Chapter 4

Identification of Novel Interacting Protein Partners of nNOS by the virtue of its PDZ domain.
4 Introduction

Proteins interact with one another to form smaller or larger complexes in a space - and time-dependent manner. Protein-protein interactions (PPIs) imply non-random physical contact between two and more proteins. Interactions are regulated depending on a particular signal, stimulus, developmental stage of the cell, cell-cycle phase, external conditions and obviously the presence of other proteins (interactors)(Ngounou Wetie et al. 2013; Phizicky and Fields 1995). The most common classification of PPIs is based on their lifetime. Protein complexes can consist of sets of more stably interacting proteins (stable PPIs) and less stably (transient PPIs) interacting or combination of both. Transient interactions are expected to control the majority of cellular processes including protein modification, transport, folding, signaling, and cell cycling and promote the interaction, such as phosphorylation, conformational changes or localization to discrete areas of the cell (Kuzmanov and Emili 2013; Phizicky and Fields 1995; Ryan and Matthews 2005; S.Fields 1995).

Proteins bind to each other through a combination of hydrophobic bonding, van der Waals forces, and salt bridges at specific binding domains on each protein. These domains can be small binding clefts or large surfaces and can be just a few peptides long or span hundreds of amino acids, and the strength of the binding is influenced by the size of the binding domain. A common surface domain that facilitates stable protein-protein interactions is the leucine zipper, Two Src homology (SH) domains, SH2 and SH3, PTB (phosphotyrosine binding), bHLH (basic helix loop helix ), LIM(Lin11, Isl-1 & Mec-3), ankyrin repeats, DNA binding domain (DBD) of receptor proteins, and PDZ(post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)). Leucine zippers are usually found as part of a DNA-binding domain in various transcription factors, and are therefore involved in regulating gene expression. Leucine zippers are found in both eukaryotic and prokaryotic regulatory proteins, but are mainly a feature of eukaryotes (Nikolaev 2009). SH2 is a structurally conserved and sequence-specific phosphotyrosine-binding module present in many signaling molecules. In cytoplasmic tyrosine kinases, the SH2 domain is located N-terminally to the catalytic kinase domain (SH1) where it mediates cellular localization,
substrate recruitment, and regulation of kinase activity. Src homology 3 (SH3) domains were initially characterized as a prevalent protein module that recognizes proline-rich sequences. Despite lacking distinguishing features, the ligand-binding surface of an SH3 domain can be molded to accommodate a variety of peptide ligands. Moreover, certain SH3 domains are capable of using surfaces distinct from the canonical ligand-binding site to engage a peptide or protein (Filippakopoulos et al. 2009; Kaneko et al. 2008). PTB domains are found in scaffold proteins that often contain additional modular domains and motifs and thereby nucleate the multiprotein complexes to organize the signaling involved in wide-ranging physiological processes including neural development, immunity, tissue homeostasis and cell growth (Smith et al. 2006; Uhlik et al. 2005). The basic helix-loop-helix (bHLH) proteins form a large superfamily of transcriptional regulators that are found in organisms from yeast to humans and function in critical developmental processes, including sex determination and the development of the nervous system and muscles (Jones 2004). The term ‘LIM’ stems from the first letters of three homoeodomain proteins in which LIM domains were originally identified, namely Linl-1, Isl-1 and Mec-3. In general, the LIM domains are 50–60 amino acids in size and share two characteristic zinc finger domains, which are separated by two amino acids. Nuclear LIM-domain proteins mainly exert tissue-specific gene regulation and cell fate determination functions through interaction with other transcription factors or cofactors, whereas LIM-domain proteins in the cytoplasm take part in cytoskeletal organization and signal transduction through interaction with cytoskeleton and cell—ECM proteins (Zheng and Zhao 2007). Ankyrin repeat, one of the most widely existing protein motifs in nature, consists of 30-34 amino acid residues and exclusively functions to mediate protein-protein interactions, some of which are directly involved in the development of human cancer and other diseases (Li et al. 2006). Nuclear receptors (NRs) are involved in many physiological processes, diseases, and therapeutic applications. They are transcription factors that contain a DNA-binding domain (DBD) which is the most conserved NR domain and consists of two α helices that are coordinated by zinc molecules, thus forming two zinc-finger modules. The first zinc finger is responsible for the recognition of AREs, while the second zinc finger is involved in DNA-dependent dimerization (Helsen et al. 2012)
PDZ domains are ubiquitous protein interaction modules that play a key role in cellular signaling. PDZ is an acronym combining the first letters of three proteins — post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1) — which were first discovered to share the domain. PDZ domains have previously been referred to as DHR (Dlg homologous region) or GLGF (glycine-leucine-glycine-phenylalanine) domains (Alderton et al. 2001; Zhou and Zhu 2009). The binding specificity involves recognition of the carboxyl-terminus of various proteins, often belonging to receptor and ion channel families. PDZ domains also mediate more complicated molecular networks through PDZ-PDZ interactions, recognition of internal protein sequences or phosphatidylinositol moieties. The domains often form a tandem of multiple copies, but equally often such tandems or single PDZ domain occur in combination with other signaling domains (for example SH3, DH/PH, GUK, LIM, CaMK). Common occurrence of PDZ domains in Metazoans strongly suggests that their evolutionary appearance results from the complication of signaling mechanisms in multicellular organisms (Jeleń et al. 2003).

Although the binding of many PDZ-containing proteins occurs by recognition of the extreme C-terminus of target proteins, some PDZ domains can also bind to internal sequences of target proteins. The most well-characterized example of this nature is the interaction between the PDZ domain of the syntrophin (or PSD-95) protein with the internal -hairpin finger structure of the nNOS protein. The N-terminal PDZ domain mediates the subcellular discrete localization of nNOS protein by interacting with adaptor proteins like: nNOS is targeted to membranes by binding to syntrophin, PSD95/SAP90, or PSD93. By anchoring nNOS to membrane or cytosolic protein via direct PDZ–PDZ domain or C-terminal-PDZ interactions, NO signaling is altered. PSD95 (post-synaptic density protein-95), a multivalent synaptic scaffolding protein and core component of the post-synaptic density, can link nNOS to N-methyl-D-aspartate receptor (NMDAR), and accounts for the efficient activation of nNOS by NMDAR stimulation (Cheah et al. 2006; Christopherson et al. 1999). Binding of nNOS to PSD95 is a determinant of post-synaptic targeting of nNOS other adapter protein, designated CAPON, which contains a C-terminal PDZ domain domain that binds to the N-terminal PDZ domain of nNOS as well as an N-terminal phosphotyrosine binding
(PTB) domain (Jaffrey et al. 1998). CAPON interacts with Dexras1, a brain-enriched member of the Ras family of small G proteins that is selectively induced by dexamethasone. The interaction of nNOS and CAPON provides a mechanism for NO delivery to Dexras1, leading to S-nitrosylation of Dexras1 on cysteine-11. It has been demonstrated that Dexras1 binds to the peripheral benzodiazepine receptor-associated protein (PAP7), and PAP7 in turn binds to the divalent metal transporter (DMT1), an iron import channel mediating iron uptake in neurons (Fang et al. 2000). Essential element iron plays a wide variety of physiological and biochemical roles. However, abnormally high cellular iron levels may lead to disordered neuronal function (Moos and Morgan 2004).

Since physical contact between proteins may trigger conformational changes or posttranslational modifications (PTMs) that modulate the activity of those proteins. Therefore, identification of the binding partners of a protein in a given cellular environment is essential since execution of a particular protein function will be strongly dependent on contact with surfaces of neighboring proteins. (Ngounou Wetie et al. 2013; Phizicky and Fields 1995; S.Fields 1995).

The common methods to detect protein-protein interaction are: protein affinity chromatography, affinity blotting, coimmunoprecipitation, and cross-linking; molecular biological methods such as protein probing, the two-hybrid system, and phage display. Stable protein-protein interactions are easiest to isolate by physical methods like co-immunoprecipitation and pull-down assays because the protein complex does not disassemble over time. Weak or transient interactions can be identified using these methods by first covalently crosslinking the proteins to freeze the interaction during the co-IP or pull-down. Alternatively, crosslinking, along with label transfer and far-Western blot analysis, can be performed independent of other methods to identify protein-protein interactions (Berggard et al. 2007; Kuzmanov and Emili 2013; Miernyk and Thelen 2008; Ngounou Wetie et al. 2013; Phizicky and Fields 1995).

Pull-down assays are similar in methodology to co-immunoprecipitation because of the use of beaded support to purify interacting proteins. The difference between these two approaches, though, is that while co-IP uses antibodies to capture protein complexes, pull-down assays use a “bait” protein to purify any proteins in a lysate that bind to the
bait. Pull-down assays are ideal for studying strong or stable interactions or those for which no antibody is available for co-immunoprecipitation (Figure 1). Besides investigating the interaction of two or more proteins, pull-down assays are a powerful tool to detect the activation status of specific proteins. For example, proteins that are activated in response to tyrosine phosphorylation can be pulled down using an immobilized SH2 domain that targets the phosphorylated tyrosine on a given protein. Additionally, GTPases, which act as molecular switches that regulate cell signaling by cycling between a GTP-bound (active) and GDP-bound (inactive) state, can be pulled down using an immobilized GTPase-binding domain of downstream proteins that are recruited to GTP-bound, activated GTPases. In both types of pull-down assays, because the specificity of the interaction is dependent on the sequence of the binding domain, these approaches are highly specific in detecting the activation of distinct proteins.

Bait proteins for pull-down assays can be generated either by linking an affinity tag to proteins purified by traditional purification methods or by expressing recombinant fusion-tagged proteins. The purified protein can be tagged with a protein-reactive tag commonly used for such labeling applications. Alternatively, if a cloned gene is available, molecular biology methods can be employed to subclone the gene to an appropriate vector with a fusion tag. Recombinant clones can be overexpressed and easily purified, resulting in an abundance of bait protein for use in pull-down assays.

Co-immunoprecipitation (co-IP) is a popular technique for protein interaction discovery with the assumption that is when associated proteins are co-precipitated these proteins are related to the function of the target antigen at the cellular level. Co-IP is conducted in essentially the same manner as an immunoprecipitation (IP) of a single protein, except that the target protein precipitated by the antibody, also called the “bait”, is used to co-precipitate a binding partner/protein complex, or “prey”, from a lysate. Essentially, the interacting protein is bound to the target antigen, which is bound by the antibody that is immobilized to the support. Immunoprecipitated proteins and their binding partners are commonly detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) and Western blot analysis (Figure 2).
Figure 1: General schematic of a pull-down assay. A pull-down assay is a small-scale affinity purification technique similar to immunoprecipitation, except that the antibody is replaced by some other affinity system. In this case, the affinity system consists of avidin agarose beads, respectively. The immobilized fusion-tagged (biotin-tag) protein acts as the “bait” to capture a putative binding partner (i.e., the “prey”). In a typical pull-down assay, the immobilized bait protein is incubated with a cell lysate, and after the prescribed washing steps, the complexes are selectively eluted using competitive analytes or low pH or reducing buffers for in-gel or Western blot analysis.

Figure 2. Schematic summary of a standard co-immunoprecipitation assay.
Present study was aimed to find the novel interacting protein partners of nNOS through its PDZ domain.

4.1 Objectives

1. To find the novel protein-protein interaction of nNOS by
   (a) Pull down assay
      (i) Generation of nNOS affinity column required for pull down assay.
      (ii) Pull down assay
   (b) Co-immunoprecipitation

4.2 Materials and Methods

Chemicals

All chemicals and biochemicals were purchased from the following standard commercial sources such as: Sigma Chemical Co., St. Louis, USA; Bio-Rad, Richmond, USA; New England Biolabs Inc, Beverly, MA, USA; Himedia India Ltd, Bangalore Genei India Pvt. Ltd., Bangalore, India. Tris Base, Agarose, glycine, and IPTG were purchased from Sigma Chemicals. Potassium dihydrogen phosphate, disodium hydrogen phosphate, monosodium dihydrogen phosphate from Qualigens. Sodium Chloride, Potassium Chloride from Merck, Taq Polymerase from Gene Aid. Coomassie Brilliant Blue, Bromophenol Blue, PMSF from Imperial Chemicals, India. Culture media including Luria Bertani Medium and Luria Bertani Agar, chloroamphinecol from Himedia India Ltd; Neutravidin agarose from Pierce biotechnology Pvt Ltd.

Clones

1. pBir A (encodes biotin ligase) was gifted by Dr Steven Polyak of School of Molecular and Biomedical Science, University of Adelaide.
2. pGroESL (Bacterial chaperones) was also a gift from the laboratory of Prof. P. J. Thomas at UT Southwestern Medical Center at Dallas, provided by Dr. Linda Millen from the same laboratory.
3. Recombinant rat nNOS was created in our own laboratory.
Antibodies

**Primary antibody:** polyclonal rabbit and mouse anti nNOS antibody from Alexis corporation and Santa Cruz Biotechnology respectively.

![Diagram](attachment:figure3.png)

**Figure 3:** Scheme for the generation of nNOS Affinity column for pull down assay

**Generation nNOS Affinity Column for Pull Down Assay (Figure 3)**

Plasmids of recombinant rat nNOS and pGroESL (Bacterial chaperones) were cotransformed in xl-blue cells and plated on LA plate containing ampicillin and chloroamphinecol. Similarly, plasmid of pBir A (encodes biotin ligase) was also transformed in xl-blue cells and plated on LA plate containing ampicillin. A Single colony was picked from co-transformed plate of recombinant rat nNOS plates and pGROESL and pBir A (biotin ligase) plate for inoculation respectively. Each colony was inoculated in a 5 ml LB tube containing antibiotics Ampicillin (100 µg/ml) for overnight at 37°C overnight. 1% of overnight culture was used for inoculation of recombinant rat nNOS and pGroESL along with pBir A (biotin ligase) in 100 ml LB
flask for each plasmid containing respective antibiotics, at 37°C at 200rpm until Absorbance at 600 reached 0.4-0.6 (approx. 2.5 hours i.e. logarithmic/ exponential phase). The culture was induced 0.5mM concentrations of IPTG for 15hrs at 37°C for biotin ligase (with biotin 500 µM and ATP 1µM) and 30°C Rat nNOS (with cofactors biotin 500 µM, ALA 450µM, Riboflavin 3µM, ATP 1µM) for in the incubator shaker. Induced culture was pelleted at 12,000 rpm for 5 mins. The supernatants were discarded completely. Pellets were stored at -20°C until use. The pellet was dissolved in 50mM Tris HCl, pH 7.4, lysozyme 1mg/ml, protease inhibitor- leupeptin 2ng/ml, pepstatin 5ng/ml, PMSF 1 mM, sodium fluoride 2mM, antipain 5ng/ml and incubated on ice with intermittent shaking for 1 hr followed by sonication at 80% power with 3 cycles of 30 seconds each and again centrifuged at 10,000 rpm for 20 min at 4°C. the supernatants thus obtained were named S1(Biotin ligase ) and S2(recombinant Rat nNOS). The clear supernatant S1 were allowed to bind with 1ml precleared affinity beads of Ni-NTA sepharose (Qiagen) for overnight shaking at 4°C. After binding Biotin ligase protein sample was centrifuged at 1,000 rpm for 10 min at 4°C, supernatant was discarded and resulting pellet containing Biotin ligase protein bound to Ni-NTA beads was allowed to biotinylate recombinant Rat nNOS protein present supernatant, supplemented with biotin 500 µM and ATP 1µM for 2-3 hrs with shaking at 4°C. After biotinylation the sample was centrifuged at 4,000 rpm for 10 min at 4°C to remove Ni-NTA bound Biotin ligase (pellet) and supernatant containing biotinylated recombinant Rat nNOS was incubated with 1ml Neutravidin agarose beads (pierce biotechnology) for overnight shaking at 4°C. The protein bound Neutravidin agarose beads were centrifuged at 1,000 rpm for 10 min at 4°C, supernatant was discarded and pellet was washed three times with sterile 50mM Tris HCl buffer pH 7.4. Recombinant Rat nNOS bound Neutravidin agarose beads was ready to be used as bait in the pull down assay.

**Pull Down Assay**

The free site on Neutravidin agarose beads were blocked with 3 x 5ml of 50 µM Biotin for 10 mins with shaking at 4°C. Excess biotin was removed by centrifugation at 1,000 rpm for 10 min at 4°C. The 100 µl of bound bait (Recombinant Rat nNOS) was
incubated with the clear rat brain tissue lysate 1mg/ml (supernatant obtained after homogenization and sonication of a normal/untreated Sprague dwaley brain) with gentle rocking at 4°C for 1hr. The sample was centrifuged and washed three times with 50mM Tris HCl buffer pH 7.4. The protein bound to Recombinant Rat nNOS were eluted by boiling the protein pellet in 3 x 100 µl elution buffer (1% SDS, 100 mM dithiothreitol (DTT), 50 mM Tris-HCl pH 7.6). The eluted proteins were acetone precipitated and dissolved in labeling buffer (described in chapter 3) for Cy dye labeling.

Co-Immunoprecipitation
1 ml of Rat brain tissue lysate 1mg/ml (obtained as described previously) was pre-cleared for overnight at 4 °C on shaking with 50 µl of protein A–agarose (santa cruz). The unbound protein sample was incubated overnight with 0.1 µg of monoclonal anti-nNOS antibody (santa cruz) at 4 °C. To recover nNOS interacting proteins, 60 µl of protein A–agarose were added and incubated for 8 h at 4 °C. After extensive washing with extraction buffer(50mM Tris HCl buffer pH 7.4), proteins were eluted at 95 °C with elution buffer [1% SDS, 100 mM dithiothreitol (DTT), 50 mM Tris-HCl pH 7.6] three times. The eluted proteins were acetone precipitated and dissolved in labeling buffer (described in chapter 3) for Cy dye labeling.

Cy Dye Labeling and DIGE
100 µg of protein each from pull down assay and Co-Immunoprecipitation was used for Cy dye labeling. Eluted protein sample from pull down assay and Co-Immunoprecipitation were labeled with 40 pmol of Cy3 and Cy5-maleimide dye respectively (Cy-Dye DIGE Fluor, GE Healthcare) at 37 °C for 30 min. The labeling was stop by adding freshly prepared 2x sample buffer (7M urea, 2M Thiourea, 4% CHAPS, and 1% (w/v) Bio-Lyte 3-10 pH ampholytes and 130 mM DTT). The Cy3 and Cy5-labeled proteins were mixed and diluted with freshly prepared 1x rehydration buffer (7M urea, 2M Thiourea, 4% CHAPS, and 1% (w/v) Bio-Lyte 3-10 pH ampholytes and 13 mM DTT) to make up the 200µl of total volume per sample and
subsequently subjected to IEF. Isoelectrically focused IPG strips were equilibrated in Equilibration buffer I containing 6 M urea (Bio-Rad), 2% SDS (Bio-Rad), 0.375M Tris-HCl pH 8.8 (Merck), 20% glycerol (Merck), 0.5% (w/v) DTT (Bio-Rad) for 10 mins and resolved using 2D gel electrophoresis by the above described method taking care of the mandatory dark condition to be maintained throughout. Fluorescence images were acquired on a Typhoon trio variable mode (GE Healthcare) scanning at 500 PMT with excitation/emission filters for Cy3 or Cy5. ImageQuant (version 5.2, GE Healthcare) was used to determine relative fluorescence intensities. Gels were post stained with colloidal coomassie blue.

**Protein Sequencing and Identification**

A Total of 6 Samples were then picked and sent to the commercial protein sequencing source (SANDOR) for the identification on 4800 MALDI TOF/TOF Analyser. (Applied Biosystems Instrument) and analysis by MASCOT. MASCOT is software used to characterize mass spectrometric data and provide a scores for the identified protein. The identified proteins with accepted significant threshold score (determined at above 60% and counter checked by the molecular mass, if confidence level of the protein identified did not matched with the molecular mass than other hits of below 60% scores was also considered) were reported in this study.

### 4.3 Results

**Labeling and Analysis of Proteins**

The eluted proteins from the pull down assay and Co-IP were successfully labeled by Cy dyes. The common proteins obtained from both methods are represented as yellowish spot in the composite overlay image from the red and green laser channel of typhoon laser scanner. Those spots which have higher yellow to whitish colour were annotated and identified by Maldi-toff (Figure 4).
Figure 4: 2-DIGE representation of co-immunoprecipitation (Co-Ip) and pull down assay. The sample contained 100 µg of protein form Co-Ip and pull down assay labeled with 40pmol Cy3 and Cy5 dye respectively. The protein spots common in both samples were annotated and identified by Maldi-tof.

Table 1: List of the proteins identified by the Maldi-tof (* Represent the lower accepted MASCOT score).

<table>
<thead>
<tr>
<th>S.No./Spot no.</th>
<th>Name of the protein</th>
<th>Molecular weight in kDa</th>
<th>pI</th>
<th>Mascot score (above 60 significant)</th>
<th>Function</th>
<th>Previously reported interaction/association with nNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Junction adhesion molecule 2, isoform CRA_a</td>
<td>23.82</td>
<td>5.19</td>
<td>66</td>
<td>May play a role in the processes of lymphocyte homing to secondary lymphoid organs. Belongs to the immunoglobulin superfamily.</td>
<td>no</td>
</tr>
<tr>
<td>2</td>
<td>Similar to Glutathione S-transferase A1 (GTH1) (HA subunit 1) (GST-epsilon)</td>
<td>25.8</td>
<td>5.41</td>
<td>35*</td>
<td>Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles.</td>
<td>yes</td>
</tr>
<tr>
<td>3</td>
<td>Junction adhesion molecule 2, isoform CRA_b</td>
<td>31.04</td>
<td>5.24</td>
<td>62</td>
<td>Same as above for Junction adhesion molecule 2, isoform CRA_a</td>
<td>no</td>
</tr>
<tr>
<td>4</td>
<td>rab GDP-dissociation inhibitor (rab GDI) beta</td>
<td>48.9</td>
<td>5.93</td>
<td>45*</td>
<td>Regulates the GDP/GTP exchange reaction of most Rab proteins by inhibiting the dissociation of GDP from them, and the subsequent binding of GTP to them.</td>
<td>yes</td>
</tr>
<tr>
<td>5</td>
<td>Keratin, type II cytoskeletal 7</td>
<td>50.7</td>
<td>5.67</td>
<td>62</td>
<td>Intermediate filament proteins responsible for the structural integrity of epithelial cells. Phosphorylation of keratins at specific sites affects their organization, assembly dynamics, and their interaction with signaling molecules.</td>
<td>no</td>
</tr>
<tr>
<td>6</td>
<td>rab GTPase-activating protein 1-like</td>
<td>92.26</td>
<td>5.21</td>
<td>62</td>
<td>Rab GTPase activator activity, involves in regulation of protein localization; positive regulation of Rab GTPase activity.</td>
<td>yes</td>
</tr>
</tbody>
</table>
Database Queries and Protein Identifications

The database like NCBI, EXPASY, BioGrid were queried for the proteins suggested by MASCOT scores obtained by Maldi-toff analysis. The Significant score of 60 were given priority while cross verifying the sequencing results but lower than 60 scores was also considered in those samples in which either the predicted protein molecular mass did not matched with the observed molecular mass or the predicted proteins is suggesting the sequence of hypothetical protein (from gene sequencing). The majority of the proteins identified represent the cytoskeletal proteins followed by signaling and anti-oxidant proteins.

4.4. Discussion

Protein-protein interaction plays a vital role in the cell-signaling. The differential subcellular localization of nNOS in particulate and soluble may contribute to its diverse functions in the brain. Proteins bearing PDZ domains can interact directly with the PDZ domain of nNOS, influencing the subcellular distribution and/or activity of the enzyme (Zhou and Zhu 2009). Till date only a few interacting domain partners of nNOS are reported which includes synaptic proteins, i.e. family members of the DPC, nicotinic acetylcholine receptor (AChR), NMDA receptor, type 1 sodium and Shaker K(+) channel proteins, and linker proteins (e.g., PSD 95, 43K rapsyn), and further suggests targeting and assembly of the NO signaling pathway at postsynaptic membrane components (Nedvetsky et al. 2002; Zhou and Zhu 2009). The interaction of nNOS and NMDA receptor is among the best studied interaction mediated by the help of adapter protein PSD-95 through the PDZ domain of nNOS (Christopherson et al. 1999; Mustafa et al. 2007).

The present study was an attempt to discover new interacting protein partner of nNOS, in the pursuit of this goal pull down as well as co-immunoprecipitation was carried out with the expectation of some new targets identified (Huang and Jacobson 2010; Miernyk and Thelen 2008). Both pull down and co-immunoprecipitation in combination with 2-DIGE have been used first time and apperared to be very effective methodology to rule out to possible false positive results. Maldi-Toff analysis revealed three important proteins: rab GDP-dissociation inhibitor (rab GDI) beta, rab GTPase-
activating protein 1-like and similar to Glutathione S-transferase A1 (GTH1) (HA subunit 1) (GST-epsilon) were identified. Rab GDP-dissociation inhibitor (rab GDI) beta and rab GTPase-activating protein 1-like belongs to Ras superfamily consists of a number of small monomeric GTPases including Ras, Rho and Rab subfamilies. These GTPases cycle between active GTP- and inactive GDP-bound states to regulate a diverse array of biological processes including cell proliferation, apoptosis, vesicular and nuclear transport. GTPases are highly regulated by multiple cellular factors. Guanine nucleotide exchange factors (GEFs) , facilitate exchange of GDP with GTP to produce the active GTP-bound form of the GTPase, whereas GTPase-activating proteins (GAPs) enhance the slow intrinsic rate of GTP hydrolysis to produce the inactive GDP-bound form of the GTPase. In addition, guanine nucleotide inhibitors (GDIs) down-regulate the activity of a subset of GTPases (e.g., Rho and Rab subfamilies) by preventing membrane association as well as inhibiting guanine nucleotide dissociation. Nitric oxide (NO) has been shown to react with Ras and other Ras-related GTPases to regulate their activity (Hess et al. 2005; John et al. 2007; Maurice et al. 2011; Raines et al. 2007).

Another important protein that was identified is glutathione-S-transferase (GST). GST is a major cellular antioxidant enzyme which catalyses the nucleophilic attack of glutathione (GSH) on electrophilic substrates. This mechanism allowed to protect a variety of cell components (protein, lipid, DNA) against reactive molecules such as electrophilic metabolites formed after xenobiotics phase I metabolism or endogenous α,β-unsaturated aldehydes and hydroperoxides formed as secondary metabolites during oxidative stress. NO is able to bind iron and two molecules of glutathiones in order to form the dinitrosyl-diglutathionyl-iron complex (DNDGIC) which, under normal conditions prevents its cytotoxic effect. But during high oxidative stress, this leads to a depletion in glutathione and could represent a key signal trigerring apoptosis (Julie Pajaud 2012; Martínez-Ruiz and Lamas 2007). Although, literature is not sufficient to conclude the direct interaction of GST with nNOS but its antioxidant role is well established and suggests that during oxidative stress, the cellular localization of GST might be modulated by some adapter proteins to bring GST in the vicinity of nNOS to exert its anti-oxidant effect.
A reduction in axonal transport has also been implicated as a cause of axonal
dystrophies and neurodegeneration including PD. Previous workers have reported that,
exposure to nitric oxide inhibited axonal transport of synaptic vesicle proteins in
cultured hippocampal neurons (Goellner and Aberle 2012; Guedes et al. 2009;
Millecamps and Julien 2013). In the present study isoforms of Junction adhesion
molecule 2 and keratin, type II cytoskeletal 7 are reported to interact with nNOS
without any previous literature to support the direct interaction of these two molecules.
However, (Ebnet et al. 2000)) has reported AF-6 (a scaffold protein containing the PDZ
domain protein) as an intracellular binding partner of the junctional adhesion molecule
(JAM), an integral membrane protein located at cell contacts. Binding of AF-6 to JAM
required the presence of the intact C terminus of JAM, which represents a classical type
II PDZ domain-binding motif. Later, (Roy et al. 2002; Su et al. 2003) showed that AF-6
regulate SAP-1(a Rap1 GTPase-activating protein) which is interact with GK domains
of PSD-95 protein which is known to interact with nNOS via PDZ domain
(Christopherson et al. 1999). Interaction of keratin, type II cytoskeletal 7 protein with
nNOS is however, unknown and not supported by literature.

Conclusively, the present study further validated two the protein-protein interaction of
important proteins rab GDP-dissociation inhibitor (rab GDI) beta and rab GTPase-
activating protein 1-like proteins with nNOS. However, the adapter protein/s that
mediates these interactions was not identified by the current study leaving the scope for
further investigation.
References


Roy BC et al. (2002) SPAL, a Rap-specific GTPase activating protein, is present in the NMDA receptor-PSD-95 complex in the hippocampus. Genes Cells. 7(6):607-17.


