP may recruit an associating protein that can regulate the GEF activity of β-Pix. Indeed signaling complexes upstream of β-Pix, Git1 and PAK can regulate β-Pix GEF activity leading to increased Rac activity and spine development (Saneyoshi et al., 2008).

Interestingly, we report that the PTB domain of NOS1AP is sufficient to localize NOS1AP to the membrane of epithelial cells, associate with Scrib, influence activation of Rac and induce process outgrowth of dendritic protrusions. This is consistent with the idea that NOS1AP-influenced Rac activation occurs through the Scrib, Pix, Git complex

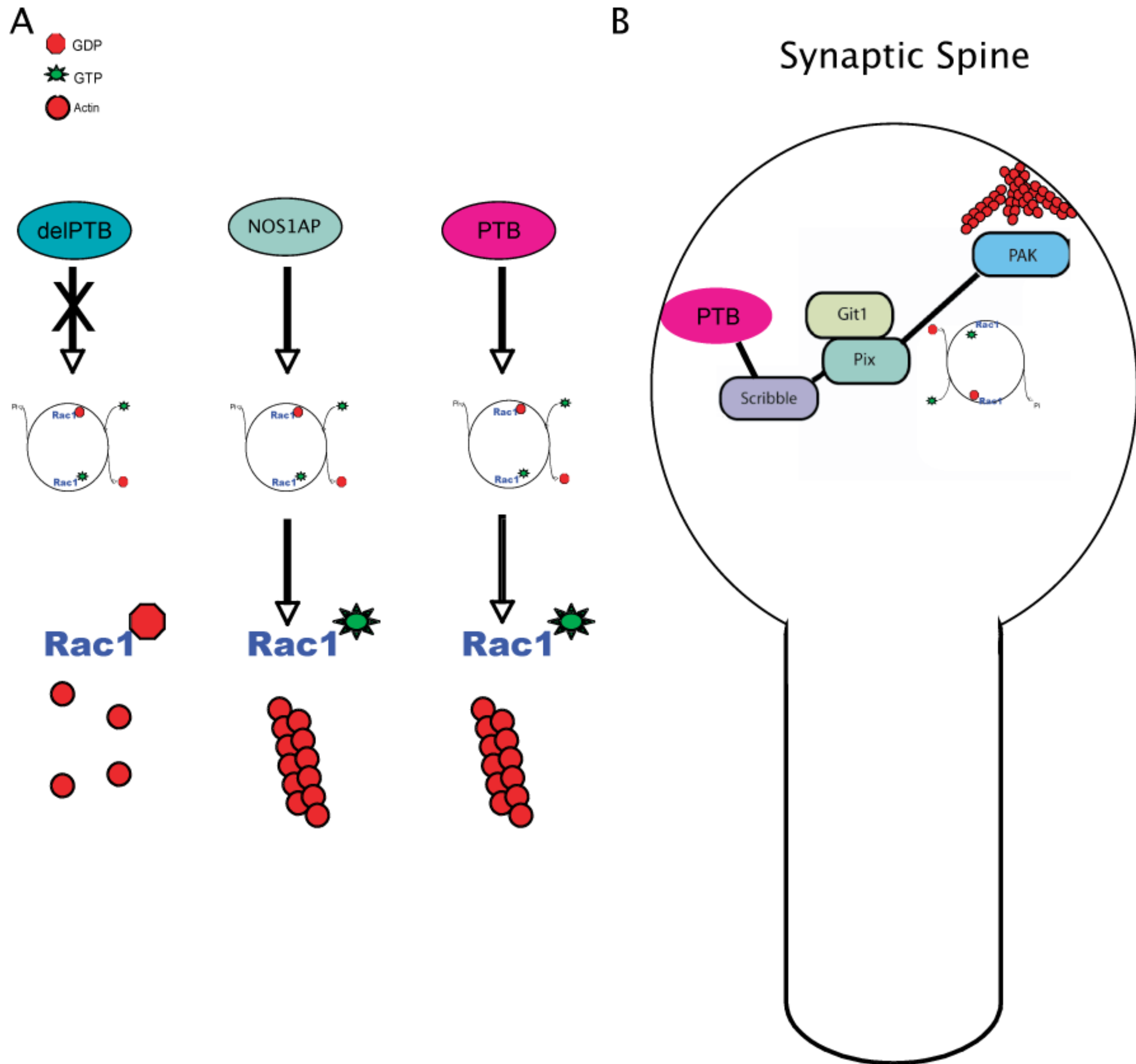


Figure 4.2 Model of the NOS1AP PTB domain. (A) Comparison of three different NOS1AP constructs and a potential role in Rac activation for each (B) A proposed mechanism of actionfor the PTB domain of NOS1AP in dendritic spines.

(Fig. 4.2). We suggest that the PTB domain of NOS1AP is important for the recruitment of NOS1AP to the leading edge of dendritic protrusions and allows for the stabilization of a Scrib complex that can influence Rac activity. Whether this domain is sufficient alone to induce the Scrib, β-Pix, Git1, and PAK complex to regulate the GEF activity of β-Pix directly or whether other PTB-associating proteins are necessary for this event remain to be determined.

4.2.3 NOS1AP and actin dynamics

Our work suggests that NOS1AP may represent the upstream component of a novel polarity complex in the cell involving Scrib, β-Pix, Git, and PAK1. What molecules function downstream to affect process outgrowth? We have identified a mechanism of action for NOS1AP in influencing Rac, a major member of the Rho GTPases. Interestingly, β-Pix has previously been shown to act as a GEF for Rac (Manser et al., 1998) and Rac has been shown to be responsible for membrane ruffling through changing actin dynamics (Ridley et al., 1992; Luo, 2000; Etienne-Manneville and Hall, 2002). Rac-induced changes in actin dynamics are thought to play a major role in formation of polarized cellular structures, such as the synapse and in neuronal development, (Luo, 2000; Nakayama et al., 2000; Etienne-Manneville and Hall, 2002). Furthermore, Git1 has been shown to co-localize with filamentous actin, a target of Rac (Botrugno et al., 2006). PAK is partially responsible for creating lamellipodia, membrane ruffles, and focal adhesions (Bokoch, 2003) and leads to polarized filopodial production by regulating actin cytoskeleton dynamics (Sells et al., 1997). Studies have shown that PAK induced changes in actin dynamics is through Lim kinase. This results in a decrease in actin catastrophe as Lim kinase phosphorylates and inactivates cofilin (Edwards et al., 1999; Bokoch, 2003). Activation of Lim kinase by PAK is dependent on Rac activity (Edwards et al., 1999; Bokoch, 2003). This provides a possible further downstream component of NOS1AP, Scrib, Git and Pix that is comprised of PAK, Lim kinase and cofilin. Further, another pathway has been elucidated consisting of Git1 and β-Pix, which act upstream of a complex involving Rac, PAK and myosin II regulatory light chain (MLC). This pathway plays a direct role in formation of dendritic spines and synapses (Zhang et al.,

2005). Signaling of PAK through MLC is thought to be in response to Rac activation, which results in changes in actin dynamics (van Leeuwen et al., 1999; Zhang et al., 2005). This provides another potential downstream actin-regulating target for NOS1AP; an area of future interest. Thus, there are two major known pathways that PAK is involved in to regulate actin dynamics; the first involves the kinase domain of PAK, which phosphorylates downstream targets (MLC); the second involves activation by Rac to target Lim kinase and cofilin (Pawson and Nash, 2000).

4.2.4 NOS1AP and Scrib at NMDA Receptors

Interesting, we have also shown that Scrib can associate with nNOS. We suggest that NOS1AP and Scrib may form a scaffolding complex and affect the interaction between nNOS and PSD-95, which in turn will affect NMDAR signaling. NMDARs are associated with NO production after calcium influx, a process requiring nNOS, which forms a ternary complex with PSD-95 (Christopherson et al., 1999). Recently, it has been proposed that NOS1AP acts to recruit nNOS away from PSD-95 at NMDAR (Jaffrey et al., 1998). Since Scrib also binds nNOS, we suspect that Scrib may also affect the involvement of nNOS at NMDAR. Recent studies have shown that both nNOS and NO production at NMDA receptors are important for synaptic dynamics (Nikonenko et al., 2008). One potential post-synaptic pathway involves NMDA receptors, β-Pix and Git1. In this model, NMDA receptors target CaMKK and CaMK1 to phosphorylate β-Pix in a complex with Git1, increasing the GEF activity of β-Pix to induce Rac activation and increase dendritic spines (Saneyoshi et al., 2008). The underlying mechanism defining how NMDAR target this downstream pathway remains to be determined. This work suggests, however, that a signaling pathway may exist involving NMDA receptor signaling through a Git1/β-Pix/PAK complex to regulate spine development and since NOS1AP is indirectly involved with NMDA receptors (Jaffrey et al., 1998) and interacts with Git1/β-Pix/PAK, it might suggest that NOS1AP is important in this pathway (Fig. 4.1).

4.3 NOS1AP IN DISEASE

4.3.1 Schizophrenia

The *NOS1AP* gene has been linked as a susceptibility locus for Schizophrenia and NOS1AP has been shown to be upregulated in patients with Schizophrenia (Brzustowicz et al., 2004; Eastwood, 2005; Zheng et al., 2005; Brzustowicz, 2008; Kremeyer et al., 2009). Furthermore, the expression of a novel isoform of NOS1AP is increased in patients with schizophrenia. Recent studies suggest that schizophrenia is a disease involving the neuron, synapse, and synaptic components (Hoffman and McGlashan, 1997; McGlashan and Hoffman, 2000; Benitez-King et al., 2004; Harrison and Weinberger, 2005). For example, in one case a susceptability gene disrupted in schizophrenia (DISC) binds to cytoskeletal proteins and is though to play a role in neuronal structure (Morris et al., 2003). Yet there is little known about the underlying role NOS1AP may play in a disorder like schizophrenia. Here we show a potential mechanism of action of NOS1AP in the post-synaptic comparment, which again raises the possibility that the post-synaptic region may be affected in patients with Schizophrenia. We also suspect that NOS1AP may be involved in pre-synaptic vessicle dynamics (Jaffrey et al., 2002b). This raises another possibility that through NOS1AP, vessicle dynamics and neurotransmitter release may be altered in patients with Schizophrenia. Still we must gain a better understanding of the importance of NOS1AP in normal development to better understand what may be occuring in the diseased state.

4.3.2 Mental Retardation

Several recent studies have put Rho GTPases at the forefront of various forms of inheritable mental retardation (MR) (Ramakers, 2000, 2002; Chechlacz and Gleeson, 2003; Dillon and Goda, 2005; van Galen and Ramakers, 2005). Human genetic diseases involving mutations in PAK3, ARHGEF (αPix), Lim kinase, and FMR-1 (Dillon and Goda, 2005), the latter involve binding partners for PAK protein (Edwards et al., 1999; Bokoch, 2003) all involve proteins that regulate or are regulated by Rho GTPases (Dillon and Goda, 2005). Patients with these forms of mental retardation show alterations in spine morphology including increases in immature spines (Dillon and Goda, 2005).

Interesting, we have shown an interaction between NOS1AP and several of these proteins. One predominant protein is a member of the PAK family, PAK3 (Allen et al., 1998; Bienvenu et al., 2000). Interestingly, a mutation in PAK3 known as R673 has been linked to a form of X-linked MR (Allen et al., 1998; Bienvenu et al., 2000). This mutation is in the p21-GTPase-binding domain (PBD) and results in decreased cdc42 binding and kinase activation, which leads to a decrease in the density of synaptic spines (Kreis et al., 2007). Indeed, mouse models using dominant negative PAK3 expression in the forebrain show decreased number of spines, increased size of spines and presynaptic terminals in cortical neurons, increased LTP, and decreased ability to perform memory tasks (Hayashi et al., 2004; Dillon and Goda, 2005). This suggests that PAK3 is a key regulator in actin dynamics, which results in control of dendritic spine morphology and loss of which results in declined memory and symptoms of MR. It will be interesting to define the influence of NOS1AP on PAK3 activity.

Williams syndrome is an autosomal dominant disease that arises from a loss of Lim kinase (Tassabehji et al., 1996). Interestingly, Lim kinase is a downstream target of PAK (Edwards et al., 1999). The prevalence of William's syndrome has been reported to be one in every 7500 (Stromme et al., 2002). William's syndrome commonly results in cardiovascular disease, gastrointestinal problems, and MR; with William's syndrome slowly progressing and worsening throughout life (Morris et al., 1988). Using a Lim kinase knockout mouse model, researchers have confirmed that Lim kinase alters actin dynamics *in vivo*, and loss of Lim kinase results in abnormalities in the development of spines and synapses, increases in LTP, similar to PAK knockout models, and changes learning behaviours (Meng et al., 2002). Thus, it will be interesting to determine if Lim kinase is a downstream target of NOS1AP.

In a similar manner, mutation in AHRGEF6 (α-Pix) leads to another form of inheritable X-linked MR (Kutsche et al., 2000). Thus, there are two primary targets of NOS1AP, PAK3, and α-Pix as well as one target of a NOS1AP-associated protein, Lim kinase, that, when mutated or lost, lead to debilitating disease. These proteins are actin regulators through association with Rho GTPases (Bagrodia et al., 1998; Manser et al., 1998;

Edwards et al., 1999), and some have been shown to cause morphological changes in dendritic spine development (Meng et al., 2002; Kreis et al., 2007). Thus, we can speculate that NOS1AP may play a similar role acting as an upstream component in these pathways and since no genetic link has been made that we are aware of to date, may play a role in non-inherited forms of MR.

4.4 A RECENT NOS1AP STUDY

Another recent study has implicated NOS1AP in dendrite development in hippocampal neurons through the binding partner carboxypeptidase E (CPE) (Carrel et al., 2009). This study showed that NOS1AP reduces the number of primary and secondary dendrites when overexpressed in dissociated hippocampal neurons between two and ten DIV (Carrel et al., 2009). Further, a short isoform of NOS1AP containing the C-terminal 113 amino acids of NOS1AP along with a novel 12 N-terminal amino acids, showed similar effects on dendrite branching, but only during five to seven DIV (Carrel et al., 2009). This phenotype was independent of both the PTB domain or PDZ motif of NOS1AP (Carrel et al., 2009). The effects of NOS1AP on dendrite branching were mediated by amino acids 181 to 307 and through CPE (Carrel et al., 2009). We show that NOS1AP acts to increase the number of dendritic protrusions along the dendrite and this phenotype is dependent on the PTB domain. Although we are analyzing a different phenotype than Carrel et al (2009), it seems slightly surprising that we would find this phenotype was dependent on the PTB domain while Carrel *et al.* (2009) report no such findings on the PTB domain. One possible explanation for this discrepancy is that we use an extended PTB domain construct that has additional sequence C-terminal to the PTB domain that could be mediating our effects. We will examine the effects of a more stringent PTB domain to confirm our results. Another explanation is that the PTB domain used in the study by Carrel *et al.* (2009) was unable to fold correctly, thus losing binding affinity for associating proteins, rendering the PTB domain unable to mediate any effects. Finally, there is also a difference on timing between the two studies. We transfected neurons 13 DIV, however, Carrel *et al., (*2009) transfected neurons between zero and ten DIV. It has been reported that dendrites develop between four and seven DIV (Dotti et al., 1988) and

dendritic spines develop after seven days and the proportion of dendritic spines increases for an additional week (Papa et al., 1995; Arimura and Kaibuchi, 2007). This raises the possibility that the phenotypic effects of NOS1AP on dendrites are timing specific. It would be interesting to express YFPNOS1AP in our system at earlier time points and note the phenotypic effects.

4.5 FUTURE DIRECTIONS

4.5.1 Rac Activation

We have shown that NOS1AP can influence the activation of Rac and speculate that it may be through the association with Scrib, Git1, the Rac GEF, β-Pix (Manser et al., 1998), and PAK. In future work, it will be important to determine if NOS1AP is regulating Rac activity through β-Pix or through an independent mechanism. In order to test this we can use a dominant negative β-Pix construct to co-express along with NOS1AP to see if (i) we no longer get Rac activation in 293T cells and (ii) if we can block the phenotypic effects of NOS1AP overexpression in hippocampal neurons similar to when we overexpressed D.N.Rac.

4.5.2 Actin Dynamics

We have shown that NOS1AP targets several proteins that target upstream components of actin and that a number of these proteins are mutated or lost in MR (Tassabehji et al., 1996; Allen et al., 1998; Kutsche et al., 2000). For example, Rac and PAK, together target Lim kinase leading to changes in cofilin-mediated actin dynamics (Edwards et al., 1999; Bokoch, 2003). β-Pix, Git1 and PAK are all upstream targets of MLC and lead to changes in actin dynamics (van Leeuwen et al., 1999; Zhang et al., 2005). It will be important to determine if any of these downstream targets of β-Pix, Git1 or PAK are also downstream targets of NOS1AP. This will be interesting and useful to determine if/how NOS1AP may be regulating the actin cytoskeleton.

4.5.3 Axogenesis

Since a number of polarity proteins, in particular the PAR complex, have been implicated in axon specification in early neuron development (Macara, 2004a; Mertens et al., 2006), it would be interesting to assess the localization and affects of overexpression of NOS1AP in early development. In addition, PAK1 acts as a polarity protein in axon projection (Jacobs et al., 2007). Unphosphorylated PAK is equally distributed throughout a developing neuron, however, phospho-PAK is enriched in one neurite, which later specifies the axon (Jacobs et al., 2007). β-Pix plays a major role in growth cone formation in the developing neuron. Specifically, Fibroblast growth factor (FGF) and nerve growth factor (NGF) are upstream of β-Pix induced rac1 activation (Shin et al., 2004). This pathway is thought to play a major role in recruiting β-Pix to lamellipodia at the growth cone, in growth cone stabilization and cytoskeletal maintenance (Shin et al., 2002; Shin et al., 2004). This raises the possibility that NOS1AP may be involved in axonal specification and growth cone formation during neuronal development and will provide an interesting future area of study.

4.5.4 NOS1APc

We have identified a novel NOS1AP isoform, NOS1APc, which lacks the C-terminal region of NOS1AP, fails to associate with nNOS, yet still associates with Scrib. Little is known about other NOS1APc associating proteins. To determine NOS1APc-associating proteins, we will conduct a proteomic screen using NOS1APc as bait. This will shed light on the role of a novel NOS1AP isoform and may provide insight into a new mechanism of action for NOS1AP proteins that is independent of nNOS.

CHAPTER 5 CONCLUSION

In summary, we have conducted a proteomic screen with a number of known polarity proteins and identified several unique interactions including a novel link between Scrib and a PTB containing protein NOS1AP. Their association is dependent on the N-terminal region containing the PTB domain of NOS1AP and the fourth PDZ domain of Scrib. Further our results suggest that NOS1AP and Scrib associate and play an important role in the synapse, both pre- and postsynaptically. We also show that Scrib functions to bridge NOS1AP to a β-Pix, Git1, PAK complex and that NOS1AP affects dendritic spine development by influencing Rac activity. Since NOS1AP affects dendrite patterning (Carrel et al., 2009) and has been linked with Schizophrenia, a disease affecting the synapse (Brzustowicz, 2008), and long QT syndrome in the heart (Aarnoudse et al., 2007; Post et al., 2007; Osawa et al., 2009), our data may shed new light on the role NOS1AP plays in disease states.

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