IDENTIFICATION AND CHARACTERIZATION OF NOS1APc INTERACTING PROTEINS

by

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Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
June 2012

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DALHOUSIE UNIVERSITY

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DALHOUSIE UNIVERSITY

DATE: June 6th, 2012

AUTHOR: Leanne Marie Clattenburg

TITLE: IDENTIFICATION AND CHARACTERIZATION OF NOS1APc INTERACTING PROTEINS

DEPARTMENT OR SCHOOL: Department of Pharmacology

DEGREE: MSc CONVOCATION: October YEAR: 2012

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ABSTRACT

The current study characterizes novel interacting proteins for the neuronal nitric oxide synthase 1 adaptor protein (NOS1AP) isoform NOS1APc. NOS1APc is a 100kDa isoform of NOS1AP (herein NOS1APa) that contains a unique 30kDa C-terminal extension that eliminates the interaction with nNOS. Immunoprecipitations using NOS1APc isoform specific antibodies revealed the potential for alternate isoforms of NOS1AP. Cloning techniques were used to identify three new NOS1AP isoforms, NOS1APd, NOS1APe and NOS1APf. All isoforms except for NOS1APf retain the ability to interact with the polarity protein Scribble. A targeted proteomic screen for NOS1APc established pyruvate carboxylase (PCB) as a potential interacting protein. Through over-expression and immunoprecipitation experiments, it was identified that NOS1APc plausibly interacts with PCB. Finally, an interaction between NOS1APc and ephrinB3 was characterized. Taken together, these data suggest differences exist between NOS1APa and NOS1APc in their ability to bind to certain proteins and therefore may act in different protein-protein complexes.

LIST OF ABBREVIATIONS AND SYMBOLS USED

5’ RACE	5’ Rapid amplification of cDNA ends
α-PIX	Alpha-p21 activated kinase-interacting exchange factor
β-PIX	Beta-p21 activated kinase-interacting exchange factor
μm	Micrometer
aa	Amino acid
Acetyl-CoA	Acetyl-Coenzyme-A
Arp 2/3	Actin related protein 2/3
ATP	Adenosine triphosphate
BC	Biotin carboxylase domain
BCCP	Biotin carboxyl carrier protein
bp	Base pair
cAMP	Cyclic adeonsine monophosphate
CAPON	Carboxy terminal PDZ ligand of nNOS
Cdc42	Cell division control protein 42
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CO_2	Carbon Dioxide
COMT	Catechol-O-methyl transferase
CPE	Carboxypeptidase E
CST	Corticospinal tract
CT	Carboxyltransferase domain
Dab	Disable
Dexras1	Dexamethasone-induced Ras protein-1
DGC	Distorphin-Distroglycan Complex
DISC1	Disrupted in schizophrenia 1
Dlg	Discs-large
DMEM	Dulbecco’s modified eagle’s medium
DMT1	Divalent metal (ion) transporter 1
DNA	Deoxyribonucleic Acid
DTNBP1	Dysbindin
DTT	Dithiothreitol
ELK1	E-twenty six (ETS)-like transcription factor-1
EMT	Epithelial-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
Eph	Erythropoietin producing human hepatocellular carcinoma
Ephrin	Eph family receptor interacting proteins
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GABA	γ-amino butyric acid
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor

GFP	Green fluorescent protein
GIT1	G-protein coupled receptor kinase-interacting protein 1
GM130	Golgi matrix protein 130
GPI	Glycosylphosphatidylinositol
GSNO	S-nitrosoglutathione
GST	Glutathione-S-Transferase
GTP	Guanosine triphosphate
HEK	Human embryonic kidney cell
HPV	Human papillomavirus
HTLV-1	Human T-cell leukemia virus type-1
ICD	Implantable cardioverter-defibrillator
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
IRS1/Dok	Insulin receptor substrate-1/Dok
IRS	Insulin receptor substrate
LAM	Lymphangioleiomyomatosis
LAP	Leucine rich repeats and PDZ domains
LDH	Lactate dehydrogenase
Lgl	Lethal-giant larvae
L-NAME	L-N^G-Nitroarginine methyl ester
LPS	Lipopolysaccharide
LQTS	Long-QT Syndrome
LRR	Leucine rich repeats
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen activated protein kinases
MI	Myocardial infarction
mRNA	messenger RNA
MTJ	Muscle-tendon junction
mTORC-1	Mammalian target of rapamycin complex-1
Nck	Non-catalytic region of tyrosine kinase adaptor protein
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS1AP	Nitric oxide synthase 1 adaptor protein
NRG1	Neuregulin-1
NTB1	Non-transferrin bound iron
N-WASP	Neuronal Wiskott-Aldrich syndrome protein
OAA	Oxaloacetate
P115	Transcytosis associating protein p115
PAK	p21-activated kinase
PAP7	Peripheral benzodiazepine receptor associated protein 7
Par3	Protease activated receptor 3
PBS	Phosphate buffered saline

PCB	Pyruvate carboxylase
PCR	Polymerase chain reaction
PDZ	Post-synaptic density 95, PSD-95; discs large, Dlg; zonlua occludens-1, ZO-1 binding motif
PEI	Polyethylenimine
pERM	Phospho-Ezrin, radixin, moesin protein family
PI3	Phosphatidylinositol 3-kinase
PMCA	Plasma membrane Ca2+ pump
PMSF	Phenylmethylsulphonyl fluoride
ppt	Precipitate or precipitated
PRODH	Proline dehydrogenase (oxidase) 1
PSD	Post-synaptic density
PSD-95	Post-synaptic density-95
PTB	Phosphotyrosine binding domain
PVDF	Polyvinyl difluoride
Rac	Ras-related C3 botulinum toxin substrate
RGS4	Regulator of G-protein signaling 4
Rheb	Ras-homologue enriched in brain
RNA	Ribonucleic Acid
RNAi	RNA interference
RT-PCR	Reverse transcription PCR
SAM	Sterile alpha motif domain
Scrib	Scribble
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SFM	Serum free media
shRNA	Short hairpin RNA
SHC	Src-homology
siRNA	Small-interfering RNA
SLAP	Src-like adaptor protein
SNP	Single nucleotide polymorphism
SR	Sarcolplasmic reticulum
Src	Sarcoma
SVZ	Subventricular zone
TAP	Transcytosis associating protein
TBST	Tris-buffered saline tween
TCA	Tricarboxylic Acid Cycle
Tf	Transferrin
TNF-α	Tumour necrosis factor-alpha
TSC-1/2	Tuberous Sclerosis-1/2
VANGL1	Van gogh like-1
XOR	Xanthine-oxidoreductase
YFP	Yellow fluorescent protein

ACKNOWLEDGEMENTS

First and most importantly, I would like to thank my supervisor **Dr. James Fawcett** for all of his support and guidance throughout my time in the lab. I would like to thank members of my thesis examination committee, **Dr. Ryan Pelis** and **Dr. Robert Rose** and members of my graduate student advisory committee, **Dr. Kishore Pasumarhi** and **Dr. Chris Sinal**. For her help with the glial cell cultures, I would like to thank **Annette Kolar**; and for her work with the cloning of NOS1AP, I owe many thanks to **Maggie Qi**. I also owe gratitude to the current and past members of the lab including; **Michael O'Brien, Lindsay Richier, Natasha Zinck, Crystal Milligan, Peter Dixon, Maggie Qi, Ciaran Lane** and **Michael Wigerius**. Their friendly presence in the lab and continued support has made for a great lab environment and made my time there very entertaining and enjoyable. A big thanks goes out to the ladies in the office – **Luisa, Sandi, Janet and Cheryl** – for all their guidance and friendly natures. To my **family** and **friends**, thank you so much for your all your support and encouragement throughout this entire process. Finally, I would like to thank the **Cancer Research Training Partnership (CRTP)** and the **Beatrice Hunter Cancer Research Institute (BHCRI)** for financial support.

CHAPTER 1 INTRODUCTION

1.1 NITRIC OXIDE SYNTASE1 ADAPTOR PROTEIN: NOS1AP

In 1998, Jaffrey et al. identified nitric oxide synthase adaptor protein (NOS1AP) also known as carboxy terminal PDZ ligand of nNOS, (CAPON). NOS1AP was identified through a yeast-2-hybrid screen as a binding partner for neuronal nitric oxide synthase (nNOS) (Jaffrey et al., 1998). *Rattus* NOS1AP contains a 2820 base pair (bp) complementary DNA (cDNA) that translates to a 503 amino acid (aa) protein. The NOS1AP protein contains a carboxyl-terminal PDZ (post-synaptic density 95, PSD-95; discs-large, Dlg; zonula occludens-1, ZO-1) binding motif and an amino-terminal phosphotyrosine-binding (PTB) domain (Jaffrey et al., 1998). NOS1AP lacks any other known consensus sequences except an 18-nucleotide stretch of CAG repeats that converts to 6 glutamines (Jaffrey et al., 1998). The NOS1AP protein and messenger RNA (mRNA) are localized throughout the central nervous system (CNS), with the highest mRNA levels in the olfactory bulbs, hippocampus, cortex and cerebellum (Jaffrey et al., 1998).

The PTB domain and PDZ interacting motifs of NOS1AP are essential for interactions with other proteins. PTB domains are found in many scaffolding proteins and are important in forming multiple protein complexes (Smith et al., 2006). Typically, PTB domains recognize specific sequences on target proteins, Asparagine-Proline-X-phosho-Tyrosine or N-P-X-pY motif where X can be any amino acid (Margolis et al., 1999). PTB domains can also recognize a non-phosphorylated N-P-X-Y motif or a N-X-X-Y motif (Forman-Kay and Pawson, 1999, Smith et al., 2006). PTB domains are separated

into three groups based on structure comparisons, the Shc-like, the insulin receptor substrate (IRS1)/Dok-like or the Disable (Dab)-like PTB domains (Uhlik et al., 2005, Smith et al., 2006). The three different classes of PTB domains lack sequence homology and IRS1/Dok-like are significantly smaller than Shc-like PTB domains; however, the 3D structure is very similar for all three (Forman-Kay and Pawson, 1999, Smith et al., 2006). The three classes share similarities in their folding patterns where a central β-sandwich is comprised of 7 anti-parallel β-strands that are capped at the C-terminal by an α-helix and a variable length α-helix at the other end (Smith et al., 2006). The three classes differ in their affinity towards a specific target sequence. The Shc- and IRS1/Dok-like PTB domains bind with higher affinity to N-P-X-pY motifs while Dab-like PTB domains are more drawn to a non-phosphorylated ligand motif, N-P-X-Y (Smith et al., 2006). Examples of proteins binding in a non-phosphorylation dependent manner include the PTB domains of X11, Fe65 and Numb proteins (Forman-Kay and Pawson, 1999).

In addition to the PTB domain, the other major protein interaction motif is the carboxyl-terminal PDZ binding motif. PDZ domains consist of five or six β-strands and two α-helices (Jemth and Gianni, 2007). PDZ domains interact with specific types of sequences found at the C-terminus of the interacting protein (Songyang et al., 1997). There are three classes of PDZ domains that are arranged according to the sequence they bind. Class I PDZ domains associate with a Serine/Threonine-X-Valine-COO- (or S/T-X-V-COO) binding motif on their interacting protein where X is any amino acid. Note, the Valine residue can be substituted for any hydrophobic amino acid (e.g. valine, isoleucine or leucine) (Ranganathan and Ross, 1997)(Fanning and Anderson, 1996). An example of

a Class I PDZ domain would be the second PDZ domain of PSD-95 that binds the E-S-D-V (Glutamic acid-Serine-Aspartic acid-Valine) sequence in the N-Methyl-D-Aspartic acid (NMDA) receptor (Hung and Sheng, 2002). Class II PDZ domains recognize the X-ϕ-X-ϕ sequence on the interacting ligand (ϕ = hydrophobic residue) (Hung and Sheng, 2002). An example of a Class II interaction occurs between PICK1 and the ephrin B1 ligand with the YYKV (Tyr-Tyr-Lys-Val) sequence (Hung and Sheng, 2002). Class III PDZ domains recognize a X-D/E-X-ϕ-COO sequence with an example being nNOS and the melatonin receptor with a V-D-S-V-COO (Valine-Aspartic acid-Serine-Valine) sequence (Hung and Sheng, 2002).

As well as binding specific C-terminal sequences, PDZ domains have the ability to dimerize and form homo- and hetero-oligomers with one another (Songyang et al., 1997, Hung and Sheng, 2002). The PDZ domain is also capable of binding internal sequences. An example of this type of interaction is between nNOS and syntrophin or PSD-95 (Hung and Sheng, 2002). Here, the nNOS protein forms a β-hairpin "finger" that docks in the peptide binding groove of the PDZ domain in syntrophin (Hung and Sheng, 2002). There is also evidence showing PDZ domains can interact with other protein binding motifs including ankyrin repeats, spectrin repeats and LIM domains (Hung and Sheng, 2002).

Because PDZ domains bind to specific recognition sequences often at the C-terminus of many trans-membrane proteins and have the ability to dimerize with other PDZ containing proteins (Fanning and Anderson, 1996), it is suggested that PDZ proteins are involved with organization at the plasma membrane in locations such as tight junctions

and are often associated with scaffold proteins at those locations (Fanning and Anderson, 1996).

1.2 NOS1AP INTERACTING PARTNERS

1.2.1 NOS1AP and nNOS interact

To date a number of proteins have been shown to associate with NOS1AP. NOS1AP was first identified as an associating protein with nitric oxide synthase (NOS) giving rise to its name. Jaffrey et al. (1998), showed a direct interaction between the C-terminal region of NOS1AP and the PDZ domain of nNOS. The interaction between NOS1AP and nNOS has been shown to function as a competitive inhibitor between nNOS and two other scaffolding proteins found in the synapse, post-synaptic density 95 (PSD-95) and PSD-93. Both PSD-93/95 proteins are found in the synapse and are involved in clustering neurotransmitter receptors and ion channels in the postsynaptic region (Jaffrey et al., 1998). Since both NOS1AP and PSD-95 bind to the PDZ domain of nNOS, Jaffrey et al. (1998) showed that increasing the amount of NOS1AP decreased the amount of nNOS associating with PSD-95. They hypothesized the net effect of this would be to change the levels of nitric oxide and thus affect synaptic efficacy (Jaffrey et al., 1998), although this was not shown. Nor was it shown whether this competition event occurred *in vivo*.

1.2.2 NOS1AP interacts with Dexras1 and nNOS to form a ternary complex

In addition to nNOS, NOS1AP has also been shown to associate with dexamethasone-induced Ras protein 1 (Dexras1). Dexras1 was identified by its ability to be induced by dexamethason, a member of the glucocorticoid steroid family (Kemppainen and Behrend,

1998). Dexras1 is a member of the Ras family of small monomeric G proteins that is enriched in the brain and is the only member of the Ras family that is induced by a steroid (Kemppainen and Behrend, 1998, Fang et al., 2000). The Dexras1 protein contains the conserved domains of typical GTPases including the guanosine triphosphate (GTP) binding domain, Mg2+ binding domain and C-terminal prenylation site, however, Dexras1 contains an extra 7kDa C-terminal tail (Cheah et al., 2006). Interestingly, Fang, et al., (2000) determined that the C-terminal region of Dexras1 was able to associate directly with the PTB domain of NOS1AP. Further, they determined that Dexras1 coupled to NOS1AP was also able to associate with nNOS forming a ternary complex (Fang et al., 2000). Because Dexras1 is a member of the Ras family of G-proteins, it cycles between an inactive, guanosine-diphosphate (GDP)-bound state and an active gunaosine-triphosphate (GTP)-bound state. The conversion between GDP- and GTP-bound states is completed by guanine-nucleotide exchange factors (GEF's) that dissociate GDP and allow GTP to bind. The conversion of Dexras1 to its 'active' GTP bound state is coupled to NMDA receptor-dependent nitric oxide (NO) synthesis. Consistent with this, NOS1AP, when associated with nNOS, was able to enhance the level of GTP Dexras1 or 'active' Dexras1 (Fang et al., 2000). It is also shown that nNOS knockout mice have impaired dendrite arborization. Since the Ras family members have been implicated in growth and differentiation of neurons, this suggests Dexras1 may be involved in neuron growth through the proper regulation of NO (Fang et al., 2000).

In 2006, Cheah et al. identified peripheral benzodiazepine receptor associated protein 7 (PAP7) as a binding partner for Dexras1 through a yeast-2-hybrid screen. PAP7 is a

protein that binds peripheral benzodiazepine receptors and cyclic adenosine monophosphate (cAMP) dependent protein kinase (Cheah et al., 2006). The interaction between Dexras1 and PAP7 was confirmed through co-immunoprecipitations and glutathione-S-transferase (GST)-pull down experiments. PAP7 is known to associate with divalent metal (ion) transporter (DMT1), an import channel localized to the plasma membrane that is the only known mammalian iron transporter associated with iron import into cells (Cheah et al., 2006). Cheah et al., (2006) wanted to determine if Dexras1 and PAP7 through DMT1 play a role in neuronal iron homeostasis. Over-expression of Dexras1 in HEK 293T cells showed a moderate increase in iron uptake through transferrin (Tf)-mediated and non-transferrin bound iron (NTBI) uptake pathways that increased substantially with co-transfection of PAP7 (Cheah et al., 2006). The S-nitrosylation of Dexras1 occurs through glutamate interacting with NMDA receptors that in turn activates nNOS to produce NO that then allows for other proteins to undergo S-nitrosylation (Cheah et al., 2006). Cheah et al., (2006) suggest that the S-nitrosylation of Dexras1 through the previously mentioned pathway allows the interaction with PAP7 and thereby DMT1 and influences iron homeostasis. Consistent with this idea, enhancing S-nitrosylation of Dexras1 in PC12 cells by S-nitrosoglutathione (GSNO) increased NTBI uptake and when Dexras1 is silenced through RNA interference in those same cells, GSNO was not able to enhance iron uptake (Cheah et al., 2006). Further, in primary cortical neuron cultures from nNOS knockout mice, NMDA treatment fails to increase NTBI uptake adding evidence that nNOS and NO are a part of NTBI iron uptake (Cheah et al., 2006). Taken together, these data suggest NOS1AP may play a role in iron uptake due to its involvement in the ternary complex that bridges nNOS and Dexras1.

1.2.3 NOS1AP, Synapsin1 and nNOS form a ternary complex important in mediating nNOS localization

Another NOS1AP associating protein identified is synapsin1. Similar to Dexras1, synapsin1, NOS1AP and nNOS have been shown to form a ternary complex. The ternary complex formed by NOS1AP, synapsin1 and nNOS is thought to be important in retaining nNOS at pre-synaptic terminals. Synapsin's I, II and III are a family of neuronal phosphoproteins associated with the pre-synaptic membrane. They act as scaffolding proteins important for neurotransmitter release, morphogenesis and synaptogenesis (Jaffrey et al., 2002, Sanchez-Islas and Leon-Olea, 2004). Although NOS1AP has the ability to bind all three synapsin proteins (Jaffrey et al., 2002), NOS1AP only forms a ternary complex with synapsin1 and nNOS (Jaffrey et al., 2002). Similar to Dexras1, the PTB domain of NOS1AP is important for the association with a conserved C-terminal region common to all synapsins (Jaffrey et al., 2002). Further, Sanchez-Islas, et al., (2004) showed that the interaction between synapsin1 and NOS1AP was dependent on the phosphorylation of synapsin1. Functionally, the interaction between NOS1AP, synapsin1 and nNOS is thought to regulate NO localization in the presynaptic space. Consistent with this, a lack of synapsin1 led to changes in the subcellular localization of nNOS and NOS1AP (Sanchez-Islas and Leon-Olea, 2004). Further, in synapsin mutant mice there is a decrease in the size of synaptic terminals and the number of vesicles per terminal and as well, the absence of NO affected the localization of synapsins at the synaptic vesicles (Sanchez-Islas and Leon-Olea, 2004).

1.2.4 NOS1AP associates with Scribble

In addition to Dexras1, synapsin1, and nNOS, recent work from the Fawcett lab has shown that NOS1AP associates with the tumor suppressor protein scribble. Here, the interaction seems to be direct between the amino-terminal region containing the PTB domain and the fourth PDZ domain of scribble.

Scribble (scrib) is a member of the LAP (leucine rich repeats and PDZ domains) family of proteins and contains 16 leucine rich repeats (LRR) at the N-terminus and four C-terminal PDZ domains (Bryant and Huwe, 2000). The scrib protein is important not only in actin dynamics but in the establishment of cellular polarity. Scrib functions to control/regulate the establishment of the apical-basal axis of epithelial cells through a complex with Discs-Large (Dlg) and lethal giant larvae (Lgl) (Humbert et al., 2003). Mutations in any of these three proteins result in the loss of apical-basal polarity (Humbert et al., 2003). As well as it's role in cellular polarity, scrib is a tumour suppressor protein since loss of scrib protein leads to the development of tumours (Humbert et al., 2008). Several studies have examined the role of scrib in tumourigenesis. Vaira et al. (2011) used small-interfering RNA (siRNA) to reduce scrib levels in lung adenocarcinoma A549 cells and showed a decrease in tumour cell migration and invasion and that there was a down-regulation of several markers of cell motility and the epithelial-mesenchymal transition (EMT) including β-catenin, focal adhesion kinase (Ptk2-Fak) and Snai2. Pearson et al. (2011) identified that heterozygous scrib mutant mice (scrib+/-) display prostate hyperplasia when compared to wild-type littermates. Further work identified that the hyperplastic lesions had mislocalized E-

cadherin, phospho-ezrin-radixin-moesin (pERM), protease activated receptor 3 (Par3) and Dlg; all important factors in cell shape and adhesion (Pearson et al., 2011). The same group also identified an increase in nuclear phospho-ERK (phospho-extracellular signal-regulated kinases) and phospho-ELK1 (phospho-E-twenty six (ETS)-like transcription factor-1), both members of the Ras/MAPK (mitogen activated protein kinase) pathway, suggesting that scrib acts as a tumour-suppressor in the mouse prostate through inhibiting the Ras/MAPK pathway (Pearson et al., 2011). Scrib has been associated with human papillomavirus (HPV) and human T-cell leukemia virus type-1 (HTLV-1) (Nakagawa and Huibregtse, 2000, Javier, 2008). The HPV E6 and HTLV-1 Tax proteins are thought to target scrib for ubiquitination and disrupt the integrity of tight junctions (Nakagawa and Huibregtse, 2000, Javier, 2008).

In addition to being a regulator of cell polarity and tumour suppressor, scrib acts as a scaffolding protein in the synapse that is important for establishing the correct localization of the synaptic proteins (Roche et al., 2002). Sun et al. (2009) identified that scrib associates with β-Catenin and this association is important in localizing and clustering synaptic vesicles to the presynaptic terminal (Sun et al., 2009). The β-Catenin/Cadherin adhesion complex is an important player in recruiting synaptic vesicles to the synapses (Sun et al., 2009). Beta-Catenin contains an N-terminal domain that interacts with α-catenin, a central armadillo domain that binds cadherin and a C-terminal PDZ binding domain that is thought to allow β-Catenin to act as a scaffold and link PDZ containing proteins to cadherin clusters in the synapse (Sun et al., 2009). Sun, et al. (2009) identified that in primary hippocampal neurons, a large portion of β-catenin

puncta colocalized with scrib puncta and that deletion of the PDZ binding motif of β-catenin prevents the association between scrib and β-catenin. Further experiments utilized RNA interference (RNAi) towards scrib in hippocampal neurons and indentified that cells expressing scrib RNAi had very few synaptic vesicle clusters compared to control (Sun et al., 2009). These data taken together suggest that scrib interacts with the β-catenin/Cadherin adhesion complex and plays a role in localization of synaptic vesicles to the synapse. However, it is still unknown as to how scrib recruits the synaptic vesicles to the synapse itself. Further work by Sun & Bamji, (2011) added a member to the β-catenin/Cadherin/Scrib interaction by determining β-PIX (p21-activated kinase-interacting exchange factor) is involved in the interaction. β-PIX is a member of the GEF family for Ras-related C3 botulinum toxin substrate (Rac) and cell division control protein 42 (cdc42) and is involved in actin dynamics (Sun and Bamji, 2011). Through co-immunoprecipitation experiments using synaptosomal factions from adult mouse brain, Sun & Bamji, (2011) determined the association between β-catenin/Cadherin/Scrib/β-PIX. Knockdown of β-PIX using siRNA in primary hippocampal neurons resulted in mis-localization of synaptic vesicles along the axon (Sun and Bamji, 2011). Similarly, in neurons transfected with short-hairpin RNA (shRNA) to scrib, a decrease in synaptic vesicle clusters and β-PIX staining occurred suggesting scrib is important for recruiting β-PIX to the synapse (Sun and Bamji, 2011). Sun & Bamji, (2011) used a mutated β-PIX where the mutation causes a lack of GEF activity and determined that without the GEF activity, β-PIX did not elicit its effect of recruiting synaptic vesicles. These results suggest β-PIX enhances actin polymerization at synapses and recruits synaptic vesicles and this is controlled through the GEF activity

of β-PIX. Since NOS1APa interacts with scrib and localizes to synaptic vesicles (Richier et al., 2010) it will be interesting to determine the role of NOS1APa in synaptic vesicle localization and release.

In astrocytes, scrib plays a key role in establishing cell polarity during migration by interacting with β-PIX and controlling the localization and activation of the small GTPase cdc42 and regulates cdc42-dependent polarization pathways (Osmani et al., 2006). The β-PIX protein is also recruited by GIT1 (G protein-coupled receptor kinase-interacting protein 1), a GTPase activating protein (Nola et al., 2008). Both β-PIX and GIT1 are involved in the formation of platforms for large macromolecular assemblies (Schlenker and Rittinger, 2009). The β-PIX-GIT1 complex was then shown to interact with PAK (p21-activated kinase), an enzyme that is activated by Rac that is involved in activation of microtubule and actin regulatory proteins (Stockton et al., 2007). The GIT1 protein is able to recruit β-PIX that then activates Rac by exchanging GDP for GTP and renders Rac responsible for activation of PAK. This cascade then has the ability to regulate proteins involved in actin dynamics at the synapse and in dendritic spine morphology (Zhang et al., 2005). Scrib has the ability to interact with the GIT1/βPIX/PAK cascade and recent work in our lab by Richier et al. (2010) identified that NOS1AP interacts with Scribble and regulates dendritic spine development in primary hippocampal neurons through scrib and its interaction with the GIT1/βPIX/PAK cascade.

1.2.5 NOS1AP interacts with Carboxypeptidase E

In addition to Scribble, NOS1AP has been shown to associate with carboxypeptidase E (CPE). Carrel, et al., (2009) performed a yeast-2-hybrid screen using aa 181 – 307 of NOS1AP, and identified CPE as an interacting partner. Carboxypeptidase E is a glycoprotein involved in the biosynthesis of neuropeptides (Manser et al., 1990). CPE is found in secretory granules where it is either membrane bound or soluble (differ by 2 – 3kDa in size) (Manser et al., 1990). CPE is expressed at high levels in the brain and pituitary gland as well as in endocrine tissues (Manser et al., 1990). The yeast-2-hybrid results were confirmed by pull-down experiments suggesting that the proteins interact directly.

To characterize the functional significance of the NOS1AP/CPE interaction, shRNA constructs against CPE were co-transfected with cDNA constructs encoding NOS1AP into hippocampal neurons. Neurons lacking CPE but expressing NOS1AP showed no change in the number of primary or secondary dendrites. However, in neurons where NOS1AP was reduced, there was an increase in primary and secondary dendrite numbers (Carrel et al., 2009). To determine if CPE mediated the reduction in primary hippocampal neuron dendrites caused by the loss of NOS1AP, the shRNA construct for CPE was co-transfected into primary hippocampal neurons along with the shRNA for NOS1AP. The co-expression of these constructs prevented the changes of NOS1AP alone suggesting that NOS1AP acts via a CPE-dependent pathway to regulate dendrite patterning (Carrel et al., 2009). However, this is likely simplified since loss of CPE alone had no effect on endogenous NOS1AP dependent neurite outgrowth. Nonetheless,

because nNOS is a binding partner for NOS1AP, the effect of nNOS activity was also tested. Neurons were transfected with the full length NOS1AP construct and then directly treated with the nNOS inhibitor L-N^{G}-nitroarginine-methyl-ester (L-NAME) to block nNOS activity. Blocking nNOS function in NOS1AP deficient neurons prevented the reduction in the number of primary dendrites (Carrel et al., 2009). These data suggest NOS1AP may play an important role in initiation, growth and maintenance of dendrite branching and this may be through a CPE dependent manor (Carrel et al., 2009). This has biological significance due to decreased dendrite numbers or branching being a characteristic of disorders such as Schizophrenia and bipolar disorder (Carrel et al., 2009).

1.2.6 VANGL1 and NOS1AP associate through scribble

Recently, Anastas, et al., (2011), identified the protein Van gogh like-1 (VANGL1) as a novel interacting partner for NOS1AP and Scribble through a mass spectrometry screen. They identified a co-localization between NOS1AP, scrib and VANGL1 along cellular protrusions in metastatic breast cancer cells (Anastas et al., 2011). Because scrib is involved in multiple protein complexes, Anastas, et al., (2011), completed several immunoprecipitations to validate scrib's involvement in several complexes. One in particular is the protein complex involving scrib and the GIT1/β-PIX/PAK cascade published previously by Richier, et al., (2010). Anastas, et al. (2011), found NOS1AP did not co-immunoprecipitate with endogenous GIT1 in a breast cancer cell line, however, scrib did. They performed a competition assay and identified that increasing levels of NOS1AP caused a reduction in the amount of endogenous GIT1 co-

immunoprecipitating with scrib suggesting that NOS1AP competitively displaces GIT1 from the scrib complex and that scrib associates independently with the GIT1/β-PIX/PAK protein complex and the NOS1AP-VANGL1 protein complex. The use of shRNA to knockdown scrib and NOS1AP identified a consistent reduction in wound closure in two different breast cancer cell lines (Anastas et al., 2011). They also identified that NOS1AP shRNAs disrupted the sub-cellular localization of scrib, whereby it failed to reach the leading edge of lamellipodia (Anastas et al., 2011). However, in normal mammary tissue, scrib and VANGL1 localized to sites of cell-cell contact whereas NOS1AP localizes to the cytosol (Anastas et al., 2011). There was no co-localization between VANGL1, scrib and NOS1AP at cell junctions in normal breast cells (Anastas et al., 2011). With that being said, the colocalization of scrib, NOS1AP and VANGL1 along the edge of cellular protrusions in invasive breast cancer cells, but not in normal mammary tissue, implies these proteins may be a complex that promotes cell migration in breast cancer cell lines.

1.3 NOS1AP AND DISEASE

Since a number of binding partners have been shown to associate with NOS1AP, it comes as no surprise that NOS1AP has been linked to a number of human disorders. These disorders include Schizophrenia (Brzustowicz et al., 2002), QT syndrome (Crotti et al., 2009), muscular dystrophy (Segalat et al., 2005), diabetes (Lu et al., 2010) as well as chronic inflammation (Shao et al., 2011). How NOS1AP is associated with or functions in these disorders remains elusive.

1.3.1 NOS1AP in Schizophrenia

Schizophrenia is a psychiatric disorder that effects approximately 1% of the population (Fang et al., 2008). Schizophrenia has been classified as predominately a genetic disorder with high heritability as shown through twin and adoption studies (Brzustowicz et al., 2004, Fang et al., 2008). The exact etiology and genetics behind schizophrenia are still unknown. Morphological changes associated with schizophrenia can range from a slight decrease in brain size to localized changes in morphology and molecular composition of specific neuronal, synaptic and glial populations in the hippocampus, dosolateral prefrontal cortex and the dorsal thalamus (Harrison and Weinberger, 2005). Alterations in synaptic plasticity, especially those affecting NMDA receptor-mediated glutamatergic transmission, have been implicated in the disease hence schizophrenia being known as a disease of the synapse (Harrison and Weinberger, 2005). Recently, several different susceptibility genes have been identified for schizophrenia and they include; disrupted in schizophrenia 1 (*DISC1*), neuregulin 1 (*NRG1*), dysbindin (*DTNBP1*), *G72/G30* gene locus, regulator of G-protein signaling 4 (*RGS4*), proline dehydrogenase (oxidase) 1 (*PRODH*), catechol-O-methyl transferase (*COMT*) and *NOS1AP* (Harrison and Weinberger, 2005). Many of these candidate susceptibility genes identified have all been located to non-coding regions except for one (Eastwood, 2005).

In 2004, Brzustowicz et al. implicated *NOS1AP* in schizophrenia. *NOS1AP* is located on chromosome 1q22 and linkage disequilibrium studies from several groups have identified 6 single nucleotide polymorphisms (SNP's) within the genomic region of the *NOS1AP* gene (Brzustowicz et al., 2004). Despite the genetic studies, no mutations in the coding

region of *NOS1AP* that would affect or alter the NOS1AP protein have been identified in patients with schizophrenia. This does not rule out mutations in the non-coding regions that may affect the transcriptional regulation of *NOS1AP*. In another study, the same group identified a novel NOS1AP isoform, NOS1APshort, containing only the last two exons of NOS1AP. The NOS1APshort protein is 211aa protein composed of exons 9 and 10 that generate a mutant protein retaining the capacity to bind nNOS (Xu et al., 2005). Since this isoform lacks the N-terminal PTB domain it is predicted that it would not interact with synapsin1, Dexras1 or scrib. Interestingly, mRNA levels of this novel NOS1AP isoform (NOS1APshort) are increased in the dorsolateral prefrontal cortex in patients with schizophrenia and bipolar disorders (Xu et al., 2005). This group hypothesized that since this NOS1APshort isoform is capable of binding nNOS, it may function as a dominant negative protein to disrupt the association between nNOS and PSD-95. Since PSD-95 couples nNOS with the NMDA receptor, the uncoupling of nNOS from PSD-95 would prevent the localization of nNOS near NMDA receptor complexes and alter levels of nitric oxide within the synapse (Xu et al., 2005). Whether this happens *in vivo* remains to be determined.

1.3.2 NOS1AP in Long-QT Syndrome

Recent research has identified the presence of the *NOS1AP* gene in the heart muscle. In particular, *NOS1AP* has been identified in a condition called Long-QT Syndrome (LQTS). The QT interval includes the beginning of the Q-wave to the end of the T-wave in the heart's electrical cycle, when the ventricle is being re-polarized (Lu et al., 2010). In LQTS, there is an abnormally prolonged QT interval duration and this is associated

with life threatening arrhythmias and sudden cardiac death (Crotti et al., 2009, Lu et al., 2010). Long-QT syndrome is a genetic disorder that is caused by a mutation that affects genes encoding ion channel subunits or proteins that indirectly modulate function of ion channels (Tomas et al., 2010). The most common form is caused by a mutation in the gene that codes for a pore forming subunit of the potassium channel responsible for slow cardiac delayed rectifier current, *KCNQ1* (Crotti et al., 2009). Currently, the treatment for LQTS is beta-blockers, unless patients are at a high risk of sudden cardiac death or do not respond to therapy, in which case they receive an implantable cardioverter-defibrillator (ICD) (Tomas et al., 2010).

Recently, a genome wide association study found an association between SNP's in the 5' region of *NOS1AP* and a prolonged QT interval (Tomas et al., 2010). It was identified that genetic variation in *NOS1AP* contributes to the QT interval duration in the general population (Crotti et al., 2009). Crotti, et al., (2009) also found that *NOS1AP* variants are modifiers of the clinical severity of LQTS and is associated with an increased chance of a more prolonged QT interval in those that carry a mutation. Lu, et al., (2010) indicated that there is evidence of an association between *NOS1AP* and the QT interval in women and in those with diabetes.

1.3.3 NOS1AP in Cardiomyocytes

Within the heart, NOS1AP protein has been localized to the cardiomyocyte. Beigi, et al., (2009) identified that nitric oxide synthase 1 (NOS1) redistributes from the sarcoplasmic reticulum (SR) to the sarcolemma membrane in the cardiomyocyte following injury and

may act to protect the heart by preventing a calcium overload. The NOS1AP-NOS1 complex under physiological conditions is present in the SR and interacts with Dexras1 and xanthine-oxidoreductase (XOR) (Beigi et al., 2009). An interaction was identified between NOS1AP and the plasma membrane Ca^{2+} pump (PMCA) protein, a sarcolemma ion pump that NOS1 binds to (Beigi et al., 2009). During a myocardial infarction (MI), NOS1 is translocated to caveolae and is accompanied by NOS1AP but if NOS1 is lacking, then NOS1AP is not redistributed after MI (Beigi et al., 2009). These results suggest that the interaction between NOS1AP and NOS1 is important when injury to the heart occurs but on a regular basis they contribute differently.

The expression of NOS1AP was also identified in the sarcolemmal membrane and the perinuclear region of guinea pig ventricular cardiomyocytes (Chang et al., 2008). Over-expression of NOS1AP caused an up-regulation of NOS1-NO signaling pathways and application of the NOS blocker, L-NAME, counteracted the effects of the over-expressed NOS1AP (Chang et al., 2008). This study identified a shortened action potential duration caused by inhibition of L-type calcium channel and activation of delayed rectifier potassium channels due to over-expression of NOS1AP that may act to stabilize NOS1 and activate NOS1-derived NO signaling cascades in ventricular cardiomyocytes (Chang et al., 2008). This information suggests a possible mechanism for NOS1AP in LQTS.

1.3.4 NOS1AP in Skeletal Muscle

In skeletal muscle, there are three different forms of NOS: a form of nNOS called nNOS-μ, endothelial NOS (eNOS) and inducible NOS (iNOS) (Chen et al., 2008). The NOS-

NO function is associated with the neuromuscular transmission, muscle contractility, mitochondrial respiration and carbohydrate metabolism (Chen et al., 2008). The nNOS-μ isoform is restricted to the sub-sarcolemmal cytoskeleton and it provides NO to the skeletal muscle through interaction with the dystrophin-dystroglycan complex (DGC) (Segalat et al., 2005). The expression of *NOS1AP* gene was identified in the satellite cells, myotubes, myoblasts, the diaphragm, dystrophic muscle, the muscle-tendon junction (MTJ) and growing or regenerating muscle fibres (Segalat et al., 2005, Chen et al., 2008). Chen et al., (2008) then went on to identify that mice with sciatic nerve injury have an increased expression of NOS1AP mRNA and protein for two weeks following injury while nNOSμ decreases for two weeks post-injury and both NOS1AP and nNOSμ return to normal following that two week period. In this instance, it is suggested that the interaction between NOS1AP, nNOSμ and the formation of a complex with either Dexras1 or synapsin1 regulates the delivery and specificity of the NO signal. Segalat, et al., (2005) suggests NOS1AP acts as an adaptor protein at specialized regions of muscle fibres and motor end plates to prevent instability of nNOSμ. Since dystrophin and DGC mutations destabilize nNOSμ in the skeletal muscle, an increased expression of NOS1AP may help attenuate the destabilization of nNOSμ since it is capable of responding to changes of nNOSμ activity and expression (Segalat et al., 2005). This interaction between nNOSμ-NOS1AP-DGC can have important implication in disease such as muscular dystrophy since NO localization is a critical factor for muscle growth, satellite cell activation and muscle regeneration (Segalat et al., 2005).

1.3.5 NOS1AP in Inflammation

A study completed by Shao, et al., (2011) identified a potential role for NOS1AP in inflammation. Nitric oxide has been implicated as an important inflammatory mediator in the CNS that responds to lipopolysaccharide (LPS) and as well, nNOS and eNOS may also contribute to the inflammatory response (Shao et al., 2011). Because NOS1AP acts as a stabilizer and a localizer for nNOS, Shao, et al., (2011) tested if NOS1AP played a role in inflammation in the brain treated with LPS. The expression of NOS1AP mRNA and protein increased in the brain after stimulation with LPS and immunocytochemistry staining showed NOS1AP localizing to the nuclei of neurons in the hippocampus and cerebral cortex after LPS treatment both *in vivo* and *in vitro* (Shao et al., 2011). These results indicate that during the inflammatory response, the localization of NOS1AP is changed from the cytoplasm to the nucleus. This may then prevent the removal of nNOS from the membrane by lack of competition with PSD-95 and cause dis-regulation of nNOS and NO production therefore changing the cellular response to inflammation.

1.4 NOS1APc

Previous work completed in the Fawcett lab had identified an alternate isoform of NOS1AP subsequently called NOS1APc (Richier et al., 2010). The NOS1APc isoform was first identified while performing Western blot analysis. A slower migrating band was seen at approximately 100kDa and was subsequently identified using bioinformatics as a longer isoform of NOS1AP known as NOS1APc. The NOS1APc isoform retains the PTB domain at the N-terminal region of NOS1AP; however, there is a 30kDa extension at the C-terminal that effectively removes the PDZ domain present in NOS1AP. Two

different isoform specific antibodies were generated to the unique 30kDa extension in NOS1APc by the Fawcett lab (see O'Brien thesis). One is a peptide antibody that targets a specific sequence in the unique region (CAFPLLDPPPPITRKRTPEAL) named PPIT-NOS1APc and the other is a GST fusion antibody generated to the entire sequence of the 30kDa unique region named GST-NOS1APc.

To better understand a potential function for NOS1APc, these antibodies were used to perform a mass spectrometry screen to identify potential interacting proteins. Clarified lysate from adult rat brain was used to capture NOS1APc specific complexes that were subsequently identified using a mass spectrometer. The screen provided three potential candidates as interacting partners, transcytosis associating protein p115 (p115), tuberous sclerosis 1 (TSC-1) and pyruvate carboxylase (PCB). These three proteins have never been validated as bona fide interacting partners.

In addition to the mass spectrometry screen, a previous honours student identified a unique staining pattern of NOS1APc in the midline of adult and neonatal rats (Peter Dixon, 4th year honours thesis, Department of Biochemistry and Molecular Biology, Dalhousie University, 2010). This staining pattern was similar to an ephrinB3-staining pattern. It was speculated that NOS1APc and ephrin could interact. Thus, part of my project was to confirm the interactions of both the proteins identified in the mass spectrometry screen and the immunohistochemical localization studies. Therefore, to gain insight towards what these potential interacting partners are involved with, background information of their known and potential roles will be provided.

1.4.1 NOS1APc Interacting partners

1.4.1 (a) Transcytosis Associating Protein p115

The transcytosis associating protein p115 (p115) is a peripheral membrane protein that localizes to the Golgi apparatus as well as the cytoplasm (Sohda et al., 1998). The p115 protein is also known as transcytosis-associating protein (TAP) (Sohda et al., 1998), a protein required for binding of transport vesicles from the ER/Golgi complex to their target membranes (Barroso et al., 1995). The TAP/p115 protein is conserved in yeast and is known as Uso1p. Uso1p plays an important role in the trafficking of cargo between the endoplasmic reticulum (ER) and the Golgi apparatus (Barroso et al., 1995). Sohda, et al., (1998) suggest that TAP/p115 exist as a homodimer and show that when phosphorylated, TAP/p115 associates with the Golgi apparatus. This indicates that complex post-translation modifications are important for the function of this protein in the biosynthetic pathway. The TAP/p115 protein has also been shown to bind the Golgi matrix protein GM130, a peripheral protein that associates with the cis Golgi membrane (Sohda et al., 1998), further implicating this protein in Golgi trafficking events.

1.4.1 (b) Tuberous Sclerosis

Tuberous sclerosis 1 and 2 (*TSC-1 and TSC-2*) are genes located on chromosomes 9 and 16 respectively (Lamb et al., 2000). The *TSC-1* gene encodes a novel 130-kDa protein known as hamartin while the *TSC-2* gene encodes the 200-kDa protein tuberin that acts as a GTPase activating (GAP) protein for the small GTPase proteins Rap1 and Rab5 (van Slegtenhorst et al., 1998). Tuberin interacts with Rheb (Ras homologue enriched in brain), a small GTPase that activates mTORC-1 (mammalian target of rapamycin

complex 1) (Inoki and Guan, 2009). The interaction between mTORC-1 and Rheb allows for the proper control of mTORC-1 function. GTPase activation of mTORC is important since mTORC plays a fundamental role in the cell, and is important for functions including protein synthesis, cell cycle progression, transcription, mRNA splicing and autophagy (Inoki and Guan, 2009). These complexes are all part of the mTOR signaling pathway, a pathway that has attracted a great deal of attention of late since it is a critical signaling pathway involved in cellular survival by detecting levels of cellular nutrient and energy levels.

Interestingly, hamartin and tuberin proteins interact *in vivo* (van Slegtenhorst et al., 1998). This interaction is important to promote GTP hydrolysis of Rheb (Inoki and Guan, 2009). Further, the interaction is important in stabilizing the tuberin protein, thus preventing ubiquitin-mediated degradation (Huang and Manning, 2008). Since the mTOR pathway is critical, Rheb is critical for detecting cellular nutrient levels, and thus is integral for cellular survival. Having an understanding how these proteins function is critical for understanding how cells survive.

Along those lines then, both hamartin and tuberin are known as tumour suppressor proteins. Tuberous Sclerosis is an autosomal dominant disorder that is caused by mutations in either the *TSC-1* or *TSC-2* genes (van Slegtenhorst et al., 1998). Loss of the *TSC* genes causes several different tissues types to become susceptible to the development of hamartoma's including brain, eyes, kidneys, heart and skin (van Slegtenhorst et al., 1998, Lamb et al., 2000). The hamartoma's that develop are typically

benign and rarely become malignant but the tumours that do emerge show abnormalities in cell migration, cell adhesion, and proliferation (Lamb et al., 2000). According to (Inoki and Guan, 2009), mTORC-1 is constitutively active in *TSC-1* and *TSC-2* mutant cells meaning the loss of *TSC-1/2* activation of Rheb and therefore regulation of mTORC-1, could be a contributing factor to the growth of the tumours associated with tuberous sclerosis. Even though the patients with tuberous sclerosis develop benign tumours, up to 80% of those patients will have complications in the CNS including autism, refractory seizures, epilepsy and mental retardation (in severe cases) (Huang and Manning, 2008, Inoki and Guan, 2009). The most lethal complications that occur with tuberous sclerosis include renal lesions and cysts that can lead to end stage renal failure with bacterial infections and severe hypertension as well as lymphangioleiomyomatosis (LAM), a rare lung disease that causes disorderly proliferation of smooth muscle in the lungs (Huang and Manning, 2008, Inoki and Guan, 2009).

1.4.1 (c) Pyruvate Carboxylase

Pyruvate carboxylase (PCB) is a mitochondrial enzyme located in several different tissues of the body. High levels of PCB can be identified in the liver and kidney with moderate to high levels identified in the brain, pancreas and adipose tissue (Jitrapakdee et al., 2006). The PCB protein is composed of three main domains, a N-terminal biotin carboxylase (BC) domain, a central carboxyltransferase (CT) domain and a C-terminal biotin carboxyl carrier protein (BCCP) domain (St Maurice et al., 2007). In solution, pyruvate carboxylase is composed of four identical subunits arranged in a tetrahedron structure (Jitrapakdee et al., 1996). Each of the subunits contains a covalently bound

biotin moiety at the C-terminus that is responsible for carrying a carbon dioxide molecule between the BC and BCCP domains (Jitrapakdee et al., 1996). The activation of PCB is under allosteric control by acetyl-Coenzyme A (acetyl-CoA) and aspartate (St Maurice et al., 2007, Jitrapakdee et al., 2008).

Pyruvate carboxylase is known as an anaplerotic enzyme because it is required for replenishing an intermediate for the Tricarboxylic Acid (TCA) Cycle. Anaplerosis is essential in the process of maintaining homeostasis of the TCA cycle and the cell itself (Murin et al., 2009). Pyruvate carboxylase catalyzes the adenosine triphosphate (ATP)-dependent fixation of carbon dioxide (CO_2) to pyruvate that yields oxaloacetate (OAA) (Waagepetersen et al., 2001, Berkich et al., 2007). It is the biotin moiety that requires the ATP to release the CO_2 molecule for fixation to pyruvate. By virtue of PCB being quite highly expressed in several different tissue types, it is involved in many cellular processes including gluconeogenesis, adipogenesis, lipogenesis, neurotransmitter synthesis and in pancreatic islet cells (Attwood, 1995, Jitrapakdee et al., 2006, Murin et al., 2009).
In the brain, PCB is important for the proper function of both astrocytes and neurons, however, PCB is only expressed in astrocytes (Hassel, 2001, Hertz et al., 2007). Immunohistochemistry has provided evidence for the notion that neurons do not contain PCB since no staining is detected in the neurons but has been found in astrocytes as well as satellite and Schwann cells in the peripheral nervous system (Jitrapakdee et al., 2006). Hertz, et al., (2007) have also shown through immunohistochemistry that neurons do not stain for PCB where as astrocytes do and PCB activity is not detectable in neurons. Neurons have a small role in the amount of glutamate that they produce; they instead rely

on glutamine released by astrocytes that they can then transform to glutamate through glutaminase activity (Berkich et al., 2007) or further convert to γ-amino butyric acid (GABA) (Jitrapakdee et al., 2008).

The *PCB* gene is important in maintaining normal cellular metabolism. Without *PCB* activity, pyruvate carboxylase deficiency shows 3 different clinical manifestations; Type A (North American phenotype), Type B (French phenotype) and Type C ('Benign' phenotype). The deficiency is caused by a rare autosomal recessive error of metabolism (Garcia-Cazorla et al., 2006). The incidence of *PCB* deficiency is approximately 1:250,000 births (Jitrapakdee et al., 2008). The Type A phenotype is characterized by mild lactic acidosis, psychomotor retardation and in some instances death occurs in the first year of life (Jitrapakdee et al., 2008). There is presence of residual PCB protein but with diminished activity. The Type B phenotype shows an early neonatal onset of 1 – 72 hours and is characterized by hypoglycemia, high lactic acidosis, ketoacidosis, an increased breathing rate and high levels of proline, citrulline and lysine (Garcia-Cazorla et al., 2006, Jitrapakdee et al., 2008). Type B can also be characterized by several neurological abnormalities including seizures, coma, macrocephaly, periventricular cysts, dystonia, and spasticity (Garcia-Cazorla et al., 2006). Patients with Type B deficiency may also suffer from liver disease and have decreasing neurological function and typically die within the first few months of life (Garcia-Cazorla et al., 2006, Jitrapakdee et al., 2008). The third type of *PCB* deficiency, Type C, is called the 'benign' phenotype because the aspect of deficiency that these patients show is a mild state of lactic acidosis with normal psychomotor development (Jitrapakdee et al., 1996).

The symptoms that occur in the Type A and B *PCB* deficiencies are due to several different factors. In a review by Jitrapakdee et al. (1996), they suggest several different hypotheses as to why these symptoms occur. The first hypothesis is that the lack of PCB causes an accumulation of pyruvate that is then converted to lactate by LDH (lactate dehydrogenase) that then leads to the lactic acidosis that characterizes the disease. The second hypothesis is that there is a limited amount of OAA present due to the lack of PCB. The limited amount of OAA would decrease neonatal gluconeogenesis and lead to the hypoglycemia characteristic of the Type B deficiency. The third hypothesis is that lack of supply from the 'glutamine-glutamate' cycle may decrease the amount of neurotransmitter substances available for synaptic transmission and therefore cause the delayed mental and impaired psychomotor development. A suggestion by Higgins, et al., (1997) is that there are flaws in the assembly of the myelin sheath in the brain due to PCB not providing citrate and acetyl-CoA as sources for fatty acid synthesis. In attempts to treat PCB deficiency, biotin replacement has been attempted but with little success (Higgins et al., 1997, Ahmad et al., 1999). Another treatment option is to supplement patients with Type B deficiency with high doses of citrate or aspartate to create OAA through other pathways (Ahmad et al., 1999). In the case presented by Ahmad, et al., (1999), the patient became metabolically stable with the addition of citrate, aspartate and arginine but still suffered from severe neurological issues including deficits in myelination of neurons.

1.4.1 (d) Eph Receptors and Ephrin Ligands

Erythropoietin producing human hepatocellular carcinoma (Eph) receptors constitute the largest family of receptor protein-tyrosine kinases (1997). There are 14 different receptors that are classified into two families, A or B, according to what ligand they preferentially bind (1997). The Eph receptors contain an extracellular N-terminal that contains an Eph ligand-binding domain and two-fibronectin type III repeats (Surawska et al., 2004). The receptor then spans the membrane and in the C-terminus contains a tyrosine kinase domain (a few exceptions here) and a sterile alpha motif (SAM) domain (Surawska et al., 2004).

The eight different ephrin (Eph family receptor interacting proteins) ligands are those that the Eph receptors will bind (1997). The ephrin ligands are segregated into the A and B families allowing for the Eph receptors to be named appropriately. The ephrin ligands are either linked to the outer leaflet of the plasma membrane via glycosylphosphatidylinositol (GPI) anchor (such is the case for the ephrinA family) or have a transmembrane domain (ephrinB family) with a C-terminal Tyrosine-Lysine-Valine (Y-K-V) target site important for binding PDZ domains of other proteins (Frisen et al., 1999, Kullander and Klein, 2002).

Typically, the receptors and ligands will interact according to what family they belong; for example, EphA receptors interact with ephrinA ligands. The exception to this rule occurs with EphA4; it has the ability to interact with ephrinB3 (1997). For signaling to occur between the Eph receptor and ephrin ligand, cell-to-cell interaction is required and

the Eph/ephrin interaction is known as bi-directional signaling. The interaction is bi-directional because the binding of the ephrin ligand to the Eph receptor causes a 'forward' signal into the cell containing the Eph receptor and initiates phosphorylation at specific tyrosine residues in the cytoplasmic portion of the receptor (Liu et al., 2004, Merlos-Suarez and Batlle, 2008). The 'reverse' signal occurs in the cell containing the ephrin ligand where in response to binding to the Eph receptor, the ligand undergoes tyrosine phosphorylation and subsequent intracellular proteins are recruited to the ligand (Liu et al., 2004, Heroult et al., 2006). The other important factor in the properties of Eph/ephrin signaling is the requirement for clustering of several of the membrane bound ephrin ligands to activate the Eph receptor (Kullander and Klein, 2002).

The effect of Eph/ephrin signaling is essential for proper functioning during embryogenesis as well as for several processes in the adult. Eph/ephrin signaling has been shown to be involved in angiogenesis, regulating cytoskeleton dynamics, cell migration, apoptosis, synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD), tumourigenesis, cell adhesion and repulsion as well as cell-cell communication (Liu et al., 2004, Ricard et al., 2006, Merlos-Suarez and Batlle, 2008). The involvement of Eph/ephrin signaling in such a wide variety of cellular functions is most likely due to the range of different downstream signaling pathways that they target. The Eph receptors have been implicated in interactions with non-receptor tyrosine kinases families of Src (Sarcoma) and Abl, phosphatidylinositol 3-kinase (PI3-kinase), Ras, Nck2 (Non-catalytic region of tyrosine kinase adaptor protein-1; Grb4), Grb2, Grb 10, Crk, p53, and SLAP (Src-like adaptor protein) to name a few (Frisen et al., 1999,

Dodelet and Pasquale, 2000). The ephrin ligands also bind a variety of downstream targets such as DOCK 180 (dedicator of cytokinesis), Rac, Nck2, cdc42, Pak, and Wnt (Noren and Pasquale, 2004, Xu and Henkemeyer, 2009). These roles for Eph/ephrin signaling have been well characterized for several cellular processes including angiogenesis, axon guidance, topographic mapping and in the intestinal epithelium but the roles of several downstream targets are still being investigated.

The repulsive effects of ephrin ligands have been established for many cellular functions including ephrinA5 and ephrinA2 in the guidance of chick retinal axons, migrating neural crest cells, in angiogenesis (Kullander and Klein, 2002, Heroult et al., 2006), in the intestine (Batlle et al., 2002), formation of the hippocampus and guidance of developing sensory and motor spinal nerves (Bergemann et al., 1998, Frisen et al., 1999). In the brain and spinal cord, ephrinB ligands expressed in cells that form the midline act as repellents for subsets of axons to prevent crossing (Palmer and Klein, 2003). A well-established example of the repelling action of ephrinB ligands exists with ephrinB3 in the corticospinal tract (CST). The CST connects the brain with the spinal cord to act as a central pattern generator that controls the rhythmic movements of the body (Kiehn and Butt, 2003). The CST neurons originate in the motor cortex of the brain and travel to the brain-spinal cord junction where they cross the midline once and descend the contralateral side of the spinal cord and synapse with motor neurons (Palmer and Klein, 2003). It was identified that ephrinB3 localized to the midline of the spinal cord where it acts as a repellent for axons containing the EphA4 receptor and prevents crossing of the midline for a second time (Palmer and Klein, 2003). In mutant mice lacking ephrinB3 or

mice with a catalytic mutant of EphA4, neurons will cross the midline a second time and cause a rabbit-like parallel hopping phenotype (Kullander and Klein, 2002, Palmer and Klein, 2003).

In the brain, Eph/ephrin signaling has been implicated in embryogenesis as well as processes in the adult such as LTP and neurogenesis. Ricard, et al., (2006) identified ephrinB1, B2 and B3 along with EphB2, B3 and A4 expression in the subventricular zone (SVZ), an area of the brain where neurogenesis persists into adulthood. They identified ephrinB1 and B2 co-stained with the astrocyte marker GFAP within the SVZ while ephrinB3 was expressed in regions that border the SVZ (Ricard et al., 2006). They hypothesize that ephrinB3 may function to inhibit cell proliferation and with Eph, up-regulate cell cycle activators such as AKT and down-regulate cell cycle inhibitors (Ricard et al., 2006). Another group showed evidence that EphA4 acted as a pro-apoptotic dependence receptor in the SVZ (Furne et al., 2009).

In the hippocampus, roles for the ephrinB family of ligands have been implicated in several different processes. Grunwald, et al., (2004) identified that in the hippocampus, the Schaffer collateral axons from the CA3 pyramidal neurons that project to the CA1 neurons express ephrinB3 in the post-synaptic density (PSD). The implication of ephrinB3 expression in the PSD is that ephrinB3 is involved in NMDA receptor-dependent forms of hippocampal LTP and LTD potentially through interactions with EphA4 (Grunwald et al., 2004). Yamaguchi, et al., (2004) also suggest that the loss of post-synaptic ephrinB2 and B3 in CA1 synapses causes strong defects in LTP and LTD.

Antion, et al., (2010) imply that ephinB3-EphB2 interaction in the excitatory synapses of the CA1 hippocampal neurons is required for proper balance of glutamate receptors. Xu, et al., (2009) suggest ephrinB3, through its interaction with Nck2 (a SrcHomolgy/SH3 adaptor protein), is involved in axon pruning. The mossy fibre axons extend from granule cell neurons found in the dentate gyrus and target the pyramidal neurons of the CA3 area and form the CA3-dentate gyrus neuronal network (Xu and Henkemeyer, 2009). Since the axons typically extend beyond their target, they need to be selectively pruned and Xu, et al., (2009) demonstrated that without the presence of ephrinB3 in the CA3 pyramidal cell layer, there is a lack of shortening of the axons. It was determined that the region of ephrinB3 required to stimulate pruning was the phosphorylation sites found on the cytoplasmic domain and the phosphorylation is required to recruit Nck2 to the ligand (Xu and Henkemeyer, 2009). Xu, et al., (2011) then went on to identify ephrinB3 expression in the CA1 pyramidal cells along with PSD-95. The ephrinB3 knockout mice showed a significant increase in the number of primary dendrites and total dendritic branches at p12 compared to their wild type littermates (Xu et al., 2011). The suggested role for ephrinB3 in this situation is as a negative regulator of dendrite growth and arborization in the CA1 pyramidal cells through a similar pruning method mentioned previously (Xu et al., 2011). Xu, et al., (2011) also suggest that Pick-1 could be the interacting partner needed for spine maturation. Noren, et al., (2004) discuss a role for EphB2 in hippocampal neurons and regulating dendritic spine morphogenesis through interactions with intersectin and cdc42 or kalirin and Rac1. There is also a suggestion that Pak interacting exchange factor, α-PIX, may play a role in the activation of Rac1 and cdc42 downstream of EphB2 (Noren and Pasquale, 2004). Noren, et al., (2004) suggest

the mediator of EphB2 activated αPIX is Nck2. It has been suggested that intersectin, the exchange factor for cdc42, promotes exchange in concert with the adaptor protein N-WASP (neuronal Wiskott-Aldrich sydrome protein) and together activate the Arp2/3 (actin related protein 2/3) complex that polymerizes the branched actin filaments and likely contributes to expansion of the dendritic spine head (Yamaguchi and Pasquale, 2004). Kullander and Klein, (2002) also discuss EphB1 and Nck2 interact to activate downstream Nck interacting protein that further activates integrins and subsequently cell adhesion.

The identification of the Eph receptor in the human hepatocellular carcinoma cell line provides the background that suggests Eph-ephrin signaling has a role in the development of cancer. The multiple roles of Eph-ephrin signaling in areas such as angiogenesis and cell proliferation provide further evidence for a role in cancer. The Eph receptors and ephrin ligands have been identified as either up-regulated or down-regulated in a multitude of cancers. The ephrinB2 ligand has been found to be over-expressed in many human colorectal cancers and may be involved in tumour angiogenesis (Liu et al., 2004). An interaction between EphB4 and ephrinB2 was found to be expressed in tissue malignancies including melanoma, neuroblastoma, prostate, lungs, colon and uterine (Heroult et al., 2006, Alam et al., 2008). Alam, et al., (2008) identified high levels of EphB4-ephrinB2 in ovarian cancer and discovered that mRNA levels of EphB4-ephrinB2 increased significantly as the cancer developed and found those with high expression had a much lower rate of survival. Heroult et al., (2006) discuss ephrinA1/B2 and EphB4 as being TNFα (tumour necrosis factor – alpha) inducible genes and being involved in

inflammation. There is also evidence EphB4 stimulation by ephrinB2 is inhibiting tumour growth through interactions with Abl-Crk that work downstream to block cell proliferation (Merlos-Suarez and Batlle, 2008).

Many of the Eph receptors have been implicated in tumour growth and development. Several different groups have identified potential roles for Eph receptors. For instance, (Surawska et al., 2004) discovered an up-regulation of EphA2 in many cancer types and more so in aggressive tumours. The EphA2-ephrinA1 signaling event is a target of the p53 (protein 53) family and phosphorylation of EphA2 due to DNA damage is thought to stabilize p53 (Surawska et al., 2004). Batlle et al., (2005) identified EphB2 to be highly reduced in adenocarcinomas (colorectal cancer) and observed a higher tumour grade was associated with highly decreased or almost absent EphB2 expression. Merlos-Suarez and Batlle, (2008) discuss EphA2 activity in breast cancer as promoting mammary tumour progression and invasion through a complex with the oncogene *ErbB2*.

In the brain, Nakada et al., (2006) discovered in *in vitro* and *ex vivo* models that over-expression of ephrinB3 in human glioma cells induced invasion and that using siRNA designed for ephrinB3 inhibits glioma invasion. (Nakada et al., 2006) studied human glioblastomas, astrocytomas and neuroblastomas and identified over-expression of phosphorylated ephrinB3 while there was little expression in normal brain controls. The hypothesis in glioblastomas is that ephrinB3 interacts with Rac1 via the adaptor protein Nck2 and promotes the increased migration and invasion of the glioblastoma cells (Nakada et al., 2006).

There are some conflicting views on the role of Eph-ephrin signaling in cancer. Further work needs to be completed to indentify the potential roles and influence therapy may have in targeting Eph-ephrin signaling.

1.5 SUMMARY AND RATIONALE

NOS1AP associates with a number of different interacting partners. In this thesis, I discuss the role NOS1AP (herein NOS1APa) has with its interacting partners including nNOS, Dexras1, Synapsin, carboxypeptidase E, scrib and the GIT1/β-PIX/PAK cascade and VANGL1. There is also evidence that NOS1APa may be involved in different complexes with regards to GIT1/β-PIX. Evidence provided by the Fawcett lab suggests NOS1APa exists in a complex with scrib and GIT1/β-PIX/PAK (Richier et al., 2010). Interestingly, Anastas et al., (2011) suggest NOS1APa associates with VANGL1 in a GIT1 independent manner. One possibility is that since we know there are different NOS1AP isoforms, it may be that the different isoforms interact with different protein-protein complexes. I now want to explore whether or not this possibility is true and NOS1APa is involved in different protein-protein complexes. Nonetheless, interactions between NOS1APa and its binding partners place NOS1APa and its isoforms in the realm of cellular polarity and synaptic function. Because of its many interacting partners, NOS1APa has been implicated in several diseases including schizophrenia, Long-QT syndrome, diabetes, muscular dystrophy and in chronic inflammation.

I have also introduced you to an isoform of NOS1APa known as NOS1APc. I have discussed the differences between NOS1APa and its longer isoform NOS1APc and how

this might affect its binding capabilities. I have also introduced several potential interacting partners for NOS1APc as determined by mass spectrometry and observational studies. These proteins are tuberous sclerosis-1/2, transcytosis associating protein p115, pyruvate carboxylase and members of the ephrinB family of ligands. However, it is unknown if any are legitimate binding partners of NOS1APa or NOS1APc. Determining whether any of these are legitimate binding partners for NOS1APa or NOS1APc will help shed light into the functional significance of NOS1APc.

1.6 HYPOTHESIS

I hypothesize that NOS1APa and NOS1APc are involved in different protein-protein complexes.

1.7 AIMS

1. Characterize the NOS1APc isoform.

2. Determine the sub-cellular localization of NOSAP isoforms using over-expression techniques.

3. Identify and characterize binding partners for NOS1APc.

CHAPTER 2 MATERIALS AND METHODS

2.1 CHEMICALS

All chemicals, unless otherwise noted were purchased from Sigma.

2.2 ANTIBODIES

Antibodies used in this body of work include: pan-NOS1AP – rabbit polyclonal antibody (Richier et al., 2010), used at 1:200 dilution for IP, 1:1000 for Western blot; NOS1APc – rabbit polyclonal peptide antibody (Richier et al., 2010), used at 1:200 dilution for IP, 1:1000 for Western blot; GST-NOS1APc – rabbit polyclonal GST fusion antibody (O'Brien thesis) used at 1:200 dilution for IP, 1:1000 for Western blot; Pan-EphrinB – rabbit polyclonal used at 1:200 dilution, 1:1000 for Western blot; Scribble – rabbit polyclonal antibody generated by Dr. Fawcett used at 1:200 dilution for IP (Richier et al., 2010); Scribble H-300 – rabbit polyclonal antibody from Santa Cruz Biotechnologies, Inc. (Cat. No. sc-28737) used at 1:200 dilution for IP, 1:500 for Western blot; Pre Immune NOS1APc – rabbit polyclonal antibody generated by Dr. Fawcett used at 1:200 dilution for IP; nNOS – mouse monoclonal antibody from BD Transduction Laboratories used at 1:350 dilution for IP, 1:1000 for Western blot; Mitochondrial Pyruvate Carboxylase – mouse monoclonal antibody from MitoSciences (Abcam; Cat. No. MS774) used at 1:200 dilution for IP; Pyruvate Carboxylase – rabbit polyclonal antibody from Santa Cruz Biotechnology, Inc. (Cat. No. sc-67021) used at 1:500 for Western blot; GFP – rabbit polyclonal antibody from Abcam (Cat. No. ab290) used at 1:2000 dilution for IP, 1:10,000 for Western blot; Flag M2 – mouse monoclonal antibody from Sigma

(Cat. No. F3165) used at 1:2000 dilution for IP, 1:10,000 for Western blot; GIT1 – mouse monoclonal antibody from BD Transduction Laboratories (Cat. No. 611396) used at 1:350 dilution for IP, 1:1000 for Western blot; β-PIX – mouse monoclonal antibody from BD Transduction Laboratories (Cat. No. 611648) used at 1:350 dilution for IP, 1:1000 for Western blot; Blotting Grade Protein A-Horseradish Peroxidase Conjugate – Secondary antibody from Bio-Rad (Cat. No. 170-6522) used at a dilution of 1:10,000 for Western blot; Blotting Grade Affinity Purified Goat Anti-Mouse IgG (H+L) Horseradish Peroxidase Conjugate – secondary antibody from Bio-Rad (Cat. No. 170-6516) used at a dilution of 1:10,000 for Western blot; c-Myc (9E10) – mouse monoclonal antibody from Santa Cruz Biotechnology, Inc. (Cat. No. sc-40) used at 1:1000 dilution for IP and Western blot.

2.3 CELL CULTURE AND TRANSFECTIONS

Human Embryonic Kidney (HEK) 293T cells were grown at 37°C, 5% carbon dioxide in Dulbecco's Modified Eagle's Medium (DMEM) (Wisent; Cat# 319-005-CL) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Wisent; Cat# 080450), 2 mM L-glutamine (Wisent; Cat# 609-065-EL), 100 U/ml penicillin and 100 µg/ml streptomycin (Wisent; Cat# 450-201-EL). A rat hepatoma cell line (Fao cells; a kind gift from Dr. Chris Sinal) were grown at 37°C, 5% carbon dioxide in DMEM supplemented with 20% heat inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

For transfection, HEK 293T cells were plated onto either 3.5 cm or 10 cm plates and allowed to reach a confluence of 50 – 70%. Cells in a 10 cm plate were transfected by combining 750 μl of serum free media (SFM; Wisent, Cat # 319-005-CL) with 5-10 μg of cDNA (for co-transfection, 5 μg of each cDNA) and 25 μl of polyethylenimine (PEI; 2 mg/ml), vortexed, left for 5 minutes at room temperature, re-vortexed and then added drop wise to cells in media containing serum (Ehrhardt et al., 2006). Transfected cells were incubated at 37°C for forty-eight hours prior to lysis. For 3.5 cm plates containing HEK 293T cells the same protocol was followed except 24 μl of PEI (2 mg/ml) was used. For transfection of Fao cells (rat liver cells), Polyplus JetPRIME transfection reagent (VWR; Cat# 89129-922) was used. The Fao cells were allowed to reach a confluence of 70% in media containing serum. Transfection was performed according to the manufacturer's protocol. Briefly, 200 μl of Polyplus buffer was combined with 5 μg of cDNA and 4 μl of polyplus jetPRIME reagent, vortexed 10 seconds, allowed to sit 10 minutes at room temperature and then added drop wise to cells.

2.4 IMMUNOPRECIPITATION

Fresh or frozen mouse or rat brains were homogenized using a Dounce homogenizer in NP40 lysis buffer (10% glycerol, 1% NP40, 20mM tris pH 8.0, 37.5mM NaCl) containing 1mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and a pinch of sodium pervanadate. The NP40 lysis buffer was combined with tissue at a 1/10 (weight/volume) ratio. Once homogenized, the lysate was nutated for 30-minutes at 4°C followed by repeated centrifugation, three times at 13,000 rpm for 40 minutes at 4°C each. The clarified top layer was collected following each spin. Whole

cell lysate samples were created by combining 30 μl of lysate (approx. 150 μg of protein) with 30 μl of 2x Sample Buffer (2xSB) consisting of 250 mM Tris pH 6.8, 4% sodium dodecylsulphate (SDS), 20% glycerol, 0.01% bromophenol blue and 0.4 M Dithiothreitol (DTT). The remaining lysate was divided into 1ml aliquots of approx. 5 mg/mL total protein and incubated with the appropriate antibody overnight on a nutator at 4°C. Following overnight incubation, samples were incubated with 100 μl of 10% protein A sepharose beads (GE Healthcare; Cat# 17-0780-01) for 1 hour on a nutator at 4°C. The beads were washed by centrifugation of the mixture for 1min at 3000 rpm. Supernatant was removed and 1ml of fresh NP40 lysis buffer was added to re-suspend the beads. This process was repeated 3 times followed by a final spin at 3000 rpm for 1 minute, removal of the supernatant and re-suspending the beads in 30 μl of 2xSB. Samples were stored at -20°C until required for western blotting.

For immunoprecipitation from transfected cell lines, a similar protocol was followed. Forty-eight hours following transfection, plates were washed two times with room temperature phosphate buffered saline (PBS). Cells were then suspended in 1ml of NP40 lysis buffer (see above) with 1mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and a pinch of sodium pervanadate. The cells were rocked for 20 minutes at 4°C, scraped off the plate and collected into an eppendorf tube. The samples were then centrifuged for 20-30 minutes at 13,000 rpm at 4°C. The supernatant was transferred to a new eppendorf tube where 30 μl of supernatant was taken and combined with 30 μl of 2xSB to create whole cell lysate. The remaining supernatant was combined with the proper antibody and placed on a nutator overnight at 4°C. Following overnight incubation samples were

incubated with 100 μl of 10% protein A sepharose beads (GE healthcare) for 1 hour on a nutator at 4°C. The beads were then washed by centrifuging for 1 minute at 3000 rpm, removing the supernatant and adding 1ml of fresh NP40 lysis buffer. The washing process was repeated 3 times followed by a final spin for 1 minute at 3000 rpm, removal of the supernatant and re-suspending the beads in 30 μl of 2xSB. Samples were stored at -20°C until required for western blotting.

2.5 WESTERN BLOTTING

For Western blot analysis, 7.5%, 10% or 12% SDS-PAGE gels were used. Prior to loading, samples were boiled at 95°C for 5 minutes. Once samples were loaded, they were electrophoresed using a Biorad Mini-Protein Gel apparatus in constant voltage mode (70V through the stack, 150V through the separation gel). Gels were transferred onto a polyvinyl difluoride (PVDF) membrane (Millipore; Cat# ISEQ00010) for 2.5 hours at 100V. Membranes were then rinsed in distilled water and blocked for 1 hour in either 5% non-fat dry milk in tris-buffered saline 0.01% Tween (TBST) solution or 3% bovine serum albumin (BSA; Wisent, Cat# 800-095-EG) in 0.1% TBST. Membranes were then placed in appropriate primary antibody overnight in 5% milk-TBST. Following overnight incubation membranes were washed 3 times for 10 minutes each in TBST, incubated one hour in the appropriate secondary antibody at a 1:10,000 dilution in TBST and then washed 3 times for 10 minutes each in TBST. Once washed, the membrane was exposed using chemiluminescence (SuperSignal® West Pico Chemiluminescent Substrate; Thermo Scientific, Cat# 34080) and visualized using an x-ray processor.

2.6 IMMUNOCYTOCHEMISTRY

Cells were grown on 18mm cover slips as outlined previously. Transfection conditions were followed for 3.5 cm plates as outlined previously. To visualize transfected cells, the cover slips were washed twice with 2 ml of 1xPBS and then fixed in 2 ml of 4% paraformaldehyde (PFA) with 4% sucrose in PBS for ten minutes at room temperature. The PFA was removed followed by washing of the cover slips 3 times in 2ml of PBS. The cover slips were then incubated for 5 minutes at room temperature in 100 μl of a BisBenzamide (Hoescht – 33258; Sigma Cat #B1155) nuclear stain at a 1:2000 dilution in PBS. Cover slips were then washed 2 times in PBS. Cover slips were then dipped in sterile double deionized water and then mounted onto glass slides (Fisherbrand; Cat #12-552-3) in 20 μl of fluoromount mounting medium (Sigma; Cat #F4680).

2.7 CLONING OF NOS1AP ISOFORMS

2.7.1 Total RNA Isolation

The total RNA isolation was completed following a modified protocol published by Denovan-Wright et al. (2003). The cerebellum and cortex were removed from the adult rat and flash frozen using liquid nitrogen. The tissue was combined with 1.0 ml of TRIzol® Reagent (Invitrogen) per 50 mg of tissue. The tissue was homogenized using a Dounce homogenizer. Following homogenization, 200 μl of chloroform was added for every 1 ml of TRIzol® Reagent used. Samples were vortexed for 15 seconds and incubated at room temperature for 3 minutes. The homogenate was centrifuged at 12,000xg for 15 minutes at 4°C. The upper aqueous layer was transferred to a new eppendorf tube and 0.5 ml of isopropanol per 1 ml of TRIzol® Reagent used for

homogenization was added. The solution was mixed and incubated at room temperature for 15 minutes. After incubation samples were centrifuged at 12,000xg for 15 minutes at 4°C. The supernatant was removed and the pellet washed 2 times with 1ml of 75% ethanol and vortexed with every new addition of ethanol. After the second wash, samples were centrifuged at 7500xg for 3 minutes at 4°C. The supernatant was removed and the pellet allowed to air dry for 10 minutes at room temperature. The RNA samples were then re-suspended in 1 μl of sterile, RNase free distilled water per 1 mg of tissue. The RNA concentration was measured using a spectrophotometer (BioSpec-Mini; Mandel). Isolated RNA was stored at -80°C until required.

2.7.2 Reverse Transcription PCR (RT PCR)

Isolated RNA was diluted to 0.5 μg in Nuclease-free water (Ambion). The diluted RNA (15 μl) was combined with 1.25 μl of Oligo (dT) primers (250 ng/μl) for a total reaction mixture of 16.25 μl. The reaction mixture was heated to 65°C for 5 minutes and allowed to cool to 25°C for 10 minutes. The program was then paused and 8.75 μl per reaction of RT Master Mix (RNase OUT, 10mM DTT, 1mM deoxyribonucleotide triphosphate (dNTP's), 10% Affinity Script buffer, 2% Affinity Script RT) was added. Once placed back in the PCR machine the reaction was continued for 1 hour at 42°C, 5 minutes at 95°C and then paused at 10°C. Once the final pause stage was reached, samples were removed and placed at -20°C overnight.

2.7.3 Polymerase Chain Reaction (PCR)

To determine the sequences for the NOS1AP isoforms, cDNA from the RT-PCR step was subjected to PCR using specific primers designed in the lab. The Expand High Fidelity PCR System from Roche (Cat. No. 11-732-650-001) was used for the PCR. The cDNA (4 μl) was combined with 2μl of dNTP's, 5 μl of Buffer 2, 2 μl of Buffer 4 (MgCl2; 2.5mM final concentration), 1 μl of Taq, 32 μl of double processed tissue culture water and 2 μl of each primer:

1. 5': TTTGCTTCGAATTCTGCAATGCCCAGCAAAACCAAGTAC
2. 3': TTTAGCGTCGACTGCCTACTCAAAGGACAGCAG

The total reaction mixture (50 μl) was subject to PCR reaction (BioRad MyCycler) as follows: 95°C for 3 minutes, [95°C for 45sec, 58°C for 45sec, 72°C for 2.5 minutes] x 35 cycles, 72°C for 10 minutes followed by storage at 4°C.

After completing a 5'Race kit (Roche; refer to Peter Dixon, 4th year honours thesis, Department of Biochemistry and Molecular Biology, Dalhousie University, 2010 for method used) and determining that there is the potential for another NOS1AP isoform, we generated another set of primers. The Expand High Fidelity PCR System was also used for these primer sets. The following primer sequences containing sites for EcoR1 (in the 5' sequence) and Sal1 (in the 3' sequence) were used:

1. 5': TTTGCTTCGAATTCTATGTGTCTTTTTCTTATTTG
2. 3': TTTAGCGTCGACTGCCTACTCAAAGGACAGCAG

The cDNA obtained from the RT-PCR step (2 μl) was combined with 1 μl of dNTP's (), 1 μl of each primer, 2.5 μl of Buffer 2 (with 15mM MgCl2), 0.5 μl of Buffer 4 (with 25mM MgCl2), 0.5 μl of Taq (Buffer 1), and 16.5 μl of double processed tissue culture water for a total reaction mixture of 25 μl. The total reaction mixture was subject to PCR reaction (BioRad MyCycler) as follows: 95°C for 2 minutes, [95°C for 45sec, 56°C for 45sec, 72°C for 1.5 minutes] x 35 cycles, 72°C for 10 minutes followed by storage at 4°C. The resulting PCR product was then sent for sequencing through the genomics company Macrogen.

CHAPTER 3 RESULTS

3.1 IDENTIFYING CHARACTERISTICS OF NOS1APc

The work included in this thesis is an extension of the work completed by Richier et al., (2010) and unpublished work completed by Michael O'Brien in Dr. Fawcett's lab. Richier et al., (2010) conducted a proteomic screen to identify interacting partners for the tumour suppressor protein scribble (scrib). The screen identified a number of novel targets including nitric oxide synthase 1 adaptor protein, (NOS1AP), also known as carboxy terminal PDZ ligand of nNOS, (CAPON). NOS1AP (herein NOS1APa) is a 70kDa adaptor protein that binds directly to neuronal nitric oxide synthase (nNOS) and regulates NO activity (Jaffrey et al., 1998). The screen also identified a second slower migrating NOS1AP isoform (NOS1APc) with an apparent molecular weight of 100kDa. NOS1APc contains the N-terminal PTB domain found in NOS1APa but has a unique 30kDa C-terminal extension that destroys the PDZ binding motif found in NOS1APa (Richier et al., 2010).

To better characterize the NOS1APc isoform, I precipitated rat brain lysate using two different NOS1APc isoform specific antibodies [a peptide antibody (PPIT-NOS1APc) or a GST fusion antibody, (GST-NOS1APc)] or a pan-NOS1AP antibody (created against a region common to NOS1APa and NOS1APc). The resulting western blots were probed with either the GST-NOS1APc (Figure 3.1A) or the PPIT-NOS1APc (Figure 3.1B) antibody. A band migrating at 100kDa was detected in the pan-NOS1AP, GST-

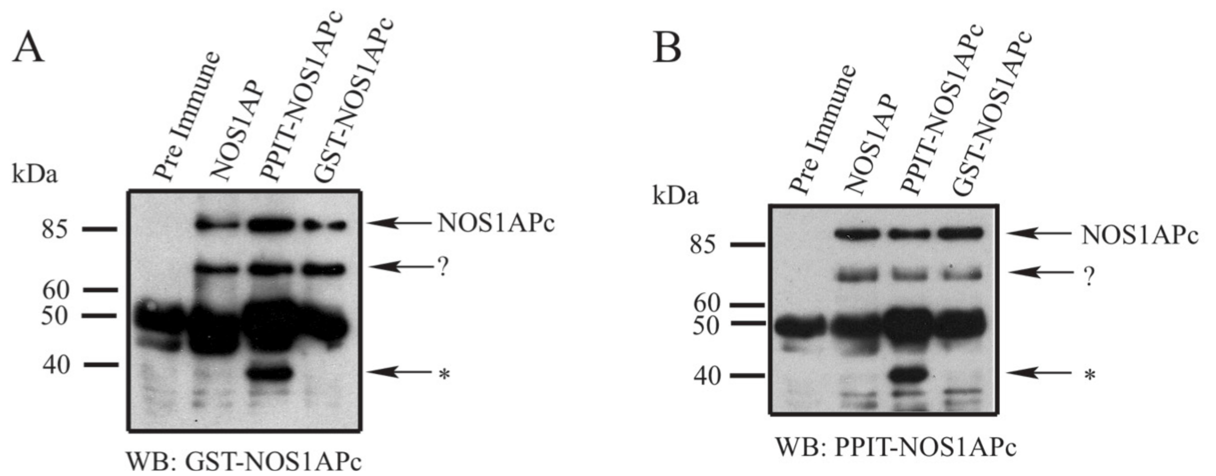

Figure 3.1 Identifying characteristics of NOS1APc. (A,B) NOS1APa and NOS1APc were precipitated (ppt) from rat brain lysate using antibodies as identified. Pre-immune serum was included as a negative control. The samples were subjected to Western blot analysis and probed with either the (A) GST-NOS1APc or (B) PPIT-NOS1APc antibody. The NOS1APc protein precipitated by all three antibodies and detected by both NOS1APc isoform specific antibodies (arrow in A/B). Interestingly, a faster migrating band at 70kDa was ppt by all three antibodies and detected by both NOS1APc antibodies (arrow with question mark in A/B). A 40kDa band was ppt by the PPIT-NOS1APc antibody and detected by both the NOS1APc isoform Ab’s (arrow with asterisk).

NOS1APc and PPIT-NOS1APc immunoprecipitations (IP's) corresponding to the expected size of NOS1APc (Figure 3.1A and 3.1B, arrow indicating NOS1APc).

Interestingly, both the NOS1APc isoform specific antibodies and the pan-NOS1AP antibody IP'd a faster migrating (70kDa) band (Figure 3.1A and 3.1B, arrow with question mark). Finally, a 40kDa band was detected. This was unique to the PPIT-NOS1APc IP (Figure 3.1A and 3.1B, arrow with astric). Taken together, these data suggest that NOS1APc contains sequences common to NOS1APa. Further, the fact that multiple bands were detected with both NOS1APc isoform specific antibodies raises the possibility that other NOS1AP isoforms containing the unique C-terminal region found in NOS1APc exist. However, I cannot rule out that the 70kDa and 40kDa bands represent degradation products. Future work will need to be completed to identify the 70kDa and 40kDa bands.

3.2 CLONING NOS1APc

The presence of faster migrating bands detected by the NOS1APc isoform specific antibodies raised the possibility that other splice variants of *NOS1AP* may exist. With the help of Maggie Qi in the lab, we set out to determine if there were novel splice variants. As a first step, a cDNA library was generated from adult rat cerebellum. This was used to amplify NOS1APc. Richier, et al., (2010), had pieced together the NOS1APc sequence bioinformatically based on mass spectrometry hits. To confirm this sequence, we first cloned NOS1APc using 5' and 3' primers based on the Richier, et al., (2010) NOS1APc sequence. A number of positive clones were identified and sequenced. Surprisingly, the amplified NOS1APc clones were different from that published by

Richier, et al, (2010) (Note: all of the NOS1APc clones selected and sequenced gave the same sequence).

Specifically, the junction between NOS1APa and the unique C-terminal region of NOS1APc was different from that published in (Richier et al., 2010) (Figure 3.2). In the published sequence the last exon of NOS1APa ends in (– GEPLLGGLELIKFRESGIA SEYESNTDESEERDSWSQEELPRLLNVLQRQELGDSLDDEIAV) and is spliced with PMAQ in the previous exon (Figure 3.2). However, in the cloned sequence from rat cerebellum, the last exon in NOS1APa is deleted and the PMAQ sequence is spliced with VDHSMFEN of NOS1APc. When examining exon-intron boundaries in the genomic sequence this new splicing fits with known splice acceptor and donor sequences.

While a number of the clones amplified and sequenced were NOS1APc, a number of other clones had deletions of other exons in the NOS1APa sequence, yet maintained the unique C-terminal region found in NOS1APc (Figure 3.3). These new isoforms have been named NOS1APd and NOS1APe (Figure 3.3). In particular, NOS1APd and NOS1APe contain the novel 30kDa C-terminal extension found in NOS1APc, however, NOS1APd and e have exon 10 deleted between the PTB domain and the C-terminal extension. The deleted exon is underlined in the NOS1APc sequence (Figure 3.2). Further, NOS1APe is similar to NOS1APd but contains a short 5 amino acid insert, leucine-leucine-leucine-leucine-glutamine, (LLLLQ), in the PTB domain.

NOS1APa

MPSKTKYNLVDDGHDLRIPLHNEDAFQHGISFEAKYVGSLDVPRPNSRVEIVAAMRRIRY
EFKAKNIKKKKVSIMVSVDGVKVILKKKKKKKEWTWDESKMLMQDPIYRIFYVSHDSQDL
KIFSYIARDGASNIFRCNVFKSKKKSQAMRIVRTVGQAFEVCHKLSLQHTQQNADGQEDGE
SERNSDGSGDPGRQLTGAERVSTATAEETDIDAVEVPLPGNDILEFSRGVTDLDAIGKDGG
SHIDTTVSPHPQEPMLAASPRMLLPSSSSSKPPGLGTGTPLSTHHQMQLLQQLLQQQQQQ
TQVAVAQVHLLKDQLAAEAAARLEAQARVHQLLLQNKDMLQHISLLVKQVQELELKLSG
QSTMGSQDSLLEITFRSGALPVLCESTTPKPEDLHSPLLGAGLADFAHPVGSPLGRRDCLV
KLECFRFLPAEDNQPMAQ**GEPLLGGLELIKFRESGIASEYESNTDESEERDSWSQEELPR
LLNVLQR QELGDSLDDEIAV**

NOS1APc

MPSKTKYNLVDDGHDLRIPLHNEDAFQHGISFEAKYVESLDVPRPNSRVEIVAAMRRIRYE
FKAKNIKKKKVSIMVSVDGVKVILKKKKKKKEWTWDESKMLVMQDPIYRIFYVSHDSQD
LKIFSYIARDGASNIFRCNVFKSKKKSQAMRIVRTVGQAFEVCHKLSLQHTQQNADGQGDG
ESERDSDGSGDPGRQLTGAERVSTATAEETDIDAVEVPLPGNDILEFSRGVTDLDAIGKDG
GSHIDATVSPHPQEPMLAASPRMLLPSSSSSKPPGLGTGTPLSTHHQTQLLQQLLQQQQQ
QTQVAVAQVHLLKDQLAAEAAARLEAQARVHQLLLQNKDMLQHISLLVKQVQELELKLS
GQSTMGSQDSLLEITFRSGALPVLCESTTPKPEDLHSPLLGAGLADFAHPVGSPLGRRDCL
VKLECFRFLPAEDNQPMAQVDHSMFENLNTTLTPKLQSSHSFPHLSRPGAPGTITPGSGE
PGGPGLRVGSSQHLRNLGKAVGAKVNDLLRRKESSSLGSVGVMEINKTAEAQMPGGEDAA
CGPWLEDERSVQEAFPLLDPPPPITRKRTPRALKTTQDMLISSQPVLSNLEYGTELSPGQA
QDSPPTAQPVSADTSRPESTTGMGEKGEALPNGEVSLLVPDLIHKNSQEESKRKATEGRK
SSSPGPIERNGLKLSLSPISLAESWENSSPPLQARTSSLENEGLHPDLLSFE

Figure 3.2 Sequences of NOS1APa and NOS1APc. The complete peptide sequences of NOS1APa and NOS1APc are shown for comparison to one another. The region coloured in red indicates the peptide sequence extension unique to NOS1APc. The sequences common to both NOS1APa and NOS1APc are shown in black. The underlined portion of the sequence in NOS1APa is the portion of sequence originally published by Richier et al., (2010) for NOS1APc. The new NOS1APc clone does not share the underlined portion of sequence with NOS1APa.

NOS1APd

MPSKTKYNLVDDGHDLRIPLHNEDAFQHGISFEAKYVESLDVPRPNSRVEIVAA
MRRIRYEFKAKNIKKKKVSIMVSVDGVKVILKKKKKKEWTWDESKMLVMQD
PIYRIFYVSHDSQDLKIFSYIARDGASNIFRCNVFKSKKKSQAMRIVRTVGQAFEV
CHKLSLQHTQQNADGQGDGESERDSDGSGDPGRQLTGAERVSTATAEETDIDAV
EVPLPGNDILEFSRGVTDLDAIGKDGGSHIDTTVSPHPQEPMLAASPRMLLPSSSS
SKPPGLGTGTPLSTHHQMQLLQQLLQQQQQQTQVAVAQVHLLKDQLAAEAAAR
LEAQARVHQLLLQNKDMLQHISLLVKQVQELELKLSGQSTMGSQDSLLEITFRSG
ALPVLCESTTPKPEDLHSPLLGAGLADFAHPVGSPLVDHSMFENLNTTLTPKLQS
SHSFPHLSRPGAPGTITPGSGEPGGPGLRVGSSQHLRNLGKAVGAKVNDLLRRKE
SSSLGSVGVMEINKTAEAQMPGGEDAACGPWLEDERSVQEAFPLLDPPPPITRKR
TPRALKTTQDMLISSQPVLSNLEYGTELSPGQAQDSPPTAQPVSADTSRPESTTG
MGEKGEALPNGEVSLLVPDLIHKNSQEESKRKATEGRKSSSPGPIERNGLKLSLSP
ISLAESWENSSPPLQARTSSLENEGLHPDLLSFE

NOS1APe

MPSKTKYNLVDDGHDLRIPLHNEDAFQHGISFEAKYVGSLDVPRPNSRVEIVAA
MRRIRYEFKAKNIKKKKVSIMVSVDGVKVILKKKKKLLLLQKKEWTWDESKML
VMQDPIYRIFYVSHDSQDLKIFSYIARDGASNIFRCNVFKSKKKSQAMRIVRTVG
QAFEVCHKLSLQHTQQNADGQEDGESERNSDGSGDPGRQLTGAERVSTATAEET
DIDAVEVPLPGNDILEFSRGVTDLDAIGKDGGSHIDTTVSPHPQEPMLAASPRMLL
PSSSSSKPPGLGTGTPLSTHHQMQLLQQLLQQQQQQTQVAVAQVHLLKDQLAAE
AAARLEAQARVHQLLLQNKDMLQHISLLVKQVQELELKLSGQSTMGSQDSLLEI
TFRSGALPVLCESTTPKPEDLHSPLLGAGLADFAHPVGSPLVDHSMFENLNTTLT
PKLQSSHSFPHLSRPGAPGTITPGSGEPGGPGLRVGSSQHLRNLGKAVGAKVNDL
LRRKESSSLGSVGVMEINKTAEAQMPGGEDAACGPWLEDERSVQEAFPLLDPPP
PITRKRTPRALKTTQDMLISSQPVLSNLEYGTELSPGQAQDSPPTAQPVSADTSRP
ESTTGMGEKGEALPNGEVSLLVPDLIHKNSQEESKRKATEGRKSSSPGPIERNGL
KLSLSPISLAESWENSSPPLQARTSSLENEGLHPDLLSFE

Figure 3.3 Sequences of NOS1APd and NOS1APe. The complete peptide sequences of NOS1APd and NOS1APe are shown for comparison to one another. The region coloured in red indicates the peptide sequence extension unique to NOS1APc as well as NOS1APd and NOS1APe. The NOS1APe peptide sequence contains a LLLLQ insert in the first section that contains the PTB domain. The LLLLQ insert is coloured in blue.

In addition to NOS1APd and e, a short novel isoform has been deposited into NCBI (accession #NP_001177388). We obtained PCR primers that would amplify this unique cDNA and amplified it from our rat cerebellar cDNA library. We were able to confirm the sequence identified by NCBI. We have re-named this NOS1APf (Figure 3.4).

The predicted molecular weight of NOS1APf is 40kDa. This migrates close to the molecular weight as the fast migrating band seen in the PPIT-NOS1APc IP (Figure 3.1A/B, see arrowhead with *). Taken together, we have further refined the NOS1APc sequence and have identified 3 potential novel transcripts for NOS1AP (see Figure 3.5 for isoform comparison). Since NOS1APc, d and e are all very similar, it will be difficult to generate isoform specific antibodies to NOS1APd and NOS1APe. Nonetheless, quantitative PCR (qPCR) analysis using isoform specific primers for NOS1APa, NOS1APc and NOS1APf show that the levels of NOS1APf are significantly lower than that of NOS1APa (the original NOS1AP) or NOS1APc (data not shown). Future work will determine the level of NOS1APd and NOS1APe transcripts in different tissues however, anecdotally given the majority of clones from our cDNA library were identified as NOS1APc, we believe the levels of NOS1APd and e mRNA are low. Whether these novel isoforms generate protein remains to be determined.

3.3 LOCALIZATION OF THE NOS1AP ISOFORMS

Since we have identified two main NOS1AP isoforms, namely NOS1APa and NOS1APc, and NOS1APa has been shown to associate with a number of proteins including nNOS,

NOS1APc

MPSKTKYNLVDDGHDLRIPLHNEDAFQHGISFEAKYVESLDVPRPNSRVEIVAA
MRRIRYEFKAKNIKKKKVSIMVSVDGVKVILKKKKKKKEWTWDESKMLVMQD
PIYRIFYVSHDSQDLKIFSYIARDGASNIFRCNVFKSKKKSQAMRIVRTVGQAFEV
CHKLSLQHTQQNADGQGDGESERDSDGSGDPGRQLTGAERVSTATAEETDIDAV
EVPLPGNDILEFSRGVTDLDAIGKDGGSHIDATVSPHPQEPMLAASPRMLLPSSSS
SKPPGLGTGTPLSTHHQTQLLQQLLQQQQQTQVAVAQVHLLKDQLAAEAAAR
LEAQARVHQLLLQNKDMLQHISLLVKQVQELELKLSGQSTMGSQDSLLEITFRSG
ALPVLCESTTPKPEDLHSPLLGAGLADFAHPVGSPLGRRDCLVKLECFRFLPAED
NQPMAQVDHSMFENLNTTLTPKLQSSHSFPHLSRPGAPGTITPGSGEPGGPGLRV
GSSQHLRNLGKAVGAKVNDLLRRKESSSLGSVGVMEINKTAEAQMPGGEDAAC
GPWLEDERSVQEAFPLLDPPPPITRKRTPRALKTTQDMLISSQPVLSNLEYGTELS
PGQAQDSPPTAQPVSADTSRPESTTGMGEKGEALPNGEVSLLVPDLIHKNSQEES
KRKATEGRKSSSPGPIERNGLKLSLSPISLAESWENSSPPLQARTSSLENEGLHPDL
LSFE

NOS1APf

MCLFLICFPPAFEPFSRTVDHSMFENLNTTLTPKLQSSHSFPHLSRPGAPGTITPGS
GEPGGPGLRVGSSQHLRNLGKAVGAKVNDLLRRKESSSLGSVGVMEINKTAEA
QMPGGEDAACGPWLEDERSVQEAFPLLDPPPPITRKRTPRALKTTQDMLISSQPV
LSNLEYGTELSPGQAQDSPPTAQPVSADTSRPESTTGMGEKGEALPNGEVSLLVP
DLIHKNSQEESKRKATEGRKSSSPGPIERNGLKLSLSPISLAESWENSSPPLQARTS
SLENEGLHPDLLSFE

Figure 3.4 Sequences of NOS1APc and NOS1APf. The complete peptide sequences of NOS1APc and NOS1APf are shown for comparison to one another. The region coloured in red indicates the peptide sequence extension unique to NOS1APc, d, e and f. The N-terminal peptide sequence of NOS1APf is short and unique to that peptide.

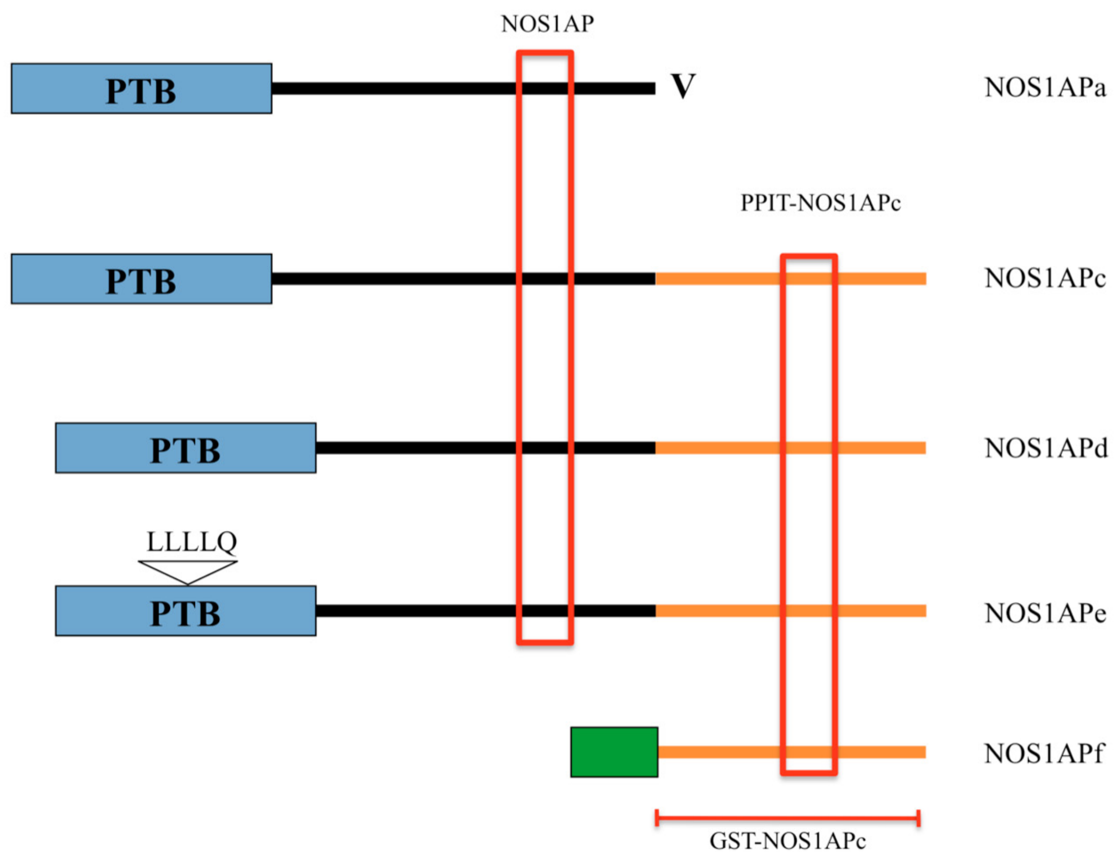

Figure 3.5 NOS1AP Isoforms. A diagram representing the NOS1AP isoforms currently identified. The PTB domain is common to NOS1APa/c/d/e however, NOS1APe contain an insert, LLLLQ. The 30kDa C-terminal extension common to NOS1APc/d/e/f is shown in orange. The NOS1APf isoform contains a unique N-terminal region shown in green. The pan-NOS1AP antibody is raised against the area boxed in red with the pan-NOS1AP label. The PPIT-NOS1APc antibody is raised against the boxed region labeled NOS1APc and the GST-NOS1APc antibody is raised against the sequence of the C-terminal extension shown in orange.

Synapsin1, Dexras1, Scribble, Carboxypeptidase E, and VANGL1, this raised the question of whether all known NOS1APa interacting proteins could also associate with NOS1APc.

As a way to characterize potential differences in the isoforms, I transfected HEK 293T cells with cDNA's that encode each of the NOS1AP isoforms and determined if they had different sub-cellular localizations. In particular, HEK 293T cells were transfected with cDNA's that encoded yellow fluorescent protein (YFP) fused in frame either with each of the NOS1AP isoforms. Forty-eight hours following transfection the cells were fixed and then imaged using confocal microscopy (Figure 3.6). NOS1APa localized at or near cell membranes (Figure 3.6, first panel). NOS1APc, d and f expression was observed within the cytoplasm and some expression was enriched in small puncta within the cell (Figure 3.6, second, third and fifth panel). Interestingly, the NOS1APe YFP-fusion protein localized at or near the membrane, similar to NOS1APa (Figure 3.6, fourth panel). Since the only difference between NOS1APd and e is a 5 amino acid insert (LLLLQ) in the PTB domain, this suggests that this small insert targets the protein to the membrane in HEK 293T cells. Further experiments are required to determine if the 5 amino acid insert is responsible for the localization cell membrane.

3.4 NOS1APc DOES NOT INTERACT WITH nNOS

The identification of novel NOS1AP isoforms and differences in their localization within the cell led me to determine if known binding partners of NOS1APa are able to bind with any or all of the novel NOS1AP isoforms as well. NOS1APa binds neuronal nitric oxide

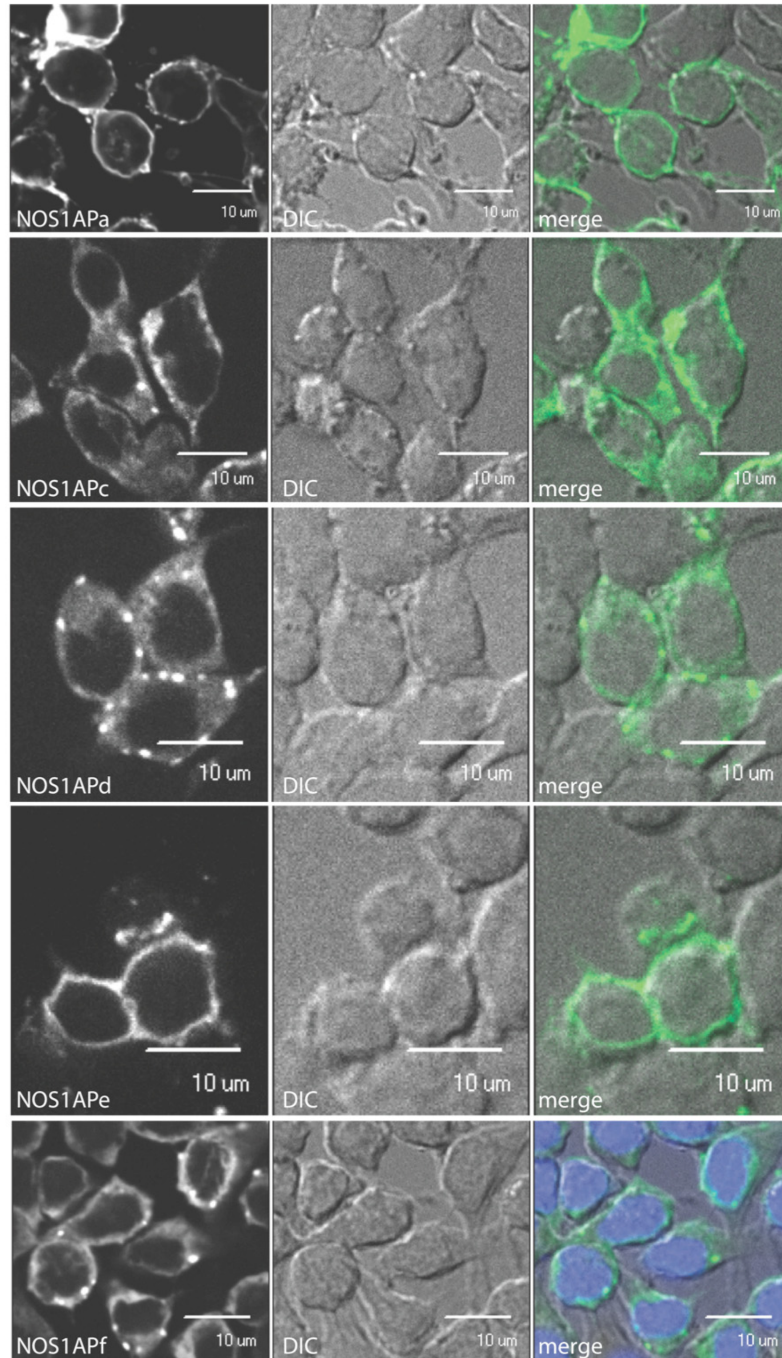

Figure 3.6 Localization of NOS1AP isoforms. The HEK 293T cells grown on cover slips were transfected with either the YFP-tagged NOS1APa, c, d, e or f construct, left 48 hours at 37°C and then fixed using paraformaldehyde. The cells were imaged using a confocal microscope. Results show NOS1APa and NOS1APe localized to the cell membrane (first and fourth panel, respectively). The NOS1APc, NOS1APd and NOS1APf isoforms appear to be localized to the cytoplasm of the cell with the appearance of small puncta (second, third and fifth panel, respectively).

synthase, (nNOS) via its C-terminal PDZ binding motif (glutamic acid-isoleucine-alanine-valine; E-I-A-V) (Jaffrey et al., 1998). Since the novel C-terminal extension in NOS1APc destroys the PDZ binding motif, I wanted to confirm that NOS1APc would not interact with nNOS. Here, I IP'd endogenous NOS1APa and NOS1APc, as well as scrib, and probed for nNOS binding. As expected, the 170kDa nNOS protein was precipitated by the pan-NOS1AP and scrib antibodies, but not with the Pre Immune or NOS1APc isoform specific antibodies (Figure 3.7A, second panel). Re-probing the blot with scrib confirmed that both NOS1APa and NOS1APc interacted with scrib (Figure 3.7A, first panel). A final re-probe using the NOS1APc peptide antibody revealed NOS1APa, NOS1APc and scrib IP's could all IP NOS1APc (Figure 3.7A, third panel, arrow). These results indicate that the unique extension in the C-terminus of NOS1APc eliminates the interaction with nNOS.

3.5 NOS1AP ISOFORMS INTERACT WITH SCRIBBLE

Previously, the lab identified an interaction between scrib and NOS1APa. Futher, they showed that the PTB domain of NOS1APa was responsible for the interaction with the fourth PDZ domain of scrib (Richier et al., 2010). Since the PTB domain of NOS1APa is responsible for the interaction with scrib, I wanted to determine if the other NOS1AP isoforms, namely NOS1APc, d, e and f, were also able to interact with scrib. For this, I co-transfected HEK 293T cells with cDNA's encoding myc-scrib along with constructs encoding either YFP-NOS1APa, c, d, e, f or YFP alone. Forty-eight hours post-transfection, clarified lysates were precipitated with a green fluorescent protein (GFP) antibody. The resulting blots were probed with a Myc antibody (Figure 3.7B and C).

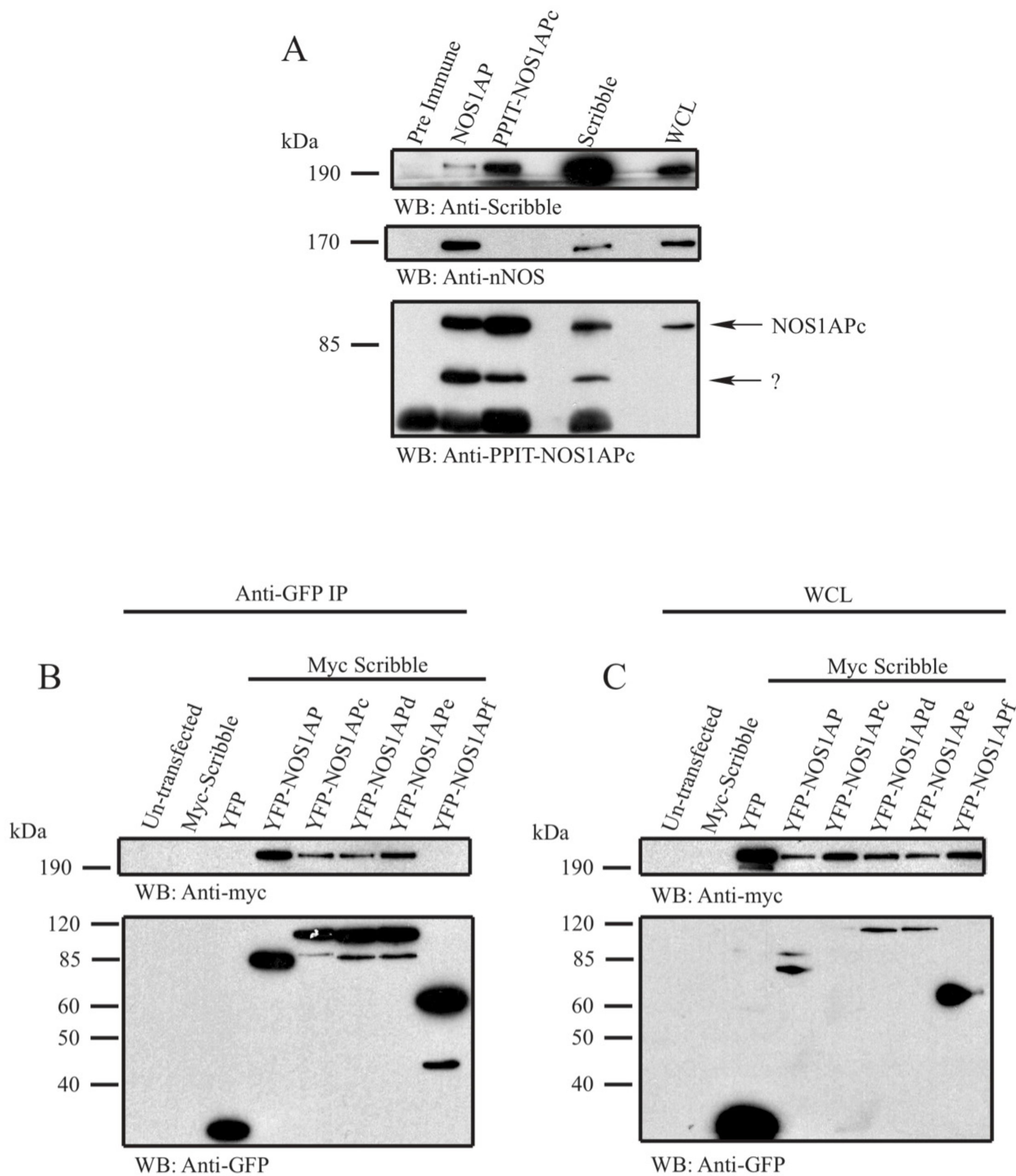

Figure 3.7 The interactions between NOS1APc, nNOS and Scribble. (A) Rat brain lysate was IP'd with pan-NOS1AP, PPIT-NOS1APc, and Scribble antibodies. Results show NOS1APa and Scribble are capable of precipitating nNOS but not NOS1APc. (B) YFP-tagged NOS1AP isoforms were co-transfected with myc-Scribble in HEK 293T cells, lysed and IP'd with a GFP antibody. The samples were subjected to Western blot analysis and probed with myc and GFP antibodies respectively. Results indicate NOS1APa, c, d and e can precipitate Scribble. (C) The whole cell lysate indicates that the proteins were over-expressed in the cells.

Scrib associated with NOS1APa, c, d and e but not NOS1APf (Figure 3.7B). The lack of interaction between scrib and NOS1APf was expected since NOS1APf does not contain the PTB domain.

3.6 NOS1APc DOES NOT INTERACT WITH GIT1 AND β-PIX

Since I had shown that scrib could interact with all the NOS1AP isoforms containing the PTB domain, yet only NOS1APa interacted with nNOS, this raised the possibility that NOS1APa and scrib may exist in different protein-protein complex than a NOS1APc and scrib complex. This is important in light of previous studies that showed scrib was necessary to bridge an interaction between NOS1APa and βPIX/GIT1, while a more recent publication showed that NOS1APa and scrib associated with VANGL1 but not β-PIX and GIT1 (Anastas et al., 2011). One possibility is that NOS1APa may interact with βPIX and GIT1 while NOS1APc interacts with VANGL1. To determine if NOS1APc precipitates β-PIX and GIT1, I immunoprecipitated rat brain lysate with either the pan-NOS1AP Ab or the two NOS1APc isoform specific Ab's. Scrib and β-PIX or GIT1 IP's were included as controls. As previously reported, the pan-NOS1AP IP showed an association with GIT1 (Figure 3.8A) and β-PIX (Figure 3.8B); however, neither of the NOS1APc isoform specific antibodies showed an association with GIT1 or β-PIX. Since the pan-NOS1AP Ab detects both NOS1APa and NOS1APc, this suggests that NOS1APc fails to interact with βPIX or GIT1. These results add support to the possibility that NOS1APa and scrib exist in a complex with GIT1 and β-PIX while NOS1APc and scrib are a part of another complex.

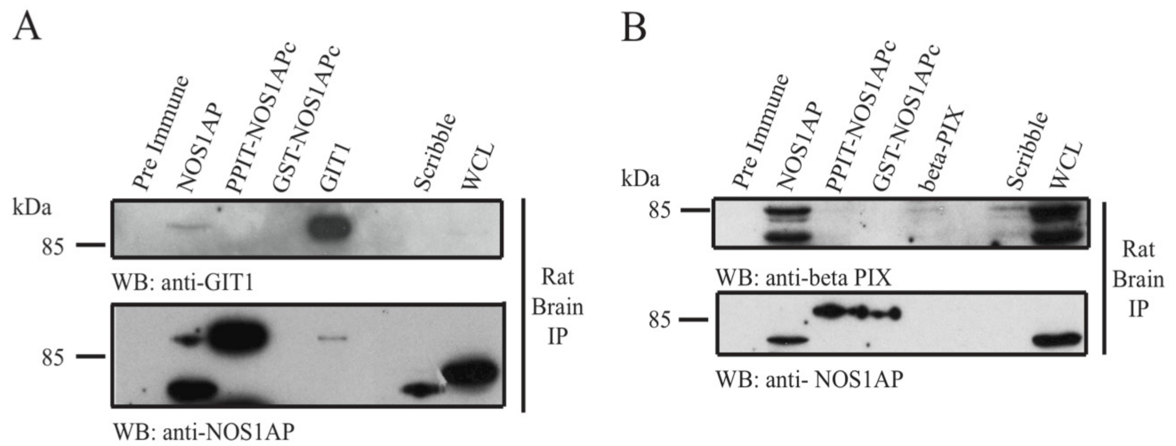

Figure 3.8 Interactions between NOS1APa and GIT1/β-PIX. (A) Rat brain lysate was immunoprecipitated with pan-NOS1AP, PPIT-NOS1APc, GST-NOS1APc, Scribble and GIT1 antibodies. The resulting blot was probed with GIT1 followed by pan-NOS1AP. Results indicate NOS1APa but not NOS1APc can precipitate GIT1. (B) Rat brain lysate was immunoprecipitated with pan-NOS1AP, PPIT-NOS1APc, GST-NOS1APc, Scribble and β-PIX antibodies. The resulting blot was probed with β-PIX followed by pan-NOS1AP. Results indicate that NOS1APa but not NOS1APc can precipitate β-PIX. These results taken together indicate NOS1APc is involved in an alternate signaling complex.

3.7 NOS1AP ISOFORMS INTERACT WITH PYRUVATE CARBOXYLASE

Because NOS1APc appears to associate in a separate complex from NOS1APa, I next wanted to determine if there were other unique binding partners between NOS1APa and NOS1APc. To address this, a previous student completed a mass spectrometery screen using the GST-NOS1APc antibody that targets the 30kDa C-terminal extension of NOS1APc. The results of the mass spectrometry screen indicated a potential interaction with three different proteins: transcytosis associating protein p115 (p115), tuberous sclerosis-1 (TSC-1) and pyruvate carboxylase (PCB).

To determine if the proteins identified by the mass spectrometry screen were legitimate binding partners, I set out to validate whether they could interact with NOS1APc. In summary, neither TSC-1 nor p115 co-precipitated with NOS1APc (data not shown). Interestingly, NOS1APc was able to precipitate PCB (Figure 3.9A/B/C/D), however PCB was not able to precipitate NOS1APc (Figure 3.9A/B/C/D).

Since endogenous NOS1APc interacted with PCB, I next wanted to determine which, if any, of the NOS1AP isoforms could interact with PCB. For this, I co-transfected HEK 293T cells with a cDNA encoding PCB along with each of the constructs encoding either YFP-NOS1APa, c, d, e, f or YFP alone. Interestingly, only NOS1APc, d and f precipitated PCB (Figure 3.10A/B). The functional significance of this interaction remains to be determined.

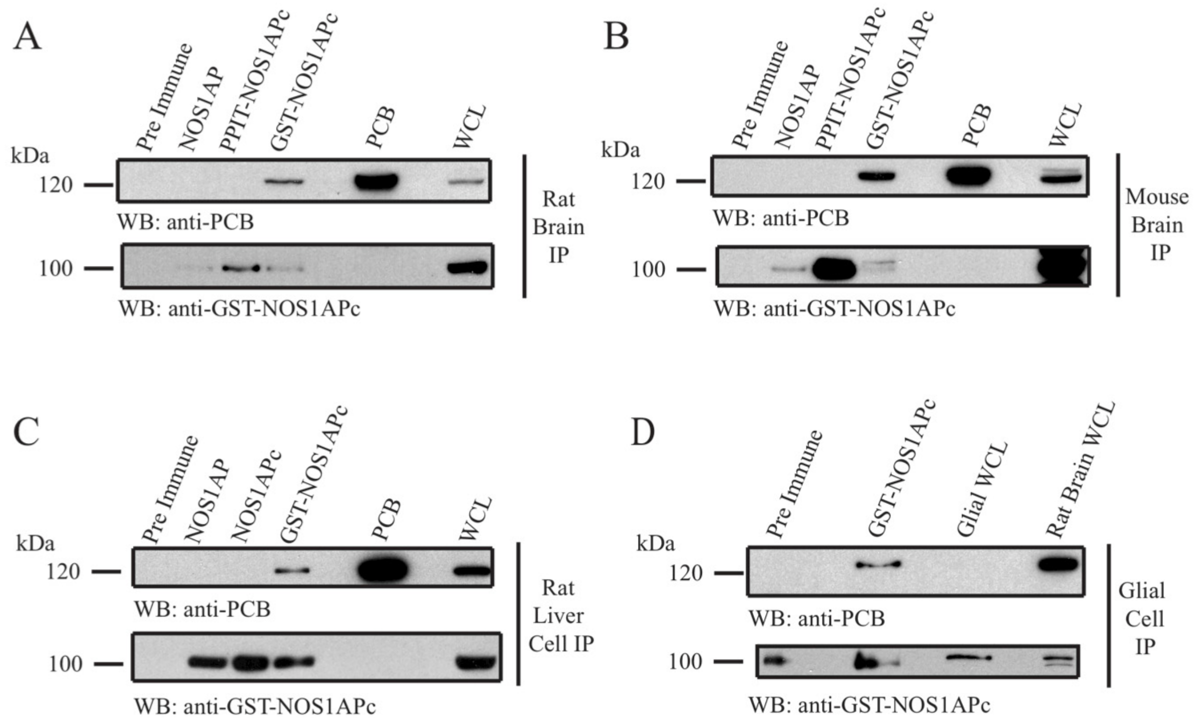

Figure 3.9 The NOS1APc isoform interacts with pyruvate carboxylase. (A) The endogenous NOS1APa, NOS1APc and pyruvate carboxylase (PCB) proteins were precipitated from rat brain lysate using specific antibodies. The samples were subjected to Western blot analysis and probed with a PCB and GST-NOS1APc antibody respectively. Results indicate that GST-NOS1APc but not NOS1APc can precipitate the PCB protein endogenously. The same experiment was completed in (B) mouse brain, (C) rat liver cells and (D) glial cells. The results for the mouse brain, rat liver cells and glial cells were the same showing GST-NOS1APc IP's able to precipitate the PCB protein but not the reverse.

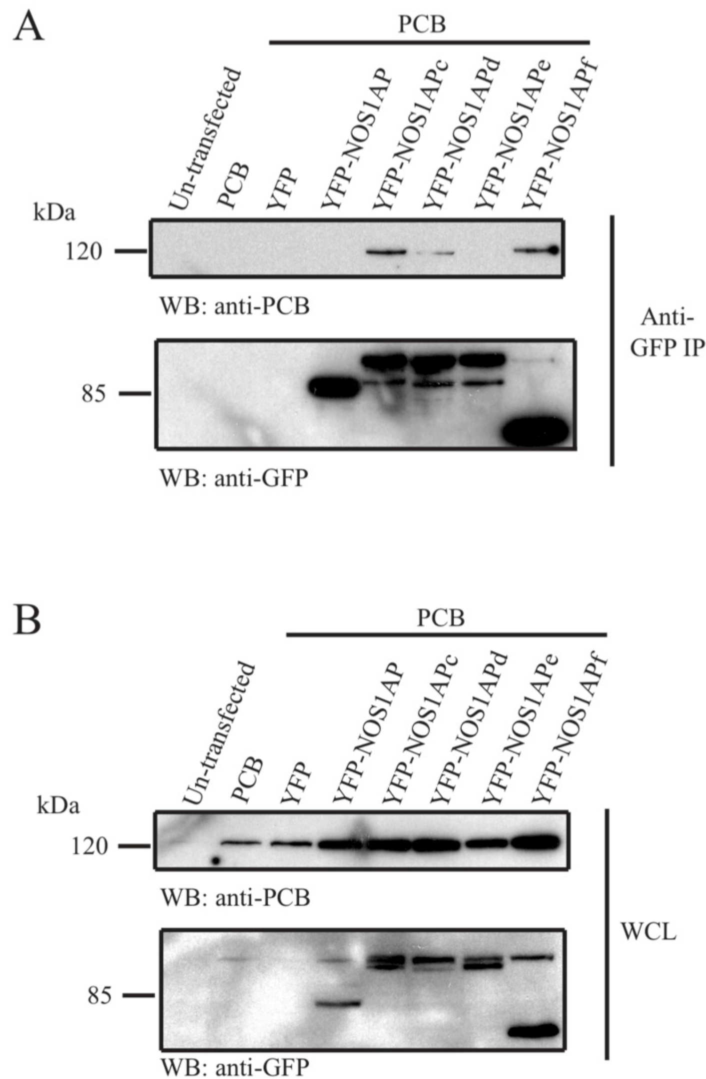

Figure 3.10 The NOS1AP isoforms interact with pyruvate carboxylase. (A) YFP-tagged NOS1AP isoforms were co-transfected with un-tagged PCB in HEK 293T cells, lysed and IP'd with a GFP antibody. The samples were subjected to Western blot analysis and probed with PCB and GFP antibodies respectively. Results indicate NOS1APc, d and f can precipitate PCB. The GFP re-probe of the blot indicates all YFP constructs were expressed. (B) The whole cell lysate indicates that the proteins were expressed in the cells.

3.8 NOS1APc INTERACTS WITH AN EPHRIN-B LIGAND

Previous work from the Fawcett lab had revealed a unique distribution of NOS1APc in the developing rat embryo. In particular, NOS1APc staining was seen in the midline of the developing and adult spinal cord similar to ephrinB3 ((Peter Dixon, 4th year honours thesis, Department of Biochemistry and Molecular Biology, Dalhousie University, 2010). Since NOS1APc and ephrinB3 co-localize in midline structures, I next wanted to test whether the two proteins could interact. Here, I precipitated endogenous NOS1APc from rodent brain lysate and probed with a pan-ephrin antibody. Consistent with the immunohistochemistry data, endogenous ephrinB was able to precipitate NOS1APc (Figure 3.11A, bottom panel). As well, NOS1APc was able to precipitate ephrinB (Figure 3.11A, top panel). To more directly test which NOS1AP isoform could interact with ephrinB3, I co-transfected constructs encoding YFP-NOS1APa, c or d with a construct encoding flag-ephrinB3. The resulting lysates were precipitated with GFP antibodies and probed with anti-flag or the pan-ephrinB antibody. Interestingly, ephrinB3 was precipitated with the NOS1APc and d isoforms but not NOS1APa (Figure 3.11B/C). Taken together, these data suggest a unique and novel interaction between NOS1APc and ephrinB3. The functional consequence of this interaction will need to be determined. Nonetheless, they further support that NOS1APa and NOS1APc function in two distinct cellular complexes within the cell.

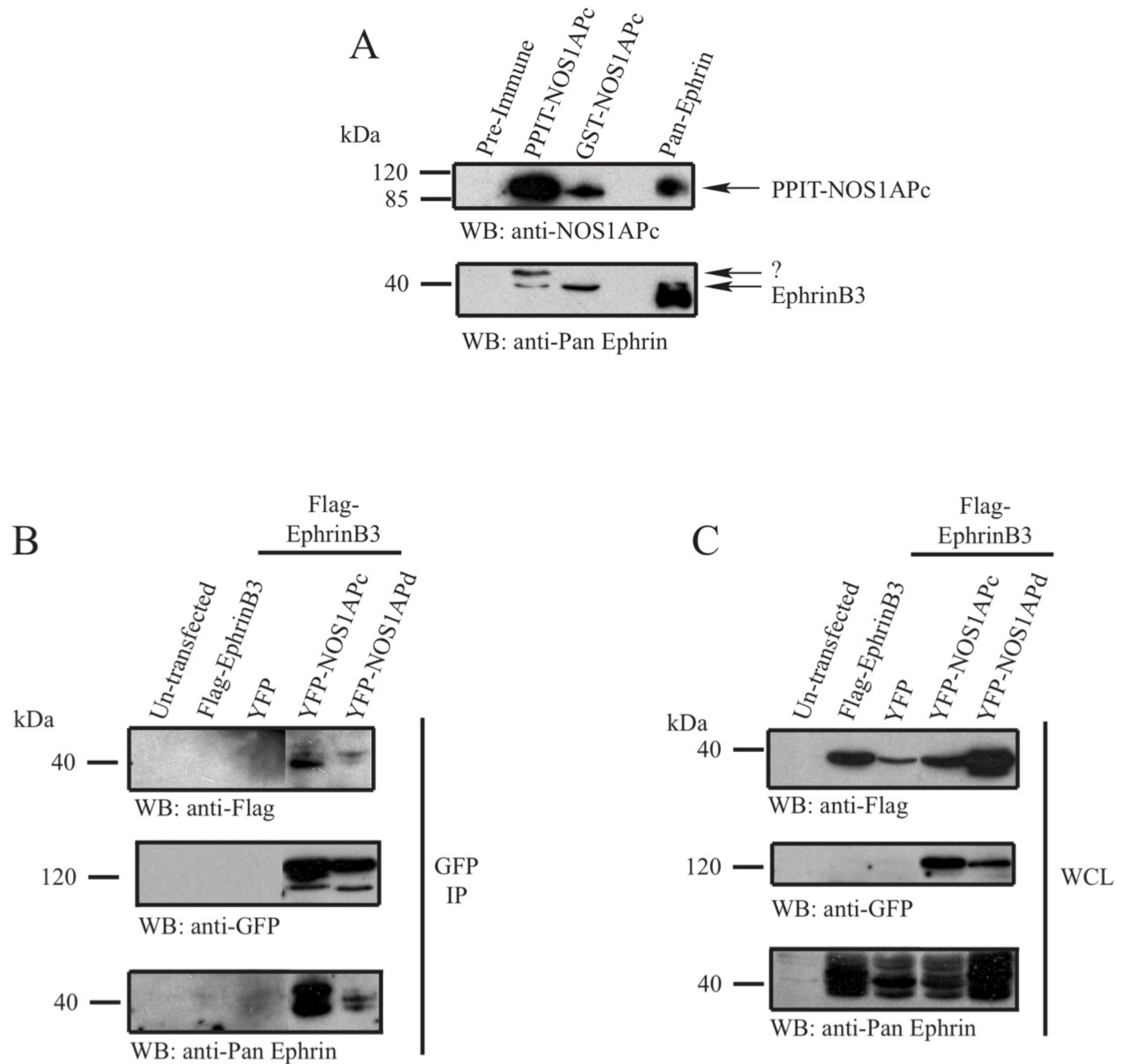

Figure 3.11 EphrinB and NOS1APc interact biochemically. (A) Endogenous NOS1AP and EphrinB proteins were precipitated from rat brain lysate using specific antibodies. Resulting blots were probed with NOS1APc and pan-EphrinB antibodies respectively. Results indicate the pan-ephrinB antibody can precipitate NOS1APc. The NOS1APc IP's precipitated a protein just below the 40kDa mark (arrow) that is also present in the ephrinB IP. The band could be EphrinB1, B2 or B3. There is another unknown band just above the 40kDa mark (arrow ?) in the NOS1APc IP and the WCL. (B) HEK 293T cells were co-transfected with Flag-ephrinB3 and the YFP-tagged NOS1AP isoforms followed by an IP with GFP. Resulting Western blots were probed with a Flag, GFP and pan-EphrinB antibody, respectively. Results indicate NOS1APc and potentially NOS1APd can precipitate ephrinB3. (C) The whole cell lysate blot showed the proteins were expressed in the cells.

CHAPTER 4 DISCUSSION

4.1 SUMMARY OF MAJOR FINDINGS

The aim of the current study was to characterize the longer isoform of NOS1AP - NOS1APc. Little is known about the function of the NOS1APc isoform. Therefore, in an attempt to help define a role for NOS1APc, my work examined potential binding partners for NOS1APc. I first obtained a proper cDNA for NOS1APc from adult rat cerebellum using RT-PCR. We also identified two alternate isoforms of NOS1AP subsequently named NOS1APd, NOS1APe and we confirmed a third isoform previously reported in NCBI, NOS1APf. To better understand the different isoforms, I over-expressed YFP fusion proteins of each of the NOS1AP isoforms and determined their subcellular localizations. NOS1APa and NOS1APe localized to the membrane while NOS1APc, NOS1APd and NOS1APf localized to the cytoplasm and intracellular puncta. Differences in the subcellular localization may be indicative of different functions within the cell. This is likely the case since I was able to show that NOS1APa but not NOS1APc interacted with β-PIX and GIT1 while NOS1APc but not NOS1APa interacted with ephrinB3. Taken together, my work suggests that NOS1APc functions as a scaffolding protein that links different protein complexes than NOS1APa. Further work will define differences in the biological significance of the NOS1APc isoform from NOS1APa.

4.2 IDENTIFICATION OF NEW NOS1AP ISOFORMS

4.2.1 Cloning NOS1APc

While completing experiments to characterize the endogenous NOS1APa and NOS1APc isoforms using a pan-NOS1AP antibody and two isoform specific NOS1APc antibodies, I identified the 100kDa NOS1APc isoform from rat brain lysate. However, I also noticed multiple faster migrating bands (Figure 3.1). This raised the possibility that there were other splice variants of NOS1AP that contained the unique C-terminal extension found in NOS1APc. To better characterize NOS1APc, I cloned NOS1APc from rat cerebellum since Richier, et al., (2010) had pieced together the NOS1APc sequence bioinformatically based on mass spectrometry. To do this, I designed 5' and 3' primers using the *rattus* NOS1APc sequence published by Richier, et al., (2010). Upon obtaining a number of clones, I was able to identify an inconsistency in the sequence from that published by Richier, et al., (2010) (Figure 3.2). In the published sequence for NOS1AP, the last exon of NOS1AP ends in (– GEPLLGGLELIKFRESGIASEYESN TDESEERDSW SQEELPRLLNVLQRQELGDSLDDEIAV) and is spliced with PMAQ from the previous exon (Figure 3.2). In the newly cloned sequence of NOS1APc, the second last exon of NOS1AP ending in PMAQ was spliced with VDHSMFEN… the beginning of the unique C-terminus of NOS1APc (Figure 3.2). We believe this newly cloned sequence is correct since 1) we obtained multiple clones, 2) the sequence fits with the genomic exon-intron boundaries and 3) when the clone is over-expressed, an appropriate band size is seen at 130kDa. Since YFP adds 30kDa this would give rise to a protein of 100kDa that matches the size of endogenous NOS1APc. Together, we are more confident in the new NOS1APc sequence.

4.2.2 New isoforms

Using NOS1APc isoform specific antibodies, as noted, we identified the presence of several unknown bands at 40kDa and 70kDa that were reactive to both our NOS1APc isoforms specific antibodies (Figure 3.1). Our pan-NOS1AP antibody also precipitated some of these unique bands. The presence of the bands led us to believe there could potentially be alternate isoforms of NOS1AP that contained the unique NOS1APc region. Since both the pan-NOS1AP antibody, which was raised against the C-terminal region of NOS1APa, and both the NOS1APc isoform specific antibodies recognized the 70kDa isoform, this suggests that this isoform likely contains the C-terminal region of NOS1APa and the unique C-terminal extension of NOS1APc. One possibility is that this transcript may lack the 30kDa N-terminal PTB domain but retain the C-terminal region of NOS1APa spliced with the NOS1APc sequence. Indeed, others have shown a unique NOS1AP transcript, NOS1APshort (Xu et al., 2005). This 125 amino acid isoform lacks the N-terminal PTB domain but contains a unique 5' stretch of 12 amino acids spliced with exons 9 and 10 that code for the C-terminal region of NOS1APa that includes the PDZ binding motif (Xu et al., 2005, Carrel et al., 2009). This NOS1APshort is higher in patients with schizophrenia and bipolar disorders (Xu et al., 2005). Further work will determine if this is indeed the case.

An alternate explanation of this unique 70kDa isoform may be that it is a degradation product of the 100kDa NOS1APc isoform (it should be pointed out that all the IP's were conducted in the presence of broad-spectrum protease inhibitors thus it is unlikely that degradation by either trypsin or serine, threonine and cystine proteases had occurred).

Consistent with this, in Northern blot analysis, using the unique C-terminal extension of NOS1APc indicates most tissues contain only one prominent band (O'Brien Thesis; Figure 1H, Richier, et al., 2010). It is possible that the 70kDa isoform exists; however, the mRNA levels may be too low to be detected using conventional Northern blot protocols.

If we believe the 70kDa isoform is a processed isoform, are there other examples of other proteins being processed? Three well-documented examples of processing occurring are Notch, p75 and β-Catenin. It has been shown that Notch, p75 and β-Catenin can be proteolytically cleaved. Upon cleavage, they translocate to the nucleus and function as transcriptional regulators. Interestingly, in preliminary results from work I performed and work from a previous student have shown that NOS1APc can be found in the nucleus (data not shown). The precise nature of this is unknown since over-expression of YFP-NOS1APc fails to enter the nucleus (Figure 3.5). It will be interesting to test whether an antibody raised against the N-terminus would detect a alternately sized product. Alternately, a fusion protein with an N-terminal GFP tag and a C-terminal RFP tag could be generated and expressed in cells to see if the GFP and RFP tags differentially separate within the cell. Finally, metabolic labeling experiments in cells endogenously expressing NOS1APc could be used to determine if cleavage of NOS1APc occurs.

During the process of confirming the sequence of NOS1APc, we identified two variants of NOS1APc subsequently named NOS1APd and NOS1APe. Both contain the 30kDa C-terminus of NOS1APc but lack exon 10 contained in NOS1APa and NOS1APc. The N-

terminal PTB domain is still present in both NOS1APd and NOS1APe, however NOS1APe contains a small 5aa insert (LLLLQ) in the PTB domain. A third isoform, NOS1APf, was also confirmed. It is unlikely that any of these isoforms account for the 70kDa isoform since their predicted molecular weight would be close to NOS1APc or approx. 40kDa for NOS1APf. Further, whether these isoforms make functional protein remains to be determined. Given the high degree of similarity to NOS1APc in amino acid sequence, it will be hard to create isoform specific reagents to these novel isoforms. Further work will be necessary to confirm if protein is translated from the mRNA and which, if any, cell types express them.

4.2.3 Localization of NOS1AP isoforms

The identification of new NOS1AP isoforms prompted us to identify their subcellular localization to determine any potential differences between them. Therefore, to determine if the different isoforms could exist in different subcellular locations, each NOS1AP isoform was exogenously expressed in HEK 293T cells. NOS1APa and NOS1APe localized to the cellular periphery where as NOS1APc, d, and f were found in the cytosol and small intracellular puncta.

The localization of NOS1APe to the cellular periphery is interesting because it contains a small 5aa insert, LLLLQ, in the PTB domain. The LLLLQ insert seems to target NOS1APe to membranes. How is a 5aa sequence able to direct NOS1APe to membranes? The simplest explanation may be that since leucine (L) is hydrophobic and glutamine (Q) is uncharged, this stretch would likely want to insert into a hydrophobic

environment such as the plasma membrane. In order for this to be the case, the small stretch would need to be exposed in the PTB structure. The PTB domain of NOS1AP has been shown to be very similar to the PTB domain of the protein Numb (Jaffrey et al., 2002) and when we align the NOS1AP sequence with the Numb structure the region where the LLLLQ insert is placed is in a β-sheet turn (Personal Communication, Dr. Jan Rainey, Dalhousie University). Thus, the LLLLQ insert would be found on an exposed surface. Both NOS1AP and Numb PTB domains lack certain residues that are expected to bind phospho-tyrosine based on the Shc-like PTB domain crystal structure (Jaffrey et al., 2002). Thus, one possible experiment to show if the LLLLQ region is sufficient to divert NOS1APe to membranes, the 5aa stretch could be substituted with alanine's to determine if that change redirects NOS1APe to the cytoplasm. Another way to test if this 5aa stretch is necessary for membrane targeting could be to insert the LLLLQ sequence into the Numb PTB domain to determine if Numb is directed to membranes. Finally, it should be noted that the LLLLQ insert is found in the published dog NOS1AP (accession #: XP_851837.2) sequence suggesting that this is a true transcript.

The localization of NOS1APe, the isoform that contains the LLLLQ insert, to the cellular periphery is also intriguing when considering the interaction between NOS1APa and scrib (Richier et al., 2010). Scrib localizes to the cellular periphery and contributes to the development of cellular polarity and in synaptic vesicle localization (Sun et al., 2009). In 2010, Richier et al. identified a complex of NOS1APa, scrib and the GIT1/β-PIX/PAK cascade that was found to be involved in dendrite development in primary hippocampal neurons. The interaction was reported to occur between the PTB domain of NOS1APa

and the fourth PDZ domain of scrib (Richier et al., 2010). Since NOS1APc can still associate with scrib, this suggests that the LLLLQ insert is not affecting the structure of the NOS1AP PTB domain. Further, it suggests the region in the PTB domain where the LLLLQ insert is found is unlikely to be the binding surface responsible for the association with scrib. Taken together, the localization studies show that NOS1APa and NOS1APc have different subcellular localizations and may therefore have different roles in the cell.

4.3 NOS1APc INTERACTING PARTNERS

4.3.1 NOS1APc does not interact with nNOS

Since NOS1APa and NOS1APc have different subcellular localization, one possibility is that the two isoforms exist in different protein-protein complexes within the cell. This appears to be the case. NOS1APa but not NOS1APc (and likely d, e and f) associates with nNOS. This is due to the fact that the splicing event that creates NOS1APc destroys that PDZ binding motif in NOS1APa. However, although NOS1APa associates with nNOS and NOS1APc doesn't, both NOS1AP isoforms and any containing the PTB domain, associate with scrib.

4.3.2 NOS1APc does not interact with β-PIX or GIT1

The fact that both NOS1APa and NOS1APc both associate with scrib but only NOS1APa associates with nNOS, suggests they are part of two separate complexes. This may explain a recent report that suggested NOS1APa and scrib did not interact with β-PIX or GIT1 but rather they associated with VANGL1 (Anastas et al., 2011). These results

differ from a paper published by the Fawcett lab by (Richier et al., 2010) who showed NOS1APa and scrib associated with GIT1/β-PIX/Pak. One possibility for the difference between the results in the two papers maybe the difference in cell lines used in the two studies. An alternate possibility is that NOS1APa associates with GIT1/β-PIX through scrib while NOS1APc associates with scrib and VANGL1. This is likely the case for two reasons; First, NOS1APa and NOS1APc have different subcellular localizations, NOS1APa is found at membranes while NOS1APc is found cytoplasmically (Figure 3.5). It is documented that the GIT1/β-PIX complex is found at the membrane (Botrugno et al., 2006) therefore, since scrib and NOS1APa are localized at membranes it would be simple to see how a complex of NOS1APa/Scrib/β-PIX/GIT1 could come together at or near the plasma membrane. Second, using isoform specific Ab's I was able to show that NOS1APc fails to interact with GIT1/β-PIX. Further, others have shown that NOS1APc localizes to the midline in the spinal cord. Both scrib and VANGL1 proteins localize to the developing spinal cord midline. Indeed, *Scrib* crc homozygous mutant mice develop a severe neural tube deficits due to the fact that during embryonic development the developing spinal cord fails to close (Murdoch et al., 2003). Interestingly, *Vangl1* and *Vangl2* mutant mice have similar defects in the developing spinal cord leading to neural tube defects (Kibar et al., 2009). This suggests that the (Anastas et al., 2011) paper may be correct; however, their results could be explained by the fact they may be precipitating NOS1APc. Further work is needed to rectify these two results.

4.3.3 NOS1APc interacts with pyruvate carboxylase

In addition to the differences between NOS1APa and NOS1APc with respect to nNOS and β-PIX/GIT1, a proteomic screen used to identify unique NOS1APc interacting proteins showed that pyruvate carboxylase (PCB) and ephrinB3 could preferentially associate with NOS1APc, d and e.

What role would NOS1AP have with pyruvate carboxylase? PCB is a mitochondrial protein; NOS1APc, d or f would have to be located in the mitochondria to directly interact with PCB. This suggests there could be another protein involved in the interaction that bridge NOS1APc and PCB, however, this is not known. What is interesting though, is the appearance of the puncta in the cells transfected with the NOS1APc, d and f isoforms. As already mentioned, the identity of these puncta are unknown but it would be interesting to know if they are associated with the mitochondria in any way.

In astrocytes, PCB is involved in the generation of oxaloacetate (OAA), an important TCA cycle intermediate, by adding a carbon dioxide to pyruvate (Murin et al., 2009). The OAA formed by the actions of PCB is then used to generate α-ketoglutarate, a TCA cycle intermediate important in the *de novo* synthesis of glutamate (Jitrapakdee et al., 2006). Because PCB activity is not detected in neurons, its presence in astrocytes is essential for providing glutamine to neurons where it is then converted to glutamate in the processes called the 'glutamine-glutamate cycle' (Jitrapakdee et al., 2006). A role for NOS1APc in astrocytes has yet to be defined but we do know NOS1APc is present in

astrocytes (O'Brien Thesis, 2011). Whether or not NOS1APc can enter the mitochondria and if the interaction between NOS1APc and PCB is direct is not known. Since NOS1APa acts as a scaffold protein, it could be that NOS1APc establishes a scaffold that provides PCB with the necessary products to function. Whether this affects glutamate synthesis remains to be tested. One interesting way to test this would be to knockdown NOS1APc in astrocytes and determine if this affects glutamate levels. Future work will focus on characterizing the relationship between PCB and NOS1APc and establishing the subcellular localization of NOS1APc to determine a role for NOS1APc in astrocytes.

4.3.4 NOS1APc interacts with a member of the ephrinB family of ligands

One interesting finding of my work was to show that NOS1APc but not NOS1APa interacts with eprhinB3. What role would NOS1APc have with ephrinB3? EphrinB3 is important for midline development and for establishing spinal circuits important for walking (Palmer and Klein, 2003). Since NOS1APc associates with ephrinB3 and is expressed in the midline similarly to ephrinB3, it is interesting to speculate that NOS1APc may function with ephrinB3 to control spinal circuits necessary for gait. However, until isoform specific knockouts are developed, this will be difficult to test. In addition to midline structures, ephrinB3 and NOS1APa have been shown to be important for synapse development (Richier et al., 2010, Xu et al., 2011). Thus, it will be interesting to test whether NOS1APc and ephrinB3 function together in synapse formation or function.

The ephrinB family of ligands has a well-conserved C-terminal cytoplasmic domain. The 33 amino acids in the C-terminal region is highly conserved and contains 5 tyrosine residues important for modulating some of the 'reverse' signaling effects (Bong et al., 2004). Because the ephrinB ligands have such a well-conserved C-terminus, there is potential for NOS1APc to bind ephrinB1 and B2 as well as ephrinB3. It would be interesting to test whether NOS1APc can bind ephrinB1 and B2, which is possible with use of specific ephrinB1, B2 and B3 antibodies. With that being said, NOS1APc could be involved in the ephrinB1 and B2 signaling pathways such as Wnt-PCP signaling (Lee et al., 2006) or in the subventricular zone (Ricard et al., 2006). Future work will focus on characterizing the relationship between ephrinB3 and NOS1APc as well as determining if a relationship exists between NOS1APc and ephrinB1 or ephrinB2. Determining these relationships will aid in determining a functional role for NOS1APc.

4.4 FUTURE DIRECTIONS

We have identified the presence of three new isoforms of NOS1AP known as NOS1APd, e and f. We have been able to show that there are differences in their sub-cellular localization and there appears to be differences in some of their binding partners, but some of their characteristics still remain unknown. Future work needs to focus on determining characteristics of these novel isoforms as well as NOS1APc. Knowing the differences in binding partners and localizations in other cell types will aid in distinguishing unique functions for these isoforms.

CHAPTER 5 CONCLUSION

In summary, we have characterized a novel isoform of NOS1AP known as NOS1APc. Through the process of characterizing NOS1APc, we have identified several alternate isoforms subsequently named NOS1APd, NOS1APe and NOS1APf. The subcellular localization of these isoforms suggests similarities between the NOS1APa and NOS1APe isoforms and similarities between NOS1APc, NOS1APd and NOS1APf isoforms. We have also identified differences and similarities between NOS1APa and NOS1APc. The NOS1APc isoform does not interact with nNOS as predicted and does not interact with β-PIX or GIT1, but retains the ability to interact with Scribble through the PTB domain consistent with that of NOS1APa. These differences suggest NOS1APa and NOS1APc may interact with Scribble in different complexes. Additionally, we have used specific antibodies towards NOS1APc to identify unique binding partners through a mass spectrometry screen and through immunohistochemistry. To date, unique interactions between NOS1APc/pyruvate carboxylase and NOS1APc/ephrinB ligand have been identified. Future work will focus on characterizing the relationships between NOS1APc and its unique binding partners to aid determining potential roles for the protein as well as characterizing the newly identified isoforms.

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