CHARACTERIZATION OF A NOVEL ISOFORM OF NOS1AP: NOS1APc

by

Michael O’Brien

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

at

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Halifax, Nova Scotia
March 2011

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DALHOUSIE UNIVERSITY

DEPARTMENT OF PHARMACOLOGY

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ABSTRACT

The current study characterizes a novel isoform of the Nitric Oxide Synthase 1 Adaptor Protein (NOS1AP), herein NOS1APc. NOS1APc was identified in a proteomic screen for Scribble interacting proteins. Northern blot analysis revealed a 7kb transcript that was present in a number of different tissues and cell lines. Highest levels were detected in the cerebellum. In situ hybridization studies revealed NOS1APc mRNA throughout the cortex and hippocampus. In addition, cerebellar Purkinje cells and different brainstem nuclei also contained NOS1APc mRNA. Antibodies directed against NOS1APc revealed a 100 kDa protein, while immunohistochemical staining revealed high levels of this protein within the molecular layer of the cerebellum. Finally, immunocytochemical studies using isolated primary astrocytes revealed a subset of BrdU positive nuclei that co-expressed NOS1APc, suggesting that this protein may function in some capacity in cell-cycle progression.
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<td>μm</td>
<td>micrometer</td>
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<tr>
<td>[α-(^{32})P]dCTP</td>
<td>alpha-(^{32})Phosphorus Deoxycytidine 5'-triphosphate</td>
</tr>
<tr>
<td>β-Pix</td>
<td>β-p21-activated kinase interacting exchange factor</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>AMPAR</td>
<td>α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionic acid receptor</td>
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<td>AP</td>
<td>Alkaline Phosphatase</td>
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<td>Adenomatous polyposis coli</td>
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<td>ARF</td>
<td>ADP-ribosilation factor</td>
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<td>Cell division cycle 42</td>
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<td>Cdk</td>
<td>Cyclin-dependant protein kinase</td>
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<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
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<tr>
<td>CFA</td>
<td>Complete Friends Adjuvant</td>
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<td>cGMP</td>
<td>cyclic Guanosine Monophosphate</td>
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<tr>
<td>CKI</td>
<td>Cyclin-dependant kinase inhibitor</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CP</td>
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<td>Dexamathasone induced Ras</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagles Medium</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>eNOS</td>
<td>endothelial Nitric Oxide Synthase</td>
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<td>Erc</td>
<td>Extracellular signal-related kinase</td>
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<td>Filamentous actin</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FMOC</td>
<td>9H-fluoren-9-ylmethoxycarbonyl</td>
</tr>
<tr>
<td>fMRI</td>
<td>functional magnetic resonance imaging</td>
</tr>
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<td>G-actin</td>
<td>monomeric actin</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GC</td>
<td>Guanylyl Cyclase</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>Git1</td>
<td>G-protein coupled receptor kinase interacting protein1</td>
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<td>GST</td>
<td>Glutathione-S-Transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks Balanced Salt Solution</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>hScrib</td>
<td>human Scribble</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediate filament</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin Receptor Substrate</td>
</tr>
<tr>
<td>Kb</td>
<td>kilo base pair</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole Limpet Hemocyanin</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine Rich Repeat</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
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<tr>
<td>MgCl2</td>
<td>Magnesium Chloride</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>MLC</td>
<td>Myosin-II regulatory light chain</td>
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<tr>
<td>MPF</td>
<td>M-phase promoting factor</td>
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<tr>
<td>M-phase</td>
<td>Mitotic phase</td>
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<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro blue tetrazolium chloride</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate Receptor</td>
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<td>Nucleation Promoting Factor</td>
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<td>NOS1AP</td>
<td>Nitric Oxide Synthase 1 Adaptor Protein</td>
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<td>NOS1APc</td>
<td>Nitric Oxide Synthase 1 Adaptor Protein c (CAPONlong)</td>
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<td>Nonident-P 40</td>
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<td>nWASP</td>
<td>neuronal Wiskott-Aldrich syndrome protein</td>
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<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDZ</td>
<td>PSD-95/ZO-1/Dlg</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PMSF</td>
<td>phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
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<td>Definition</td>
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</tr>
<tr>
<td>PSD</td>
<td>Post-synaptic density</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding</td>
</tr>
<tr>
<td>pY</td>
<td>phosphorylated tyrosine</td>
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<tr>
<td>Rac</td>
<td>Ras-related C3 botulinum toxin substrate</td>
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<td>RhoA</td>
<td>Ras-related homologue member A</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>SHD</td>
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CHAPTER 1  INTRODUCTION

1.1 NOS1AP

Previously, the Nitric Oxide Synthase 1 Adaptor Protein (NOS1AP) also known as carboxyl-terminal PDZ ligand of neuronal Nitric Oxide Synthase (nNOS) (CAPON) has been shown to regulate nitric oxide (NO) synthesis (Jaffrey et al., 1998), iron homeostasis (Cheah et al. 2006) and synapse morphology (Cheah et al., 2006; Richier et al., 2010). More recently, other isoforms of this adaptor protein have been identified, yet their function has not been well characterized. In this study, I report the characterization of a novel isoform of NOS1AP, NOS1APc (Richier et al., 2010).

1.1.1 NOS1AP Domain Architecture

NOS1AP is a 70 kDa protein that contains an amino-terminal (N-terminal) phosphotyrosine binding (PTB) domain and a carboxyl-terminal (C-terminal) PDZ (postsynaptic density 95, PSD-95; discs-large, Dlg; zonula occludens-1, ZO-1) binding motif (Jaffrey et al., 1998). PDZ domains are approximately 90 amino acids in length and are generally found in scaffolding proteins (Sheng and Sala, 2001). They can be characterized into four different classes based on their interactions. Most commonly, PDZ domains recognize a conserved PDZ binding motif at the C-terminal region of their binding partner, however they can also recognize internal peptide motifs (Pawson and Nash, 2000; Feng and Zhang, 2009). Other examples of PDZ interactions include recognition of lipids and PDZ-PDZ dimerization (Pawson and Nash, 2000, 2003; Feng and Zhang, 2009). An example of PDZ-PDZ interaction is the interaction between PSD-
95 and nNOS, which acts to localize nNOS at the postsynaptic region (Christopherson et al., 1999).

PTB domains are often 100-150 amino acids in length and commonly bind to recognition sequences that contain phosphorylated tyrosine residues; most commonly Asp-Pro-X-pTyr motifs (N-P-X-Y), where X represents any amino acid (Kavanaugh et al., 1995; Pawson and Scott, 1997). PTB domains can be classified as one of two types, Shc-like or Insulin Receptor Substrate (IRS)-like, depending on the respective protein in which they were first identified (Margolis et al., 1999). These two types share a low sequence homology and differ in their binding specificity. PTB domains confer target specificity and binding affinity based on the 5-8 amino acids that lie N-terminally to the core pTyr residue (Pawson and Scott, 1997). More recently, it has been shown that the PTB domain of some proteins such as Dab and Numb are able to recognize N-P-X-Y motifs, independent of tyrosine phosphorylation (Dho et al., 1998; Stolt et al., 2003). Furthermore, Numb PTB domains have also been shown to bind motifs devoid of tyrosine residues (Yaffe, 2002). The PTB domain of NOS1AP has been identified as most similar to the PTB domain of mouse Numb, indicating they may contain a similar fold (Jaffrey et al., 2002b). Recently, the PTB domain of Numb has also been shown to bind PDZ domains (Nie et al., 2004). Furthermore, previous work from our lab has shown that the PTB domain of NOS1AP associates with the PDZ domain of the polarity protein, Scribble (Richier et al., 2010). Taken together, these findings demonstrate the dynamic nature and high specificity of binding through PTB domains.
1.1.2 NOS1AP as an nNOS-targeting protein

NOS1AP was first identified as a binding partner to nNOS through the interaction of its C-terminal PDZ binding motif with the PDZ domain of nNOS (Jaffrey et al., 1998). nNOS belongs to the family of NOS enzymes, which also includes endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) (Knowles and Moncada, 1994).

nNOS signaling has been implicated in several forms of synaptic plasticity by exerting its effects on nitric oxide (NO) generation (Jaffrey et al., 1998). NO is produced from l-arginine by means of NOS enzymes, therefore its spatial activity within the nervous system is dependent on the subcellular location of nNOS (Knowles and Moncada, 1994). NO is found throughout the nervous system and has been shown to have biphasic effects. At low levels, it modulates both dendritic spine growth and neurotransmitter release, while elevated levels are capable of exerting neurotoxic effects (Dawson et al., 1993, 1994; Roskams et al., 1994). nNOS-dependant activity is of great physiological importance when considering the high reactivity and neurotoxicity of unregulated NO activity. NO has been shown to elicit its effects on synaptic plasticity as a retrograde signaling molecule that promotes the production of cyclic guanosine monophosphate (cGMP) through enhancing guanylyl cyclase (GC) activity (Dawson et al., 1994). Activity-dependant synaptic remodeling can be blocked either by the addition of nNOS inhibitors or the NO scavenging molecule, haemoglobin (Dawson et al., 1994).
nNOS has recently been shown to elicit its effects as part of a ternary complex including PSD-95 and N-methyl-D-aspartate receptors (NMDAR), where the PDZ domain of nNOS binds to the C-terminal region of PSD-95 (Brenman et al., 1996; Christopherson et al., 1999). This complex has been identified as an important regulator of synapse morphology both pre- and post- synaptically (Nikonenko et al., 2008). nNOS localization, however, is also mediated by a number of adaptor proteins, such as NOS1AP that associate through an interaction with its PDZ domain (Jaffrey et al., 1998; Fang et al., 2000). As an adaptor protein, NOS1AP is thought to function in the formation of protein complexes through specific protein-protein interactions. Work by Jaffrey et al., (1998) showed that NOS1AP acts to sequester nNOS away from NMDAR through competition with PSD-95 for the PDZ domain of nNOS. This finding implicates NOS1AP in developmental synaptic remodeling through its role as an nNOS-targeting protein. Despite this discovery, the functional consequences of this molecular event have never been fully characterized in vivo.

1.1.3 NOS1AP and nNOS Function through the Formation of Two Separate Ternary Complexes

NOS1AP and nNOS have been proposed to elicit their effects on neuronal development through the formation of two separate ternary complexes. The first involves a direct association between the PTB domain of NOS1AP and a dexamathasone induced Ras family member (Dexras1) (Kemppainen and Behrend, 1998; Fang et al., 2000). Interestingly, Dexras1 has no requirement for pTyr in order to bind NOS1AP (Fang et al., 2000). In order to be activated within this complex, Dexras1 must be converted from an inactive GDP-bound to an active GTP-bound state (Fang et al., 2000; Jaffrey et al.,
This is achieved following an NMDAR/nNOS-mediated NO release, where NO-mediated S-nitrosylation converts Dexras1 to a GTP-bound state (Fang et al., 2000; Jaffrey et al., 2002a). This nNOS-dependant activity of Dexras1 is enhanced in the presence of NOS1AP, as NOS1AP is thought to juxtapose these two molecules in a manner that promotes interaction (Fang et al., 2000; Jaffrey et al., 2002a). Additionally, Dexras1 activity is significantly reduced in nNOS-deficient mice, further evidence that its activity is regulated by NO (Fang et al., 2000). Interestingly, Dexras1 was shown to be dominantly expressed in apoptotic CNS neurons, and it co-localized with NOS1AP and nNOS within these cells (Li et al., 2008). This implies a function for this complex in neurotoxicity as excess NO signaling is widely known to play a role in glutamate-mediated cell death. This is consistent with previous findings that demonstrate a role for Dexras1 in NMDAR-mediated neurotoxicity (Cheah et al., 2006). To complicate these findings, Fang et al. (2000) challenge the idea that NOS1AP and PSD-95 compete for nNOS binding. They conceive that as nNOS exists as a dimer, one monomer may bind to PSD-95 and the other to NOS1AP/Dexras1. This suggests a potential biphasic role for NOS1AP as an adaptor protein that regulates both NMDA-mediated synaptic plasticity and NMDA neurotoxicity through a NO-dependant mechanism.

NOS1AP has also been shown to associate with another complex involving NOS1AP, nNOS and synapsin I (Jaffrey et al., 2002b). Synapsin I is a member of the synapsin family of neuronal phosphoproteins consisting of synapsins I, II and III, and it has long been implicated in synaptogenesis and neurotransmitter release (De Camilli et al., 1990; Greengard et al., 1993; Fdez and Hilfiker, 2006). Synapsin I has been shown to associate
with the PTB domain of NOS1AP, however, like Dexras1 the association between NOS1AP and synapsin I is not dependent or regulated by tyrosine phosphorylation (Jaffrey et al., 2002b). This suggests that phosphorylation is not a necessary event for NOS1AP to stabilize various protein complexes. NOS1AP has also been identified as a binding partner of synapsins II and III, although it has not been shown to form a complex with these proteins (Jaffrey et al., 2002b). While nNOS coupling to NMDAR through PSD95 is targeted exclusively to postsynaptic sites, synapsins are enriched presynaptically and have been shown to colocalize with NOS1AP in this region (Fletcher et al., 1991; Jaffrey et al., 2002b). This suggests a potential role for NOS1AP as a determinant for nNOS functioning at presynaptic sites. Furthermore, its ability to shuttle between complexes suggests that it is an adaptor protein capable of regulating multiple cellular events.

The synapsin family of proteins have been found to independently contribute to the assembly of synaptic vesicle clusters, neurite outgrowth, as well as synaptogenesis (Li et al., 1995; Jaffrey et al., 2002b). Interestingly, double knockout-mice lacking both synapsin I and II genes exhibit subcellular changes in both nNOS and NOS1AP expression, suggesting that the formation of this complex determines its intracellular localization (Jaffrey et al., 2002b). Furthermore, both nNOS- and synapsin-deficient mice show similar deficits in dendritic morphology and neurotransmitter release, suggesting that these deficits in neurite outgrowth may reflect a lack of nNOS targeting to presynaptic sites (Chin et al., 1995; Inglis et al., 1998). This NOS1AP/nNOS/synapsin I
ternary complex has, therefore, been accepted as a likely contributor to presynaptic growth and morphology.

1.1.4 NOS1AP Associates with a Scribble, Git1, β-Pix, PAK Complex to Regulate Dendritic Spine Morphology

Recently, through a proteomic screen, NOS1AP was identified as a binding partner of Scribble (Richier et al., 2010). Scribble, a known tumor suppressor and polarity protein, contains a number of identified signaling domains, including 16 N-terminal leucine rich repeats (LLR) and four PDZ domains (Bilder and Perrimon, 2000). A direct interaction has been reported between the fourth PDZ domain of Scribble and the PTB domain of NOS1AP (Richier et al., 2010). These domains were identified as both necessary and sufficient for Scribble to associate with NOS1AP (Richier et al., 2010).

NOS1AP was also shown to associate with the proteins G-protein coupled receptor kinase interacting protein1 (Git1), β-p21-activated kinase interacting exchange factor (β-Pix) and p21-activated kinase (PAK) (Richier et al., 2010). Git1 is composed of an N-terminal ADP-ribosylation factor (ARF)-GAP domain followed by ankyrin repeats, and a Spa2 homology domain (SHD) (Zhang et al., 2005). It has been shown to bind β-PIX that, in turn, binds PAK (Zhang et al., 2005). Recently, Scribble has been identified as a scaffolding protein responsible for bridging NOS1AP with the Git1/β-Pix/PAK complex (Richier et al., 2010). This is congruent with the role of LRR and PDZ-containing scaffolding proteins in the development and functioning of the synapse. The Git1/β-Pix/PAK complex has been found to contribute to dendritic spine morphology by acting through myosin-II regulatory light chain (MLC) (Zhang et al., 2003; Zhang et al., 2005).
Furthermore, NOS1AP and Scribble colocalize and are enriched in dendritic spines, suggesting that this complex plays a role at the postsynaptic region to control spine morphology (Richier et al., 2010). Interestingly, overexpression of NOS1AP leads to an increased outgrowth of spines in addition to influencing Ras-related C3 botulinum toxin substrate (Rac) activity (Richier et al., 2010). It has been speculated that a NOS1AP-Scribble interaction may elicit its effects on dendritic spine morphology by modulating nNOS-mediated NO production. This possibility has been discounted, however, by the finding that NOS1AP has the same effect on spine morphology in the absence of its nNOS binding region (Richier et al., 2010). Taken together, this has led the model in which NOS1AP stabilizes a Scribble, Git1/βPix/PAK complex which influences Rac activity and a subsequent outgrowth of actin filaments. It is through this mechanism that NOS1AP is believed to control the growth of dendritic spines.

In addition to their postsynaptic localization, NOS1AP and Scribble have been shown to colocalize presynaptically, identifying potential roles for these proteins at both ends of the synapse (Richier et al., 2010). Proteomic screening identified a number of interacting proteins with Scribble that are involved in the cytoskeletal matrix at the active zone (CAZ), including GIT1, β-Pix, liprin-α and Erc1b (Richier et al., 2010). Scribble has also been implicated in a pathway downstream from β-catenin that is responsible for the organization of vesicles at the presynaptic region (Sun et al., 2009). Consistent with this role is the finding that NOS1AP binds synapsin I, a protein involved in linking vesicles to the actin cytoskeleton (Jaffrey et al., 2002b). This evidence suggests a potential role for
NOS1AP and Scribble in regulating vesicle dynamics in addition to contributing to presynaptic organization.

1.1.5 NOS1AP Isoforms and Disease Linkage

NOS1AP mRNA is widely expressed in neuronal structures including the cortex, cerebellum, hippocampus, olfactory bulb and striatum (Jaffrey et al., 1998). More recently, its expression has also been revealed in other regions including heart, gut, skeletal muscle and spinal cord (Segalat et al., 2005; Chang et al., 2008). To date, Northern blot analysis has revealed several mRNA transcripts of NOS1AP, however only two have been reported as coding transcripts for NOS1AP isoforms. Jaffrey et al. (1998) reported the original isoform, CAPON, which has also been termed NOS1AP (Richier et al., 2010) or NOS1AP-L (Carrel et al., 2009). This isoform has been implicated in QT interval extremes (long- and short-QT syndromes) (Arking et al., 2006; Aarnoudse et al., 2007; Chang et al., 2007; Post et al., 2007) and schizophrenia (Brzustowicz et al., 2004; Zheng et al., 2005). Of particular interest to the present study is the finding that the NOS1AP gene is associated with susceptibility to schizophrenia. Moreover, NOS1AP has recently been shown to be significantly downregulated exclusively within the cerebellum of patients with this disease (Hadzimichalis et al., 2010). This comes with a recently growing body of evidence for a role of this brain region in various neuropsychiatric disorders (Andreasen et al., 1998; Daskalakis et al., 2005; Andreasen and Pierson, 2008).
A second isoform, NOS1AP-S, was reported that contains the last two exons of the
NOS1AP gene with a unique 5’ sequence (Xu et al., 2005). This protein maintains the
PDZ-binding domain that is sufficient for binding nNOS, however lacks the N-terminal
PTB domain responsible for all other known interactions with NOS1AP (Xu et al., 2005).
Recent studies have also identified a link between NOS1AP-S and schizophrenia,
suggesting a role for the C-terminal region in disease progression (Hadzimichalis et al.,
2010). This is of particular interest since this region is known to be responsible for the
association of nNOS with PSD-95 at NMDAR, and NMDAR are thought to play an
integral role in the synaptic defects associated with schizophrenia (Harrison and
Weinberger, 2005). Furthermore, elevated levels of both nNOS and NOS1AP have been
reported in the prefrontal cortex of affected individuals (Baba et al., 2004; Xu et al.,
2005). It is widely accepted that deficits in synaptic plasticity within this brain region are
strongly associated with the pathophysiology of various psychiatric disorders, including
schizophrenia. Further evidence for an nNOS-mediated dysregulation of the synapse
comes from the finding that increased allelic frequencies of a polymorphism of the
human NOS1 gene have been identified in schizophrenic patients compared to controls
(Shinkai et al., 2002).

Recently, through proteomic analysis of Scribble-associating proteins, we have identified
a third novel isoform, herein NOS1APc. This ~100kDa protein contains an
approximately 30kDa extension beyond the C-terminal valine residue found in NOS1AP
(Richier et al., 2010). This extension likely blocks an interaction between NOS1APc and
nNOS. This novel isoform maintains the N-terminal PTB domain and has a short proline-
rich region in the novel extension (Richier et al., 2010). As nothing is presently known about this novel protein, the basis of my work has been to focus on its characterization using a rodent model.

Since NOS1AP has been implicated in a number of complexes involved in regulating neuronal development and functioning, I will introduce various aspects of central nervous system (CNS) development and function to give context to the underlying role of NOS1AP in these events.

1.2 NOS1AP within the CNS
1.2.1 Components of a Synapse

Neurons are the functional unit of the nervous system. They typically contain a cell body, an axon which functions to transmit electrochemical signals from one neuron to another, and one or many dendrites, which are designed to receive these signals (Arimura and Kaibuchi, 2007). Adjacent neurons can communicate through a specialized cell-cell junction called a synapse (Arimura and Kaibuchi, 2007). The synapse consists of a presynaptic region where the axon terminal of one neuron meets with an adjacent target neuron and a postsynaptic region where the target neuron receives this signal through its dendritic projection (Arimura and Kaibuchi, 2007). This signal is often mediated across the synapse as a chemical message, through the release of neurotransmitters from the presynaptic region (Dillon and Goda, 2005; Arimura and Kaibuchi, 2007). These neurotransmitters bind to specific receptors on the postsynaptic compartment where they initiate the flow of ions that creates an electrical signal to be propagated through the
dendrite toward the cell body (Dillon and Goda, 2005; Arimura and Kaibuchi, 2007). As actin dynamics is largely responsible for the proper development and functioning of individual synapses, an introduction to this topic is necessary.

1.2.2 Actin Dynamics

Actin is a structural protein that plays an integral role in axon and dendrite formation, axonal pathfinding during development and the overall organization of the synapse (Dillon and Goda, 2005). It also plays a major role in maintaining cell shape and cell motility (Dillon and Goda, 2005). The development of a functional synapse, therefore, depends largely on an event called actin dynamics - the growth and catastrophe of actin filaments (Dillon and Goda, 2005). Actin can be found in two forms, filamentous (F-actin) and monomeric (G-actin), where individual G-actin molecules bond together to form filaments (F-actin) (Dillon and Goda, 2005). These filaments undergo lengthening at the growing (or barbed) end and catastrophe at the pointed end (Dillon and Goda 2005). This growth and catastrophe is a very dynamic process, where cleaved G-actin molecules at the pointed end are recycled for addition at the growing end (Dillon and Goda 2005).

The concept of plasticity and activity-dependant learning within the synapse has only recently eclipsed the former notion of a stable synapse. This can be largely attributed to the discovery of actin as a major component in the synapse (Dillon and Goda, 2005). Much of what is understood about this activity-dependant learning comes from the well-understood role of actin dynamics within dendritic spines at excitatory synapses (Dillon
and Goda, 2005). Matus and colleagues first demonstrated the highly dynamic nature of dendritic spines by using green fluorescent protein (GFP)-labeled actin within dissociated hippocampal neurons (Fischer et al., 1998). This group was able to show that dendritic spines can remodel as much as 30% of their cytoskeletal length in a matter of seconds (Fischer et al., 1998). This has since been identified using in vivo studies (Lendvai et al., 2000). To date, much less is known about the phenomenon of actin-based remodeling of the presynaptic terminal (Dillon and Goda, 2005). To illustrate the role of actin in this highly dynamic process, I will therefore focus on the signaling pathways involved in activity-dependant learning within dendritic spines.

1.2.3 Dendritic Nucleation Model (Actin Dynamics)

Activity-dependant learning involves the dynamic balance between the growth of newly formed and existing synapses and the activity-dependant reduction of others (Dillon and Goda, 2005). These two forms of synapse remodeling are known as long-term potentiation (LTP) and long-term depression (LTD), respectively (Dillon and Goda, 2005). Synaptic remodeling is regulated by glutamate activity (Fischer et al., 2000). Specifically, it is controlled by the cooperative effects of glutamate acting on both α-amino-3-hydroxyl-5-methyl-4-isoxazote-propionic acid receptors (AMPAR) and NMDAR (Murphy et al., 1997; Malinow and Malenka, 2002). Many factors, such as the level of activation of individual receptors and past synaptic activity confer specificity to different downstream signaling pathways (Dillon and Goda, 2005). It is this extensive level of input that allows these receptor types to couple synaptic activity with a diverse
number of molecular candidates, leading either to the growth or reduction of individual synapses (Dillon and Goda, 2005).

Events leading to LTP can either result in the strengthening of existing synapses or the formation of new ones. At established dendritic spines, LTP results from a process called actin nucleation, an increase in F-actin through either the sequestering of synaptic or dendritic G-actin or an increase in translation of β-actin within the spine (Welch and Mullins, 2002; Tiruchinapalli et al., 2003; Dillon and Goda, 2005). Actin nucleation is thought to involve the following proteins; actin, actin-related protein 2 and 3 (Arp2/3) complex, an Arp2/3 complex-activating nucleation promoting factor (NPF), a barbed-end capping protein, coflin and profilin (dos Remedios et al., 2003; Winder, 2003; Dillon and Goda, 2005; Pollard, 2007). In the dendritic nucleation model, individual G-actin monomers are bound to profilin, effectively inhibiting nucleation from occurring (Pollard, 2007). Profilin acts to prevent the addition of actin monomers at pointed ends, allowing it to occur only at barbed ends (Pollard, 2007). To promote the nucleation of new filaments, Arp2/3 complexes act to mimic a pointed end (Pollard, 2007). To do this, however, they must be activated by the binding of NPF and they must be anchored to the side of a pre-existing filament (Pollard, 2007). As this complex is able to promote nucleation at both the barbed end of the ‘mother filament’ as well as the pointed end of the attached ‘daughter filament’, this results in dendritic branching; these branches generally occur at 70° angles (Pollard, 2007). As the ‘mother filament’ continues to grow at its barbed end, capping proteins such as F-actin capping protein (CP) or Gelsolin eventually prevent the addition of new actin monomers to prevent unregulated growth of
individual filaments (Machesky and Insall, 1999; Dent and Gertler, 2003; Cooper and Sept, 2008). Changes in dendritic spine morphology, therefore, are controlled by the growth and catastrophe of individual actin filaments, an event that is regulated by external signaling molecules. As actin dynamics is under the control of signaling molecules such as Arp2/3 and cofilin, these signals are, in turn, under the control of various molecular switches.

1.2.4 Rho GTPases in Actin Dynamics

There are a number of proteins that are responsible for directly controlling the cycling activity of filamentous actin. These proteins include neuronal Wiskott-Aldrich syndrome protein (nWASP), cofilin, Arp2/3, Gelsolin, and Lim kinase (Dillon and Goda, 2005). These proteins are in turn, regulated by the Rho family of small GTPases, which act as molecular switches to control the assembly and disassembly of actin filaments (Dillon and Goda, 2005). The three most common RhoGTPases acting on this pathway are; Ras homologue member A (RhoA), Rac1 and cell division cycle 42 (cdc42) (Miller and Kaplan, 2003; Govek et al., 2005; Tada and Sheng, 2006). Generally, Rac has been shown to promote the growth of actin filaments, while RhoA is thought to promote catastrophe (Etienne-Manneville and Hall, 2002; Miller and Kaplan, 2003).

Rac activity is controlled by the binding of guanosine nucleotides. It is active in its GTP-bound state, and is converted to an inactive state following the hydrolysis of GTP to GDP. This process is under the control of guanine nucleotide exchange factors (Rho-GEFs) and GTPase activating proteins (Rho-GAPs) (Etienne-Manneville and Hall, 2002;
Miller and Kaplan, 2003). Rho-GEFs increase the affinity for GTP to bind Rac, effectively enhancing its activity and allowing it to interact with a series of downstream actin effectors (Luo, 2000; Etienne-Manneville and Hall, 2002). Rho-GAPs oppose these actions by hydrolyzing GTP bound to Rac, thereby decreasing its activity and preventing an interaction with downstream effector proteins (Luo, 2000; Etienne-Manneville and Hall, 2002).

Recently, a number of synaptic proteins have been identified that regulate Rac activity through exerting their effects on Rho-GEFs and Rho-GAPs. For example, the Git1/β-Pix/PAK complex is localized in the postsynaptic region and has been identified as an important synaptic regulator through its effects on actin dynamics (Zhang et al., 2005). It has been hypothesized that postsynaptic Rac activity can be attributed to the activity of this ternary protein complex as β-Pix is a Rho-GEF protein that locally activates Rac in dendritic spines (Zhang et al., 2005). While this mechanism is not fully understood, it is believed that Git1 may be involved in localizing Rac adjacent to β-Pix and PAK at the postsynaptic region where they can exert their effects on actin dynamics (Richier et al., 2010). Interestingly, synaptic abnormalities can arise from the deletion of either Git1, β-Pix or PAK. For example, mislocalization of Git1 at the postsynaptic region leads to a lack of dendritic spine formation (Fiala et al., 2002; Zhang et al., 2005). Furthermore, mutations in either β-Pix or PAK have been associated with non-syndromic mental retardation (Fiala et al., 2002; Zhang et al., 2005). Taken together, the Git1/β-Pix/PAK protein complex has been identified as an important regulator of actin remodeling and the subsequent development of dendritic spines. An understanding of how these proteins
function with one another is, therefore, fundamental in creating a working model for actin dynamics.

Several examples of Rho-dependant effects on actin dynamics have been well characterized. PAK, for example, is known to have specific effects on actin catastrophe. Upon binding active Rac, PAK can begin to elicit kinase activity, which subsequently activates Lim kinase through a phospho-dependant mechanism (Edwards et al., 1999; Bokoch, 2003). Once activated, Lim kinase phosphorylates cofilin. In a phosphorylated state, cofilin is unable to bind with actin, losing its ability to promote actin catastrophe (Maciver and Hussey, 2002; Bokoch, 2003). Another protein, nWASP, has been shown to act through a Rho-dependant mechanism to have opposite effects on actin dynamics. nWASP is activated by the RhoGTPase, cdc42, allowing it to associate with Arp2/3 and effectively leading to the growth of filamentous actin (Luo, 2000).

Actin dynamics, therefore, is a fundamental process in the growth and development of dendritic spines that is under the control of various molecular switches, such as Git1 and Rac. As NOS1AP has been shown to stabilize a Scribble, Git1/βPix/PAK complex ultimately leading to an increase in Rac activity, it has direct implications in actin remodeling and the subsequent development of dendritic spines.

1.3 The Cerebellum

Recent reports have identified a significant reduction in NOS1AP levels within the cerebellum of patients with schizophrenia (Hadzimichalis et al., 2010). Furthermore, a
growing body of evidence suggests that cerebellar abnormalities occur in schizophrenia (Andreasen et al., 1998; Daskalakis et al., 2005; Andreasen and Pierson, 2008). As we are currently unaware of the specific role of NOS1APc within this brain region, it is necessary to provide a general introduction to the cerebellum.

1.3.1 Anatomy and Circuitry

The cerebellum is a distinct part of the brain in all vertebrates and its histological structure is nearly indistinguishable in all mammals. The cerebellum is divided into two hemispheres that are separated by a narrow midline zone called the vermis. Deep fissures that extend across the cerebellum further subdivide these regions into ten lobules (Glickstein and Voogd, 1995; Voogd and Glickstein, 1998). The most superficial tissue is a thin cortical sheet that is comprised entirely of grey matter. Beneath the cortical layer is the inner white matter, which houses the deep cerebellar nuclei (Glickstein and Voogd, 1995; Voogd and Glickstein, 1998). The cerebellum is widely accepted as a key regulator of motor-related functions, as cerebellar lesions are most often associated with defects in coordination, equilibrium and fine motor control. More recently, however, the cerebellum has also been accepted as contributing to more complex cognitive functions, such as; attention, language and timing (Wolf et al., 2009).

Unlike the cerebral cortex, which has very obvious structural differences among different regions, the cerebellar cortex is divided into a highly regular three-layered arrangement. This layer of cortical tissue consists of a single row of Purkinje neurons between an outer molecular layer and an inner granule layer (Glickstein and Voogd, 1995; Voogd and
The lower granule cell layer consists of tiny, densely packed granule cells, the most abundant cell type in the human brain. The Purkinje layer consists entirely of Purkinje cell bodies, while the superficial molecular layer houses the flattened dendritic projections from these cells in addition to a large number of parallel fibres (Glickstein and Voogd, 1995; Voogd and Glickstein, 1998).

There are two sources of sensory input into the cerebellar circuit: climbing fibres or mossy fibres, both of which terminate on Purkinje cells. Mossy fibres constitute the main source of input into the cerebellum forming both direct and indirect connections with the deep cerebellar nuclei (Mason and Gregory, 1984; Voogd and Glickstein, 1998). The direct connection is excitatory and it projects from collateral branching of mossy fibres, while the indirect connection is inhibitory and it begins with mossy fibres synapsing on granule cells (Herrup and Kuemerle, 1997; Voogd and Glickstein, 1998). These granule cells send axons called parallel fibres which enter the molecular layer and bifurcate, forming excitatory connections with the dendritic projections of a large number of Purkinje cells (Herrup and Kuemerle, 1997; Voogd and Glickstein, 1998). The arrangement of the molecular layer allows for many sources of mossy fibre input to contact a single Purkinje cell, resulting in a widespread activation. Thus, Purkinje neurons, which are the sole motor output of the cerebellum are controlled by both excitatory and inhibitory inputs and these cells are responsible for integrating vast amounts of sensory information and relaying this into a coordinated outgoing motor response (Glickstein and Voogd, 1995; Herrup and Kuemerle, 1997; Voogd and Glickstein, 1998).
1.3.2 NO Activity in the Cerebellum: Implications with Schizophrenia

NOS activity, particularly nNOS has higher levels of activity within the cerebellum than any other CNS structure (Manto and Fatemi, 2004). Specifically, it acts to regulate cGMP levels in both granule cells and Purkinje cells. Activity of parallel fibres within the molecular layer are known to result in NO release, which leads to an increase in cGMP levels in adjacent cells to regulate synaptic morphology (Manto and Fatemi, 2004). This is of particular importance when considering the level of plasticity that is required for motor learning at synapses between parallel fibres and Purkinje cells. NO activity has also been identified as a critical messenger for inducing LTP at mossy fibre-granule cell synapses, acting as a retrograde signal to increase presynaptic excitability (Maffei et al., 2003).

Interestingly, NO activity has been implicated in the pathological involvement of schizophrenia, a disorder often characterized by defects in synaptic activity (Karson et al., 1996; Bernstein et al., 1998; Shinkai et al., 2002; Yanik et al., 2003; Baba et al., 2004; Manto and Fatemi, 2004). This is supported by findings that demonstrate increased allelic frequencies of a polymorphism of the human NOS1 gene in schizophrenic patients compared to controls (Shinkai et al., 2002). As NOS1AP has recently been shown to be downregulated in the cerebellum of patients with schizophrenia, this identifies a potential role for NOS1AP-mediated nNOS signaling in the pathophysiology of this disease (Hadzimichalis et al., 2010).
1.3.3 The Cerebellum in Schizophrenia: Human Studies

For many years, the cerebellum has been widely accepted as a coordinator of motor and vestibular function. Much of this knowledge has been attained from lesion studies in humans. Depending on the anatomical location of lesions within the cerebellum, various defects can be identified, including; disturbances in gait, control of limbs, articulation and smooth pursuit of eye movements (Bastian et al., 1998; Blazquez et al., 2004; Thach and Bastian, 2004). In recent years, it has become increasingly evident that the cerebellum may be involved in many higher cognitive functions through its reciprocal connections with the cerebral cortex. This growing body of evidence suggests that cerebellar malfunction may play a role in various cognitive deficits associated with schizophrenia (Akshoomoff and Courchesne, 1992; Kim et al., 1994; Allen et al., 1997; Seidler et al., 2002).

As it is now widely accepted that the cerebellum has a role in facilitating various cortical tasks, malfunction of these circuits is thought to contribute to various cognitive dysfunctions seen in schizophrenia. In addition to changes in cerebellar size, experimenters have used positron emission tomography (PET) imaging to demonstrate an overall reduction in relative and absolute metabolic rates within the cerebellum of schizophrenia patients (Volkow et al., 1992; Loeber et al., 2001; James et al., 2004). Abnormalities have also been widely reported from neuropathological findings that show a significant reduction in the size and decreased linear density of Purkinje cells (Reyes and Gordon, 1981; Tran et al., 1998). These findings are particularly important since
Purkinje cells provide the major output of the cerebellum. Using functional magnetic resonance imaging (fMRI) and PET imaging, abnormalities have been noted in both vermis and cerebellar hemispheres relating to various mental tasks. Compared to control subjects, schizophrenic patients showed abnormalities in blood flow to the vermis in response to studies of emotion, tasks that require input from limbic regions (Andreasen et al., 1996; Muller et al., 2002; Paradiso et al., 2003). Additionally, in studies using higher cortical regions, such as memory and retrieval tasks, schizophrenic patients showed abnormalities in more lateral regions of cerebellar hemispheres (Crespo-Facorro et al., 2001; Walter et al., 2007).

1.3.4 Schizophrenia: Synaptic Implications within the Cerebellum

Schizophrenia has often been characterized by dysfunctional synaptic activity, particularly within the prefrontal cortex. This knowledge stems mainly from a number of susceptibility genes for schizophrenia that are known to be involved in synapse regulation (Eastwood et al., 2001; Harrison and Eastwood, 2001; Andreasen and Pierson, 2008). For example, dysbindin, a known synaptic regulator, has been shown to be significantly downregulated within the prefrontal cortex and hippocampus of schizophrenia patients (Chen et al., 2008; Zhao et al., 2009). Interestingly, within the CNS, dysbindin has dramatically elevated levels of expression in mossy fibres of the cerebellum and hippocampus (Benson et al., 2001). This suggests a role for this protein at very large synaptic terminals, most notably within the cerebellum. Furthermore, in an effort to identify aberrant activity of synaptic proteins within the cerebellum of schizophrenic patients, experimenters were able to show that synaptophysin and Complexin II showed a
reduced immunoreactivity of 31% and 36% respectively, compared to control patients (Eastwood et al., 2001). These findings support a potential role for decreased synaptic activity within the cerebellum of schizophrenic patients.

Recently, dysbindin has been shown to be a nucleocytoplasmic shuttling protein and that this shuttling activity directly regulates the levels of synapsin I (Fei et al., 2010). Upon translocation to the nucleus, dysbindin has been shown to directly activate synapsin I transcription (Fei et al., 2010). As previously mentioned, the synapsin family of proteins has been implicated in vesicle release. Additionally, synapsin I has been shown to act in a complex with NOS1AP and nNOS to regulate presynaptic morphology (Jaffrey et al., 2002b). As NOS1APc maintains the PTB domain necessary for binding to synapsin I, this identifies a potential link for this novel protein in the development of schizophrenia within the cerebellum.

1.4 Glial Cells

Glial cells are non-neuronal cells found in the nervous system that act as a support system for neurons. They can be subdivided into two groups based on their developmental origin. Microglia are small glial cells that originate from the mesoderm and differentiate in bone marrow (Chan et al., 2007). The remaining types of glial cells are derived from neuroepithelial progenitor cells and include: ependymal cells, oligodendrocytes, radial glia, Schwann cells, satellite cells and astrocytes (Zhang, 2001; Chan et al., 2007; Allen
and Barres, 2009). As we have recently identified NOS1APc within the nucleus of a subpopulation of astrocytes, it is necessary to introduce the function of these cells.

1.4.1 Astrocyte Characterization and Identification

Astrocytes are the most abundant type of glial cell in the nervous system (Montgomery, 1994; Sofroniew and Vinters, 2009). They are found ubiquitously throughout the CNS and are easily identifiable by their characteristic star-like shape (Montgomery, 1994). Astrocytes give rise to extensive and variable branching, depending on which neuronal region they are found (Sofroniew and Vinters, 2009). Branching, in turn, gives rise to end feet which often terminate on synaptic junctions (Sofroniew and Vinters, 2009). In highly plastic brain regions such as the hippocampus or cortex, a single astrocyte is estimated to contact over 100 000 synapses (Oberheim et al., 2006).

Expression of glial fibrillary acidic protein (GFAP) has become a widely used marker for immunohistochemical detection of astrocytes (Sofroniew and Vinters, 2009). GFAP is a member of the type III family of intermediate filament (IF) proteins (Pekny and Pekna, 2004). There are several known isoforms of this protein, including GFAP α, β, γ, δ and κ, however the distribution and function of these isoforms is poorly understood (Sofroniew and Vinters, 2009). While GFAP is not necessary for the proper functioning of most astrocytes, it is essential for glial scar formation and the process of reactive astrogliosis making it a sensitive marker of CNS neuronal injury (Pekny and Pekna, 2004; Sofroniew and Vinters, 2009).
1.4.2 Astrocytes and NO Signaling

Astrocytes have been long been identified as having active roles in response to CNS damage. Reactive astrogliosis is a well-identified pathological marker for diseased or damaged neuronal tissue (Sofroniew, 2009; Sofroniew and Vinters, 2009). While the characteristic qualities of reactive astrogliosis vary widely in relation to the extent of injury, they almost always include cell hypertrophy, proliferation and dramatic changes in cell signaling and gene expression (Sofroniew, 2009; Sofroniew and Vinters, 2009). These cellular changes can result in either beneficial or detrimental effects on surrounding tissue depending on the context and extent of injury. In severe injury, upregulation of GFAP has been shown to have pronounced effects on tissue reorganization (Sofroniew, 2009; Sofroniew and Vinters, 2009). Other signs of extensive neuronal damage include the formation of glial scars, which is shown to have neuroprotective effects in creating a barrier between inflammatory cells or infectious agents and adjacent healthy tissue (Sofroniew, 2009; Sofroniew and Vinters, 2009; Voskuhl et al., 2009). In other cases, elevation of certain signaling molecules, such as NO, glutamate, or various cytokines can have neurotoxic effects (Sofroniew, 2009).

Reactive or ‘activated’ astrocytes generally show an overexpression of inflammatory mediators. Generally, iNOS is produced in response to inflammatory conditions, however eNOS and nNOS have also been shown to be inducible with a longer temporal pattern of expression (Hirsch et al., 1993; Murphy et al., 1993; Ma et al., 1994). This variability in temporal expression profiles may be suggestive of a correlation with the level of injury in which these cells are responding to. As severe reactive astrogliosis requires more long-
term restructurings of tissue architecture, a molecule such as nNOS or eNOS with a longer temporal expression would be favorable when compared to the more short-lived effects of iNOS. Regardless, these various NOS enzymes elicit their function through the production of NO, which is known to have biphasic effects on neuronal tissue (Calabrese et al., 2007). At normal physiologic concentrations, NO is neuroprotective and acts to promote cell proliferation, while at higher concentrations it has the opposite effects, causing cell death (Calabrese et al., 2007). This biphasic role for NO is consistent with the spectrum of effects that can arise from reactive astrogliosis. As previously mentioned, the variability in signaling changes resulting from CNS injury can lead either to proliferative or neurotoxic effects.

Recently, a role for NOS1AP has been identified in astrocytes relating to the pathological conditions arising from NO activity. Following overexpression of NO in rat primary astrocytes, experimenters were able to show that NOS1AP mRNA and protein was significantly upregulated (Jiang et al., 2010). Furthermore, when compared to untreated astrocytes, where NOS1AP immunoreactivity was localized mainly within the cytoplasm, NOS1AP translocates to the nucleus following treatment with NO donors. Following NO treatment, NOS1AP also colocalized with nNOS in the nucleus of these cells (Jiang et al., 2010). Interestingly, another study has identified a case where NOS1AP translocates to the nucleus following treatment with lipopolysaccharide (LPS) (Shao et al., 2011). LPS is a key mediator of the immune response in reactive astrogliosis, as it induces the expression of both cytokines and NOS enzymes (Sharma et al., 2000; Marchini et al., 2005; Geoghegan-Morphet et al., 2007). In this study, varying levels of LPS were
injected into cultures containing either primary cortical or hippocampal neurons. This coincided with a dose-dependant increase in NOS1AP mRNA and protein, also causing shuttling of the protein to the nucleus (Shao et al., 2011). Taken together, these findings imply a role for NOS1AP in mediating inflammatory reactions within the injured CNS. As the C-terminal extension of NOS1APc destroys the nNOS-binding domain, it is possible that this protein may be acting as a dominant negative form of NOS1AP in relation to NO activity. This is supportive of the biphasic role of NO in reactive astrogliosis, as its levels must be carefully regulated to avoid unwanted cell death. Of interest to the present study is the fact that the NOS1AP antibody used in these studies was raised to a region common to both NOS1AP and NOS1APc. As neither of these studies included overexpression experiments to see if NOS1AP selectively enters the nucleus under such conditions, it is unclear which isoform was being detected in the nucleus. This identifies a potential link for NOS1APc as a mediator of the inflammatory response in reactive astrocytes.

1.5 The Cell Cycle

Recently, Scribble, an interacting protein with NOS1AP, has been shown to be a negative regulator of cell-cycle progression (Nagasaka et al., 2006). This suggests that NOS1AP may play a role in the cycling activity of cells. As NOS1APc maintains the essential binding domain necessary for an interaction with Scribble, this identifies a potential link for NOS1APc within the cell cycle. An introduction to this topic is, therefore, necessary.
1.5.1 Phases of the Cell Cycle

The cell-division cycle is a ubiquitous sequence of orderly events in which the cell duplicates its contents then divides; a process that underlies the growth and development of all organisms (Schafer, 1998). In eukaryotic cells, this cycle can be divided into two broad phases: interphase, where the cell grows and duplicates its genetic content – and the mitotic (M) phase, where the cell splits into two genetically identical daughter cells (Schafer, 1998; Vermeulen et al., 2003). To control these events, the cell cycle control system responds to a series of molecular switches, receiving signals from inside and outside the cell (Schafer, 1998; Vermeulen et al., 2003).

It is now widely accepted that interphase can be further subdivided into three distinct phases: G1 phase, S (synthesis) phase and G2 phase, which combined with mitosis makes up the cell cycle (Schafer, 1998; Vermeulen et al., 2003). At each phase, different steps are necessary for coordinated cell division to occur. For example, during S phase, the cell duplicates its genome, and cell division subsequently occurs during M phase (Schafer, 1998; Vermeulen et al., 2003). G1 and G2 constitute the “gap” phases and they occur prior to S phase and M phase, respectively (Schafer, 1998; Vermeulen et al., 2003). During these gap phases the cell continues to grow; however during these phases the cell is able to determine if the environmental conditions are appropriate before committing to the increased metabolic demands of DNA replication (S phase) and cell division (M phase) (Vermeulen et al., 2003). Gap phases, therefore, act as checkpoints that allow for the prevention of DNA replication or division in a cell where DNA damage may have occurred or the cells metabolic demands cannot be met. In such a case, cell cycle arrest
occurs at either $G_1$ or $G_2$ allowing for the cell to repair itself prior replicating its DNA or before entering mitosis (Schafer, 1998; Vermeulen et al., 2003). This information, therefore, directs cells to proceed, pause or exit the cell cycle by temporarily or permanently entering a state of quiescence or cell cycle arrest; a phase known as $G_0$ (Schafer, 1998; Vermeulen et al., 2003).

1.5.2 Cyclins and Cyclin-dependant Protein Kinases

The regulation of the cell cycle depends on a number of cyclically activating proteins and protein complexes that initiate and control DNA replication or mitosis. These events are controlled by a specific set of protein kinases called cyclin-dependant protein kinases (Cdks) (Schafer, 1998; Vermeulen et al., 2003). Protein kinases are enzymes that carry out phosphorylation reactions, whereby they add phosphate groups to a variety of protein substrates that control processes in the cell cycle (Schafer, 1998; Vermeulen et al., 2003). While Cdks are found in relatively stable levels throughout the cell cycle, their activation is dependant on another set of proteins called cyclins, whose levels fluctuate throughout the cell cycle (Schafer, 1998; Vermeulen et al., 2003). Activation of Cdks are dependant on the formation of cyclin-Cdk complexes (Schafer, 1998; Vermeulen et al., 2003). At high levels, cyclins bind to Cdks and complex formation results in the activation of Cdk active sites (Schafer, 1998; Vermeulen et al., 2003). It is the cyclic activity of cyclin molecules, therefore, that triggers various cell cycle events through the formation of cyclin-Cdk complexes.
1.5.3 Scribble Negatively Regulates Cell Cycle Progression

Cyclin-Cdk complexes have long been known to be under the regulation of inhibitory proteins called cyclin-dependant kinase inhibitors (CKIs). CKIs are involved in cell cycle arrest at the G1 phase and they can act to inhibit most cyclin-Cdk complexes (Shackelford et al., 1999). For example, p21 is a CKI that binds to cyclinE/Cdk2 and prevents G1 -S phase transition (Sherr and Roberts, 1999). While cyclin E is known to be significantly downregulated in cells arrested in G1, little is known about the upstream signals that regulate its transcription (Brumby et al., 2004). In order to identify genetic interactors of cyclin E in *Drosophila*, Brumby et al. (2004) utilized a genetic screen for dominant modifiers of a hypomorphic cyclin E mutation, *DmcycEjp* (Brumby et al., 2004). In this screen, they identified Scribble as a novel negative regulator of G1 -S phase transition.

Recently, a study by Nagasaka et al. (2006) identified a homologous role for human Scribble (hScrib) in negatively regulating cell cycle progression from G1 -S phase (Nagasaka et al., 2006). In this study, hScrib was transfected into mammalian epithelial cells and was shown to suppress cell proliferation through its effects on the cell cycle. Through analysis of the critical domains of Scribble, it was identified that hScrib requires at least LRRs and a single PDZ domain to exert its effects on cell cycle regulation (Nagasaka et al., 2006). Previous studies identified that *Drosophila* Scribble requires these same two domains for its proper localization at the septate junction of epithelial cells (Albertson et al., 2004). Loss of either LRRs or PDZ domains results in the mislocalization of Scribble in the nucleus or cytoplasm, respectively (Albertson et al.,...
A two-step model was proposed, where LRRs are responsible for targeting Scribble to the plasma membrane, while PDZ domains enhance Scribble binding to septate junctions (Albertson et al., 2004). Nagasaka et al (2006) identified a similar mechanism for hScrib using mammalian epithelial cells (Nagasaka et al., 2006). They suggest that LRRs are responsible for the localization of hScrib at the plasma membrane and that PDZ domains limit hScrib to adherens junctions where it can convey extracellular signals to the intracellular environment.

Previously, PDZ1 of hScrib was shown to be both necessary and sufficient in binding the tumor suppressor protein Adenomatous polyposis coli (APC) (Takizawa et al., 2006). Furthermore, in cells transfected with hScrib it was confirmed that the disruption of cell cycle entry from G₁-S phase was dependant on the upregulation of APC (Takizawa et al., 2006). Interestingly, human discs large (hDlg), which exists as part of a complex with hScrib, has also been shown to bind APC through its PDZ domain (Matsumine et al., 1996). Previously, hDlg has also been shown to cooperate with APC in cell cycle regulation (Matsumine et al., 1996). Taken together, this suggests an important role for the interaction of these proteins in the negative regulation of G₁-S phase transition. As NOS1APc is likely an interacting protein with Scribble, and it has recently been identified in the nucleus of cycling cells, this identifies a potential link for this protein in the cell cycle.
1.6 Summary and Rationale

NOS1AP has been identified as an adaptor protein that is able to shuttle between various protein complexes. Within these complexes, it has been implicated in various roles within the nervous system. In this thesis, I have discussed the role of NOS1AP in synapse morphology, and how it can shuttle between various complexes to elicit this function. I also argued that through its role as a synaptic regulator, NOS1AP is implicated in the pathogenesis of schizophrenia. Specifically, I discussed a potential role for NOS1AP within the cerebellum in the development of this disease.

I have also discussed a potential role for NOS1AP within activated astrocytes, as an nNOS-targetting protein. In this role, I have argued that it may exert biphasic effects following CNS injury, through a NO-dependant mechanism.

Finally, I have discussed a potential role for NOS1AP in the regulation of the cell cycle through an interaction with Scribble. These issues are important in light of the recent evidence showing that a novel NOS1AP isoform, NOS1APc, might exist. The aim of this thesis is to characterize the novel NOS1APc isoform and determine where it is expressed in order to give context to what roles, if any, this novel isoform play in the cellular events described above.
1.7 Hypothesis

*I hypothesize that there are novel isoforms of NOS1AP that exist within the developing CNS.*

1.8 Objectives

1. Characterization of NOS1APc mRNA and protein using isoform-specific reagents.

2. Determine the localization of NOS1APc protein and mRNA using isoform-specific reagents.

3. Determine the subcellular localization of NOS1APc within various cell types, combined with various markers
2.1 Cell culture

Human Embryonic Kidney (HEK) 293T cells, rat primary liver cells and astrocyte cultures were grown at 37 °C, 5% carbon dioxide in Dulbecco’s Modified Eagles Medium (DMEM) (Wisent; Cat #319-005-CL) or horse serum (Invitrogen; Cat # 16050-114) with 10% heat-inactivated fetal bovine serum (FBS) (Wisent Cat #090150), 2mM L-glutamine (Wisent; Cat #609-065-EL), 100 U/mL penicillin and 100 mg/mL streptomycin (Wisent; Cat #609-065-EL).

2.2 Northern Blotting for NOS1APc RNA

2.2.1 Isolation of Tissue

RNA was extracted from wild-type rats provided from Jackson Laboratories. Adult rats (n = 5) were sacrificed through cervical dislocation, and various tissues were surgically removed under a dissecting microscope; genotype and sex were not considered. This procedure was completed according to ethics protocol no. 10-031 from the Carleton animal care facility at Dalhousie University.

2.2.2 Isolation of Cell Lines

For RNA extraction from various cell lines, cells were grown to 70% confluence, media was removed through aspiration and cells were washed twice with 10 mL PBS.
2.2.3 Isolation of RNA from Animal Tissue and Cell Lines

Total RNA was extracted from either brain tissue or cultured cell lines using phenol saturated TRIzol (Invitrogen; Cat #15596-26). For rat tissues, 100 mg of the desired tissue was homogenized in 1 mL of TRIzol. For cell lines, 2 mL of TRIzol was added for each 10 cm plate and cells were collected by scraping. After a 5 minute incubation at room temperature, 200 μl of chloroform (Fisher) was added per mL of TRIzol reagent. After a short vortex, the samples were left to sit for 5 minutes, then centrifuged at 12 000 x g for 15 minutes at 4 °C. The top aqueous phase was removed and added to new tubes containing 500 μl of isopropanol (Fisher). Following a 10 minute incubation at room temperature, the samples were centrifuged at 12 000 x g for 10 minutes at 4 °C. The resulting supernatant was removed and discarded, while the resulting pellet was washed with 1 mL of 70% ethanol (Fisher). After centrifugation for 5 minutes at 7 500 x g (4 °C), the supernatant was removed, and the resulting pellet air dried for 5-10 minutes. The dried pellet was resuspended into 50 μl of distilled RNase free water (Ambion; Cat # AM9937) and quantified using a Spectrophotometer (Shimadzu BioTech-mini) according to manufacturers protocol. Optical density ratios (260nm/280nm) between 1.7 and 2.0 were considered acceptable.

In order to isolate poly A+ messenger RNA (mRNA) from total RNA, an Oligotex Direct mRNA kit (Qiagen; Cat #70022) was used following manufacturers guidelines. Briefly, total RNA (250 μg) was brought up to a volume of 250 μL with RNase-free water. An equal volume of Buffer OBB was added, followed by the addition of preheated (37 °C)
Oligotex suspension (15 μL). To disrupt the secondary structure of the RNA, the sample was then heated for 3 minutes at 70°C, followed by a 10 minute incubation at 30°C to allow hybridization between the oligo dT$_{30}$ of the Oligotex particle and the poly-A tail of the mRNA. This complex was collected by centrifugation for 2 minutes at 13 000 x g. The Oligotex:mRNA pellet was resuspended in 400 μL of Buffer OW2, then pipetted onto a small spin column and centrifuged at 13 000 x g for 1 minute. The spin column was transferred to a new microcentrifuge tube and once again washed with Buffer OW2. To elute poly A+ mRNA, the spin column was transferred to a new tube; 70 μL of hot Buffer OEB (70°C) was added directly to the column then centrifuged for 1 minute to yield the final product.

2.2.4 Northern Blotting

Total RNA (10 μg) or Poly A+ mRNA (1 μg) was dissolved in sample buffer containing 57 % deionized formamide (Fisher), 20% formaldehyde (37%) (Fisher), 11.4% 10X MOPS buffer (Sigma), 5.7% RNase-free glycerol (Fisher), 5.7% Bromophenol blue. Samples were brought up to 60 μl using the appropriate volume of sample buffer, heated (65 °C for 15 minutes) then loaded onto an agarose gel containing 1.5% formaldehyde. The gel was prepared by dissolving 3.75g of agarose in 25 mL of 10X MOPS buffer and 180 mL of distilled water. After heating the agarose solution to adequately dissolve the agarose, the mixture was cooled to 50 °C and 2.2 M Formaldehyde was added. Once hardened, samples were loaded onto the gel and electrophoresed overnight at 5 V/cm in 1X MOPS running buffer.
RNA was then transferred to a Nylon membrane (Ambion; Cat #10104) according to the following protocol. The gel was removed from the running buffer and the formaldehyde was removed from the gel by two 15-minute washes in 20X saline sodium citrate (SSC) (Sigma). The downward transfer apparatus (from Schleicher & Schuell) was set up as follows. Twenty Whatman 3M filter papers were laid down into the transfer apparatus. Two blotting papers were then laid on top, followed by a positively charged nylon membrane (presoaked in double distilled water) then the formaldehyde gel containing migrated RNA. To complete the sandwich, three blotting papers presoaked in 2X SSC were placed on top. A buffer wick resting in a pool of 2X SSC was placed over top of these layers. The gel was left to transfer overnight. The following day, the membrane was lifted from the transfer apparatus, rinsed in distilled water and washed once using 2X SSC to remove any excess salt. The membrane was then air-dried and the RNA was immobilized using a UV Stratalinker (120 mJ for 1 minute).

2.2.5 Generation of a NOS1APc-specific cDNA Probe

A NOS1APc-specific DNA probe was made by subcloning the unique sequence (amino acids 506-773; GenBank: ADE96994) of NOS1APc into a pSPORT vector using the following primers: 5’ TTTTCGAATTCTATGTTTGAGAATTG’; 3’ GCGGCGCGCTACTCAAAGG-ACAG. Primers were designed to contain EcoR1 and Not1 restriction sites at the 5’ and 3’ end, respectively. To generate a cDNA probe, 10 μg of this construct was digested with EcoR1 and Not1 enzymes (Roche). The digestion was run on a 1% agarose gel and the 807 base pair fragment was excised and gel purified using a QIAGEN Gel Extraction kit (Cat #; 20021). Briefly, three volumes of Buffer
QX1 were added per volume of gel. After the addition of 20 μL of QIAEX II beads, the mixture was placed at 50 °C for 10 minutes in order to solubilize the agarose fragment and bind DNA to the QIAEX II particles. Following this step a one minute centrifugation at 13 000 x g was performed to collect the DNA. The resulting pellet was then washed once with Buffer QX1, and twice with Buffer PE (with each subsequent wash requiring a centrifugation step to collect the pellet). Following the final wash step, the resulting pellet was air dried for 15 minutes and eluted with 20 μL of Tris-EDTA buffer. A probe specific for β-actin was also generated using this method with the purposes of using it as a loading control.

2.2.6 Labeling of cDNA Probes and Hybridization

The gel purified NOS1APc template DNA or β-actin (Dr. Chris Sinal) control was radiolabeled using the Prime It RmT random primer labeling kit (Stratagene; Cat #300392-1). Here, 50 ng of DNA was brought to a final volume of 42 μL with distilled water and added to a single-use reaction tube. This mixture was heated to 100 °C for 5 minutes and its contents were collected by brief centrifugation. To radioactively label the probe, [α-32P] dCTP (Perkin Elmer) and magenta DNA polymerase (Stratagene kit) were added at concentrations of 3000 Ci/mmol and 4 U/ μL, respectively. This reaction was incubated at 37 °C for 10 minutes. To stop the mix, 2 μL of stop mix (provided with the kit) was added to each reaction tube. The specific activity of the probe was measured using a scintillation counter (Dr. Jonathan Blay, Department of Pharmacology). Here, 2 μL of labeled probe was added to a vial of scintillation fluid and the automatic counting option for α-32P determined the number of counts per minute (specific activity of the
probe). After prehybridization of the RNA-containing nylon membrane for 30 minutes (using 15mL PerfectHyb Plus Hybridization Buffer (Sigma; Cat #H7033)), $10^7$ counts of either the NOS1APc or β-actin radiolabeled probe was added and left to hybridize overnight. These steps were performed at 65 °C in cylindrical glass containers (Fisher; Cat #13-247-300) using a revolving hybridization oven. Following the overnight incubation, the blot was subjected to three posthybridization washes at 65 °C using 2X SSC containing 0.5% sodium dodecyl sulfate (SDS). The blot was then encased in Saran Wrap and exposed to X-ray film (Kodak; Cat #864 6770) in the presence of an intensifying screen for 3 days at -80 °C to visualize the bands.

2.3. *In situ* Hybridization

2.3.1. Tissue preparation

Brains were removed from wild-type female rats ($n = 5$) obtained from Jackson Laboratories. Cerebella were removed by making a coronal section between the cortex and cerebellar fissure. The cerebella were then rapidly frozen by dropping them into liquid nitrogen. Cerebella were kept at -80 °C until needed, then mounted in Tissue-Tek media (Sakura; Cat #4583) in a Tissue-Tek cryomold (Sakura; Cat # 4566) for sectioning on a cryostat. Sections (20μm) were collected on SuperfrostPlus glass slides (Fisher; Cat # 12-550-15) and immediately placed in 4% paraformaldehyde (PFA) at room temperature for 1 hour. [Note: All solutions used were treated with 0.1% diethyl pyrocarbonate (DEPC) (Sigma; Cat #D5758) and glassware was treated with RNase ZAP (Sigma; Cat#R2020)]. Sections were subsequently washed three times in 0.1M PBS, then once in distilled water. Acetylation was performed by placing slides in 1%
triethanolamine (Sigma; Cat #T1377)/0.25% acetic anhydride (Fisher; Cat # 351001) in water. The sections were then washed twice in 0.1M PBS, once in 2X SSC and placed in prehybridization buffer (50% deionized formamide, 25% 20X SSC, 12% ddH2O, 10% Denhardtts solution (Sigma; Cat #30915), 2% yeast RNA (Roche; Cat #10 109 223 001), 1% salmon sperm (Sigma; Cat #31149) for 4 hours. Prehybridization and Hybridization steps were carried out at 58 °C in a humid chamber, with a reservoir of 5X SSC/50% formamide located on the bottom.

2.3.2 Generation of NOS1APc-specific RNA Probes

Antisense and sense strand RNA probes were made against the 807 base pair unique C-terminus of NOS1APc (GenBank: ADE96994); this was done using a digoxigenin-labeling method. The unique NOS1APc sequence was subcloned into the PT7T3-pac cloning vector, containing EcoR1 and Not1 restriction sites at the 5’ and 3’ end, respectively. Briefly, 6 μg of this construct was linearized using either EcoR1 or Not1 restriction enzymes to yield templates for generating antisense or sense strand probes, respectively. Linearized templates were then purified through phenol/chloroform extraction, according to the following protocol; DNA digests were brought up to a volume of 300 μL with nuclease-free water containing 0.3M sodium acetate. This mixture was added to a phenol/chloroform solution (50% saturated phenol, 45% chloroform, 5% amyl alcohol), briefly vortexed, then centrifuged at 12 000 x g for 5 minutes. Following centrifugation, the top aqueous layer was removed, and precipitated with 3 equal volumes of 100% ethanol (overnight at -20 °C). The following day, precipitated plasmid DNA was recovered by centrifugation (12 000 x g for 30 minutes at
4°C) and washed using RNase-free 70% ethanol to remove any residual salt from the nucleic acid pellet. After a final centrifugation step to collect the washed pellet, it was resuspended at a final concentration of 0.5 μg/μL in RNase-free water.

The following protocol was used to generate a digoxigenin (DIG)-labeled RNA probe according to manufacturers’ guidelines (Roche Cat. No. 11 031 163 001). Briefly, to 1 μg of template DNA, 2 μL of 10X transcription buffer, 2 μL 10X DIG RNA labeling mix, 2 μL of either T3 or T7 RNA polymerase and 12 μL nuclease-free water were added. [Note: T3 RNA polymerase was used to transcribe antisense RNA strand from EcoR1 digested plasmid DNA, while T7 was used to transcribe sense strand RNA from Not1 digested plasmid DNA.] Transcription reactions were then carried out at 37°C for 2 hours, followed by the addition of 1 μL turbo DNase (Ambion; Cat # AM2238) for 15 minutes at 37°C. 10mM EDTA was added to stop the reaction. Digoxigenin-labeled probes were stored at a temperature of -80 °C until used.

2.3.3 Hybridization and Detection
RNA probes (20-200ng) were denatured by heating at 80°C for 10 minutes, then 150μL of hybridization mixture was added per slide and covered with Parafilm. The following day, post-hybridization wash buffers were equilibrated to hybridization temperature (58 °C), and each wash was carried out for 5 minutes in the following order (5X SSC, 2X SSC, 0.2X SSC, 50% formamide/0.2X SSC). After the final wash at 58 °C, slides were transferred to 0.2X SSC, then washed twice in 1X detection buffer (each for 5 minutes at room temperature). Sections were then blocked for 1 hour at room temperature in 1X
blocking buffer (88% detection buffer (1M Tris-Cl/1.5M NaCl, pH 7.5), 10% blocking reagent (Roche; Cat #11 096 176 001), 2% sheep serum (Sigma; Cat #G9023), 0.1% Triton X-100 (Sigma; Cat #X100). After blocking step, sections were incubated in with anti-DIG antibody-alkaline phosphatase (AP) conjugate (1:1000 in 1X blocking buffer) (Roche; Cat #11 093 274 910) for 1 hour at room temperature. Slides were then rinsed in 1X detection buffer then submerged in 1X detection buffer. This step was repeated twice for 15 minutes at room temperature. Following these washes, the slides were submerged into equilibration buffer (5% 1M Tris buffer pH 9.5, 0.5% MgCl2, 0.3% NaCl in distilled water) for 5 minutes at room temperature. After equilibration, slides were then developed in a solution containing 0.36 % nitro blue tetrazolium chloride (NBT) and 0.33% 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in equilibration buffer until desired colorimetric reaction was reached. Three subsequent washes in 10 mM Tris (pH 8.0) were performed for 10 minutes each. Slides were then rinsed in distilled water and air-dried. Fluoromount media (Sigma; Cat #F4680) and glass coverslips were placed on slides, which were subsequently stored in the dark at room temperature.

2.4. Cortical Astroglia Cultures

Brains were harvested from one-day old rat pups and were placed in a dish containing Hanks Balanced Salt Solution (HBSS; Invitrogen Cat # 14175). Meninges were removed to prevent the overgrowth of fibroblasts in the culture. Cerebral hemispheres were removed, finely chopped with scissors then placed into a volume of 12 ml HBSS containing 1.5 mL of each 2.5% trypsin (Gibco; Cat #10345) and 1% DNase; this mixture was then incubated at 37°C for 5 minutes with occasional swirling. Careful mechanical
trituration was then performed in order to dissociate the tissue, and the mixture was returned to 37°C for 10 minutes with occasional swirling; this step was repeated once. Undissociated tissue was excluded after running through a cell strainer; dissociated cells were collected in 15 mL of glial medium (Kaech and Banker, 2006). This mixture was collected in a 50 mL conical tube and a centrifugation step was performed (120 x g) to remove enzymes and lysed cells present in the supernatant. The resulting pellet was resuspended in 15 mL of glial medium, and 10μL of resuspension mix was added to a hemacytometer in order to count cell density (four chambers were counted in order to give an average). Cells were plated at a density of 7.5 x 10⁶ cells per 75-cm² flask and 15 mL of glial media was added. Cultures were fed every 2-3 days with fresh glial media. Before removing old media, the flask was shaken 5-10 times to dislodge loosely attached cells. Once astroglia had reached confluence (after approximately 10 days in culture) they were harvested by the addition of 5 mL trypsin/EDTA at 37°C for 2 minutes. To stop trypsinization, 5 mL of glial media was added. Cells were removed by repetitive pipetting, transferred to a conical centrifuge tube and collected by centrifugation at 120 x g for 5 minutes. Finally, cells were prepared and coverslipped according to Kaech & Banker (2006).

Once coverslipped, cells were allowed to grow in culture for an additional 14 days, or until desired maturity. At this point, cells were washed three times using sterile 1X PBS, blocked for 1 hour using 1X PBS (5% goat serum (Sigma; Cat # G9023), 0.1% Triton X-100), then incubated in primary antibody overnight at 4°C. A NOS1APc-specific Protein-A purified antibody and GFAP antibody (BD BioSciences; Cat # 556327) were used at
concentrations of 1:5000 and 1:2500 in block, respectively. The following day, cells were washed three times using 1X PBS, then stained using Alexa Fluor secondary antibodies for 1 hour at room temperature (Invitrogen). Goat anti-rabbit (Alexa Fluor 594; Cat #A11037) was used for detection of NOS1APc, while goat anti-mouse (Alexa Fluor 488; Cat # A11029) was used to detect the GFAP antibody; both antibodies were used at concentrations of 1:2000 in PBS (2% goat serum) Briefly, cells were washed twice in PBS, then incubated for 5 minutes at room temperature in BisBenzimide (Hoechst 33258; Sigma Cat # B1155) at a concentration of 1:10 000 in PBS. Again, cells were briefly washed twice in PBS, then mounted onto slides using Fluoromount mounting media and observed under fluorescence microscopy.

2.5.BrdU labeling

Following two weeks in culture, primary glial cell cultures were pulsed with 10μM 5-bromo-2-deoxyuridine (BrdU) for 1 hour at 37°C then washed once with 1 X PBS. Cells were fixed with PBS containing 4% paraformaldehyde (PFA) and 4% sucrose for 10 minutes at room temperature. The PFA solution was removed and cells were washed three times with 1X PBS. Cells were blocked and permeabilized by the addition of PBS containing 5% goat serum and 0.1% Triton X-100 for 1 hour at room temperature. This was followed by the addition of NOS1APc Protein A-purified antibody (1:5000) in PBS (5% goat serum). After this overnight incubation at 4°C, cells were washed three times in PBS, then incubated for 1 hour at room temperature in secondary antibody (goat anti-rabbit Alexa Fluor 594) at a concentration of 1:2000 in PBS (2% goat serum). Cells were then washed two times in PBS, and incubated in 2N HCl for 45 minutes at room
temperature. This was followed by two 15-minute washes in 0.1M Borate Buffer (pH 8.3), then three washes in PBS. Rat anti-BrdU (AbCam; Cat #ab6326) was then added at a concentration of 1:200 in PBS (2% goat serum) and allowed to incubate overnight at 4°C. The following day, cells were washed three times in PBS, then incubated for 1 hour at room temperature in secondary antibody (goat anti-mouse alexa 488, Invitrogen; Cat # A11029) at a concentration of 1:2000 in 1ml PBS (2% goat serum). Following two washes in PBS, BisBenzimide was used as a nuclear stain by diluting 1: 10 000 in PBS and incubating with cells for 5 minutes at room temperature. Cells were then washed three times with 1 ml PBS and rinsed using double deionized water. Coverslips were mounted on slides using Fluoromount mounting medium and observed under fluorescence microscopy.

2.6. Antibodies

2.6.1 Generation of a NOS1APc-specific GST-fusion Antibody

A GST-fusion antibody was created to recognize the unique NOS1APc sequence (amino acids 506-773; GenBank: ADE96994). Oligonucleotide primers (5’, TTTTCGAATT-CTATGTTTGAGAAT-TTG; 3’, TTTGTCGACTGGTTACTACTCAAAGGACAG) were used to amplify this unique sequence through polymerase chain reaction (PCR). Primers were designed to generate an amino terminal EcoR1 site and a carboxyl-terminal Sal1 site. The resulting PCR product was digested with EcoR1 and Sal1 then subcloned into EcoR1/Sal1 sites of PGEXT3 placing the coding region of NOS1APc in frame with the GST vector. The cDNA was then transformed into BL21 competent bacteria. A single positive colony was picked and then grown overnight in a 5mL culture of Lysogeny broth
(LB) containing 50 μg/mL Ampicillin (Wisent; Cat #400-110-EG). The following day, this culture was added to 100 mL of LB/Amp solution and shaken at 37 °C. Cultures were grown until they reached an optical density (600nm) of 0.6-0.8, then cells were induced with a final concentration of 5mM isopropyl-β-D-thio-galactoside (IPTG) (Fermentas; Cat #R0393). Induction was carried out for 4 hours at 37 °C, followed by centrifugation for 20 minutes at 3 000 x g to harvest the bacterial cells. The resulting pellet was resuspended in an NP40 lysis buffer containing 37.5 mM NaCl, 20mM Tris pH 8.0, 10% glycerol, 1% NP40 with 1mM phenylmethylsulphonylfluoride (PMSF), 10mg/ml leupeptin (Sigma; Cat #M92884), 10mg/ml aprotinin (Sigma; Cat #A1153), and 10mg/ml Benzamidine (Sigma). Following a 30-minute incubation on ice, the resuspended bacterial cells were lysed by sonication. Lysed cells were then centrifuged at 13 000 x g for 20 minutes, and the supernatant collected for further processing.

Glutathione sepharose beads were added (100 μl of a 50% slurry was added for every 10 ml of lysed bacteria) to the supernatant containing our protein of interest. Following an incubation at 4 °C for 30 minutes, this mixture was washed three times in NP40 lysis buffer then once 1X PBS. Following the final wash, the mixture was centrifuged at 3 000 x to pellet the beads, resuspended in a solution of 0.1 M reduced glutathione (Sigma) and allowed to incubate on ice for 30 minutes. Beads were then separated by centrifugation and the supernatant (containing a Glutatione-S-Transferase (GST)-fusion protein) was dialyzed overnight in 1X PBS at 4 °C. The following day, GST-fusion protein was concentrated and purified in Centricon tubes (Millipore) to a final concentration of 200 μg/mL.
To generate the GST-NOS1APc antibody, 100 μg of purified GST fusion protein was resuspended in 600 μl of 1X PBS, then mixed with 600 μl of complete Freunds adjuvant (CFA; Santa Cruz). Tituration of this mixture was performed to generate an emulsion, which was then injected subcutaneously into adult New Zealand white rabbits obtained from Charles River laboratories, according to animal ethics protocol (06-119) at the Carleton Animal Care Facility. Boosts were conducted at intervals of six weeks, where incomplete Freund’s adjuvant was substituted for CFA. Serum samples were collected fourteen days following each boost.

2.6.2 Generation of a NOS1APc-specific peptide antibody

A 20 amino acid peptide antibody (CAFPLLDPDPITRKTPEAL, herein PPIT peptide) was generated against an amino acid stretch in the unique region of NOS1APc; this was performed using FMOC (9H-fluoren-9-ylmethoxycarbonyl) synthesis with an N-terminal cysteine residue. Two μg of this peptide was then conjugated to Keyhole Limpet Hemocyanin (KLH) using sulfhydryl reactive protocols according to the manufacturer (Pierce Kit No. 77614). KLH conjugated PPIT peptide was generated in aliquots of 50 μl. For antibody production, aliquots were brought up in volume to 600 μl in PBS and added to 600 μl of complete Freund’s adjuvant. Immunization and collection of serum were performed as outlined above.
2.7 NOS1APc Immunohistochemistry

Adult female rats (n = 5) from Jackson Laboratories were euthanized by injecting sodium pentobarbital at a concentration of 2mL/4.5 kg of body weight. Rat tissues were then perfused using 4% PFA in PBS, then brains were surgically removed and post-fixed for 24 hours. Cryoprotection was subsequently performed by sinking brains overnight in PBS containing 30% sucrose. The cerebellum/brainstem region was surgically removed from the brain then cut down the midsagittal plane. Each hemisphere was placed in a Tissue-Tek cryomold and rapidly frozen on dry ice in an embedding medium consisting of 2 parts OCT (Sakura, Cat # 4583): 1 part PBS (20% sucrose). Embedded brain tissue was then mounted on a chuck at the desired cutting temperature (-22°C) such that midsagittal sections would be obtained first. Sections were cut were at 20 μm, collected on SuperfrostPlus glass slides and allowed to dry for 1 hour at room temperature.

Two separate methods of immunostaining were used for the detection of NOS1APc protein; fluorescence staining and Diaminobenzene (DAB) staining (according to manufacturers protocol (Vectastain ABC kit; Vector Labs Cat # PK-6102)). For fluorescence staining, sections were washed three times in PBS then blocked and permeabilized in PBS containing 5% goat serum and 0.1% Triton X-100 for 1 hour at room temperature. This was followed by the addition of NOS1APc Protein A-purified antibody (1:5000) in PBS (4% goat serum). After an overnight incubation at 4°C, sections were washed three times in PBS, then incubated for 1 hour at room temperature in secondary antibody (goat anti-rabbit Alexa Fluor 594) at a concentration of 1:2000 in PBS (2% goat serum). Following two washes in PBS, BisBenzimide was used as a
nuclear stain by diluting 1:10 000 in PBS and incubating with sections for 5 minutes at room temperature. Slides were then washed three times in PBS, dipped quickly in double deionized water, and coverslipped using Fluromount mounting media. Sections were observed under fluorescence microscopy.

2.8 NOS1APc Immunoprecipitation and Western Blotting

Adult rat brains were lysed in appropriate amounts of an NP40 lysis buffer (1ml/mg of tissue), using a dounce homogenizer. Clarified lysate was collected from the supernatant of homogenized tissue following centrifugation at 13 000 x g for 15 minutes at 4°C. For whole cell lysate, 40μL of supernatant was removed and added to a 2X SDS sample buffer containing 250mM Tris pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue, 0.4M Dithiothreitol (DTT). The remaining cleared lysate was then incubated overnight with the desired antibody. The following day, lysate was incubated on a nutator with 100μL of a 10% slurry of Protein A Sepharose (GE Healthcare; Cat #17-0963-02). The beads (containing linked proteins) were then washed four times using NP40 lysis buffer and centrifugation was performed (4000 x g for 1 minute) before each wash to collect the beads. After the final wash, buffer was removed and beads were resuspended in 40μL of 2X SDS sample buffer. Samples were then eluted by boiling at 100°C for 5 minutes, prior to being run on a 7% SDS-PAGE and subjected to western blot analysis.

After running the gel, proteins were transferred to a Millipore PVDF membrane (presoaked in methanol) (Cat #IPVH00010) using a wet transfer apparatus. Following transfer, the membrane was washed in 10 mL of Tris-buffered saline Tween 20 (TBST).
The membrane was then blocked for 1 hour in 15 mL TBST containing 5% milk powder, and incubated with the desired primary antibody overnight at 4 °C. All NOS1APc antibodies were used at a concentration of 1:1000 in blocking solution. The following day, membranes were washed three times for 10 minutes in 10 mL TBST. After the third wash, membranes were incubated for one hour in 10 mL of TBST containing 1 μl Protein A conjugated to HRP (horseradish peroxidase) (abcam; Cat #ab7245). Again, three 10-minute washes were performed using 10 mL TBST.

Following treatment with Protein-A-HRP, membranes were blotted onto filter paper to remove excess liquid. Chemiluminescence was then performed using SuperSignal West Pico solutions (ThermoScientific) according to manufacturers’ protocol. Membranes were then exposed onto X-Ray film and autoradiography was performed. Exposure times for NOS1APc antibodies ranged from 30 seconds to 10 minutes for enhanced exposure.
CHAPTER 3       RESULTS

3.1 Identification of NOS1APc

The work in this thesis extends a finding in Richier et al., 2010. In Richier et al., (2010) a proteomic screen was conducted to identify Scribble interacting proteins. One of the proteins identified was a Nitric Oxide Synthase1 Adaptor Protein (NOS1AP). In addition to this adaptor protein, a second, slower migrating band of 100kDa was identified that contained peptides for NOS1AP. The band also contained peptides for a second unknown protein with a predicted molecular weight of 30kDa. Since neither NOS1AP (70kDa) nor this 30kDa could account for the apparent 100kDa size, this prompted us to consider the possibility that these two proteins might be a novel NOS1AP isoform. We tested this in silico by merging the two cDNA’s and blasting this sequence to determine if there was a potential cDNA in the database that would correspond to this unique protein.

Surprisingly, the entire in silico fusion cDNA mapped to exons within the same locus on chromosome 13 (Rattus norvegicus) as NOS1AP suggesting that it was a unique cDNA (Richier et al., 2010). Despite the in silico evidence, there is little information about this isoform.

To confirm the existence of this novel isoform, PCR amplification was performed using primers specific to the last exon for NOS1AP and the predicted first exon of the novel region (Richier et al., 2010). A cDNA library generated from adult rat brain was used as the template. The resulting PCR product was then sequenced, confirming this novel
splice variant. As this was the third isoform of NOS1AP to be discovered, we have called it NOS1APc (GenBank: ADE96994) (Fig 3.1). NOS1APc is predicted to contain the N-terminal PTB domain and has a novel C-terminal peptide extension that destroys the PDZ binding motif of NOS1AP. This novel isoform also contains a short proline-rich region found within the C-terminal peptide extension (Figure 3.1, highlighted region).

3.2 Northern blotting confirms the existence of a novel NOS1APc isoform

To confirm this novel isoform exists in vivo, I performed Northern blot analysis. Total or polyA+ mRNA was isolated from a number of different cell lines and tissues (Figure 3.2) and probed with a cDNA probe generated against the unique region of NOS1APc (base pairs 1518-2319, GenBank: ADE96994).

Total RNA isolated from a number of different tissues showed a band at approximately 7 kilo base pairs (kb). This 7 kb band was detected in neuronal (hippocampus, cortex, cerebellum and whole brain) (Figure 3.2A, lanes 1-4 (arrow)) and non-neuronal tissues (heart, kidney, lung and skeletal muscle) (Figure 3.2A, lanes, 2-4 & 6 (arrow)). Little to no expression was seen in the pancreas or spleen (Figure 3.2A, lanes 5 & 7). Interestingly, an elevated level of expression was evident in the cerebellum when compared to other tissues (Figure 3.2A, lane 3 (arrow)).

In addition to the 7 kb band, a second faint band was detected at approximately 5kb just below the 28 S band (Figure 3.2A, arrowhead). To confirm whether this band corresponded to a legitimate transcript, a series of Northern blot experiments were then
Figure 3.1. Complete peptide sequence of NOS1APc. Highlighted (red) region corresponds to the C-terminal peptide extension unique to NOS1APc. This was adapted from Richier et al. (2010)
Figure 3.2. (A-C) Identification of NOS1APc transcript through Northern blot
(A) Total RNA was extracted from various tissues, run on a denaturing gel and transferred
to a nylon membrane. This membrane was subsequently probed using a NOS1APc-
specific cDNA probe. Arrow represents NOS1APc transcript at 7kb (with an elevated
level of expression evident in the cerebellum). Also, note the presence of a faster
migrating band at 5kb (arrowhead). (B,C) messengerRNA was isolated from total RNA
of various tissues using a PolyA+ extraction. mRNA was run on a denaturing gel,
transferred to a nylon membrane and probed using a NOS1APc-specific DNA probe.
These blots were subsequently re-probed with beta-actin as a loading control. Arrows
represent NOS1APc transcript at 7kb. Note the presence of a faster migrating (5kb) band
in RNA extracted from rat liver cells (arrowhead; Fig 3B lane 2).
performed on tissues in which polyA+ mRNA was isolated. Similar to the total RNA Northern, a band was detected at approximately 7 kb in various tissues and cell lines (Figure 3.2B, lanes 1 & 2 (arrow); Figure 3.2B, lanes 3 & 4; Figure 3.2 C, lanes 1-3 (arrow)). As seen in the total RNA Northern blot, levels of the 7kb band were higher in the cerebellum than in other regions of the CNS including the hippocampus and cortex (Figure 3.2B, lane 2; Figure 3.2C lanes 2 & 3 (arrows)). This was confirmed since the tubulin reprobe showed equal loading of mRNA (Figure 3.2B,C; lower panels). An elevated level of expression was also evident in rat primary liver cells grown in primary culture (Figure 3.2B, lane 2 (arrow)). In addition to the 7 kb transcript, a 5 kb transcript was evident in a liver cell line (Figure 3.2B, lane 2 (arrowhead)). This suggests that a second transcript of NOS1APc may exist, although at lower levels.

3.3 In situ hybridization: NOS1APc is enriched in the adult cerebellum

I next wanted to localize NOS1APc mRNA by in situ hybridization using an anti-sense RNA probe directed against the unique 3’ region of NOS1APc (corresponding to the entire unique C-terminus). Sagittal sections of adult rat brains were probed with our NOS1APc-specific probe (Figure 3.3). Staining was observed in forebrain regions, including the hippocampus and cerebral cortex (Figure 3.3 B-D,G-I). Punctate staining was observed in the CA1, CA2, CA3 and dentate gyrus (DG) of the hippocampus (Figure 3.3B-D). As well, different layers of the cerebral cortex showed staining (Figure 3.3G-I). No staining was detected in adjacent tissues using a sense probe (Figure 3.3E,J).
Figure 3.3 (A-J) Identification of NOS1APc mRNA within the hippocampus and cortex of adult rat. (A) Sagittal section of adult rat brain. Boxed region indicates hippocampus expanded in B-E. Scale bar = 6.0 mm. (B) Antisense staining reveals positive cells within the CA1, CA2, CA3 and dentate gyrus of the hippocampus. Upper and lower boxed regions indicate CA1 and CA2, respectively. Scale bar = 0.7 mm. (C) Magnified view of CA2 region from (B). Scale bar = 140 µm. (D) Magnified view of CA1 region from (B). Scale bar = 140 µm. (E) Sense probe lacked any positive staining. Scale bar = 0.7 mm. (F) Sagittal section of adult rat brain. Boxed region indicates cerebral cortex expanded in G-J. Scale bar = 6.0 mm. (G) NOS1APc within the cortex. Scale bar = 80 µm. (H) Magnified view of boxed region from (G) indicating NOS1APc-positive cells. Scale bar = 200 µm. (I) Magnified view of boxed region from (H) indicating NOS1AP-positive cells within layers III and IV of the cortex. Scale bar = 140 µm. (J) Sense staining, indicating no positive staining with the cortex. Scale bar = 80 µm.
Moving caudally, robust positive staining was in the cerebellum (Figure 3.4B,D) and brain stem (Figure 3.5A-F). Within the cerebellum the staining was localized to the Purkinje cell layer of the cerebellar cortex (Figure 3.4B,D). A sense-probe revealed no staining in this region confirming the specificity of our NOS1APc probe (Figure 3.4C). To confirm that NOS1APc mRNA was localized to Purkinje cells, we co-stained sections with a Purkinje cell specific marker, Calbindin – a calcium binding protein (Christakos et al., 1989) (Figure 3.4B, lower right panel). Cells containing NOS1APc mRNA were also Calbindin positive, confirming these to be Purkinje cells.

In addition to the cerebellar staining, NOS1APc mRNA was also evident in cells within the brain stem, in particular the pons and medulla (Figure 3.5A-F). Positive cells were seen in the medial vestibular nucleus and intermediate reticular nucleus (Figure 3.5C,F). Taken together, this suggests that NOS1APc mRNA is distributed within cells in the forebrain, brain stem and Purkinje cells of the cerebellum.

3.4 NOS1APc protein is expressed in the adult rat brain

Northern blotting and in situ hybridization experiments confirmed the existence of a unique NOS1APc isoform. To confirm that NOS1APc protein was being expressed, a number of antibodies were generated. One against the carboxy-terminal region (amino acids 365-503, Gene ID: 192363) of Rattus NOS1AP (pan NOS1AP; described in Richier et al. 2010), a second against a peptide unique to the NOS1APc region and a third against a fusion protein generated by linking glutathione S-transferase in frame with the unique C-terminal region of NOS1APc (amino acids 506-773; GenBank: ADE96994).
Figure 3.4. Localization of NOS1APc mRNA in adult Rattus cerebellum (A) Mid-sagittal section from adult rat brain. Boxed area indicates the location of the cerebellum. Scale bar = 5.0 mm (B-E) NOS1APc staining within the cerebellum revealed positive staining in (B) Purkinje cells of the cerebellum (using an antisense probe). Scale bar = 100 µm. NOS1APc staining within (D) presumptive Purkinje cells was confirmed by (E) Calbindin immunohistochemistry (green) Scale bars = 14 µm. As a control, (C) sense probe was used to stain adjacent sections, and revealed no positive staining. Scale bar = 100 µm.
Figure 3.5 Localization of NOS1APc mRNA in adult Rattus brainstem. (A-F) NOS1APc in situ hybridization of sagittal sections from the adult rat cerebellum, pons and medulla. (A,D) Sagittal section of adult rat cerebellum and brainstem. Boxed areas indicate regions within the pons and medulla. Scale bar = 2.0 mm. (B,E) Magnified view of boxed regions from (A & D, respectively) indicating NOS1APc positive cells within the pons and medulla. Scale bar = 200 µm. (C,F) Magnified view of boxed regions from (B & E, respectively). Note the punctate staining pattern of NOS1APc within the identified regions. Scale bar = 100 µm. IRN, intermediate reticular nucleus; LC, locus coeruleus; LRN, lateral reticular nucleus; MVe, medial vestibular nucleus; Sol, nucleus solitary trract; SpVe, spinal vestibular nucleus.
To test the specificity of the NOS1APc peptide antibody, adult rat brain lysate was precipitated with either a pre-immune antibody or the NOS1APc peptide antibody. The immunoprecipitations (IP), along with whole cell lysate was then separated through SDS-PAGE, transferred and probed with the NOS1APc peptide antibody (Figure 3.6A). Of interest, the whole cell lysate detected only one band at the same molecular weight as the IP (100kDa) using the NOS1APc peptide antibody. No band was detected in the pre-immune lane confirming the specificity and legitimacy of our antibody (Figure 3.6A, lane 3 (arrow)).

3.5 Characterization of various NOS1APc-specific antibodies

Since it had previously been shown that the NOS1AP antibody, in addition to detecting NOS1AP, had detected a slower migrating band (Richer et. al. 2010), I wanted to test whether this slower migrating band was in fact the NOS1APc isoform. Therefore, I precipitated endogenous NOS1AP proteins from rodent brain lysate using either the NOS1AP peptide or NOS1AP-GST fusion antibodies and probed the IPs with either the NOS1APc peptide or NOS1APc GST antibody. Interestingly, a band migrating at approximately 100kDa was seen with each of the three antibodies but not the pre-immune control when probed with either the peptide (Figure 3.6A, lane 2-3 (arrow); Figure 3.6 B & C, lanes 2-4 (arrow)) or GST antibody (Figures 3.6A,B & C, lane 1). Interestingly, all three antibodies precipitated a faster migrating band (approximately 70kDa) (Figure 3.6A,B & C (arrowhead)). The 70kDa band was detected with both the NOS1APc peptide and GST-fusion antibody. This band is either another novel isoform containing the novel C-terminal region of NOS1APc or a cross-reacting protein. This remains to be
Figure 3.6 Characterization of various NOS1APc antibodies. (A) NOS1APc was precipitated from rat brain lysate using a NOS1APc peptide antibody. This immunoprecipitation (IP), along with whole cell lysate, was subjected to Western blot analysis and probed with the peptide antibody. In both cases, NOS1APc was recognized (arrow). Note the presence of a faster migrating band in the NOS1APc IP (arrowhead).

(B,C) NOS1APc was precipitated from rat brain lysate using three different antibodies; a pan-NOS1AP antibody, a NOS1APc peptide antibody (PPIT) and a GST-fusion NOS1APc antibody. Pre-immune serum was also included as a negative control. Precipitated proteins were then probed using either the (B) peptide or (C) GST-fusion antibody. NOS1APc was recognized under all conditions (arrows). Of interest were two faster migrating bands; one at approximately 70kDa (arrowheads) that was precipitated by all three antibodies and recognized following Western blot using either the peptide or GST antibody. The other faster migrating band at approximately 40 kb (asterix) was detected only by IP with NOS1APc peptide antibody, following WB for either the peptide or GST-fusion antibody.

(D) Western blot elucidating the distribution of NOS1APc expression in the adult rodent brain. Homogenized brain regions were probed with the NOS1APc peptide antibody and reprobed for tubulin as a loading control. Note the presence of NOS1APc specifically within the cerebellum.
tested. In addition to the 70kDa band, the NOS1APc peptide antibody detected a rapidly migrating band at approximately 40kDa that was revealed by both the NOS1APc peptide antibody and the NOS1APc GST fusion antibody (Figures 3.6B,C (asterix)). The identity of this band remains unknown. Taken together, the peptide and GST-fusion NOS1APc antibodies detect a protein at approximately 100kDa that is also precipitated by the pan-NOS1APc antibodies. In addition the 100 kDa isoform, both NOS1APc specific antibodies detected two faster migrating bands, one at 70 kDa the other at 40kDa. Whether these are other NOS1AP isoforms or cross-reacting bands remains to be determined (see discussion).

3.6. NOS1APc is enriched in the cerebellum

Since our NOS1APc specific antibody revealed a single band running at 100kDa, similar to the predicted molecular weight of the NOS1APc specific isoform, I wanted to determine if this was reflected in protein expression. Northern blot analysis revealed different levels of NOS1APc mRNA in different brain regions. To determine the level of NOS1APc protein within the CNS, equal amounts of protein from various brain regions were probed using the NOS1APc peptide antibody. Consistent with the Northern blotting, NOS1APc protein was highest in the cerebellum (Figure 3.6D, lane 2). Following a longer exposure time, detectable levels were apparent in other regions including the hippocampus, cortex and brainstem (data not shown).

To gain a better perspective of NOS1APc protein expression within the adult rat CNS, NOS1APc immunoreactivity was performed on sagittal sections of adult rat brain using
the NOS1APc peptide antibody (Figure 3.7A-H). Positive staining was restricted to the cerebellum (Figure 3.7A, lower panel), specifically within projections of Purkinje cells within the molecular layer (Figure 3.7B-D (arrowhead). No staining was seen using a blocking peptide (Figure3.7A, middle panel) or in the absence of primary antibody (Figure 3.7A, upper panel), confirming the specificity of the staining. To confirm NOS1APc staining within Purkinje cells, sections were co-stained with Calbindin (Figure 3.7F). Both antibodies showed a pattern of staining, confirming NOS1APc expression within Purkinje cell dendrites (Figure 3.7E,F,H). Little staining was seen in other brain regions; this may be due to our antibody recognizing only the 100 kDa NOS1APc isoform (see discussion).

3.7 NOS1APc is located in the nucleus of a subpopulation of proliferating astrocytes

Since we had identified NOS1APc mRNA expression in a number of cell lines, I next decided to examine the distribution of NOS1APc in cells. I chose primary glial cells since they were readily available from another project ongoing in our lab. Interestingly, the NOS1APc peptide antibody detected NOS1APc in the nuclei of a subpopulation of glial cells (Figure 3.8A-C). Punctate nuclear staining, in addition to a diffuse cytoplasmic stain, was also evident using the NOS1APc antibody raised against the GST-fusion protein (Figure 3.8D-F). Since two antibodies detected NOS1APc staining in the nucleus of a subpopulation of primary glial cells, we believe the nuclear staining is legitimate. To further confirm the specificity of the NOS1APc peptide antibody staining, two control experiments were conducted. In the first, we added the immunogenic peptide in excess to
Figure 3.7. NOS1APc is localized in the molecular layer of the rodent cerebellum. (A) DAB immunohistochemistry on sagittal sections reveals that NOS1APc is expressed in the molecular layer of the adult rodent cerebellum. (A) Secondary only (top) and peptide competition (middle) controls were compared to sections stained using a NOS1APc peptide antibody (bottom). Scale bar = 3.0 mm. (B, C and D) Images of increasing magnification of adult rat cerebellum stained for NOS1APc indicate punctate expression that lays along fibers (arrowhead). (B) Scale bar = 100 µm (C) Scale bar = 50 µm (D) Scale bar = 25 µm (E) NOS1APc is located in Purkinje cells in the adult rat cerebellum. Mid-sagittal sections were obtained from adult rat cerebellum and stained using (E) a NOS1APc antibody (red), (F) Calbindin antibody (green), (G) a Hoechst nuclear stain (blue) and (H) merged. Note the localization of NOS1APc in the outer molecular layer of the cerebellum. GCL = Granule Cell Layer, ML = Molecular Layer, PC = Purkinje Cell Layer. Scale bar = 120 µm
Figure 3.8. NOS1APc is localized in the nucleus of glial cells. Glia were isolated from dissociated hippocampal cells and cultured for 13 days. Astroglia were stained using a (A) Protein-A purified peptide antibody specific for NOS1APc (PPIT) (red), (B) Hoechst (blue) and (C) merged. This nuclear expression was also evident using a (D) GST-fusion NOS1APc antibody followed by Hoechst nuclear stain (E) and merged (F). Scale bar = 10 μm.
the peptide antibody prior to staining; this abolished the staining of NOS1APc in the nucleus (Figure 3.9G-I). As a second control, to rule out the possibility that the secondary antibody was cross-reacting with nuclear proteins, we incubated some of the cultures without the primary antibody. Under these conditions, no such staining was identified (Figure 3.9D-F). To confirm that NOS1APc localized specifically within the nucleus of glial cells, we co-stained the primary glial cultures with glial fibrillary acidic protein (GFAP), an astrocytic marker (Figure 3.10A-D (arrow)); this confirmed that NOS1APc is enriched in the nucleus of a subpopulation of astrocytes.

3.8 NOS1APc localizes to the nucleus of cycling cells

As noted, we saw that NOS1APc was present in only a subpopulation of astrocytes, raising the possibility that NOS1APc was enriched in cycling cells. To test this, I pulsed the glial cells with BrdU. Following a one-hour labeling with BrdU, the cells were fixed and co-stained with a BrdU antibody and the NOS1APc peptide antibody. In the fields examined, all cells that were NOS1APc positive had incorporated BrdU into the nucleus (3.11A-D (arrows)). Interestingly, a population of cells that were BrdU positive were also weakly positive for NOS1APc (Figure 3.11A,B (arrowhead)) and some BrdU positive cells were NOS1APc negative (Figure 3.11A,B (asterix)). This suggests that perhaps NOS1APc may change its localization during different phases of the cell cycle. This remains to be tested. Taken together this data suggests that NOS1APc or one of the other isoforms detected by our NOS1APc peptide antibody can be found within the nucleus of cycling cells.
Figure 3.9 NOS1APc is confirmed in the nucleus of glial cells. Glia were isolated from dissociated hippocampal cells and cultured for 13 days. Cells were stained using (A) a Protein-A purified NOS1APc peptide antibody (PPIT) (red), (B) Hoechst nuclear stain (blue) (C) and merged. As a negative control, (D) no primary antibody was used and followed with (E) a nuclear stain, then (F) merged. (G) Peptide competition with the NOS1APc antibody was used as an additional negative control and followed by (H) Hoechst stain, (I) then merged. Scale bar = 10 μm.
Figure 3.10. NOS1APc is confirmed in the nucleus of astroglia. Astroglia were isolated from dissociated rat hippocampal neurons and cultured for 13 days. Cells were co-stained using (A) a NOS1APc Protein-A purified peptide antibody (PPIT) (red), (B) a GFAP antibody (green) and (C) Hoechst nuclear stain (blue). (D) Merged image. Note cells positive for NOS1APc (A) are also GFAP positive (B) (arrows). Scale bar = 5 μm.
Figure 3.11. NOS1APc is expressed in a subpopulation of proliferating astroglial cells. Astroglia were isolated from dissociated hippocampal cells and cultured for 13 days. Cells were stained using (A) a Protein-A purified NOS1APc peptide antibody (PPIT) (red) following (B) a 1-hour pulse with BrdU (green). (C) A subsequent Hoechst nuclear stain was then performed (blue). (D) Merged image. Note cells that are expressing barely detectable levels of NOS1APc that are also BrdU positive (arrowheads) and cells expressing BrdU that are NOS1APc negative (asterix). Scale bar = 5μm.
CHAPTER FOUR       DISCUSSION

4.1 Summary of major findings
The current study characterizes of a novel NOS1AP isoform, NOS1APc. To date, little is known about NOS1APc, therefore, the focus of my investigation has been to create various reagents specific to this isoform and focus on the distribution of mRNA and protein in the rodent CNS and in various cell lines. To date, we have successfully created a NOS1APc mRNA probe and three different antibodies that detect this novel isoform. Using these reagents we have localized a NOS1APc transcript within various neuronal and non-neuronal structures. Of note, high levels of NOS1APc mRNA were detected in the cerebellum; this was confirmed by in situ hybridization and antibody studies. In addition to the localization studies, I have shown that our NOS1APc antibodies detect a protein that localizes to the nucleus of dividing glial cells. Finally, I report that there may be other NOS1APc isoforms.

4.2 NOS1APc is a legitimate NOS1AP isoform distributed throughout multiple tissues
In this work I have identified a novel NOS1AP isoform – NOS1APc. What evidence is there that this is a legitimate isoform? First, I have used Northern blotting using a specific probe against the unique region of NOS1APc and have detected a band that migrates at approximately 7kb. Second, I have used in situ hybridization protocols to show that NOS1APc is found in a number of different cell types in the adult rat CNS. Third, I have used a number of different antibodies to detect an endogenous protein of approximately
100kDa, corresponding to the predicted molecular weight of the NOS1APc isoform. Taken together these three pieces of evidence extend the findings of Richier et al., (2010) arguing that this isoform exists.

4.3 NOS1APc is a NOS1AP isoform

I have argued that NOS1APc is a fusion of the NOS1AP protein with a unique C-terminal extension, suggesting that NOS1APc is an isoform of the NOS1AP gene. Evidence in favour of this comes from Figure 3.6 (A-C). Here I used either an antibody generated against the NOS1AP protein or two different NOS1APc specific antibodies to precipitate endogenous NOS1AP proteins from rat brain. All three antibodies precipitated a 100kDa band that was detected with both of the NOS1APc specific antibodies. Therefore, since the NOS1AP antibody was directed against peptides unique to the NOS1AP region, yet it was able to precipitate a band of the same molecular weight as the NOS1APc antibodies, this argues that the 100kDa protein must contain peptides unique to the NOS1AP protein; thus NOS1APc is an isoform of NOS1AP.

4.4 NOS1AP is widely distributed in the CNS

4.4.1 NOS1APc mRNA

Initially, NOS1AP mRNA and protein were reported as being enriched in neuronal structures, showing no expression outside of the nervous system (Jaffrey et al., 1998). Recently, however, NOS1AP has been implicated in other tissues such as the heart and liver as it has been linked with both long-QT syndrome and diabetes (Hu et al., ; Aarnoudse et al., 2007). Interestingly, in my studies I have demonstrated that NOS1APc
mRNA (by both Northern blotting and *in situ* hybridization studies) is ubiquitously expressed within various tissues in the body, and shows enrichment in the cerebellum. Of all the tissues from which RNA was extracted, only the pancreas and spleen failed to show NOS1APc transcript. This, however, may be misleading since these tissues are very high in nucleases and are notoriously difficult to perform RNA extractions from (Li et al., 2009). Nonetheless, this suggests that NOS1APc is expressed ubiquitously throughout the nervous system and in multiple tissues.

4.4.2 NOS1APc Protein

Both Western blotting and immunocytochemical studies show that NOS1APc protein is expressed mainly in the cerebellum. More specifically, the NOS1APc-peptide antibody showed that the NOS1APc protein was restricted to the dendrites of Purkinje cells in the cerebellum. There was little, if any, protein expression in cell bodies or structures outside of the cerebellum. What could account for the distribution of NOS1APc protein in the dendrites of cerebellar neurons? Previously, others have shown that NOS1AP protein is localized within dendrites of dissociated hippocampal neurons (Carrel et al., 2009; Richier et al., 2010). Thus, it is consistent that the NOS1APc isoform could be directed to dendrites. How might NOS1AP proteins be directed to membranes? One possibility is that NOS1AP may be recruited to membranes through its associating protein, Scribble. Scribble has been shown to associate with membranes through its leucine rich repeat region, and since NOS1AP associates with Scribble though a PTB/PDZ4 interaction (Richier et al., 2010), it is possible that the NOS1AP proteins are directed to dendrites through Scribble. Importantly, both NOS1AP and NOS1APc contain the PTB domain, so
both are expected to bind Scribble. This has been confirmed in our lab by Leanne Clattenburg (personal communication). Alternatively, NOS1AP has been shown to bind the low-density lipoprotein (LDL) receptors (Megalin and ApoER2), and since LDL receptors are found at membranes (Gotthardt et al., 2000), this may account for NOS1AP proteins being found at membranes. Whether the LDL receptors are exclusively localized to the dendrites of cerebellar neurons in vivo remains to be determined. Nonetheless these possibilities could explain the staining of NOS1APc in the dendritic region of Purkinje cells in the cerebellum.

4.5 Identification of novel NOS1APc isoforms?

One of the puzzling results from the immunocytochemical studies was the fact that NOS1APc staining was so strong in the cerebellum, yet absent from the rest of the CNS, despite there being mRNA for NOS1APc in other regions of the CNS. What could account for this inconsistency? One possibility is that there may be other isoforms of NOS1APc. Consistent with this, both the mRNA and protein studies suggest there may be other NOS1AP isoforms containing the unique C-terminal region of NOS1APc. Northern blotting experiments showed a faster migrating band at 5kb (Figure 3.2A (arrowhead); Figure 3.2B, lane 2 (arrowhead)), while both the peptide and GST-fusion antibodies consistently detected, in addition to the 100kDa isoform, two faster migrating bands at approximately 70 kDa and 40kDa. While these bands may be degradation products of NOS1APc, it is unlikely to be the case since a cocktail of protease inhibitors were used in the lysis buffer. Is there other evidence that other NOS1AP isoforms exist? Examination of the NCBI database using the unique C-terminal peptide of NOS1APc
identified a hypothetical peptide that contained the entire C-terminus of NOS1APc with a short N-terminal extension of 15 amino acids. (NCBI Reference Sequence: NM_001190459). This hypothetical protein has a predicted molecular weight of approximately 40 kDa. Consistent with the existence of this hypothetical isoform, others in the lab have used 5’ RLM-RACE technology (Ambion; Cat #AM1700) with primers specific to the unique C-terminus of NOS1APc, and have cloned out this hypothetical cDNA (Jiansong Qi and Leanne Clattenburg, personal communication). This may account for the faster migrating band seen in our NOS1APc IPs, however, this remains to be tested.

What can account for the 70kDa isoform? Interestingly, another group has shown that a short isoform of NOS1AP, called NOS1AP-short (NOS1AP-S), exists (Xu et al., 2005). This polypeptide contains 113 amino acids in common with the C-terminus of NOS1AP but has 12 unique amino acids N-terminally to this region (Xu et al., 2005). NOS1AP-S maintains the PDZ binding domain necessary for an association with nNOS, however it lacks the PTB domain (Xu et al., 2005). As various cases of alternative splicing have been reported for the NOS1AP gene, it is possible that the 70kDa isoform we have reported is a result of splicing between NOS1AP-S and the 40kDa hypothetical protein that we have also detected in our study. This remains to be tested. Nonetheless, it appears that there may be other NOS1AP isoforms that contain the unique C-terminal region of NOS1APc.
Thus, the staining we see for NOS1APc in the cerebellum may reflect the 100kDa NOS1APc isoform. Consistent with this is the Western blot of different brain regions showing that this isoform is highest in the cerebellum with only low levels of this protein being found in other brain regions. The wide distribution of mRNA staining from the in situ experiments may reflect a mixture of the 100kDa, 70kDa and 40kDa isoforms, since they are all predicted to contain the unique C-terminal region of NOS1APc and our probe would not distinguish them. Why, however, did our NOS1APc antibody not detect these smaller isoforms in the immunocytochemical studies? One possibility is that the levels of expression of the 70 and 40 kDa isoforms are too low to be detected using this antibody. Alternatively, these smaller isoforms may contain associating proteins that block the epitope of the NOS1APc peptide antibody, while the 100kDa NOS1APc protein associates with a different protein complex that does not mask the epitope of the NOS1APc peptide antibody. Until we have reagents specific for these isoforms, we will not be able to determine the localization of these other potential isoforms.

4.6 NOS1AP isoforms in the nucleus

The identification of different NOS1AP isoforms that contain the unique C-terminal extension may also help explain why the NOS1APc peptide antibody shows positive staining in the nucleus of dividing cells. I have argued that the NOS1APc 100kDa isoform is likely recruited to membranes. How might this isoform also be found in the nucleus, and what evidence is there that NOS1APc might be localized to the nucleus? First, NOS1AP has been localized to the nucleus following administration of NO donors or LPS stimulation (Shao et al., 2011). Second, the PTB domain of NOS1AP and
NOS1APc contains a stretch of lysine residues, a known nuclear localization signal. A combination of these may direct NOS1AP and NOS1APc into the nucleus. Alternatively, the other suspected isoforms of NOS1AP that contain the NOS1APc unique region may be localized to the nucleus. This will be tested once isoform specific reagents are generated.

4.7 NOS1AP isoforms in the cell cycle

What might be the function of NOS1APc, or a NOS1AP isoform containing the unique C-terminal extension of NOS1APc, in glial cells? One function may be to regulate cell-cycle progression. Scribble, a NOS1APc-associating protein, has been shown to negatively regulate the progression of the cell cycle from G₁ to S phase through an APC-dependent pathway (Matsumine et al., 1996; Takizawa et al., 2006). Thus, NOS1AP proteins may function in some capacity with Scribble to negatively regulate cell cycle progression in glial cells. Alternatively NOS1AP proteins may function as scaffolding proteins to bring other proteins necessary for cell cycle progression into the nucleus, independent of Scribble. These possibilities remain to be tested.

4.8 NOS1AP isoforms: nuclear nitric oxide regulators?

Another potential role for NOS1APc within the nucleus of glial cells comes from the finding that NOS1AP has been reported to translocate to the nucleus of these cells as part of an inflammatory response (Jiang et al., 2010). This study reported that following treatment with either NO donor or LPS, NOS1AP expression was upregulated and it colocalized with nNOS in the nucleus of these cells (Jiang et al., 2010). Here,
experimenters hypothesized that NOS1AP is expected to regulate NO activity, which has biphasic effects on cell proliferation and cell toxicity following CNS injury. As previously mentioned, however, the NOS1AP antibody used in these studies was raised to a region that NOS1APc has in common. If NOS1AP serves to mediate NO signaling within the nucleus of activated astrocytes, there is a potential for NOS1APc to serve as a dominant negative version of NOS1AP as it lacks an nNOS-binding domain. Together, these two isoforms may be involved in precisely regulating NO levels within the nucleus through competitive inhibition of one another.

4.9 NOS1APc isoforms in disease

NOS1AP has recently been implicated in a number of disease processes including schizophrenia, long Q/T syndrome and diabetes. How might the same protein be implicated in so many different disorders?

4.9.1 Schizophrenia

The results we have obtained from in situ hybridization and immunoreactivity using NOS1APc-specific reagents clearly identifies a role for this protein within the cerebellum. More specifically, NOS1APc is highly enriched within the molecular layer in Purkinje cell dendrites. These projections are responsible for receiving vast amounts of information relating to cerebellar motor movement and vestibular input and coordinating them into a motor response. It is also responsible for sending inputs to higher brain regions within the cerebral cortex to modulate cortical activity. The molecular layer, therefore, is an area that undergoes a tremendous amount of synaptic pruning and
dendritic spine morphology. Interestingly, NOS1AP has been identified as a regulator of spine morphology specifically within the hippocampus, another region associated with high levels of synaptic activity.

These findings provide an interesting framework for the involvement of NOS1AP and NOS1APc in the pathogenesis of schizophrenia. As previously mentioned, it is possible that these proteins are eliciting antagonistic roles to one another by acting as competitive inhibitors. This hypothesis is supported by the fact that while NOS1AP levels are downregulated in the cerebellum of schizophrenia patients, the levels of NOS1AP-S (a protein missing the PTB domain yet retaining the PDZ binding motif) have been shown to be upregulated within the prefrontal cortex. Interestingly, in patients affected with schizophrenia these two brain regions have both been identified as having defects in synaptic activity. This suggests that NOS1AP isoforms are highly regulated proteins and that regional imbalances within the prefrontal cortex and cerebellum can contribute to the development of this disease, likely through dysfunctional synapse regulation. It is currently unclear, however, whether these various isoforms play separate roles within individual brain regions. As we have identified that NOS1APc has dramatically elevated levels within the cerebellum, this yields further evidence for a role of this protein within this brain region.

4.9.2 Long Q/T syndrome and Diabetes
Recently, using a genome-wide association study in a general population, an association was identified between a common genetic variant of the NOS1AP gene (single nucleotide
polymorphism: rs10494366) and extremes in QT-interval duration, resulting from defects in cardiac repolarization (Arking et al., 2006; Chang et al., 2007). The involvement of NOS1AP in cardiac repolarization is further supported from a finding that identified abnormal cardiac contractility in nNOS-deficient mice (Ashley et al., 2002; Barouch et al., 2002). Moreover, it is known that NOS1AP can interact with NOS1 in heart tissue to inhibit L-type calcium channels, effectively altering the rate of cardiac repolarization (Leonoudakis et al., 2001; Murata et al., 2001; Chang et al., 2008). Together, these findings identify an association between NOS1AP and both long and short Q/T syndrome in a mechanism that is believed to be dependant on intracellular calcium signaling.

Interestingly, a genetic association has also been identified between the same genetic variant of NOS1AP (rs10494366) and the incidence of type II diabetes in users of calcium channel blockers (Becker et al., 2008; Chu et al., 2010). As it is known that calcium signaling plays a major role in insulin secretion (Becker et al., 2008), this implies a potential link between NOS1AP and these two diseases. It is currently unknown what signaling domains of NOS1AP are responsible for its involvement in long Q/T syndrome and diabetes, implying a potential role for NOS1APc in these disease processes. This is supported by our finding that NOS1APc mRNA is expressed in both heart and liver tissue (an organ with known implications in diabetes through glucose metabolism (Collison et al., 2009)) (Figure 3.2A, lane 5; Figure 3.2B, lane 2). As we have identified two potential transcripts of NOS1APc within both heart and liver tissue, it is unclear which variant of NOS1APc is being expressed, the 7kb or 5kb isoform. Further exploration of the multiple NOS1AP isoforms in these diseases is therefore warranted.
CHAPTER 5  CONCLUSION

In summary, we have confirmed the existence of a novel NOS1AP isoform – NOS1APc. We have generated various reagents, including a NOS1APc mRNA probe and three different antibodies that recognize NOS1APc. Using these reagents, we have localized this novel isoform to various tissues. Through in situ hybridization and immunohistochemical staining, we identified elevated levels of NOS1APc within the rodent cerebellum. Specifically, using our NOS1APc peptide antibody, we were able to localize this protein to the membrane of Purkinje cell dendrites. Additionally, we identified NOS1APc in the nucleus of dividing glial cells. As we have identified two potential novel isoforms containing the unique C-terminal region of NOS1APc, it is unclear which isoform is being expressed within these different cell types. Future directions are to utilize isoform-specific reagents for these newly discovered NOS1APc isoforms to determine their localization and elucidate potential functions in various disease states.
REFERENCES


