ROLE OF SRC SPLICE VARIANTS IN NERVE TERMINAL FUNCTION

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Declaration

I, Taher Mohamed Abdelhameed, candidate for a PhD in Biomedical Sciences, declare that this thesis has been composed solely by myself, and that the work described is my own, except where indicated, and that this work has not been submitted for any other degree or professional qualification.

Taher Abdelhameed

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Abstract

Src is a 60 kDa tyrosine kinase that is expressed in most of animal tissues. Src has three splice variants, C-src, which is ubiquitously expressed, and N1- and N2-src, which are neuronal specific splice variants. The srcs are differentially spliced at their SH3 domains, therefore the hypothesis is that this splicing allows them to have different binding partners and perform different roles in neurons. The aim of this project is to identify new interactions for the three src splice variants in neurons and their possible functional roles. The SH3 domains, kinase active truncated proteins (Δ80) and kinase dead mutant full length versions of the three splice variants of src were cloned from a rat brain cDNA library into bacterial expression vectors. GST-pull downs from nerve terminal lysates showed that different src splice variants had different binding partners. These partners were identified by mass spectrometry and confirmed by western blotting. C-src binding partners included dynamin, synapsin, and synaptojanin, while N2-src binding partners included synaptophysin, Munc18-1, and NSF. The interaction between N2-src and Munc 18-1 was characterized further; however a number of *in vitro* interaction assays and kinase assays showed that Munc 18-1 may not be a direct binding partner for N2-src or substrate.

N1-src displayed a stimulation-dependent interaction with dynamin I. This was shown to be phosphorylation-dependent in contrast to C-src binding. The major phosphorylation sites on dynamin I, S774 and S778, were not involved in the regulation of N1-src binding. The binding site for N1-src on dynamin I was different to C-src, with extensive mutagenesis studies suggesting that the interaction site is at the tail of the dynamin I xa splice variant, which has an additional two phosphorylation sites.

List of abbreviations

A	Absorbance
A	adenine
A	alanine
AA	amino acid
ACh	acetylcholine
ADBE	activity-dependent bulk endocytosis
ADP	adenosine diphosphate
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
APS	ammonium persulfate
AP2	adaptor protein 2
ATP	adenosine triphosphate
αSNAP	alfa soluble NSF attachment protein
BAR	(Bin-Amphiphysin-Rvs) domain
bp	base pair
BSA	bovine serum albumin
C	cytosine
°C	degree Celsius
C2	calcium binding domain 2
Ca^{2+}	calcium ion
CaMK	Ca^{2+}-calmodulin dependent kinase
CDK5	Cyclin-dependent kinase 5
cDNA	complementary DNA
CME	clathrin mediated endocytosis

CNS	central nervous system
Csk	C-terminus src kinase
C-src	cellular src
C-terminus	carboxy terminus (the end of protein)
DCS	downstream control sequence
dH_2O	distilled water
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
Δ80	Truncated versions that starts at AA 80
dATP	Deoxyadenosine triphosphate
dNTPs	Deoxyribonucleotides
DynI	dynamin I
$DynI^{dmA}$	dynamin I double mutation (SS774/8AA)
$DynI^{dmE}$	dynamin I double mutation (SS774/8EE)
E	glutamate
ECL	enhanced-chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
ERK	extracellular signal-regulated kinase
FRET	fluorescence resonance energy transfer
FGF	fibroblast growth factor
FAK	focal adhesion tyrosine kinase

g	g-force
G	guanidine
GAPDH	glycerylaldehyde-3-phosphate dehydrogenase
GABA	gamma-aminobutyric acid
GED	GTPase effector domain
Grb2	Growth factor receptor-bound protein 2 also
GST	Glutathione S-transferase
GTP	Guanine triphosphate
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
Hrs	hours
IL	interleukin I
IP	immunoprecipitation
IPTG	isopropyl-beta-D-thiogalactopyranoside
Kb	kilobase
KCl	potassium chloride
kDa	kilo daltons
KDEL receptor	Lys-Asp-Glu-Leu receptor
KO	knock out
l	liter
LB	Luria-Bertani medium

LTP	long-term potentiation
M	molar
MALDI-tof MS	matrix-assisted laser desorption ionisation time-of-flight mass spectrometry
MAP	Mitogen-activated protein
MAPK	mitogen-activated protein kinase
MeOH	methanol
Mg	milligrams
µg	microgram
MHz	megahertz
Min	minutes
Ml	millilitre
µl	microliter
MLCK	myosin light chain kinase
mM	millimolar
µM	micromolar
MNB	Minibrain kinase
mRNA	Messenger ribonucleic acid
mV	millivolts
mw	molecular weight
N-terminus	amino-terminus
N1-src	src neuronal isoform 1
N2-src	src neuronal isoform 2
NaCl	sodium chloride

Ni^{+2}	nickel ion
ng	nanogram
nm	nanometer
NMDA	N-methyl-D-aspartic acid
NSF	N-ethylmaleimide sensitive factor
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC12	pheochromocytoma cells
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PI3K	phosphatidylinositol 3 kinase
PKA	protein kinase A
PH domain	pleckstrin homology domain
PKC	protein kinase C
PLCγ	phospholipase Cγ
PMSF	phenylmethanesulphonylfluoride
PNS	peripheral nervous system
PP	phosphoprotein
PP2	(4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d]pyrimidine
PRD	proline rich domain
PTB	polypyrimidine tract binding protein
PTB	phosphotyrosine binding domain
PTP	protein tyrosine phosphatase

PVP	Polyvinylpyrrolidone
QKI	quaking homolog KH domain RNA binding
rpm	round per minute
RPTP	Receptor Protein Tyrosine Phosphatase
S	serine
SDM	site directed mutagenesis
SDS	sodium dodecyl sulfate
SH	src homology
Shc	(Src homology 2 domain containing) transforming protein
SFK	src family kinases
SNAP-25	Synaptosome-associated protein of 25,000 daltons
SNARE	SNAP (Soluble NSF Attachment Protein) Receptors
Stat3	Signal transducer and activator of transcription 3
SV	synaptic vesicle
T	thymine
TEMED	Tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
Tween-20	polyoxyethlene-sorbitan monolaurate
U	Uracil
UV	ultraviolet
VAMP	Vesicle-associated membrane protein
V	volt
Vs	versus

v/v	volume per volume
w/v	weight per volume
WT	wild type
Y	tyrosine

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Chapter 1

Introduction

1.1 Introduction

Phosphorylation (adding a phosphate group to an amino acid of a protein) and dephosphorylation (taking away the phosphate group) are well known mechanisms in cell signalling and function. Enzymes that utilize high energy donor molecules such as ATP to phosphorylate other proteins are known as protein kinases, while proteins that dephosphorylate these proteins are known as protein phosphatases (Tatosyan and Mizenina, 2000). The amino acids that can be phosphorylated and dephosphorylated are serine, threonine, tyrosine, and histidine (Steeg et al., 2003; Roskoski, 2005).

Tyrosine phosphorylation is an important mechanism that regulates many important processes in all cell types including, the central nervous system (Purcell and Carew, 2003; Alonso et al., 2004). Protein tyrosine kinases are involved in many cell activities including, signalling, motility, proliferation and differentiation (Sandilands and Frame, 2008). There are approximately 90 protein tyrosine kinase in the human genome (Manning et al., 2002). There are two groups of protein tyrosine kinases that are classified: 1) receptor tyrosine kinases, which are trans-membrane proteins that contain an extra-cellular domain that interacts with ligands from outside the cell, and 2) non-receptor tyrosine kinases, which are cytosolic proteins (Tatosyan and Mizenina, 2000; Bromann et al., 2004; Cohen, 2005; Roskoski, 2005; Sandilands and Frame, 2008).

1.2 The src family of protein tyrosine kinases (SFKs)

The src family kinases (SFKs) is an important group of non-receptor tyrosine kinases that contains 11 members: src, fyn, yes, yrk, lyn, hck, fgr, lck, blk, frk/rak and iyk/bsk (Engen et al., 2008). The members of the src family share amino acid

homology and domain architecture (Superti-Furga, 1995; Thomas and Brugge, 1997; Summy et al., 2000). The src family is present in all metazoan cells, however they have different distribution patterns in different tissues, and some of them have splice variants that are found in specific tissues (Engen et al., 2008). Src, fyn and yes are expressed in all tissues, however src has two neuro-specific isoforms with fyn having one T cell specific isoform. Yrk is expressed in all tissues but only in chicken. Lyn, hck, fgr, blk, and lck are expressed mainly in blood related tissues, and frk and iyk are expressed only in epithelial cells (Bolen and Brugge, 1997; Thomas and Brugge, 1997; Parsons and Parsons, 2004; Engen et al., 2008). The distribution of these kinases suggests the presence of several src family members in any cell type but at possibly different locations (e.g. while src is found at the cell membrane or endosomes, fgr is found at the nucleus) and also more than one isoform from some members in the same cell (Robinson et al 1995; Li et al 1996; Thomas and Brugge 1997; Engen et al 2008).

SFKs are now known to be widely expressed in the CNS of adult mammals and are involved in a range of neuronal functions (Kalia et al., 2004). The activity of these kinases is critical for normal cell function, abnormal conditions and appropriate cell responses to external stimuli and is regulated by several factors including growth factors, cytokines, adhesion, and antigen receptors (Zamoyska et al., 2003; Boggon and Eck, 2004; Lowell, 2004; Cohen, 2005; Okutani et al., 2006; Rivera and Olivera, 2007; Abram and Lowell, 2008; Engen et al., 2008; Sandilands and Frame, 2008; Kim et al., 2009). The SFKs family members share similar multi-domain structure with molecular weights ranging from 52 kDa to 62 kDa (Tatosyan and Mizenina, 2000).

1.3 Src

Src was first charactarized as a cellular protein similar to the Rous sarcoma virus gene product (Collett et al., 1978; Collett et al., 1979; Oppermann et al., 1979). Src has a molecular weight of about 60 kDa and was referred to as $PP^{60c\text{-}src}$ (PP = phosphoprotein) (Bunte and Moelling, 1983). There are some differences between v-src and C-src in activity, substrates and in amino acid sequence (Vojtechova et al., 2004). Src is expressed in most of vertebrate cells; however neurones, osteoclasts, and platelets express high levels of src that could be 5-200 times more than other cell types (Golden et al., 1986; Horne et al., 1992; Brown and Cooper, 1996). The location of src within the cell is not restricted to one area but it is usually found attached to membranes via myristoylation, using fibroblasts as an example; src can be found binding to endosomes, perinuclear membranes, secretory vesicles, and the cytoplasmic surface of the plasma membrane, where it can interact with growth factors and integrin receptors (Roskoski, 2004).

The role of src is usually related to its kinase activity. Activated src can phosphorylate a variety of proteins associated with intracellular signalling and cell structure. This includes the non-receptor focal adhesion tyrosine kinase (FAK), and the cytoskeleton associated proteins vinculin, talin, tubulin, cortactin, and actin filament-associated protein-110 (Ito et al., 1983; Thomas and Brugge, 1997; Lodyga et al., 2002; Destaing et al., 2008). Even though src is expressed in most of cell types, it is expressed in high levels in cells that are specialized for regulated secretion such as neurons and is concentrated at the site of exocytosis in nerve terminals (Foster-Barber and Bishop, 1998).

1.3.1 Structure of src

The src family members share a common modular structure, and C-src is considered as the prototype of this family (Schwartzberg, 1998; Engen et al., 2008). C-src in all species has the same structure with some amino acid differences (Lowell, 2004). Since C-src was first discovered in chicken, the amino acid numbering of chicken C-src is usually used by researchers. C-src contains an N-terminal 14 carbon myristoyl group (SH4), a unique segment, a src homology 3 (SH3) domain, a src homology 2 (SH2) domain, a protein-tyrosine kinase domain or src homology 1 (SH1) domain, and a C-terminal regulatory tail (figure 1.1 A) (Superti-Furga, 1995; Tatosyan and Mizenina, 2000; Boggon and Eck, 2004; Lowell, 2004; Roskoski, 2005; Sandilands and Frame, 2008). The most obvious structural difference between C-src and the viral homologue v-src is that v-src lacks the final 7 C-terminal amino acids (Martin, 2001).

1.3.1.1 The src N-terminus

The N-terminal region, which is also called the src homology 4 (SH4) domain is the myristoylation region, where a myristoyl group is added to src through an amide bond on a glycine residue (Boutin, 1997; Sicheri and Kuriyan, 1997; Roskoski, 2004; Engen et al., 2008). Myristoylation facilitates src attachment to membranes, and it is also required for src function in the cell (Resh, 1994; McLaughlin and Aderem, 1995; Song et al., 1997; Roskoski, 2004). The initial 7 amino acids of the N-terminal begin with glycine and these are essential for myristoylation. A relationship has been proposed between membrane association via N-myristoylation and the ability of v-

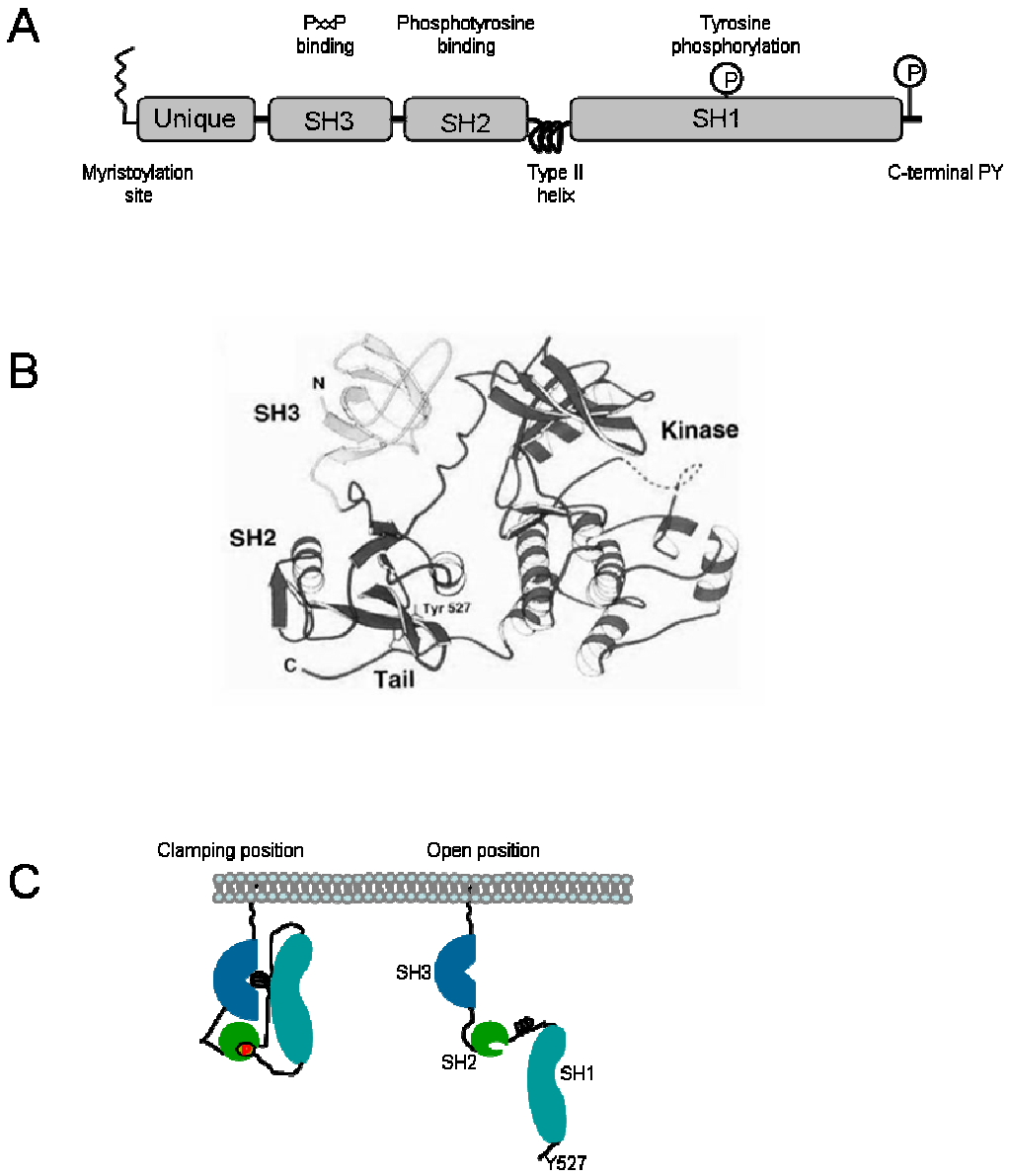


Figure 1.1- *Structure of src*

A) Organization of Src. Different domains are shown and different phosphorylation sites are also indicated (Boggon and Eck 2004).

B) Crystal structure of C-src showing the positions of different domains in the, closed, inactive state. The region before SH3 is not expected to have a role in the protein conformation (Xu et al 1997).

C) Illustration of the clamping position, where both SH2 and SH3 domains are not accessible (left) versus the open position where both SH2 and SH3 domains are exposed (right) in src (Yeatman 2004)

src to transform the cell into a neoblast, however myristoylation does not guarantee membrane association, suggesting there might also be specific binding proteins for the src N-terminal region that enhances its localisation (Brown and Cooper, 1996; Roskoski, 2004). The v-src interaction with calcium-bound calmodulin is regulated by myristoylation (Hayashi et al., 2004), which supports this assumption.

1.3.1.2 Src SH3 domain

The src homology 3 (SH3) domain is a common protein recognition module that has 60-80 amino acids and is seen widely in different types of proteins. Proteins that contain an SH3 domain are involved in many cellular activities including signal transduction, cytoskeleton organization, and membrane traffic (Koyama et al., 1993; Feller et al., 1994; Weng et al., 1995; Birge et al., 1996; Dalgarno et al., 1997; Camara-Artigas et al., 2009). The src SH3 domain binds to proline rich domains (PRD) with a left handed helical conformation. There are two types of PRDs that bind to src. The first (Class I src SH3 domain ligand) has the preferred structure of RPLPPLP, while the second (Class II src SH3 Domain ligand) has the preferred structure of ΦPPLPXR (where Φ is a hydrophobic amino acid, L is leucine and X is any amino acid) (Feng et al., 1994; Tatosyan and Mizenina, 2000; Roskoski, 2004).

The SH3 domain exhibits a number of possible regulatory roles in src interactions and function that can be summarized in four main points. First, it constrains the enzymatic activity of src by an intramolecular interaction with the linker between the SH1 small lobe and the SH2 domain (Gonfloni et al., 1997; Xu et al., 1997; Brabek et al., 2002; Boggon and Eck, 2004) . The linker does not have the structure of an SH3 binding motif; however it does have a left handed helical conformation allowing

the SH3 domain to bind (Xu et al., 1997; Brabek et al., 2002; Boggon and Eck, 2004). The crystal structure of src in the closed conformation illustrates the binding between the SH3 domain and the linker (Figure 1.1 B). This negative regulation means that mutations in the SH3 domain would cause src activation, and v-src indeed has several point mutations within its SH3 domain (Parsons and Weber, 1989; Schwartzberg, 1998; Roskoski, 2004). Second, proteins that have SH3 binding motifs can bind to the SH3 domain of src, where they can be attracted to specific cellular locations. Third, by displacing the intramolecular SH3 domain-linker interaction, proteins can enhance src kinase activity (Brabek et al., 2002; Roskoski, 2004; Onofri et al., 2007). Fourth, proteins that bind to the src SH3 domain may also be substrates for its tyrosine kinase activity (Onofri et al., 1997; Roskoski, 2004; Onofri et al., 2007).

1.3.1.3 Src SH2 domain

The src homology 2 (SH2) domain is a protein recognition module that recognises short phospho-tyrosine containing sequences (Schlessinger, 1994; Garcia-Echeverria, 2001; Bradshaw and Waksman, 2002). SH2 domains are about 100 amino acids long, and share a common structure. They contain one β-sheet that is flanked from both sides with α helices. The pockets between these sheets are the binding site for the phospho-tyrosine containing sequence (Waksman et al., 2004; Machida and Mayer, 2005). The amino acid sequence C-terminal to the phospho-tyrosine residue contributes to the binding specificity of the SH2 domain (Yaffe, 2002). These sequences vary from three to six amino acids in length (Songyang et al., 1993). The src SH2 domain binds with a preferred sequence of pYEEI (where pY is a

phosphorylated tyrosine). This is the same sequence that is preferred by some other members of src family such as Fgr, Fyn, and Lck (Songyang and Cantley, 1995; Waksman et al., 2004). The SH2 domain can bind other phospho-tyrosine sequences, such as the C-terminus of the src protein (Tatosyan and Mizenina, 2000; Zheng et al., 2000). More than 90% of src is phosphorylated at tyrosine residue 527 of the C-terminus during basal conditions (Zheng et al., 2000). SH2 binding to this intramolecular site is not strong, because it is not its preferred ligand. Thus binding is strong enough to keep the protein in its inactive state, but weak enough to allow src activation by other proteins (Porter et al., 2000; Yaffe, 2002; Waksman et al., 2004). SH2 domain binding to phospho-tyrosine 527 has two possible roles; the first is to regulate kinase activity via intramolecular binding to the C-terminal region of the src molecule, and the second is to promote interactions with other components of the cell (Yaffe, 2002; Waksman et al., 2004; Machida and Mayer, 2005). In a study on one of the src family members (Hck) by Porter and co-workers (2000), the intramolecular binding site of SH2 was mutated to pYEEI, which resulted in a form of the protein that could not be activated by exogenous SH2 binding sequences (Porter et al., 2000). Thus the SH2 domain shares a regulatory function with the SH3 domain in both kinase activity and protein-protein interactions (Schwartzberg, 1998; Boggon and Eck, 2004; Roskoski, 2004).

1.3.1.4 Src SH1 domain

The tyrosine kinase domain, src homology 1 (SH1) domain is highly conserved in the src family (Brown and Cooper, 1996; Tatosyan and Mizenina, 2000). It consists of a bi-lobal architecture, where the first lobe (the N-lobe) is smaller (about 71 amino

acids from 267 to 337). The second lobe (the C-lobe) is approximately 180 amino acids (from 341 to 520) with the catalytic site between these two lobes (Hubbard, 2002; Boggon and Eck, 2004; Roskoski, 2004). The kinase domain plays a role in substrate specificity (Brown and Cooper, 1996). The site for ATP binding and the phosphotransferase reaction is very similar in all tyrosine kinases (Tatosyan and Mizenina, 2000). The two lobes can close the cleft containing the catalytic site, by moving toward each other. While opening the cleft is necessary to allow ATP to access the catalytic site and also to release ADP, closing brings the substrates to the catalytic site. Preventing opening or closing of the cleft is inhibitory for kinase activity. Each lobe has a polypeptide segment that has active and restrained positions (Kefalas et al., 1995). The amino acid sequence that is preferably phosphorylated by src is EEEIY$^{G}/_{E}$EFD (Tatosyan and Mizenina, 2000). There is a tyrosine residue (Y416) in the kinase domain that can be phosphorylated; phosphorylation of Y416 stimulates kinase activation and provides a binding site for other proteins such as the phosphotyrosine binding domain (PTB) of Cb1 (Sanjay et al., 2001).

1.3.2 Activity and regulation

Src has two important phosphorylation sites that regulate its tyrosine kinase activity. Auto-phosphorylation of Y416 stimulates kinase activity, while phosphorylation of Y527 inhibits kinase activity (Yaciuk and Shalloway, 1986; Gould and Hunter, 1988; Okada and Nakagawa, 1989; Cary et al., 2002; Sun et al., 2002). The result is a restrained form of the enzyme (closed conformation) (Figure 1.1 C) (Kefalas et al., 1995). In the restrained form, neither the SH3 nor the SH2 are accessible to their ligands, and thus not only the kinase activity is affected but also protein-protein

interactions via SH3 and SH2 domains (Schwartzberg, 1998; Roskoski, 2004). Binding to phosphorylated Y527 by the SH2 domain forms a latch, which stabilizes the attachment of SH2 with the large lobe (Tatosyan and Mizenina, 2000; Brabek et al., 2002; Yeatman, 2004). Mutation of Y527 to phenylalanine produced an active oncogenic protein, highlighting the importance of this residue in src function regulation (Hunter, 1987).

1.3.2.1 Src inhibition

The kinase that phosphorylates Y527 is called C-terminus src kinase (Csk), which is a cytoplasmic kinase that is expressed in most of cell types. Y527 is also phosphorylated by Csk homologus kinase (Chk) which has limited expression to some tissue types including neurons and breast cells (Okada and Nakagawa, 1989; Zrihan-Licht et al., 1997; Bjorge et al., 2000; Kawabuchi et al., 2000; Takeuchi et al., 2000; Dey et al., 2005; Roskoski, 2005). The phosphorylation at Y527 resulted in decreasing src activity by at least 80 %. Furthermore, Csk is very specific in its phosphorylation of src where mutation of Y527 to phenylalanine resulted in a 500 fold decrease in src phosphorylation by Csk (Cole et al., 2003; Roskoski, 2005). Chk can also inhibit src activity via direct binding to the src SH1 domain inhibiting src auto-phosphorylation at Y416 (Chong et al., 2004). Src can also be phosphorylated at Y213 in the SH2 domain and at Y138 in the SH3 domain by the PDGF receptor. This results in reducing SH2 domain binding to phospho-Y527 and SH3 domain binding to the linker between the SH2 domain and the SH1 domain, resulting in unclamping src and overriding the inhibitory effect of Csk phosphorylation (Stover et al., 1996; Broome and Hunter, 1997).

1.3.2.2 Src activation

Dephosphorylation of phospho-Y527 is the major activator of src kinase activity by shifting the clamp conformation of src to open and thus activating src. There are several phosphatases that can dephosphorylate P-Y527 including protein tyrosine phosphatase-1B (PTP1B), SH2 domain-containing tyrosine phosphatases 1 and 2 (Shp1 and Shp2), CD45 (cluster of differentiation) and protein tyrosine phosphatase-α (PTPα) (Roskoski, 2005). PTP1B is the main tyrosine phosphatase for phospho-Y527, in fact more than 50% of src Y527 dephosphorylation is catalysed by PTP1B activity (Roskoski, 2005). PTPα, a transmembrane protein that is abundant in brain, is known to dephosphorylate src Y527 and regulate src kinase activity (Su et al., 1999; Zheng et al., 2000; Brandt et al., 2003).

Dephosphorylation of phospho-Y416 results in decreasing src activity. PTP-BL, a cytosolic tyrosine phosphatase, dephosphorylates Y416 (Palmer et al., 2002). The phosphatases that catalyse the dephosphorylation of Y138 in the SH3 domain and Y213 in the SH2 domain have not yet been identified (Roskoski, 2005). Src might also be activated by displacing the SH3 domain or the SH2 domain or both from their intramolecular binding sites via binding to their ligands in other proteins (Brabek et al., 2002; Onofri et al., 2007).

1.4 Src function in non-neuronal cells

Src can be found in two distinct locations in the cell. The first is non-raft regions on the plasma membrane and this population can be rapidly activated upon receptor stimulation. The second population is found in perinuclear endosomes and can be

translocated to lipid rafts after receptor stimulation (Cary et al., 2002; Yeatman, 2004; Seong et al., 2009). Many signal transduction cascades are mediated by tyrosine phosphorylation of intracellular proteins; for example src activation by receptor tyrosine kinases such as the platelet derived growth factor (PDGF) receptor and epidermal growth factor (EGF) receptor. PDGF binding to its β-receptor results in auto-phosphorylation of its cytoplasmic region at several tyrosine residues. The SH2 domain of src then binds to these phosphotyrosine residues causing src activation and subsequent src tyrosine phosphorylation of many proteins in the cell such as Shc ((Src homology 2 domain containing) transforming protein) which will result in stimulation of DNA synthesis and cell growth (Kefalas et al., 1995; Courtneidge, 2002; Waters et al., 2005). Src activation by PDGF is required for endothelial cell proliferation and migration, and src kinase activity is necessary for cells during mitosis (Mureebe et al., 1997). The role of src in mitosis and cell cycle and its involvement in gene expression is most likely mediated by phosphorylation of Sam 68 (Taylor et al., 1995; Bjorge et al., 2000). Furthermore src plays an important role in PDGF β-receptor endocytosis (Ahn et al., 2002; Waters et al., 2005). Internalization of the PDGF receptor leads to association of mitogen-activated protein kinase (MAPK) with the complex on the endocytosed vesicle and results in activation of MAPK (Waters et al., 2005).

Activation of src by different signalling results in activation of different downstream proteins. Src activation by EGF receptor led to phosphorylation of Stat3 (Signal transducer and activator of transcription 3) and cortactin, whereas src activation by β-adrenergic receptor caused phosphorylation of Shc and subsequent ERK (extracellular signal-regulated kinase) activation (Goi et al., 2000).

1.4.1 Src function in vesicle trafficking

Activated src is internalized from the plasma membrane either constitutively or in association with growth factor receptors such as the EGF receptor. EGF receptor internalization regulates the duration and location of signal transduction and it is co-internalized with src (Donepudi and Resh, 2008). Overexpression of active src prolongs the activity of EGF receptor inside the cell, which might explain the role of src activity in cancer (Donepudi and Resh, 2008).

Src also plays an important role in agonist mediated angiotensin II receptor internalization. Src binding to β-arrestin and angiotensin II receptor complex results in the binding of the clathrin adaptor protein AP2 to β-arrestin. This recruits clathrin and results in internalization of the angiotensin II receptor (Fessart et al., 2005). Src binding to the AP2 and β-arrestin complex induces phosphorylation of the β_2-adaptin subunit of AP2 by src and results in dissociation of the AP2 - β-arrestin complex (Fessart et al., 2007; Zimmerman et al., 2009). Src mediated phosphorylation of AP2 is a common mechanism for ligand-induced receptor internalization for many receptors such as EGF receptor and angiotensin II receptor (Zimmerman et al., 2009).

Src is also involved in endosomal trafficking in many cell types, an important process for internalizing and recycling membrane proteins and many receptor types, including hormone receptors as well as membrane lipids (Sandilands and Frame, 2008). Src also plays a role in caveolae mediated endocytosis in internalizing substances such as albumin. Albumin receptors, for example, will cluster upon binding to its ligand and then move to caveolae to activate their endocytosis

(Sverdlov et al., 2007). Addition of cargo results in activation of src and caveolae mediated endocytosis, which is dependent on tyrosine phosphorylation by src, since a dominant negative src or kinase inhibitors such as PP2 blocked caveolae endocytosis (Sverdlov et al., 2007). This role is mediated by src phosphorylation of several proteins including caveolin 1 and 2 and dynamin II (Sverdlov et al., 2007). Caveolin 1 (which is probably the primary src substrate in caveolae) is phosphorylated at Y14 (Orlichenko et al., 2006). Mutations in this site resulted in decreased caveolae endocytosis (Li et al., 1996), whereas src overexpression or application of vanadate, a phosphatase inhibitor, resulted in increased endocytosis, suggesting that caveolin 1 tyrosine phosphorylation by src is essential for caveolae endocytosis (Botos et al., 2007). There have been several proposed functions for caveolin-1 phosphorylation (including regulation of its oligomerization) but there are only two proteins that bind to caveolin 1 in a phosphorylation dependent manner Csk and Grb7 (Lee et al., 2000; Cao et al., 2002). Caveolin 2 is not essential for caveolae formation, but it influences caveolae size by forming heteromers with caveolin 1. It is also phosphorylated by src at Y19 and Y27 and this phosphorylation affects its oligomerization with caveolin 1 (Lee et al., 2002a; Wang et al., 2004). Dynamin 2 phosphorylation by src at Y231 and Y579 results in increased GTPase activity (Ahn et al., 1999; Ahn et al., 2002), and binding to caveolin 1, resulting in directing dynamin 2 to the caveolae neck and consequently caveolae endocytosis (Oh et al., 1998; Kim and Bertics, 2002; Yao et al., 2005).

Src is also involved in Golgi apparatus structure and regulation and KDEL receptor transport to endoplasmic reticulum. Src KO cells have a compact Golgi apparatus with bloated cisternae that can be restored to normal structure by expressing active

src (Bard et al., 2003). When trafficking from the endoplasmic reticulum to cis-Golgi increases, src is activated and binds to the cytoplasmic region of the KDEL receptor. This binding results in src activation and subsequent phosphorylation of several proteins including the KDEL receptor, atypical protein kinase C ι/λ and glycerylaldehyde-3-phosphate dehydrogenase (GAPDH) which will interact with rab 2 and stimulate COPI-vesicle formation (Tisdale and Artalejo, 2007; Asp and Nilsson, 2008).

1.4.2 Src function in permeability

In addition to its role in vesicle trafficking, src regulates the permeability of endothelial cells in response to inflammatory signals via phosphorylation of myosin light chain kinase (MLCK). The phosphorylated myosin light chain then initiates cross bridge formation between myosin and actin which results in cell contraction, cytoskeleton rearrangement and shape changes that will lead to increased endothelial (Birukov et al., 2001; Hu et al., 2008; Kim et al., 2009). Src also phosphorylates junctional proteins such as β-catenin which alters the interaction between these junctional proteins and the cytoskeleton again increasing endothelial permeability (Hu et al., 2008).

Focal adhesion kinase (FAK), located in the focal adhesion complex, is also a src substrate; FAK phosphorylation by src is essential for integrin-dependent FAK attachment to actin stress fibers, which increases vascular endothelial cell permeability (Mitra and Schlaepfer, 2006; Hu et al., 2008). Src phosphorylation of integrin also promotes cell migration (Cary et al., 2002). Sex-steroid hormones such as estradiol and progesterone stimulate src activity through their receptors in many

cell types including prostate cells, vascular endothelial cells, fibroblasts and platelets (Migliaccio et al., 2007).

1.4.3 Src function in migration and proliferation

Src plays a major role in cell migration and proliferation. Smooth muscle cell migration is one of the cell activities that require activation of src. Src mediates its effect by causing focal adhesion complex dissociation and reformation and its interaction with cytoskeleton (Mureebe et al., 1997). Osteoclast cells express high levels of src, with most associated with intracellular organelle membranes (Horne et al., 1992). Osteoclast migration and normal bone resorption require both src presence and activity; Cb1 phosphorylation by src and the subsequent activation of phosphatidylinositol 3 kinase (PI3K) causes cytoskeleton reorganization and results in osteoclast cell migration (Miyazaki et al., 2006). Even kinase dead src could still restore some osteoclast functions in src KO cells, suggesting that src might be acting as a scaffolding molecule as well as its function as a tyrosine kinase (Miyazaki et al., 2006). Src also plays a role in shape changing and cell migration mediated by fibroblast growth factor 1 (FGF1). This role involves cortactin phosphorylation by src that increases its association with cytoskeleton and actin filaments (Liu et al., 1999).

1.5 Src splice variants

As described above C-src has numerous essential roles in non-neuronal cells. In neurons there are two neuronal specific splice variants of src; N1-src which has 6 extra amino acids, and N2-src which has 17 extra amino acids (Levy et al., 1987;

Pyper and Bolen, 1990; Matsunaga et al., 1993; Chan and Black, 1995). These extra amino acids are inserted in the SH3 domain between exon 3 and exon 4 (Figure 1.2) (Pyper and Bolen, 1990; Wagner and Garcia-Blanco, 2001). The N1-src splice variant has higher expression in the CNS compared to the PNS. On the other hand cancer cells such as neuroblastoma cells express all src splice variants (Bjelfman et al., 1990; Matsunaga et al., 1993). The majority of N1-src mRNA is found in the mesencephalon, cerebellum, pons, medulla and telecephalon, while C-src has a different distribution pattern with its mRNA levels highest in hippocampus, thalamus and cerebellum (Sugrue et al., 1990; Yagi, 1994). Immunohistochemical analysis also showed that N1-src and C-src proteins have different patterns of distribution within neurones (Sugrue et al., 1990; Yagi, 1994; Mukherjee et al., 2003). N1-src is localized in the cell soma, dentritic processes, and synaptosomal membrane; however C-src and N1-src are both expressed in the synaptic region. This suggests that if N1-src has a role in synaptic transmission, it is most likely to be different from C-src (Sugrue et al., 1990; Yagi, 1994; Onofri et al., 2007), Even though both isoforms are found in growing tips and growth cones of neurons (Worley et al., 1997), C-Src and N1-Src differentially modify axonogenesis and intracellular phosphotyrosine levels, providing evidence that the activities of C-Src and N1-Src perform non-equivalent roles (Worley et al., 1997). In contrast, there is very little information about N2-src expression or distribution.

Splicing of src is controlled by a sequence that flanks the N1-src intron called downstream control sequence (DCS). This sequence is CUCUCU and it binds to splicing repressor proteins (Chan and Black, 1995; Chou et al., 2000). The protein that represses N1-src splicing is polypyrimidine tract binding protein (PTB), also

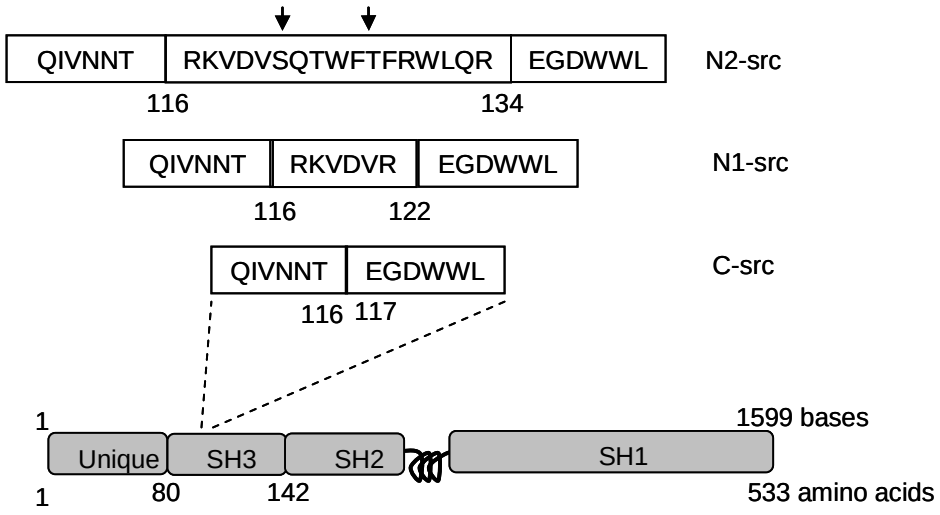


Figure 1.2- ***The Differences between various src splices.***
C-src amino acids in the bottom, N1-src extra six amino acids and N2-src extra seventeen amino acids are shown above. Extra amino acids that are inserted in SH3 domains of N1-src and N2-src share 5 amino acids and the sixth one was changed from arginine in N1-src to serine in N2-src. Arrows on top of N2- src show the possible phosphorylation sites that are inserted.

called heterogeneous nuclear ribonucleoprotein 1 (hnRNP 1) (Chan and Black, 1997; Chou et al., 2000; Grabowski and Black, 2001). In neuronal cells, a neuron specific form of PTB dissociates from the binding site in the presence of ATP and allows N1-src splicing (Chou et al., 2000; Grabowski and Black, 2001). There is a complex of proteins that binds to the DCS to allow splicing; this complex includes KH-type splicing regulator protein (KSRP), nPTB, (hnRNP F) and hnRNP H (Chou et al., 1999; Grabowski and Black, 2001). Although these proteins are expressed in many cell types they act as splicing regulators only in neuronal cells, which might be related to a neuronal specific splice variant of PTB that enhances complex formation (Grabowski and Black, 2001). It seems to be essential to have the N1-src insertion when expressing the N2-src splice variant. It is not clear why there is no splice variant of src that has the N2-src insert of 11 amino acids while skipping only the N1-src intron but not N2-src intron (Pyper and Bolen, 1990).

1.6 Src function in the nervous system

Since src has two neuronal-specific splice variants, it suggests src has additional neuronal functions. C-src is expressed in high amounts in neurons (Cotton and Brugge, 1983). Embryonic brain expresses higher amounts of C-src as well as higher kinase activity than the adult brain, suggesting a role of src in brain development, proliferation and differentiation (Cartwright et al., 1988; Grandori and Hanafusa, 1988; Kalia et al., 2004). Neuronal differentiation was accompanied by an increase in src activity that is mediated by C-src activation by RPTPα (Receptor Protein Tyrosine Phosphatase α) (den Hertog et al., 1993). Growth cone membranes contain nine fold more C-src than the subcellular fractions accompanied with an elevated

kinase activity, which suggests an important role in growth cone mediated neurite extention (Maness et al., 1988; Bixby and Jhabvala, 1993). C-src involvement in lipid rafts at the growth cones is necessary for its activation and function in triggering neurite outgrowth (Zhao et al., 2009). Furthermore, induction of neurobastoma cells to neuronal differentiation resulted in elevated expression levels and activity of both C-src and N-src, suggesting that neuronal differentiation is accompanied by elevated expression and activity of src splice variants (Bjelfman et al., 1990). The same elevation for both C-src and N1-src expression levels and activity was observed in embryonic neuronal cell differentiation (Cartwright et al., 1987). In some areas of the brain such as the hippocampus, src expression increases after birth and remains high even in adult (Cartwright et al., 1988). In fact, in the hippocampus region of rat brain, src expression at day two after birth is twice as high as its expression at embryonic day 18 (Cartwright et al., 1987). The high expression of src in hippocampus after birth and through the life of the animal might reflect its importance in this region which is involved in learning and memory

1.6.1 Src function in learning and memory

Src kinase activity is essential for normal brain development and function. One of these functions is in learning and memory formation. Src is required for both short term and long term memory, since src inhibitors blocked both (Bevilaqua et al., 2003). C-src activation is essential and sufficient for long term memory formation since blocking src by using an anti-src1 antibody prevented LTP in CA1 pyramidal cells (Lu et al., 1998). Src activation also enhanced AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) receptor and NMDA (N-methyl-D-aspartic acid)

receptor response (Lu et al., 1998). In agreement with a role for C-src, its expression increases during memory formation. For example, C-src mRNA levels in the CA3 region of rat hippocampus were increased after maze learning (Zhao et al., 2000). Furthermore, C-src protein levels in hippocampal synaptosomes were increased in parallel to an increase in its kinase activity (Zhao et al., 2000). In addition C-src association with and phosphorylation of the presynaptic nerve terminal proteins synapsin 1 and synaptophysin and also the postsynaptic NR2 subunits of the NMDA receptor increased after maze learning, suggesting that C-src is involved in the learning process and is activated as a tyrosine kinase both presynaptically and postsynaptically (Zhao et al., 2000).

1.6.2 Src function in nerve regeneration

C-src and N1-src are also involved in nerve regeneration and myelination. Both C-src and N1-src levels and activity were increased during peripheral axonal regeneration (Le Beau et al., 1991). Furthermore, Schwann cells also expressed high amounts of C-src at the same time of myelination in the regenerated nerve; Schwann cells usually express only low levels of C-src, suggesting that C-src is important in differentiation during regeneration (Le Beau et al., 1991). However, when C-src and N1-src were overexpressed in retinal cells, both impaired axonogenesis. This may has been due to uncontrolled activity of the kinase which resulted in untargeted activity, or an excess of phosphorylation with respect to axonogenesis (Worley et al., 1997). Similar results with abnormal dendritic and growth cone formation were observed in cerebral Purkinje cells overexpressing wild-type N1-src but the effect

was more severe with constitutively active N1-src. These abnormal features were rescued using the selective src inhibitor PP2 (Worley et al., 1997).

C-src is also important in maintaining the neuromuscular junction by phosphorylating the acetylcholine (ACh) receptor and maintaining receptor clusters and interaction with the cytoskeleton (Sadasivam et al., 2005). The presence of C-src is able to stabilize ACh receptor clusters in postsynaptic neuromuscular junctions even during PP2 treatment (Smith et al., 2001). C-src also phosphorylates the C-terminal region of QKI (quaking homolog KH domain RNA binding) a signal transduction activator of RNA, to regulate its binding to mRNA; QKI is an mRNA binding protein that regulates myelin production during brain development by enhancing myelin basic protein production through its binding to mRNA to protect it from degradation (Zhang et al., 2003).

1.6.3 Src function in neuro-inflammation

Src is also involved in inflammation, infection and injury healing in the brain. For example, the amount of C-src and its activity were both increased up to three fold after sciatic nerve injury (Zhao et al., 2003). Schwann cells, which play a scaffolding role for the injured nerve regeneration, also showed high expression levels and activity of src distal to the site of injury. This resulted in the activation of adhesion sites and cytoskeleton assembly to generate a pulling force for the newly formed growth cone (Zhao et al., 2003). Furthermore, active C-src was located at the sites of injured axons and after the injured nerve was healed, src levels returned to normal. PDGFβ receptors might have an essential role for C-src activation in injured

neurones. The receptor may direct C-src to the plasma membrane of the injured neurones and activate the pathway for nerve regeneration (Isozumi et al., 1997).

C-src is also involved in interleukin I (IL-1) signalling of inflammation. C-src is activated in hypothalamic neurones by IL-1 receptor activation. This activation of C-src results in hyperpolarization and decreased firing rates of these neurones, which is most likely related to ion channel regulation. Furthermore, this inhibition can be blocked by using the src inhibitor PP2 (Davis et al., 2006). On the other hand, activation of the IL-1 receptor and subsequent activation of C-src in hippocampal neurons resulted in phosphorylation of NMDA receptors and potentiation of neuronal damage, which was blocked by the src inhibitor PP2 (Viviani et al., 2003). NMDA phosphorylation by C-src in interleukin I receptor activation resulted in increasing Ca^{2+} influx and subsequent hyperexitability, seizures and a proconvulsive effect (Balosso et al., 2008). Furthermore, C-src is activated in NMDA receptor induced hyperalgesia in the spinal area, and this effect can be overcome by using the src inhibitor PP2 (Sato et al., 2003).

1.6.4 Src function in the synapse

1.6.4.1 Post-synaptic function of src

One well established function of C-src in neuronal cells is its role in upregulating post synaptic NMDA glutamate receptor function (Yu and Salter, 1999; Kalia et al., 2004). The NMDA receptor consists of four subunits. Two subunits are represented by the NR2 subunit, which has four splice variants (A-D), and is the binding site for glutamate, and the other two are NR1 subunits which are the binding site for the co-agonist glycine (Furukawa et al., 2005). Immunoprecipitation of either NR1 or C-src

resulted in coprecipitation of the other protein, suggesting that src is associated physically with the NR1 subunit of the NMDA receptor (Yu et al., 1997). However the NR1 subunit has only one tyrosine residue in its cytosolic C-terminal region, with no evidence of tyrosine phosphorylation (Salter, 1998). The NR2A, NR2B and NR2D subunits of the NMDA receptor have a very long intracellular C-terminal tail of more than 600 amino acids (Salter, 1998; Salter and Kalia, 2004). NR2B is mainly phosphorylated by Fyn, whereas NR2A is phosphorylated by C-src at multiple sites (Y1292, Y1325 and Y1387) (Salter and Kalia, 2004).

C-src is implicated in the enhancement of NMDA receptor function during LTP (Lu et al., 1998). There are several ways that C-src is proposed to upregulate NMDA receptor function. For example, NMDA receptor phosphorylation by src increases its signal transduction and permeability (Kohr and Seeburg, 1996; Salter, 1998; Lu et al., 2000). Another possible mechanism involves the phosphorylation of other proteins in the NMDA receptor complex; one of which is the scaffolding protein PSD93, which might alter NMDA receptor channel gating (Salter and Kalia, 2004). Alternatively, phosphorylation of NR2A and NR2B subunits of the NMDA receptor could recruit signalling proteins that contain SH2 domains, such as PLCγ and PI3 kinase, to the plasma membrane (Salter and Kalia, 2004). Tyrosine phosphorylation of these subunits protects NMDA receptors from degradation by Ca^{2+}-activated proteases (Salter and Kalia, 2004).

A consequence of C-src phosphorylation of the NMDA receptor is induction of LTP (Salter and Kalia, 2004). This is mediated by increasing the number of NMDA receptors at the plasma membrane (Grosshans et al., 2002), since C-src mediated tyrosine phosphorylation of NMDA receptor subunits stabilize the receptor at the

plasma membrane via decreasing its association with the clathrin-mediated endocytosis machinery (Grosshans et al., 2002; Salter and Kalia, 2004).

What are the mechanisms via which C-src is activated to phosphorylate the NMDA receptor? Activation of G-protein coupled receptors such as the acetylcholine M1 muscarinic receptor potentiates NMDA receptor responses in hippocampal neurons (Salter and Kalia, 2004). This potentiation can be blocked by src inhibitors, suggesting that M1 muscarinic receptor potentiation of NMDA receptor function is mediated by C-src (Salter and Kalia, 2004). C-src activation can also be induced by brain ischemia (Liu et al., 2001). Activation of C-src via both routes involves both PKC and the FAK (focal adhesion kinase) CAKβ (also known as Pyk2). CAKβ auto-phosphorylation at Y402 creates a binding site for the C-src SH2 domain, which results in src activation and subsequent NMDA receptor potentiation (Henley and Nishimune, 2001; Liu et al., 2001; Salter and Kalia, 2004). In support of this, NMDA receptor NR2B subunit phosphorylation at Y1472 after tetanic stimulation occured concurrently with CAKβ and C-src activation in the CA1 region of the hippocampus (Ali and Salter, 2001).

Src activation also leads to enhanced AMPA receptor function (Grosshans et al., 2002). AMPA is a glutamate receptor ion channel that consists of four subunits in a heterodimer structure of GluR1-GluR4 (Song and Huganir, 2002). Most AMPA receptors contain GluR2 which gives them Ca^{2+} impermeability (Song and Huganir, 2002). The AMPA receptor GluR2 subunit is phosphorylated by C-src at Y876 and this phosphorylation is essential for regulating AMPA receptor internalization and also may contribute to stimulation dependent long term plasticity (Hayashi and Huganir, 2004). GluR2 subunit tyrosine phosphorylation is required for either insulin

or low frequency stimulation-dependent AMPA receptor endocytosis and long term depression an effect blocked by the src inhibitor PP2 (Ahmadian et al., 2004).

1.6.4.2 Pre-synaptic functions of src

C-src is also found in the pre-synaptic region and there are many proteins that both interact with src and are phosphorylated by src. In fact both C-src and N1-src are found in all synaptic preparations, and C-src is the major src family kinase member in synaptic vesicle preparations, suggesting it has roles at the synaptic vesicle (Onofri et al., 2007). In support of this, C-src is 4-5 times more concentrated in purified synaptic vesicles than homogenized whole brain (Greengard et al., 1993). The concentration of N1-src is higher in synaptic membrane preparations compared to synaptic vesicles suggesting a role for N1-src at the plasma membrane (Onofri et al., 2007).

Src activity has previously been implicated in synaptic vesicle turnover in nerve terminals. The src inhibitors PP1 and PP2 caused an increase in Ca^{2+}-dependent glutamate release from rat brain nerve terminals and dopamine release from PC12 cells suggesting a negative regulatory role of src (Ohnishi et al., 2001; Baldwin et al., 2006). On the other hand, experiments using PP2 resulted in a suppression of glutamate release in cerebrocortical synaptosomes suggesting a positive role of src in synaptic vesicle exocytosis (Wang, 2003). The conflicting results from these experiments suggest that there are differences in the response mechanisms since different types of cells and stimulation conditions were used in these reports. Stimulation conditions in experiments showing inhibitory role of src involved a substantial increase in Ca^{2+} concentration whereas the stimulation in the other

experiment is more likely to produce a physiological like Ca^{2+} concentration. Src also activates L-type Ca^{2+} channels in cerebellar granule neurons after depolarization and enhances neurotransmitter release, which requires a prior tyrosine phosphorylation of FAK and vinculin (Evans and Pocock, 1999). Using a prolonged KCl stimulation or a tyrosine phosphatase inhibitor, such as vanadate, enhanced L-type Ca^{2+} activation and subsequently increased neurotransmitter release suggesting a regulatory role for src in neurotransmitter release (Evans and Pocock, 1999).

1.7 The synaptic vesicle life cycle

Nerve terminals are secretory machines that are engaged in repeated cycles of neurotransmitter release. The synaptic vesicle, where the neurotransmitter is stored, is the smallest eukaryotic organelle with an average diameter of 42 nm in the mammalian CNS (Jahn and Sudhof, 1994; Rizzoli and Betz, 2004; Smith et al., 2008). The synaptic vesicle cycle involves both fusing the vesicle membrane with the plasma membrane to release neurotransmitter (exocytosis) and retrieving this membrane back to form a new vesicle (endocytosis) (Figure 1.3) (Heuser and Reese, 1973; Gandhi and Stevens, 2003).

Synaptic vesicles are clustered close to the pre-synaptic membrane (De Camilli and Takei, 1996; Schweizer and Ryan, 2006). This clustering involves actin filaments and the synaptic vesicle protein synapsin, where synapsin is attached to both the vesicle membrane and actin filaments to keep vesicles in position (Hirokawa et al., 1989; Evergren et al., 2007). The vesicles then dock at the membrane of the active zone and then undergo a series of priming reactions after which they fuse with the plasma membrane and release neurotransmitter cargo. Vesicle fusion occurs in

response to action potential stimulation and a subsequent increase in Ca^{2+} concentration in the nerve terminal due to voltage gated Ca^{2+} channel opening (Nofal et al., 2007; Rizo and Rosenmund, 2008). There are a number of different routes through which a synaptic vesicle can recycle back to the nerve terminal. The first two pathways could be named flicker fusion, where vesicle retrieval happens very quickly (Smith et al., 2008); the first is called kiss and stay, where the vesicle is retrieved from the plasma membrane after fusion pore opening to allow neurotransmitter release (Sudhof, 2000; Bonanomi et al., 2006). The second pathway is called kiss and run, where the vesicle is also retrieved after fusion pore opening, but it does not stay in the docked position (Valtorta et al., 2001; Rodal and Littleton, 2008; Zhang et al., 2009). The third pathway involves full fusion of the vesicle with plasma membrane followed by clathrin-mediated endocytosis (Jarousse and Kelly, 2001; Dickman et al., 2005; Cousin, 2009). Finally a bulk endocytosis of large membrane areas can also occur, which is a way of quickly retrieving vesicle membrane after fast repeated stimulations that other retrieval mechanisms do not have the capacity to deal with (Matthews, 2004; Clayton et al., 2008; He et al., 2009).

1.7.1 Synaptic vesicle exocytosis

Proteins that are involved directly in vesicle membrane fusion with the plasma membrane are called SNARE proteins (SNAP (Soluble NSF Attachment Protein) Receptors). There are three SNARE proteins, two of which are found in the plasma membrane (syntaxin and SNAP25) while the third one is a vesicular membrane

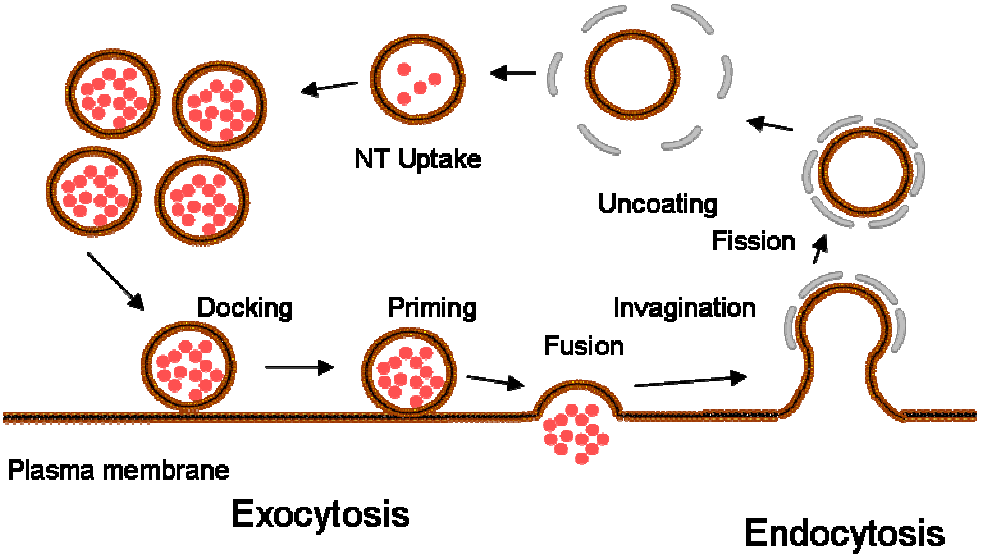


Figure 1.3- ***Illustration of the synaptic vesicle cycle***
Different steps involved in the vesicle cycle. Arrows indicate directions of vesicles towards or backwards from the membrane.

protein (VAMP) (Chen et al., 2001; Jahn and Scheller, 2006; Sudhof and Rothman, 2009). Syntaxin is a transmembrane protein, while SNAP-25 is a palmitoylated peripheral membrane protein. VAMP is a transmembrane protein (Bauerfeind et al., 1996; Hu et al., 2002; Jahn and Scheller, 2006). Both syntaxin and VAMP have only one SNARE motif while SNAP-25 has two, and all of these SNARE motifs interact with each other to form the SNARE complex (Chen and Scheller, 2001; Sudhof, 2004). SNARE motifs consist of a homologus coiled-coil region of about 60 amino acids (Fasshauer, 2003). SNARE complex formation drives the vesicular and the plasma membranes to become closer to each other and then fuse forming a fusion pore that allows neurotransmitter release (Figure 1.4) (Sritharan et al., 1998; Parpura and Mohideen, 2008).

1.7.2 SNARE Proteins

VAMP is a small synaptic vesicle membrane protein with a molecular weight of 18 kDa (Baumert et al., 1989). There are two isoforms of VAMP (VAMP1 and 2) which are both found in the central nervous system synaptic regions with an overlapping distribution, but VAMP 2 is the dominant isoform in most neurons (Raptis et al., 2005). VAMP consists of three domains: the N-terminal SNARE motif, a trans-membrane region, and a highly charged internal region (Trimble et al., 1988).

SNAP 25 (synaptosome-associated protein of 25 kDa) is a 25 kDa protein that is anchored to the cytoplasmic face of the plasma membrane via palmitoylation at several cysteine residues at the centre of the protein. SNAP 25 has two SNARE motifs that contribute to the SNARE complex for vesicle fusion (Hodel, 1998). There are two isoforms of SNAP 25 (SNAP 25 A and B), which both are present in

neurons. SNAP 25 binds to syntaxin in a 1:1 ratio and their SNARE motifs will form a 3 helical bundle and this bundle is the acceptor complex for the VAMP SNARE motif (Rizo and Rosenmund, 2008).

Syntaxin 1 is found in the nerve terminal as a trans-membrane protein. There are three functional domains in syntaxin: the C-terminal trans-membrane domain, the SNARE motif and the N-terminal regulatory domain (Bennett et al., 1993). There are two conformations for syntaxin: the first is when the regulatory domain binds to the SNARE motif causing syntaxin to be in a restrained closed conformation that is not available for SNARE complex formation. This closed conformation binds to the protein Munc 18. The other is the open conformation where the regulatory domain and SNARE motif are freed, allowing the SNARE motif to bind other SNAREs (Rizo and Rosenmund, 2008).

There are several other proteins which are involved in regulated exocytosis such as the synaptotagmins (the primary Ca^{2+} sensor) that couple Ca^{2+} entry into the nerve terminal with exocytosis (Yoon and Shin, 2009). There are two cytoplasmic C2 domains in synaptotagmin that bind to Ca^{2+}, The C2A binds to 3 Ca^{2+} ions, while C2B binds to 2 Ca^{2+} ions (Sudhof, 2004). Synaptotagmin binding to phospholipids is Ca^{2+} dependent, however the Ca^{2+}-dependency of its binding to the SNARE complex is controversial (Sudhof, 2004). The SNARE complexes form a ring shape at the fusion area and synaptotagmin binding to phospholipids inside this ring results in local positive membrane curvature that brings the two membranes close to each

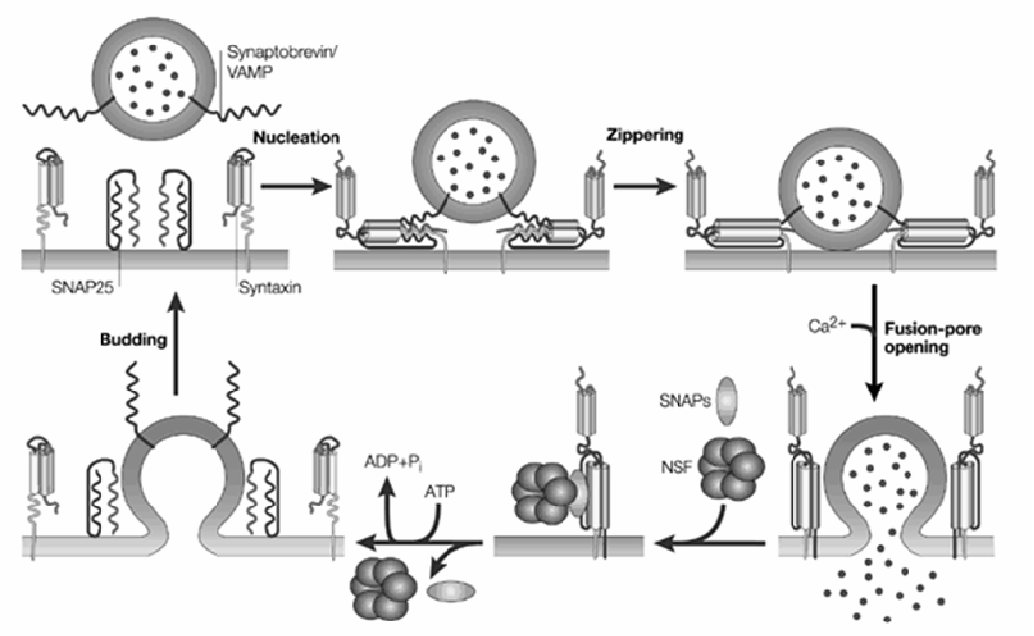


Figure 1.4- ***SNARE proteins function in vesicle fusion***
SNARE proteins (VAMP, syntaxin and SNAP 25) interaction and pore formation. Different steps in vesicle fusion are illustrated including SNARE proteins assembling and disassembling which requires αSNAP and NSF ATPase activity (Rizo and Südhof 2002).

other, which will facilitate and stabilize the pore opening in exocytosis (Sudhof, 2004; Martens et al., 2007).

1.7.3 Synaptic vesicle endocytosis

Synaptic vesicles are endocytosed and locally recycled after exocytosis, which is essential to maintain synaptic activity, and the recycled vesicles have to be identical to the originals to maintain the same function (Bonanomi et al., 2006; Schweizer and Ryan, 2006). After full fusion of a vesicle, a sorting process is needed to retrieve vesicle proteins (Sankaranarayanan and Ryan, 2001; Bonanomi et al., 2006; Rizzoli and Jahn, 2007). Clathrin mediated endocytosis performs this role (Figure 1.5 A) (Bonanomi et al., 2006; Granseth et al., 2007; Smith et al., 2008). The vesicle protein synaptotagmin attracts the cytosolic protein AP2 which initiates the recruitment of the endocytic machinery and clathrin cage formation (Bonanomi et al., 2006; Smith et al., 2008). After formation of the synaptic vesicle, the large GTPase dynamin fissions the vesicle from the plasma membrane (Slepnev and De Camilli, 2000; Bonanomi et al., 2006). The vesicle is then uncoated by removing the clathrin coat in an ATP dependent process. The vesicle is then reacidified and refilled with neurotransmitter ready to join the recycling vesicle pool (Bonanomi et al., 2006; Smith et al., 2008).

1.7.3.1 Clathrin coat formation

Clathrin cage formation involves several proteins. The adaptor protein complex (AP2) which is a tetrameric heterodimer consisting of two large subunits of about 100 kDa (α and β), a medium size subunit of about 50 kDa (μ) and a small subunit of

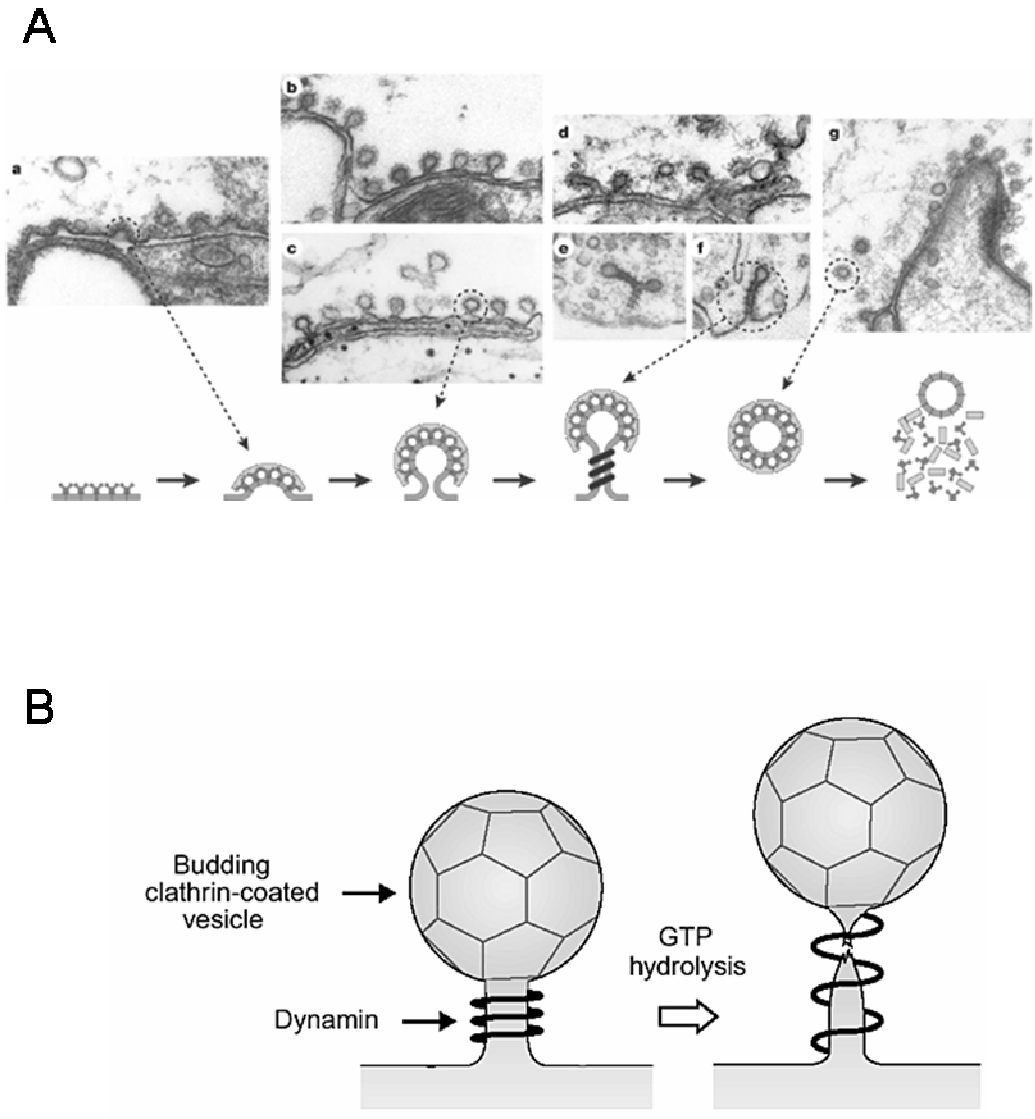


Figure 1.5- ***Clathrin mediated synaptic vesicle endocytosis***

A) Different steps in clathrin mediated synaptic vesicle endocytosis, start from coat assembly, vesicle formation, dynamin mediated vesicle fission and vesicle clathrin coat disassembly as imaged from synapses and illustrated (Slepnev and Di Camilli 2000).

B) Dynamin mechanochemical role in vesicle fission. Illustration of dynamin assembly around the vesicle neck and force generation by utilising ATP to cause vesicle fission (Stowell et al 1999).

about 20 kDa (σ) (Edeling et al., 2006). There are two sequences to which AP2 binds (YXXΦ and D/EXXXLL/I) (X is any amino acid and Φ is a large hydrophobic residue) which are considered signals for endocytic membrane proteins (Edeling et al., 2006). AP2 also binds to phosphoinositides such as phosphoinositol-4, 5-bisphosphate which are important signals for membrane identity to recruit AP2 (Edeling et al., 2006). A direct interaction between AP2 and the cytosolic domain C2B of synaptotagmin triggers the assembly of the clathrin coated pit, which suggests that synaptotagmin might be a signalling protein for both exocytosis and endocytosis (Schmid, 1997; Bonanomi et al., 2006). Endocytic motifs enhance this interaction (Haucke and De Camilli, 1999). The other adaptor protein that is involved in synaptic vesicle endocytosis is the neuronal specific protein AP180 (about 90 kDa) which also binds to phosphoinositides (Schmid, 1997; Bonanomi et al., 2006). AP180 binds to both AP2 and clathrin, which makes both AP2 and AP180 act as adaptors to assemble clathrin at the vesicle membrane (Cremona and De Camilli, 1997; Kirchhausen, 2000).

Clathrin assembly by these proteins triggers vesicle formation. The clathrin coat consists of triskelia and each triskelion consists of three clathrin heavy chains (about 190 kDa) and three clathrin light chains (25-29 kDa) that form a stable oligomeric complex (Slepnev and De Camilli, 2000; Edeling et al., 2006).

Two other proteins (endophillin and amphiphysin) which bind to inwardly curved membrane will be recruited to the curved vesicle membrane (Kirchhausen, 2000; Brett and Traub, 2006). They both have N-BAR (Bin-Amphiphysin-Rvs) domains which is an N-terminal amphipathic α-helix. These BAR domains are inserted into the membrane for optimal engagement while the core BAR domain stabilizes

vesiclular membrane curvature (Brett and Traub, 2006). Amphiphysin binds to both AP2 and clathrin making an adaptor for the assembled proteins (Slepnev et al., 1998; Farsad and De Camilli, 2002). Both endophillin and amphiphysin also contain SH3 domains that bind to dynamin and synaptojanin proline rich domains (PRD) (Kirchhausen, 2000).

Dynamin also binds to phosphoinositides through its PH domain and synaptojanin has a lipid modification activity as an inositol 5-phosphatase (Kirchhausen, 2000). Dynamin binding to the phosphoinositides promotes its oligomerization and assembly at the clathrin coated pit (Doherty and McMahon, 2009). In fact, dynamin assembly forms rings around the clathrin coated pit and membrane squeezing, by making the rings tighter, will cause the membrane to come in close proximity forming the vesicle neck. Further squeezing and twisting by dynamin causes vesicle fission and pinching off of the vesicle from the plasma membrane (Bonanomi et al., 2006; Doherty and McMahon, 2009). Dynamin effects on membrane fission requires energy to cause fission of the lipid bilayer and energy is mainly provided by dynamin GTPase activity (Figure 1.5 B) but also could involve the cytoskeleton (Doherty and McMahon, 2009). Phosphoinositide dephosphorylation decreases lipid binding proteins affinity to the lipid bilayer which acts as a signal for clathrin coat disassembly. Synaptojanin's dual phosphatase activity results in phosphoinositide dephosphorylation and promotes clathrin coat disassembly (Ryan, 2006; Zanazzi and Matthews, 2007). In fact, deletion of either of the phosphatase domains from synaptojanin resulted in accumulation of coated vesicles in the synaptic terminals suggesting a dual phosphatase activity of synaptojanin is required for vesicle uncoating (Ryan, 2006; Zanazzi and Matthews, 2007). Auxilin is another protein

that binds to clathrin and is recruited to the coated vesicle (Edeling et al., 2006). The J domain of auxilin binds to the ATPase chaperone HSC70 and stimulates its ATPase activity (Edeling et al., 2006). HSC70 also binds to clathrin heavy chain and destabilize triskilions interactions, promoting triskilion release from the cage and initiating clathrin coat dissociation from the coated vesicle (Schmid, 1997; Edeling et al., 2006).

1.7.4 Proteins involved in synaptic vesicle cycle that may interact with src or src substrates

Src has previously been shown to either interact with or phosphorylate a number of nerve terminal proteins that are involved in synaptic vesicle recycling. These proteins have different functions across the vesicle life cycle from maintaing synaptic vesicle pools, vesicle docking and clathrin uncoating of the endocytosed vesicle.

1.7.4.1 Synapsin

The synapsins are peripheral vesicle proteins (Murthy, 2001; Fdez and Hilfiker, 2006). They help in synapse formation and maintenance by maintaining the reserve vesicle pool (Murthy, 2001; Ferreira and Rapoport, 2002; Fdez and Hilfiker, 2006). There are five synapsin proteins expressed from three genes (synapsin Ia, Ib, IIa, IIb, and III) (Ferreira and Rapoport, 2002; Fdez and Hilfiker, 2006; Evergren et al., 2007). SynapsinI and synapsinII are expressed in most nerve terminals, while synapsinIII is less abundant, but can be found either in the postsynaptic region or other parts of neuronal cells (Ferreira and Rapoport, 2002; Cousin et al., 2003; Evergren et al., 2007).

All synapsins have eight different domains (A-H), but have a similar structure in three domains: the N-terminal domain A, the central domain C, and the C-terminal domain E. The other domains however, display a diverse variation in structure across different species (Hosaka et al., 1999; Ferreira and Rapoport, 2002; Evergren et al., 2007). Domain C interacts with synaptic vesicle phospholipids, actin filaments and ATP, and is a substrate for C-src (Evergren et al., 2007). The proline rich domain D is involved in synapsin interactions with many SH3 containing proteins including src (Evergren et al., 2007). Synapsins bind to the lipid surface of the synaptic vesicle via their N-terminal domain and middle portions (Ferreira and Rapoport, 2002).

Synapsin I interacts with many SH3 containing proteins and it is a major binding partner of the C-src SH3 domain in the nerve terminals (Onofri et al., 1997; Onofri et al., 2000). In agreement the C-src SH3 domain has higher affinity to synapsin I than many SH3 domain containing proteins such as amphiphysin I and II, Crk, and phospholipase Cγ (Onofri et al., 2000).

1.7.4.1.1 Synapsin phosphorylation

Synapsin I can be phosphorylated by several kinases such as PKA (protein kinase A), CaMK I and II (Ca^{2+}-calmodulin dependent kinases), MAP kinase and Cyclin-dependent kinase 5 **(**CDK5) (Fdez and Hilfiker, 2006). Synapsin I phosphorylation at domain A by PKA or CaMK I and II causes a decrease in its affinity to synaptic vesicles which results in its dissociation from synaptic vesicle (Hosaka et al., 1999; Fdez and Hilfiker, 2006; Evergren et al., 2007). This phosphorylation also releases synapsin from actin filaments allowing the synaptic vesicle to be available for exocytosis. Synapsin I phosphorylation by MAP kinase does not affect its synaptic

vesicle association, but decreases its binding to actin, which might play a role in recruiting recycled vesicles to the reserve pool (Fdez and Hilfiker, 2006). Synapsin is also phosphorylated by CDK5 at S551 and S553 (Figure 1.6A) and this phosphorylation does not seem to affect its affinity to actin (Matsubara et al., 1996). However CDK5 might play a role in synapsin clustering (Yamashita et al., 2007).

1.7.4.1.2 Synapsin tyrosine phosphorylation

Synapsin is also tyrosine phosphorylated by C-src at a single major site (Y301) in domain C (Figure 1.6A) that is conserved in all isoforms (Onofri et al., 1997; Onofri et al., 2007). Since the C domain is involved in synapsin interactions with synaptic vesicles, actin and dimerization, there is a possibility that synapsin phosphorylation by C-src regulates some aspects of the synapsin cycle or vesicle trafficking (Onofri et al., 2007). C-src phosphorylation of synapsin I provides a binding site for the C-src SH2 domain. C-src binding to synapsin via both the SH3 and SH2 domains results in a several fold increase in C-src kinase activity in phosphorylating other substrates such as synaptophysin (Onofri et al., 1997; Onofri et al., 2007). Synapsin II may also interact with and activate C-src (Onofri et al., 1997). In fact, depletion of synapsin I results in a decrease in C-src kinase activity in synaptic vesicles, suggesting that synapsin I might be a key src activator in the presynaptic region (Onofri et al., 1997; Onofri et al., 2007). In synapsin I KO mice, both src activity and tyrosine phosphorylation of synaptic vesicle proteins were depressed, even though src expression was not affected (Onofri et al., 2007). In addition, since synapsin serine phosphorylation regulates synapsin association with the synaptic vesicle, it might also regulate C-src association with synaptic vesicles and phosphorylation of its

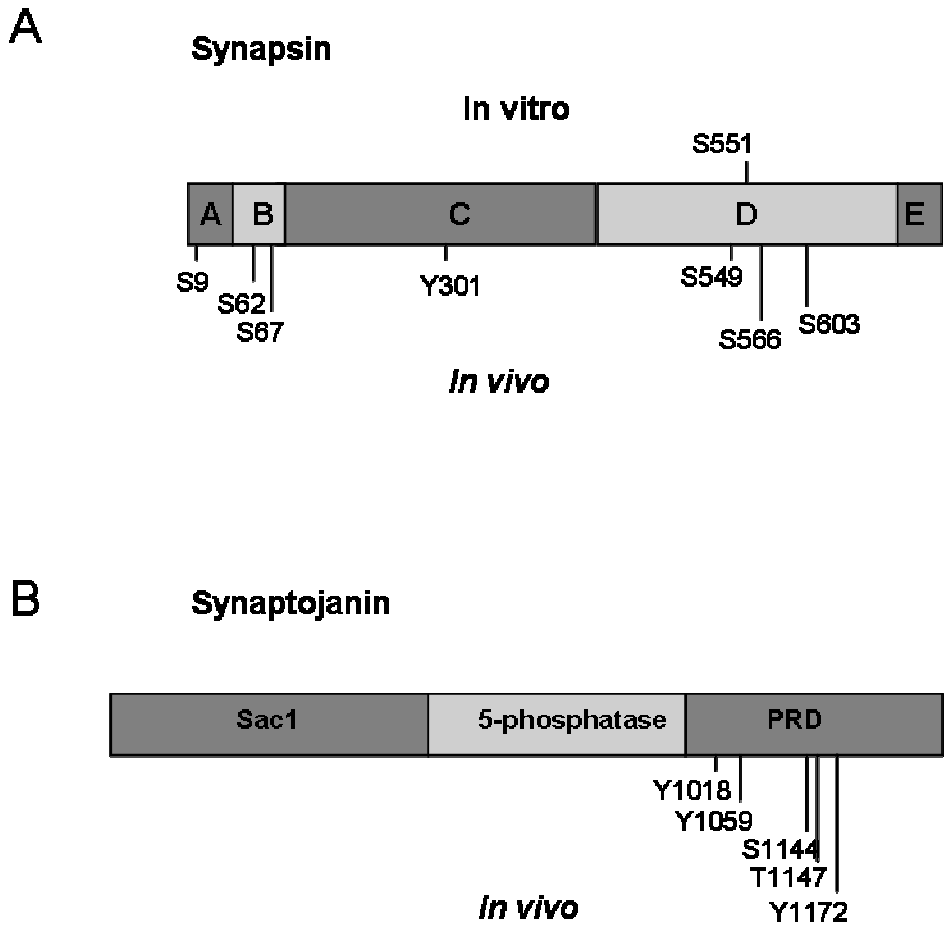


Figure 1.6- ***Src substrates and their phosphorylation sites (a)***

A) Synapsin Ia structure and phosphorylation sites. Illustration of synapsin Ia showing both serine and tyrosine phosphorylation sites. Synapsin domains were shown as (A-E), domain A involved in vesicle interaction, domain B is involved in synapsin targeting to the synaptic region, domain C is involved in interaction with actin filaments, SV phospholipids, ATP and dimerization, domain D is proline rich and involved in protein-protein interactions, domain E is involved in targeting synapsin to the nerve terminal (Evergren et al 2007). Synapsin serine phosphorylation results in conformational changes and modulates its interaction (Evergren et al 2007).

B) Synaptojanin I structure and phosphorylation sites. Illustration of synaptojanin I structure showing the functional domains and serine, threonine and tyrosine phosphorylation sites. There are at least three other phosphorylation sites for minibrain kinase that are not yet mapped (Adayev et al 2006).

substrates through synapsin association and dissociation from the vesicle membrane (Onofri et al., 2007).

1.7.4.2 Synaptojanin

Vertebrates express two genes for synaptojanin: synaptojanin 1 which is a neurospecific protein and synaptojanin 2 which is expressed ubiquitously (Rosivatz, 2006). Synaptojanin 1 has two isoforms, a 145-kD protein that is enriched in the nerve terminal and a 170-kDa isoform only expressed in embryonic tissue (de Heuvel et al., 1997; Rosivatz, 2006). Synaptojanin consists of 3 domains: two of which are lipid metabolizing domains known as the Sac 1 domain, the polyinositide phosphatase domain, and the C-terminal proline rich domain (PRD) (Hopper and O'Connor, 2005; Mani et al., 2007). Synaptojanin I is required for clathrin mediated synaptic vesicle endocytosis and vesicle reavilability due to its role in synaptic vesicle uncoating (Cousin and Robinson, 2001; Kim et al., 2002; Mani et al., 2007). Synaptojanin KO neurons displayed a smaller functional synaptic vesicle pool and a large fraction of the recycled membrane became trapped in functionally inert compartments after a prolonged stimulation (Kim et al., 2002). In fact, synaptojanin I KO resulted in accumulation of free clathrin coated vesicles in the synaptic area as well as deep invaginations (Kim et al., 2002; Mani et al., 2007).

Endophillin is the major binding partner for synaptojanin 1 in nerve terminals (Micheva et al., 1997; Cestra et al., 1999; Rosivatz, 2006). Synapses that lack synaptojanin and endophillin show a defect in synaptic vesicle recycling (Dickman et al., 2005). Synaptojanin also interacts with the C-src SH3 domain, but the function of

C-src interaction with synaptojanin I is not known (Onofri et al., 1997; Onofri et al., 2000).

1.7.4.2.1 Synaptojanin phosphorylation

Synaptojanin is one of the dephosphins that are dephosphorylated by calcineurin upon stimulation in a Ca^{2+}-dependent manner (Cousin et al., 2001; Smillie and Cousin, 2005), while it is rephosphorylated by cyclin-dependent kinase 5 (CDK5) under basal conditions (Lee et al., 2004; Smillie and Cousin, 2005). Synaptojanin phosphorylation by CDK5 at S1144 (Figure 1.6B) inhibits its interaction with endophillin (Lee et al., 2004). Minibrain kinase (MNB) also phosphorylates synaptojanin at multiple sites that are dephosphorylated by calcineurin (Adayev et al., 2006).

1.7.4.2.2 Synaptojanin tyrosine phosphorylation

Synaptojanin is tyrosine phosphorylated at multiple sites (Y1018, Y1059, and Y1172) on its PRD (Figure 1.6B) by the receptor tyrosine kinase ephrin B (EphB) receptor (Irie et al., 2005). Tyrosine phosphorylation of synaptojanin resulted in reduction of endophillin binding (Irie et al., 2005). Since endophillin binding is required for synaptojanin localization to synaptic vesicle (Schuske et al., 2003), reduction of endophillin binding decreases its recruitment to synaptic vesicles. Furthermore, tyrosine phosphorylation resulted in decreasing its phosphatase activity suggesting a regulatory role for synaptojanin phosphorylation on its function (Irie et al., 2005). This was supported by the fact that EphB stimulation results in an increase in phosphoinositide levels within the cell and the lack of changes after EphB

stimulation when synaptojanin tyrosine residues were mutated (Irie et al., 2005). Similarly, since AMPA receptor internalization is clathrin mediated, synaptojanin tyrosine phosphorylation also regulated ligand induced AMPA receptor internalization (Irie et al., 2005). In support of this, the expression of both the kinase dead EphB and tyrosine mutated synaptojanin resulted in inhibited ligand induced AMPA receptor internalization (Irie et al., 2005).

1.7.4.3 Synaptophysin

Synaptophysin 1 (p38) is a 38 kD protein that is an integral synaptic vesicle protein with four intramembrane loops (De Camilli and Jahn, 1990; Bonanomi et al., 2007). Synaptophysin's role in the synaptic vesicle life cycle is not clear since there are other isoforms such as synaptophysin 2 and synaptogyrin that might have overlapping roles. Synaptophysin KO mice showed no distinct phenotype suggesting that it has no distinct function in the synaptic region (Arthur and Stowell, 2007). However when both synaptophysin I and synaptogyrin I were deleted, short term and long term plasticity were affected (Janz et al., 1999). In synaptophysin KO mice retinal photoreceptor cells, which do not express synaptophysin II, showed a decrease in the number of synaptic vesicles, suggesting an important role for synaptophysin in synaptic vesicle formation. Injection of the synaptophysin C-terminus in the squid giant synapse showed a defect in synaptic vesicle endocytosis with an increase in clathrin coated vesicles, suggesting that synaptophysin might be involved in clathrin independent endocytosis (Spiwoks-Becker et al., 2001). In addition synaptophysin KO hippocampal neurones showed an impaired ability to

form synapses compared to wild type, suggesting a role of synaptophysin in synapse formation (Tarsa and Goda, 2002).

1.7.4.3.1 Synaptophysin interactions

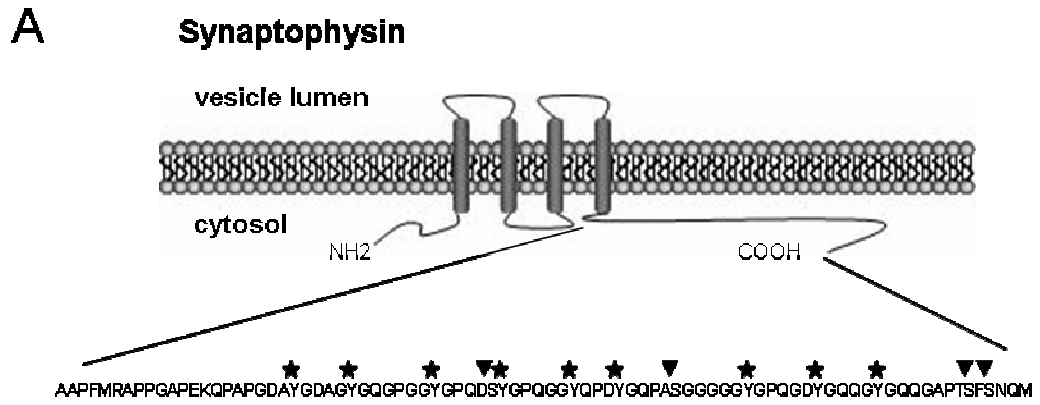
Synaptophysin is the major binding partner for cholesterol in synaptic vesicles, which might be involved in vesicle protein sorting in the plasma membrane before endocytosis, since partial depletion of cholesterol blocks endocytosis, as well as causing membrane curvature that starts vesicle budding (Thiele et al., 2000). Synaptophysin I forms oligomers by binding to other synaptophysin I molecules via their transmembrane domains (Pennuto et al., 2003). Synaptophysin also binds to VAMP via its transmembrane domains in the synaptic vesicle membrane during basal conditions. Their complex dissociates upon stimulation, suggesting this binding might be important in the regulation of SNARE complex formation since VAMP can not participate in SNARE complex when bound to synaptophysin (Becher et al., 1999; Pennuto et al., 2002; Yelamanchili et al., 2005). Synaptophysin binding to VAMP also plays an important role in VAMP targeting to the synaptic vesicle both from plasma membrane and intracellular compartments (Pennuto et al., 2003; Bonanomi et al., 2007). Synaptophysin binds to dynamin in a Ca^{2+} dependent manner at its C-terminus which is also negatively affected by synaptophysin binding to VAMP, suggesting that synaptophysin plays a role in dynamin targeting in endocytosis (Daly and Ziff, 2002).

Synaptophysin co-precipitates with C-src suggesting a direct interaction between these two proteins (Barnekow et al., 1990; Linstedt et al., 1992; Zhao et al., 2000). In support of this, FRET studies showed that synaptophysin interacts with C-src via its

SH2 domain (Felkl and Leube, 2008). The C-terminal tyrosine residues of synaptophysin are thought to mediate binding to the C-src SH2 domain, because removing these residues abolishes their interaction (Felkl and Leube, 2008).

1.7.4.3.2 Synaptophysin tyrosine phosphorylation

The tyrosine rich C-terminus of synaptophysin with nine tyrosine residues (Figure 1.7 A) forms a perfect target for tyrosine kinases, explaining why it is the major tyrosine phosphoprotein on the synaptic vesicle membrane (Pang et al., 1988). Synaptophysin is tyrosine phosphorylated by C-src in vitro and also when C-src is over expressed in cells (Barnekow et al., 1990; Evans and Cousin, 2005). Synaptophysin was also phosphorylated by either C-src or Fyn when coexpressed in COS cells, suggesting that synaptophysin is a substrate for these two members of the src family (Janz et al., 1999). There is no evidence of which of the nine tyrosine residues is phosphorylated in the synaptophysin C-terminus which might be due to the lack of a trypsin cleavage site, producing a large peptide that can not be analysed using mass spectrometry (Evans and Cousin, 2005). There are most likely, however, to be multiple phosphorylation sites in the synaptophysin C-terminus which is common for src substrates (Evans and Cousin, 2005). There is no evidence for a stimulation dependent change in synaptophysin tyrosine phosphorylation, suggesting that synaptophysin tyrosine phosphorylation is not stimulation dependent (Onofri et al., 2007), however C-src interaction with synaptic proteins might be negatively regulated by intracellular Ca^{2+} and PKC activity (Zhao et al., 2000). Tyrosine phosphorylation of synaptophysin increased after long term potentiation suggesting



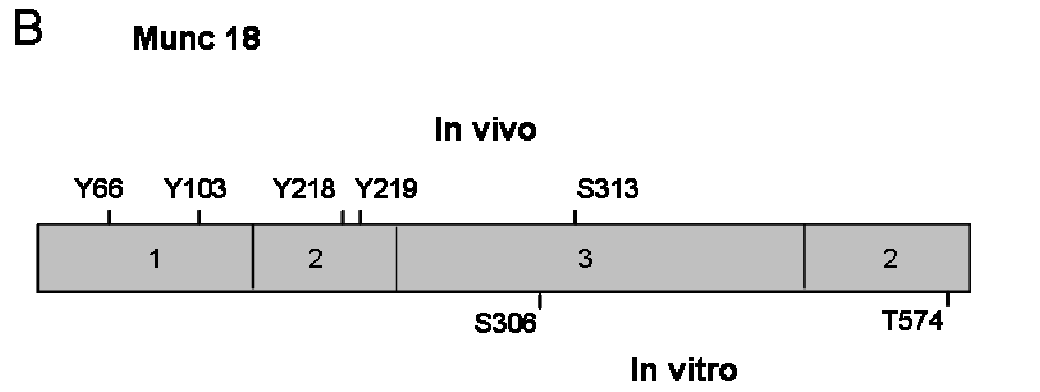


Figure 1.7- ***Src substrates and their phosphorylation sites (b)***

A) Synaptophysin structure. Illustration of synaptophysin structure showing the transmembrane loops and the C-terminus tail with the nine tyrosine residues that are expected to be phosphorylated by src and four serine residue that are also expected to be phosphorylated (▼ = serine residue, ★ = tyrosine residue) (Evans and Cousin 2005).

B) Munc 18 structure and phosphorylation sites. Illustration of Munc 18 showing both serine phosphorylation sites and tyrosine phosphorylation sites in Munc 18 (Rizo and Südhof 2002).

that this event might be an important aspect in long term potentiation (Evans and Cousin, 2005).

1.7.4.3.3 Synaptophysin serine phosphorylation

Synaptophysin is serine phosphorylated in a Ca^{2+} dependent manner by CaM kinase II in vivo, in vitro and in purified synaptic vesicles even though none of the four serine residues in the synaptophysin C-terminus match the preferred sequence (RXXS) for CaM kinase II (Rubenstein et al., 1993). Synaptophysin is also serine phosphorylated by PKC on purified synaptic vesicles; however, synaptophysin phosphorylation by CaMK II is three times higher than that of PKC (Rubenstein et al., 1993). Furthermore CaMK II inhibitors decreased synaptophysin phosphorylation in synaptosomes, suggesting that CaMK II is the main serine kinase for synaptophysin (Rubenstein et al., 1993). No important role of this phosphorylation has been shown.

1.7.4.4 Munc 18

Munc 18-1, also called n-sec1, plays an important role in the vesicle life cycle (Burgoyne et al., 2009). Munc18 KO mice showed normal brain development, and also neuromuscular junction formation that requires long axonal formation, including normal assembly of synapses and post synaptic function (Verhage et al., 2000). However, there was no neurotransmitter release observed even after strong electrical stimulation, suggesting that even though Munc 18 was not required for neuronal development, it was essential for neurotransmitter release (Verhage et al., 2000).

Munc 18 forms a complex with syntaxin when syntaxin is in the closed conformation (Dulubova et al., 2007), and also plays a role in syntaxin and Munc 18 stabilization (Dulubova et al., 2005; Gerber et al., 2008). Munc 18 binding to closed syntaxin allows syntaxin's passage from the endoplasmic reticulum to the plasma membrane without being engaged in ectopic SNARE complexes outside the nerve terminal (Rickman et al., 2007). This binding suggests that Munc 18 plays a regulatory role in SNARE complex formation (Dulubova et al., 1999; Carpp et al., 2006). However, Munc 18 also binds to the syntaxin N-terminus when it is in an open conformation or involved in the SNARE complex (Rizo and Rosenmund, 2008; Burgoyne et al., 2009; Johnson et al., 2009). Munc 18 binding to the assembled SNARE complex is reduced by mutation at D214. The D214N mutant binds to open and closed syntaxin but has an impaired binding to the assembled SNARE complex (Graham et al., 2009). This mutation slows the fusion kinetics and increases the quantal size of the vesicles. In *C. elegans* this mutant reduces the ethanol excitatory (at low concentration) and sedatory (at high concentration) effect on locomotion suggesting that ethanol may act at the fusion stage (Graham et al., 2009).Munc 18 binding to the syntaxin N-terminus is essential for neurotransmitter release since Munc 18 mutation (F113R) that inhibits open syntaxin binding did not rescue neurotransmitter release from Munc 18 null mutants (Johnson et al., 2009).

Munc 18 binding to open syntaxin mediates vesicle priming since mutations in Munc 18 that inhibited its binding to open syntaxin disrupted synaptic vesicle priming (Deak et al., 2009). Synaptic vesicle docking might also be mediated by Munc 18 and again involves Munc 18 binding to syntaxin (Okamoto and Sudhof, 1997). Munc 18 binding to syntaxin in both its open and closed conformations might be two

aspects of the same function where Munc 18 prevents inappropriate SNARE complex formation and regulates SNARE complex formation to be in the right location and right time for regulated vesicle fusion (Burkhardt et al., 2008). This regulation involves Munc 18 mediated docking and subsequent SNARE motif interaction and vesicle tethering (Burkhardt et al., 2008). Munc 18 may also regulate the readily releasable pool size and synaptic plasticity since an increase in the number of docked vesicles as well as an increase in the total number of vesicles was observed when Munc 18 was overexpressed (Weimer et al., 2003; Toonen et al., 2006; Toonen and Verhage, 2007).

1.7.4.4.1 Munc 18 phosphorylation

Munc 18 is serine phosphorylated at S306 in vitro and S313 (Figure 1.7B) both in vitro and in vivo by PKC (Fujita et al., 1996; de Vries et al., 2000; Barclay et al., 2003). PKC phosphorylation of Munc 18 in vivo is stimulation-dependent, where Ca^{2+} influx triggers this phosphorylation (de Vries et al., 2000). Munc 18 phosphorylation by PKC decreases its interaction with syntaxin and enhances secretion in nerve terminals (de Vries et al., 2000). Munc 18 phosphorylation by PKC results in decreasing the release time and increases catecholamine release from adrenal chromaffin cells (Barclay et al., 2003). Munc 18 phosphorylation by PKC also potentiates vesicle pool replacement after depleting stimulation (Nili et al., 2006). Munc 18 is also threonine phosphorylated by CDK5 in vitro at T574 (Fletcher et al., 1999), however CDK5 inhibitors had no effect on Munc 18 phosphorylation in vivo suggesting T574 phosphorylation by CDk5 does not occur in vivo since crystal structure shows that T574 is not accessible (de Vries et al., 2000). In contrast PKC

inhibitors blocked Munc 18 phosphorylation suggesting that PKC is the major Munc 18 kinase (de Vries et al., 2000).

1.7.4.4.2 Munc 18 tyrosine phosphorylation

Munc 18c (the nonneuronal form of Munc 18) is tyrosine phosphorylated in pancreatic β cells on four residues in the basal condition: Y66, Y103, Y218 and Y219 (Figure 1.7 C) (Oh and Thurmond, 2006). After glucose induced stimulation, only Y219 had an increased phosphorylation status, suggesting that it is the primary glucose stimulation dependent phosphorylation site (Oh and Thurmond, 2006). Tyrosine phosphorylation resulted in a decreased binding affinity of Munc 18c to syntaxin 4, suggesting a regulatory role for tyrosine phosphorylation in Munc 18 function in a similar way to serine phosphorylation (Oh and Thurmond, 2006). In fact, Y219 is not accessible to phosphorylation when Munc 18c is bound to syntaxin 4 in its closed conformation but it becomes available when syntaxin 4 is in an open conformation (Jewell et al., 2008). Munc 18 tyrosine phosphorylation results in an increased affinity for Doc2β and a decreased affinity for syntaxin 4 which results in facilitating SNARE assembly and promotes exocytosis (Oh and Thurmond, 2006; Jewell et al., 2008).

1.7.4.5 NSF

N-ethylmaleimide sensitive factor (NSF) is a member of AAA family of ATPase proteins that uses ATP to alter the comformation of a protein (May et al., 2001; Dalal et al., 2004; Zhao et al., 2007; Malsam et al., 2008). NSF has three domains; an N domain which is required for αSNAP/SNARE binding, a D1 domain with ATPase

activity and a D2 domain that is involved in oligomerization (May et al., 2001; Morgan and Burgoyne, 2004). NSF utilizes ATP to disassemble the SNARE complex and thus allows SNARE protein sorting and recycling (Matsushita et al., 2005; Zhao et al., 2007). NSF requires the adaptor protein αSNAP (soluble NSF attachment protein) to bind to the SNARE complex. In fact NSF does not bind to αSNAP when αSNAP is not engaged in binding with the SNARE complex or when NSF is not oligomerized or not bound to ATP (Hanson et al., 1997; Malsam et al., 2008). NSF ATPase activity is very low when it is alone but it is maximized when NSF is engaged with αSNAP/SNARE complex (Barnard et al., 1997; Haynes et al., 1998; Zhao et al., 2007). NSF also binds to a variety of proteins other than SNARE complex including receptor proteins such as GABA and glutamate receptors and cytosolic proteins such as Rab proteins (Nishimune et al., 1998; Hanley et al., 2002; Morgan and Burgoyne, 2004).

1.7.4.5.1 NSF phosphorylation

NSF is phosphorylated by PKC both in vivo and in vitro with S237 (Figure 1.8 A) in the catalytic D1 domain being the main phosphorylation site (Matveeva et al., 2001). Phosphorylation of NSF by PKC is Ca^{2+}-dependent and decreases its association with the αSNAP/SNARE complex which suppresses its action in SNARE disassembly during exocytosis (Matveeva et al., 2001). NSF is also phosphorylated by pictaire 1 (a CDK related kinase that is phosphorylated and activated by CDK5) at S569 in the D2 domain which decreases its ability for oligomerization, suggesting a regulatory role of S569 phosphorylation on NSF function (Liu et al., 2006).

1.7.4.5.2 NSF tyrosine phosphorylation

NSF is tyrosine phosphorylated by the tyrosine kinase Fes at Y83 (Figure 1.8 A) which results in increased ATPase activity and decreased binding to αSNAP, suggesting a regulatory role for tyrosine phosphorylation in NSF function instead of constitutive activation (Huynh et al., 2004). Furthermore, NSF dephosphorylation by either PTP-MEG2 or PTP1B results in NSF activation and SNARE complex disassembly (Huynh et al., 2004; Zarelli et al., 2009). Inhibition of NSF dephosphorylation or overexpression of aY83E phosphomimetic mutatnt resulted in accumulation of dead-end *cis* SNARE complexes and exocytosis inhibition (Huynh et al., 2004). PTP1B also dephophorylates NSF in sperm and this dephosphorylation results in NSF activation in disassembling SNARE complexes (Zarelli et al., 2009). Tyrosine phosphorylated NSF was unable to dissociate *cis* SNARE complex and also inhibition of PTP1B resulted in inhibition of sperm exocytosis that could be rescued by using nonphosphorylated NSF (Zarelli et al., 2009). This suggests that NSF function is negatively regulated by tyrosine phosphorylation rather than being constitutively active (Zarelli et al., 2009). Unlike neurons, SNARE proteins are assembled in *cis* complexes in sperm under resting condition and activation will result in their disassembly to be available for exocytosis (Zarelli et al., 2009).

1.7.4.6 Dynamin

As membrane fusion between synaptic vesicles and the plasma membrane requires SNARE proteins, membrane fission of the recycled synaptic vesicle requires dynamin (Smillie and Cousin, 2005). The importance of dynamin in endocytosis was first discovered when a temperature sensitive mutant of dynamin *(Shibire)* was

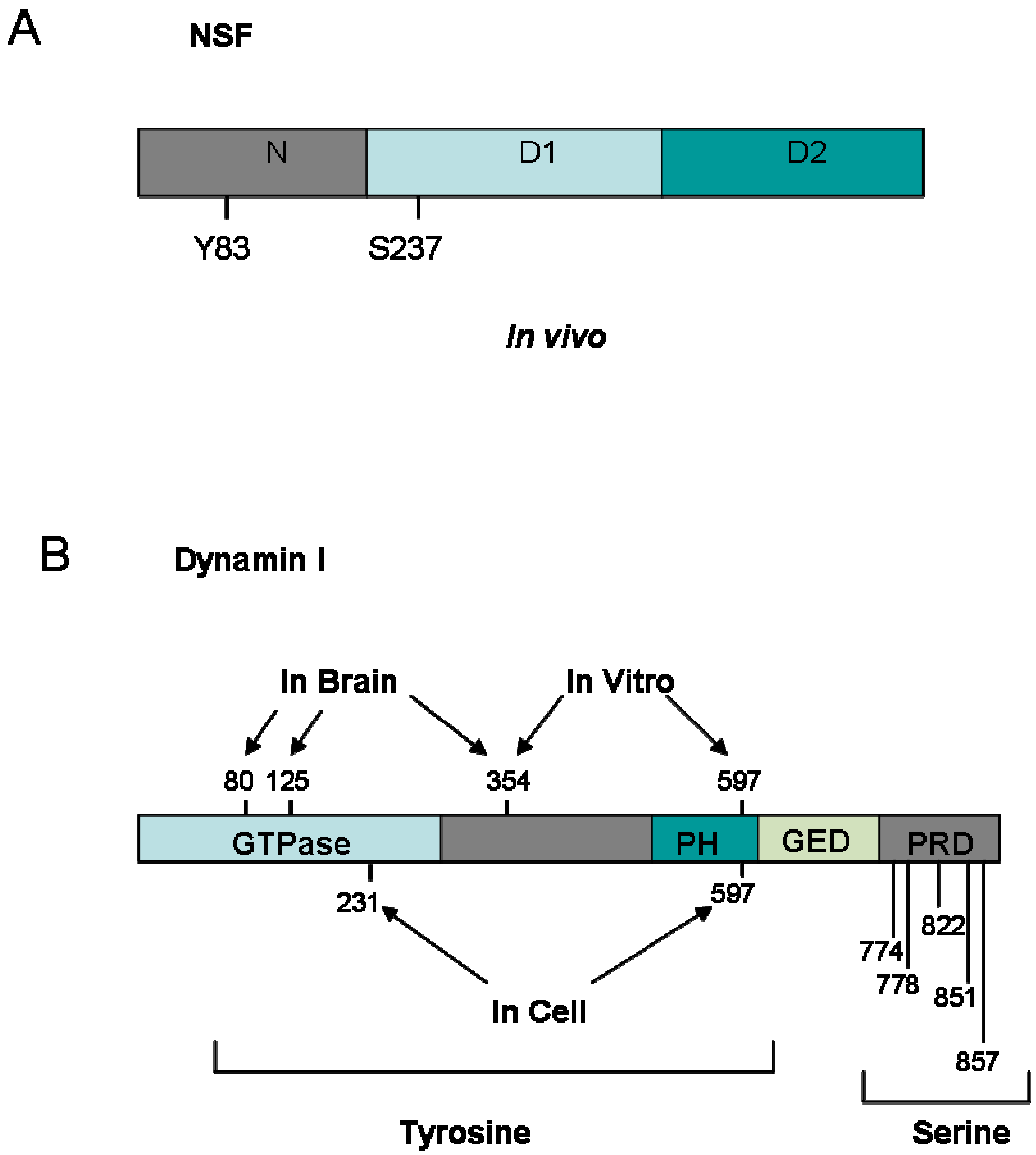


Figure 1.8- ***Src substrates and their phosphorylation sites (c)***

A) NSF structure and phosphorylation sites. Illustration of NSF showing both serine and tyrosine phosphorylation sites (N = N-terminal domain required for SNAP/SNARE binding, D1 and D2 = ATPase domains).

B) Dynamin structure and phosphorylation sites. Illustration of dynamin structure showing the five functional domains of dynamin and both serine and tyrosine phosphorylation sites. Different tyrosine residues that are phosphorylated in vitro, in non neuronal cells and in the whole brain are shown. Serine phosphorylation sites in the PRD were also shown.

shown to cause a paralysis in *Drosophila* (Grigliatti et al., 1973; Poodry and Edgar, 1979). There are three isoforms of dynamin: dynamin I is a brain specific isoform that is enriched in the pre-synaptic region, dynamin II is expressed in all tissues, and dynamin III is expressed in testis and brain, mainly in the postsynaptic region (Rappoport, 2008). Dynamin is a 96 kDa protein (Figure 1.8 B) with a large GTPase domain (Praefcke and McMahon, 2004; Peplowska and Ungermann, 2005). GTPase activity can be stimulated by oligomerization (tetramers) of the protein that is mediated by the interaction between the GTPase domain, and the GTPase effector domain (GED) (Praefcke and McMahon, 2004; Smillie and Cousin, 2005). In addition to these domains, dynamin has a pleckstrin homology (PH) domain (Praefcke and McMahon, 2004) between the middle domain and the GED, and a proline rich (PRD) domain at its C- terminus (Praefcke and McMahon, 2004). The PRD plays an important role in protein-protein interactions and is also a site of splicing (Cao et al., 1998; Smillie and Cousin, 2005).

Dynamin binds to negatively charged lipids, especially inositol phospholipids, with low affinity through its PH domain, but this binding becomes stronger after dynamin recruitment and oligomerization which controls dynamin action in membrane fission and GTPase activity (Takei et al., 2005; Konopka et al., 2006). Dynamin targeting to its site of action is mediated by its PRD binding to proteins containing SH3 domains such as amphiphysin and endophillin (Schmid et al., 1998; Praefcke and McMahon, 2004). Dynamin is also associated with clathrin independent endocytosis such as caveolae-mediated endocytosis and RhoA dependent endocytosis. However, some endocytotic pathways are dynamin independent, such as fluid-phase uptake at the non-permissive temperature for Hela cells expressing the temperature sensitive

dynamin mutant (G273D) (Damke et al., 1995), and also *Drosophila* hemocytes expressing the temperature sensitive dynamin mutant (Guha et al., 2003). Even though there were several proposed models for dynamin mechanism in vesicle fission, the accepted model is that dynamin acts in a mechanochemical way to squeeze the vesicle's neck until the vesicle pinches off (Praefcke and McMahon, 2004; Bonanomi et al., 2006), whereas a new theory suggests that squeeze alone is not enough to cause fission. Therefore squeeze and pull are needed to create a membrane tension that causes membrane breakdown and vesicle fission (Roux et al., 2006).

1.7.4.6.1 Dynamin PRD

The dynamin I PRD contains several PxxP sequences that are capable of binding to SH3 domain containing proteins. Among these proteins is amphiphysin 1, which is a 125 kDa protein, essential for directing dynamin to coated pits by simultaneously binding to dynamin and the clathrin adaptor AP2 (Grabs et al., 1997; Solomaha et al., 2005; Anggono and Robinson, 2007). Amphiphysin is another dephosphin that is dephosphorylated by calcineurin upon stimulation and its dephosphorylation is essential for its function since its phosphorylation at S376 and S285 by CDk5 reduces its binding to AP2 and lipid membranes and affects its recruitment to the coated pit (Liang et al., 2007; Craft et al., 2008). Endophillin, which is a 40 kDa protein that mediates lipid curvature and vesicle formation, is another protein that interacts with dynamin PRD via its SH3 domain (Solomaha et al., 2005; Anggono and Robinson, 2007; Graham et al., 2007). Both Amphiphysin and endophillin contain an N-BAR domain that binds to curved membranes and this might be

important for recruiting dynamin to the clathrin coated pit (Brett and Traub, 2006). Syndapin, which is a 52 kDa protein, is also a binding partner for the PRD via its SH3 domain in a phosphorylation dependent manner (Anggono and Robinson, 2007; Graham et al., 2007). Syndapin also binds to proteins involved in cytoskeletal assembly such as N-WASP where actin polymerization plays a role in linking endocytotic machinery with the cytoskeleton (Kessels and Qualmann, 2004).

There are many other SH3 containing proteins that are known to bind dynamin PRD including cortactin, profillin, myosin 1E, Grb2, phospholipase Cγ (PLCγ) and Abp1 (Orth and McNiven, 2003; Solomaha et al., 2005; Zhu et al., 2005; Konopka et al., 2006; Krendel et al., 2007; Sandvig et al., 2008). Intersectin, which binds to several other proteins through its other domains, SPIN90, which has an SH3 domain and three PRD, and Nck, which is a scaffolding protein, are also binding partners of dynamin (Orth and McNiven, 2003; Kim et al., 2005; Solomaha et al., 2005; Konopka et al., 2006).

1.7.4.6.2 Dynamin phosphorylation

Dynamin is one of the dephosphins which is dephosphorylated upon nerve terminal stimulation by calcineurin. Dynamin dephosphorylation results in relocation from the cytosol to plasma membrane, which is an important regulator for its function (Smillie and Cousin, 2005; Ryan, 2006; Graham et al., 2007). Dynamin I is phosphorylated *in vivo* on seven serine residues, two of which are found in the middle domain (S347 close to GTPase domain and S512 close to PH domain) and the other five sites found in the PRD (Figure 1.8 B) (S774, S778, S822, S851, and S857) with S774 and S778 being the major phosphorylation sites (Graham et al., 2007). CDK5 is the major

dynamin kinase (Tan et al., 2003), however, minibrain kinase (MNB) also phosphorylates dynamin at S857 in a stimulation independent manner, which negatively regulates amphiphysin binding (Huang et al., 2004). This phosphorylation also enhanced Grb2 binding, suggesting that it might regulate dynamin assembly to different protein clusters or locations (Huang et al., 2004). S857 phosphorylation represents about 12 % of the total dynamin phosphorylation and thus it might have a minor role in dynamin regulation (Graham et al., 2007). Dynamin is also phosphorylated by PKC at S795 (Powell et al., 2000); however, phosphorylation of this site does not occur in vivo (Graham et al., 2007). Dynamin phosphorylation at S774 and S778 regulate its function and inhibit syndapin binding (Tan et al., 2003; Anggono et al., 2006). Both S774 and S778 phosphorylation individually regulate dynamin's interaction with syndapin suggesting a cooperative role for their phosphorylation on the dynamin and syndapin interaction (Anggono et al., 2006). Dynamin dephosphorylation by calcineurin is required for activity-dependent bulk endocytosis (ADBE) but not for clathrin mediated endocytosis (CME) (Clayton et al., 2009). The phosphorylation dependent dynamin interaction with syndapin is also essential for ADBE but not CME (Clayton et al., 2009). Furthermore expressing a peptide that encodes the binding region for syndapin (dynamin I 769-784) greatly interfered with ADBE but not CME supporting the suggestion that dynamin interaction with syndapin is required only for ADBE, which also supported by the fact that syndapin silencing using shRNA resulted in the same effect on ADBE (Clayton et al., 2009). Thus even though dynamin is required for both types of endocytosis, dynamin dephosphorylation and syndapin binding are only required for ADBE (Andersson et al., 2008; Clayton et al., 2009).

1.7.4.6.3 Dynamin tyrosine phosphorylation

C-src interacts with both the PRD of dynamin 1 and dynamin 2 (Solomaha et al., 2005). This is a selective interaction since the SH3 domain of N1-src does not bind (Onofri et al., 1997; Foster-Barber and Bishop, 1998). C-src is suggested to have several binding sites (PxxP motifs 2, 6, and 8) on both dynamin I and dynamin 2 PRD (Solomaha et al., 2005). C-src phosphorylates dynamin 1 and 2 at Tyr597 in the PH domain, which results in an induction of it's self-assembly and an increase in its GTPase activity (Ahn et al., 1999; Ahn et al., 2002). This suggests src may activate dynamin supported by the fact that in non neuronal tissues, C-src-mediated tyrosine phosphorylation of dynamin is required for its function in G protein-coupled clathrin mediated endocytosis of β_2-adrenergic and M1 muscarinic acetylcholine receptors (Ahn et al., 1999; Ahn et al., 2002). In addition, dynamin, C-src, and Cb1 co-ordinate in formation of a signalling complex that affects osteoclast migration and adhesion suggesting a role of C-src in modulating dynamin function (Bruzzaniti et al., 2005).

Dynamin I is phosphorylated at two sites (Figure 1.8 C) by src in non-neuronal cells. The first site is at Y231 in the GTPase domain which might be important in regulating its GTPase activity, and the second is at Y597 in the PH domain which might play a role in dynamin interaction with lipids (Ahn et al., 1999; Ahn et al., 2002). When transfected into non-neuronal cells, dynamin I tyrosine phosphorylation regulates its self assembly and ligand induced endocytosis as well as β_2 adrenergic receptor internalization, supporting the suggestion that dynamin tyrosine phosphorylation modulates its functions in non neuronal cells (Ahn et al., 1999; Ahn et al., 2002). Dynamin II is activated by tyrosine phosphorylation by C-src in

endothelial cells and increases its association with caveolin I suggesting that dynamin II phosphorylation by C-src is essential for its function in caveolae endocytosis (Shajahan et al., 2004). Dynamin I is phosphorylated in the brain at three sites (Figure 1.8 C) Y80 and Y125 in the GTPase domain and Y354 in the middle domain (Ballif et al., 2008). These sites are different from the sites mapped previously, which suggests different function of tyrosine phosphorylation in neurons from that in the non-neuronal cells.

1.8 Aims and hypothesis

The src splice variants N1- and N2-src are neuron specific where as C-src is expressed ubiquitously. These splice variants are differentially spliced at their SH3 domains, therefore the hypothesis is that this splicing allows them to have different binding partners and perform different roles in neurons. The aim of this project is to identify new interactions for the three src splice variants in neurons and their possible functional roles.

Chapter 2

Materials and Methods

2. Materials and methods

2.1 Materials

2.1.1 Chemicals

Chemicals are listed below according to the manufacturers in alphabetical order.

ABcam, Cambridge, UK

Primary antibodies

ABgene, Epsom, UK

PCR tubes.

Alphalaboratories, Hampshire, UK

SDS-PAGE gel loading tips (1-200µl).

Amersham Biosciences Ltd, Bucks, UK

Glutathione beads, G25 spin columns, pGEX-T vectors.

AMS Biotechnology, Abingdon, UK

Primary antibodies (VAMP).

BD Biosciences, Erembodegem, Belgium

Quick clone rat brain cDNA library, Yeast Extract.

Biolabs (NEB), Hertfordshire,UK

Restriction enzymes (BglII, BbvCI).

Bioline, London, UK

Hyperladder I markers (200-10000 b).

Bio-Rad, Hertfordshire, UK

Transfer membrane (trans-blot) 0.45 µM and 0.2 µM. Precision plus protein markers (unstained and Kaleidoscope).

Calbiochem, San Diego,CA.USA

Dioxan-free IPTG (isopropyl-beta-D-thiogalactopyranoside).

Cogenic, Essex, UK

Sequencing.

Corning Incorporated, NY,USA

Spin-X centrifuge tube filter.

Fermentas, York, UK

Restriction enzymes (BamHI, SalI, BstXI).

Genscript Corporation, Piscataeay, NJ, USA

Glutathione beads.

Greiner bio-one, Stonehouse, UK

Filter tips, Easy loading tips, Falcon tubes (15 ml and 50 ml), Eppendorfs (1.5 ml).

Invitrogen, Strathclyde, UK

TOPO PCR4 vector, Top10 bacteria, Agar, SYBR® safe.

Merck KGaA, Darmstadt, Germany

Tryptone.

MWG biotech, London, UK.

Primers.

Novogen, Berkshire, UK

pET-28a (+).

Pechiney Plastic Packaging, Chicago, USA

Pechiney Parafilm M.

Pierce, Rockford, IL, USA

ECL reagent, Colloidal Coomassie blue G-250 simplyblue safestain.

Promega Corporation UK, Southampton, UK

Taq DNA polymerase, pfu DNA polymerase, DNA ligase, pGEM-T Easy, restriction enzymes (BamHI, SalI, BstXI, DpnI).

Qiagen Ltd, Surrey, UK

QIA quick extraction kit, Mini prep kit, pQE-30, Ni-NTA beads.

Roche Diagnostics Ltd, East Sussex, UK

Expand DNA polymerase, Complete Protease inhibitor cocktail tablets **(**4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin, and EDTA (sodium salt)).

SantaCruz Technologies, inc. Heidelberg, Germany

Primary antibodies

Sigma-Aldrich Chemicals Company, Dorset, UK

N2_Src kinase (human), IPTG, crystal violet, ampicillin, Dialysis tubing, Developer, Fixer, Kodak biomax film, leupeptin, PMSF (phenylmethanesulphonylfluoride), protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin), thrombin, all other basic chemicals.

Stratagene, La Jolla, CA, USA

Pfu turbo DNA polymerase, BL21 bacteria.

Swan Morton, Sheffield, UK

Surgical blades.

Synaptic Systems, Goettingen, Germany

Primary antibodies

VWR, Lutterworth, UK

Latex gloves.

2.1.2 Growth media and solutions

All growth solutions were sterilized in the autoclave (20 min at 120 C°).

LB media

10 g NaCl, 10 g tryptone and 5 g yeast extract in a total volume of 1 litre of dH_2O. The pH was adjusted to 7 using HCl.

Super media 1

Super media 1 was prepared by dissolving 25g yeast extract, 15g of tryptone, and 5g NaCl in one litre of dH_2O.

Super Media 2

Super media 2 was prepared by dissolving 10g yeast extract, 15g tryptone, and 10g NaCl in one litre of dH_2O.

Polyacrylamide separating gels

Polyacrylamide gels were prepared as shown (v/v) in the table below

	7.5%	**10%**	**12.5%**	**15%**	**16%**	**Stacking**
30% bis-Acrylamide	25%	33.4%	41.6%	50%	53.3%%	15 %
dH2O	50%	40.2%	31.8%	10%	0.667%	60 %
0.5 M Tris pH 6.8	-	-	-	-	-	25%
1.5 M Tris	25%	25%	25%	25%	-	-
2.5 M Tris	-	-	-	-	40%	-
Glycerol	-	-	-	15%	-	-
10% SDS	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%
APS	0.04%	0.04%	0.04%	0.04%	0.04%	0.04%
TEMED	0.08%	0.08%	0.08%	0.08%	0.08%	0.08%

2.1.3Antibodies

The following chart summarizes all the primary antibodies that are used.

	Antibody	**Species**	**Dilution**	**Manufacturer**
1	Amphiphysin	mouse	1:1000	Abcam
2	Amphiphysin	Goat	1:1000	Santa Cruz Biotechnology Inc
3	Dynamin	goat	1:1000	Santa Cruz Biotechnology Inc
4	His	mouse	1:1000	Sigma
5	Endophillin	goat	1:1000	Santa Cruz Biotechnology Inc
6	Munc18	mouse	1:3000	BD Biosciences
7	NSF	rabbit	1:1000	Synaptic systems
8	P-Dynamin 774	sheep	1:1000	Abcam
9	P-Dynamin 778	sheep	1:1000	Abcam
10	PY20	mouse	1:1000	Sigma
11	RGS-His	mouse	1:1000	Qiagen
12	SRC	mouse	1:100	Abcam
13	SNAP25	mouse	1:1000	Sigma
14	Syntaxin	mouse	1:1000	Sigma
15	Synapsin	mouse	1:1000	Synaptic systems
16	Synaptojanin	goat	1:500	Santa Cruz Biotechnology Inc
17	Synaptojanin	rabbit	1:2000	Synaptic systems
18	Synaptophysin	rabbit	1:1000	Synaptic systems
19	Syndapin	mouse	1:2000	BD Biosciences
20	VAMP	rabbit	1:1000	Ams Biotechnology
21	GST	goat	1:2000	Amersham

The following chart summarizes the secondary antibodies that were used

	Antibody	**Species**	**Dilution**	**Manufacturer**
1	Anti-Goat / Sheep	Mouse	1:10000	Sigma
2	Anti-Mouse (IgM)	Goat	1:10000	Sigma
3	Anti- Rabbit (IgM)	Goat	1:10000	Sigma

2.1.4 Vectors

The following chart summarizes the vectors (Figure 2.1) that were used in this project

	Vector	**Use**	**Manufacturer**
1	pCR®4Blunt-TOPO	Blunt ligation	Invitrogen
2	pGEM-T Easy	T-A ligation	Promega
3	pGEX 4T-1	GST tag	Amersham Biosciences
4	pQE-30	His tag	QIAGEN
5	pET-28a(+)	His tag	Novogen

TOPO vector and pGEM vector were used to amplify DNA, while pGEX was used to express GST tagged proteins, pQE30 and pET-28a were used to express the HIS tagged proteins.

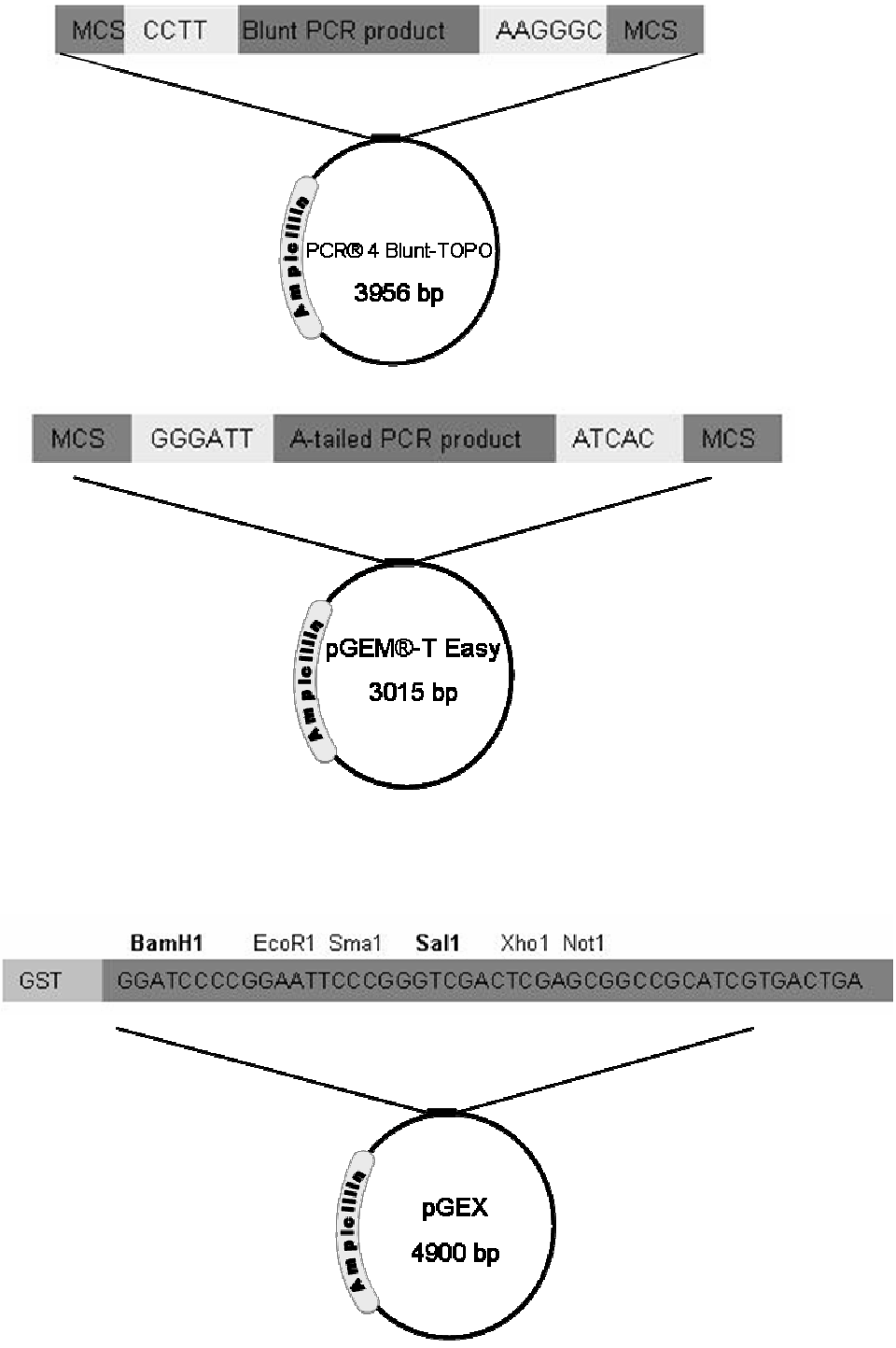


Figure 2.1 *Structure maps for TOPO, pGEM and pGEX vectors*
Maps for TOPO vector (top), pGEM vector (middle) and pGEX vector (bottom) showing the antibiotic resistance and the multiple cloning sites of the vectors (MCS = multicloning site). All vectors have ampicillin resistance.

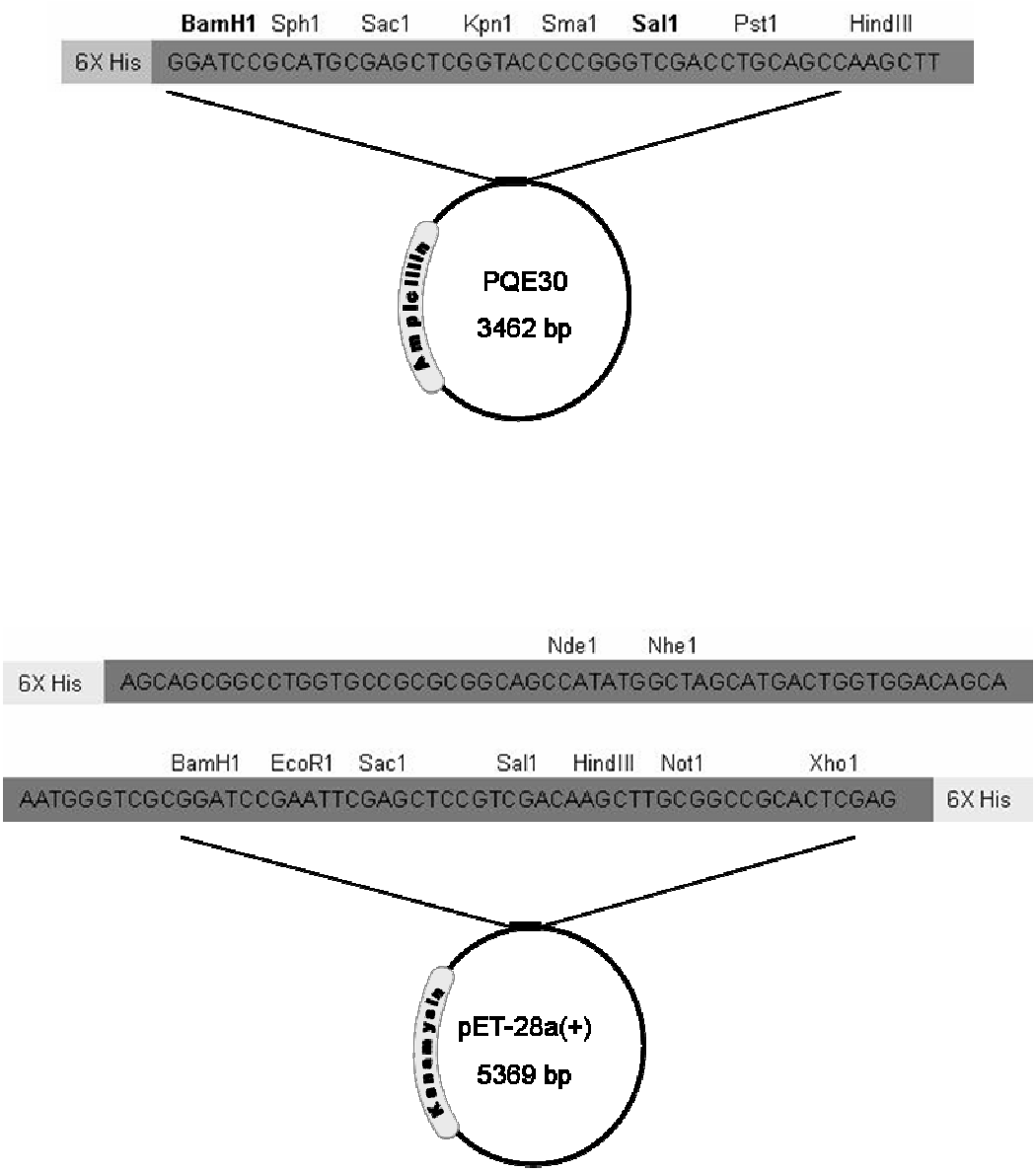


Figure 2.2 Structure maps for pQE30 and pET-28a(+) vectors
Maps for pQE30 vector (top), and pET-28a(+) (Bottom) showing the antibiotic resistance and the multiple cloning sites of the vectors. pQE30 has ampicillin resistance whereas pET-28a(+) has Kanamycin resistance.

2.1.5 Buffers

Buffers that were used are shown below

Lysis buffer: 1% triton X-100, 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1mM EGTA, 1 mM EDTA, 20 µg/ml leupeptin, 1 mM PMSF, complete protease inhibitor tablet.

PBS 10X buffer pH 7.3: 1.37 M NaCl, 40 mM KCl, 200 mM Na_2HPO_4, adjust pH using $NaH_2PO_4.H_2O$.

Transfer buffer: 25 mM Tris, 192 mM Glycine, 20 % methanol.

TBS 10X buffer: 12.15 mM Tris, 1.5 M NaCl.

Upper electrode buffer pH 8.3: 250 mM Tris, 200 mM Glycine, 1 % SDS.

Lower electrode buffer pH 8.3 : 54 mM Tris

Stop solution (3X laemmli buffer): 40 ml of 0.5 M Tris pH 6.8, 28 ml Glycerol, 228 mg EGTA, 6 g SDS, 70 mg bromophenol blue, 600 µl B-Mercaptoethanol, 19 ml dH_2O.

Coomassie stain: 0.15 % (w/v) Coomassie Blue G-250, 20 % (v/v) methanol, and 10% (v/v) acetic acid.

SDS PAGE destain: 40 % Methanol, 10 % Acetic Acid Glacial, 50 % dH_2O

Ponceaus stain: 0.5 % ponceau S stain in 1 % acetic acid

TFB1 buffer: 30 mM CH_3CO_2K, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl, 15 % v/v Glycerol, pH 5.8**.**

TFB2 buffer: 10mM MOPS, 75 mM CaCl2, 10 mM RbCl, 15 % v/v Glycerol, pH 6.5.

TBE buffer: 89 mM Tris, 89 mM Boric Acid, 2 mM EDTA.

Binding buffer A: 20 mM HEPES, 100 mM NaCl, 1 mM EDTA, pH7.4

Binding buffer B: 20 mM Tris, 100 mM NaCl, 1 mM EDTA, pH7.4

Glutathione elution buffer: 10 mM reduced glutathione, 50 mM Tris, pH 8

Bradford reagent: 0.02 g Brilliant blue G, 10 ml Methanol, 2 ml Orthophosphoric acid in a total volume of 200 ml (filter sterile before use)

Breaking buffer: 100 mM HEPES, 500 mM KCl, 2 mM β-mercaptoethanol, pH 7

Buffer A: 20 mM HEPES, 200 mM KCl, 50 mM imidazole, 2 mM β-mercaptoethanol, 10 % v/v glycerol, 1 % v/v triton X 100, pH 7.

STE buffer

10 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA.

His tag elution buffer: 20 mM HEPES, 200 mM KCl, 300 mM imidazole, 2 mM β-mercaptoethanol, 10 % v/v glycerol, 1 % v/v triton X 100, pH 7

Kinase reaction buffer: 100 mM Tris, 25 mM $MgCl_2.6H_2O$, 2 mM EGTA, 2mM DTT, 250 µM Na-VO_4, pH 7.2, after pH adjusted add 5 mM $MnCl_2$.

Kinase storing buffer: 50 mM Tris, 1 mM NaCl, 0.05 mM EDTA, 1mM DDT, 10 % glycerol, 1 mg/ml BSA, 0.05 % NP-40, pH 7.5.

ECL solution 1: For 100 ml, 100 mM Tris, 45 mg of luminal dissolved in 1 ml DMSO, 440 µl of (37 mg of p-Coumaric acid dissolved in 2.5 ml DMSO)

ECL solution 2: For 100 ml, 100 mM Tris, 60 µl H_2O_2

2.1.6 Primers and primer design

All primers were produced using the same protocol (figure 2.2). Usually primers had 6 random bases in the beginning, followed by 6 bases for the restriction enzyme, and then complementary bases to adhere the primer to the template of the required DNA sequence (27-30 bases). The antisense primers for the src SH3 domains also have a

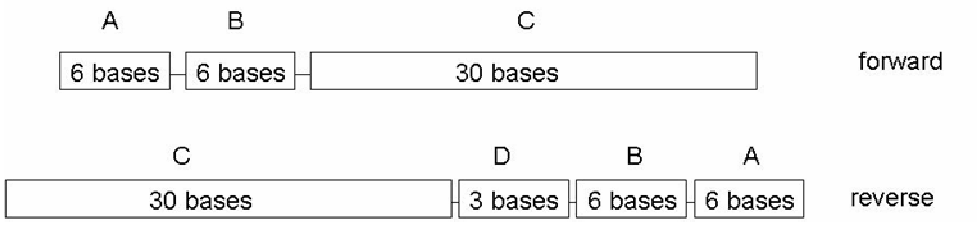


Figure 2.3 Primer design
The main structure of primers that were used to clone DNA from the rat brain cDNA library or from a vector. A: random bases to protect the restriction site, B: the restriction site for the required restriction enzyme, C: bases of the start or the end of the required DNA, D: a stop codon for the reverse primer.

stop codon between the DNA sequence and the restriction site. DNA encoding full length srcs do not need this insertion, since they already have a stop codon in their DNA sequences. For site directed mutagenesis, primers were designed identical to the template sequence except for mutagenic substitutions.

All primers that are used for DNA cloning are summarized in the following table

	Protein expressed	**Direction**	**Sequence**	**Restriction**
1	Src SH3 domains	antisense	CAC AGC GTC GAC TCA CTC CTC AGC CTG GAT GGA GTC GAA	Sal I
2	Src SH3 domains	sense	CCG CGT GGA TCC GGT GGG GTG ACT ACC TTT GTG GCC	BamHI
3	Full length src	antisense	CCG CGT GTC GAC CTA TAG GTT CTC CCC GGG CTG GTA CTG CGG	Sal I
4	Full length src	sense	CCG CGT GGA TCC ATG GGC AGC AAC AAG AGC AAG CCC AAG GAC	BamHI
5	Full length src repair primer	antisense	CCC AAA *GTC GGC* CAC TTT GCA CAC CAG GTT CTC GCC AAC	-
6	Full length src repair primer	sense	GTT GGC *GAG AAC* CTG GTG TGC AAA GTG GCC GAC TTT GGG	-
7	SDM to induce BglII restriction site	antisense	CCC TTT CTT GAA GGA CAG ATC TGT CTC TGT CCG TGA CTC	BglII
8	SDM to induce BglII restriction site	sense	GAG TCA CGG ACA GAG ACA GAT CTG TCC TTC AAG AAA GGG	BglII
9	Δ80 src	antisense	CCG CGT GTC GAC CTA TAG GTT CTC CCC GGG CTG GTA CTG CGG	SalI
10	Δ80 src	sense	CCG CGT GGA TCC GGT GGG GTG ACT ACC TTT GTG GCC	BamHI
11	Full length Synaptophysin	antisense	GTC GAC TTA CAT CTG ATT GGA GAA GGA GGT G	SalI
12	Full length Synaptophysin	sense	GGA TCC ATG GAC GTG GTG AAT CAG CTG GTG G	BamHI
13	SDM for Dynamin PRD S822A	antisense	GCC GAA AGG GTC AGG *GGC* AGC CCC CGG CCT GGA	-
14	SDM for Dynamin PRD S822A	sense	TCC AGG CCG GGG GCT *GCC* CCT GAC CCT TTC GGC	-

15	SDM for Dynamin PRD S822E	antisense	GCC GAA AGG GTC AGG *CTC* AGC CCC CGG CCT GGA	-
16	SDM for Dynamin PRD S822E	sense	TCC AGG CCG GGG GCT *GAG* CCT GAC CCT TTC GGC	-

2.1.7 Protein expression plasmids

All proteins that are used in this research were expressed in BL21 bacteria using either the pGEX-T, pET28a or pQE30 vector. The following table summarizes all the proteins that were expressed and used in this project

	Name	**Amino acids encoded**	**Species**	**Vector**	**From**
1	GST C src SH3 domain	81-142	rat	pGEX	This study
2	His C src SH3 domain	81-142	rat	pQE30	This study
3	GST N1 src SH3 domain	81-148	rat	pGEX	This study
4	His N1 src SH3 domain	81-148	rat	pQE30	This study
5	GST N2 src SH3 domain	81-159	rat	pGEX	This study
6	His N2 src SH3 domain	81-159	rat	pQE30	This study
7	His Δ80 C src	81-533	rat	pQE30	This study
8	His Δ80 N1 src	81-539	rat	pQE30	This study
9	His Δ80 N2 src	81-550	rat	pQE30	This study
10	His Full length C src	1-533	rat	pQE30	This study
11	His Full length N1 src	1-539	rat	pQE30	This study
12	His Full length N2 src	1-550	rat	pQE30	This study
13	GST Dynamin PRD xa	751-864	rat	pGEX	P. Robinson
14	GST Dynamin PRD xb	751-851	rat	pGEX	P. Robinson

15	GST Dynamin PRD xa 2C (P786A)	751-864	rat	pGEX	P. Robinson
16	GST Dynamin PRD xa 3B (P793A)	751-864	rat	pGEX	P. Robinson
17	GST Dynamin PRD xa 9A (R838E)	751-864	rat	pGEX	P. Robinson
18	GST Dynamin PRD xa PB2 (RR772/3EE)	751-864	rat	pGEX	P. Robinson
19	GST Dynamin PRD xa AA (SS774/8AA)	751-864	rat	pGEX	P. Robinson
20	GST Dynamin PRD xa EE (SS774/8EE)	751-864	rat	pGEX	P. Robinson
21	GST Dynamin PRD 3L	751-832	rat	pGEX	P. De Camilli
22	GST Dynamin PRD xa (S822 E)	751-864	rat	pGEX	This study
23	GST Dynamin PRD xa (S822 A)	751-864	rat	pGEX	This study
24	GST Dynamin PRD B6A (S822 E)	751-851	rat	pGEX	This study
25	GST Dynamin PRD B6B (S822 A)	751-851	rat	pGEX	This study
26	GST Syntaxin	1-288	rat	pGEX	A. Morgan
27	GST Amphiphysin SH3 domain	596-683	rat	pGEX	P. De Camilli
28	GST Synaptophysin C terminus	224-308	mouse	pGEX	G. Evans

29	His Synaptophysin C terminus	224-308	mouse	pET28 a	G. Evans
30	GST Synaptophysin C terminus AA (PP232/5AA)	224-308	mouse	pGEX	G. Evans
31	His Synaptophysin C terminus AA (PP232/5AA)	224-308	mouse	pET28 a	G. Evans
32	His Full length Synaptophysin	1-308	mouse	pQE30	This study
33	His Munc 18	1-593	rat	pQE30	A. Morgan
34	GST	1-239		pGEX	Amersham

2.2 Methods

2.2.1 PCR

Polymerase Chain reaction (PCR) was used to clone different SRC SH3 domains, Δ80 src and full length splice variants from a rat brain cDNA library. In addition it was used for site directed mutagenesis (SDM), cloning full length synaptophysin from various templates, and for colony screening.

The following chart summarizes the DNA polymerases that were used in this project

	DNA Polymerase	**Manufacturer**	**Main use**
1	Taq DNA polymerase	Fermentas Promega	Colony screen, rat brain library, A-tailing
2	Go-Taq DNA polymerase	Promega	Colony screen, A-tailing
3	Pfu DNA polymerase	Fermentas Stratagene	Rat brain library, template
4	Pfu turbo DNA polymerase	Stratagene	Rat brain library, template, SDM
5	Pfu Ultra DNA polymerase	Stratagene	SDM
6	Expand DNA polymerase	Roche	Template with A-tailing

All PCRs were performed using a GeneAmp PCR system 9700 PCR machine (Applied Biosystems California, USA). A standared PCR protocol is shown below:

1- First melting time 95 °C for one minute

2- Cycles usually between 28-35 cycles (for site directed mutagenesis 18 cycles were used)

   a) Melting: 95°C for 30 seconds

b) Annealing: 55 °C for 30 seconds

c) Extension: 72°C for 1 min/1 kb for taq and 2 min/1 kb for pfu

3- Extension time: 72 °C for 10 minutes

4- End at 4°C

The total volume of PCR reactions was 50 µl for all types of PCR reaction. PCR tubes were used, since these tubes have thin walls to conduct heat efficiently. The contents of the PCR reaction and their amounts are summarized in the table below.

	Components	**Normal PCR**	**Colony screen**
1	dH_2O	make up volume to 50 µl	make up volume to 50 µl
2	10X buffer	5 µl	5 µl
3	$MgCl_2$ 1M (for Taq only)	3 µl	3 µl
4	dNTPs 100ng/µl	1 µl	1 µl
5	anti-sense primer 100µM	0.5 µl	0.5 µl
6	sense primer 100µM	0.5 µl	0.5 µl
7	rat brain cDNA library or plasmid 1:10 diluted	1-2 µl	One colony from agar plate
8	DNA polymerase 2.5U/µl	1 µl	1 µl

The PCR tubes were kept on ice while the components were added to minimize the enzyme activity and to decrease the possibility of degradation.

Colony screening was performed by adding all the components apart from the templates and then a bacterial colony from a previous bacterial culture on agar plate was picked by a pipet tip emerged into the PCR reaction tube and then recultivitated

on a new agar plate. Dipping the the colony on pipet tip inside the PCR reaction tube provides enough template for the PCR reaction.

In certain cases, when PCR was performed with non-taq polymerases such as pfu, an A-tailing reaction was performed. A- tailing is a PCR reaction using Taq polymerase with only dATP and the PCR product (no primers). A-tailing adds A bases to the end of the PCR product which is essential for ligating the PCR product into the pGEM T easy vector (pGEM vector has T bases at both ends). The A-tailing reaction was performed in a total volume of 10.6 µl (PCR product 7µl, 10X buffer 1µl, $MgCl_2$ 0.6µl, Taq polymerase 1µl, 100ng/µl dATP 1µl). Thetube was incubated at 70ºC for 30 minutes.

2.2.2 Ligation

PCR products were separated using either 0.7 % (for PCR products of about 1kb or more) or 2 % (for PCR products of smaller than 1k) agarose gels with either 0.05% Crystal Violet, ethidium bromide, or SYBR® safe, to visualize DNA. Electrophoresis was run at 180V for 20 min. The bands were cut with a scalpel on a white light box for Crystal violet or on an ultra violet light box for either the SYBR® safe or ethidium bromide stained gels. The product was purified using a QIA quick extraction kit (Protocol followed according to the manufacturer's instructions). The purified PCR product was then ligated into a TOPO vector using blunt-end ligation (4µl of purified PCR product, 1µl of 1M NaCl solution, 1µl of TOPO vector, and 4 µl dH_2O and incubation for 20 minutes at room temperature). TA ligation was used for pGEM T easy vector, where the PCR product has to be A-tailed (using a Taq polymerase) and then ligated to pGEM T easy vector (3µl of A-tailed PCR product,

5µl of 2X ligase buffer, 1µl of pGEM vector, and 1µl of ligase enzyme, incubated at room temperature for 1 hour or at 4 °C overnight).

2.2.3 Agarose gel production

DNA agarose gel was prepared by mixing 0.7 % (w/v) agarose powder with 1X TBE and heating until the agarose was desolved (the percentage of the agarose was varied according to the size of the DNA). The agarose was cooled before adding 0.5 µg/ml ethidium promide or SYBR® safe, poured into moulds and allowed to set at room temperature.

2.2.4 Bacterial transformation

Ligated vectors were transformed into bacteria using the following protocol. Competent bacteria (100 µl) were thawed on ice (bacteria are stored at -70 °C) then either 2 µl of ligation product or 1 µl of mini prep was added, and incubated on ice for 30 minutes to allow binding of DNA to the cells. The cells were then heat shocked for 45 seconds at 42 °C to allow entry of DNA, and incubated on ice for a further 2 minutes. Bacteria were then added to 400 µl of LB media, incubated for 60 minutes at 37 °C/ 200 rpm, and cultured on agar plate containing the appropriate antibiotic (100µg/ml ampicillin, 50µg/ml kanamycin or both).

2.2.5 Restriction enzyme digestion

Restriction enzymes digests were used for the following reasons

1- Cutting a ligated vector to insure the insertion of the correct product before sequencing.

2- Transferring a DNA insert from one vector to another (such as from pGEM to pQE30 or from TOPO to pGEX).

The restriction digestion reaction usually takes one hour at the temperature of the enzyme's optimal activity (usually at 37 °C). If the two enzymes have different temperatures for optimal activities, the reaction will be incubated for one hour at the lower temperature and then another hour at the higher temperature. When a double digest was performed, combatable buffers were used according to the manufacturer's recommendations.

The table below summarizes the amounts of the components of the three digestion reactions. The test digestions were used to confirm that the conditions used are optimal on a small proportion of DNA before proceeding to digest the whole sample, reducing the loss of DNA samples.

	Single digest test	**Double digest test**	**Double digest**
dH2O	Up to 20 µl	Up to 30 µl	Up to 60 µl
10 X buffer	2 µl	3 µl	6 µl
BSA (1mg/ml)	2 µl	3 µl	6 µl
Enzyme 1	1 µl	1 µl	2 µl
Enzyme 2	-	1 µl	2 µl
DNA	2 µl	3 µl	40 µl

2.2.6 Mini prep

A mini prep is used to extract plasmid DNA from bacterial overnight cultures. To prepare the bacterial culture, 5 ml LB media with the appropriate antibiotic in a 30

ml culture tube was seeded with a single colony from transformed bacteria (or a scrape from a glycerol stock). The seeded 5 ml LB media was then incubated at 37 °C, 200 rpm overnight. The overnight culture was spun down and the supernatant discarded. The bacteria were then minipreped according to the manufacturer's instructions (QIAgen) and the DNA plasmid eluted in a total volume of 50 µl dH_2O. The plasmid concentration was estimated by running 5 µl of the mini prep on an agarose gel with SYBR® safe, and either visualized using ultraviolet light using known concentration on the marker, or by estimation of its optical density.

2.2.7 Glycerol stocks

One ml of the overnight culture was taken and mixed with one ml of 50% v/v glycerol and dH_2O in a 2 ml tube. The tube was then labelled and stored at -70 C° to be used when needed. This mixture will allow the bacteria to freeze slowly which will not cause any damage to the bacteria and it stays active for several months. When needed, a scrape with the tip of a pipette tip is enough to seed an overnight culture.

2.2.8 Preparation of competent cells

A scrape of competent cells was taken using a sterile pipette tip, and added to 5ml sterile LB media (For competent cells already containing a chaperone, a bacterial colony grown on agar plate was added to 5 ml LB media with the appropriate antibiotic). The LB media was then incubated overnight at 37 °C, 200 rpm. 1 ml from this was then added to 100 ml of sterile LB media (for competent cells containing a chaperone, the appropriate antibiotic was added to this mix). Filter

sterile $MgSO_4$ was added to a final concentration of 20 mM and incubated at 37 °C, 200 rpm until the optical density was between 0.4 and 0.6 (measured using Biowave CO800 cell density meter (WPA, Cambridge UK)) at 600 nm. The bacterial culture was then poured in a sterile 250 ml container and centrifuged at 4648 g for 5 minutes at 4 °C. The supernatant was discarded and the pellet resuspended in 40 ml of ice cold filter sterile TFB1 buffer and transferred to 50 ml falcon tube. The bacteria were centrifuged at 4648 g for 5 minutes at 4 °C. The supernatant was discarded and the pellet resuspended in 4 ml ice cold filter sterile TFB2 buffer and incubated on ice for 1 hour. The bacteria were then aliquoted into 100 ml aliquots in 1.5 ml eppendorf tubes and snap frozen by immersing them in a dry ice and isopropanol bath. The bacterial aliquots was then stored at -70 °C until use.

2.2.9 Preparation of agar plates

Agar stock was prepared by adding 1.5% agar to LB media in a total volume of either 250 ml or 500 ml followed by autoclaving and storage at room temperature until use. Agar stock was heated in a microwave for approximately 10 minutes until the agar was melted. After cooling, antibiotic was added (ampicillin 1:1000 of 100 mg/ml or kanamycin 1:1000 of 50 mg/ml or both) in front of a Bunsen burner. The agar was then poured into plates (in front of Bunsen burner) until the bottom of the plate was covered. The Bunsen flame was moved over the agar surface to sterilize the surface and to remove any air bubbles. The plates were left to cool to allow the agar to solidify, and then the plates were labelled, sealed with Parafilm and stored at 4 °C until use.

2.3 Protein Biochemistry

2.3.1 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were prepared using a mini-protean III kit from Bio-Rad (Hertfordshire, UK). The percentage of gel poured was dependent on the molecular weight of the proteins analyzed. When proteins with low molecular weight (10 to 40 kDa) were analysed, a 16 % SDS-PAGE gel was used while proteins with a mass of about 100 kDa were run on a 10 % SDS-PAGE gel allowing good separation. Recipes for stacking and separating buffers are displayed in section (2.1.2). Gels were run in a Tris/Glycine/SDS running buffer (upper electrode buffer) as defined in section (2.1.6) at 160 V for 60 minutes or until the front dye reached the bottom of the gel. For detection, protein gels were stained with Coomassie blue stain for 30 minutes (section 2.1.6). Gels were destained using a MeOH/acetic acid destaining solution overnight with shaking (section 2.1.6).

2.3.2 SDS-PAGE Large Gradient gel

Large gradient gels were used to separate proteins for mass spectrometry analysis. The gradient gel was made using 15% and 7.5% polyacrylamide stock solutions (section 2.1.2). A gradient former (Bio-Rad) was used to pour the gel. Gels were run on a Protein II system (BioRad). The gel apparatus was assembled and the upper and lower electrodes were filled with their respective buffer. The gel was run overnight at 100 Volts with cold water circulating in the surrounding water jacket. The gel was handled with extreme care minimizing keratin contamination. To ensure this, a face mask and nitrile gloves were used, all the equipment and instruments were cleaned with methanol and a new blade was used for each band. The gel was washed 3 times

with distilled water for 5 minutes to remove SDS and buffer salts from the gel (which interfere with dye binding to the proteins), and then stained using Colloidal Coomassie blue G-250 simplyblue safestain (Pierce) for one hour. The gel was destained with distilled water for one hour, and the bands of interest cutout with a new scalpel blade on top of a light box and stored in new eppendorf tubes. The bands were then sent for identification using MALDI-tof MS spectrometry in the laboratory of Prof. Phillip Robinson (CMRI, Sydney).

2.3.3 Western Blotting

For Western Blotting, SDS-PAGE was performed as described in section 2.3.1. After removal of the gel from the Protein III system, the gel was equilibrated in transfer buffer (section 2.1.5). The nitrocellulose membrane was soaked in water and transfer buffer for 10 minutes each. Transfer was performed in a mini trans blot cell (BioRad). The apparatus was assembled according to the manufacturer's manual (the gel and the nitrocellulose membrane were surrounded by whatman 3 MM paper and fiber pads and then assembled in a holder cassette and then placed in the transfer tank with an ice pack to prevent overheating). Transfer was typically performed for 2 hours at 100 volts on ice or alternatively overnight at 30 Volts. After transfer, membrane was stained with Ponceaus stain (section 2.1.5) to assess the transfer efficiency, and then destained with water. The membrane was incubated for 30 minutes in blocking buffer (PBS, 5% low fat milk powder, 0.5% PVP-40) to stop non-specific binding. The membrane was then incubated for 1-2 hours in primary antibody (diluted as in 2.1.3. in PBS + 0.5% PVP-40). The membrane was then washed 3 times with PBS for 5 minutes, followed by incubation for 30-60 minutes in

the secondary antibody (diluted as in 2.1.3 in PBS + 0.5% PVP-40). The membrane was then washed 3 times with PBS for 10 minutes and then incubated in ECL-substrate solution for 1-2 minutes. The membrane was placed between two filter papers to remove excess ECL, covered with cling film, and secured in an X-ray cassette. The membrane was exposed to X-Ray film in the dark room for an adequate time to allow bands to be visualized. The film was then incubated in developer (Sigma) for about 30 seconds, washed with water, incubated in fixer (Sigma) for about 30 seconds and washed with water again.

2.3.4 Expression of recombinant proteins

To express large quantities of recombinant proteins, a culture tube containing 5 ml LB media was seeded with a small scrape of bacteria from a glycerol stock. The appropriate antibiotic was used, and the culture was incubated at 37 ºC 200 rpm over night. The next day the 5 ml culture was seeded into one litre of super media (super media 1 is used usually unless otherwise is mentioned) in a 2.5 L flask containing the appropriate antibiotic (100 mg/L for ampicillin, and 50mg/L for kanamycin). The one liter culture was incubated at 37 ºC 200 rpm until the optical density was between 0.6 and 1.2 (usually after 2.5 – 3 hours). IPTG was then added to a final concentration of 1 mM to induce protein synthesis, and the culture was incubated again for 3 hours at 37 ºC 200 rpm or overnight at 16 ºC 200 rpm (dependent on which conditions gave higher protein expression). The culture was then centrifuged at 4648 g, the supernatant was discarded and the pellet was resuspended in a 50 ml falcon tube in a total volume of 40 ml of either STE buffer (section 2.1.5) for GST-tagged proteins or breaking buffer (section 2.1.5) for His-tagged proteins. The tube

was then centrifuged at 4648 g, the supernatant was discarded and the dry pellet stored at -70 ºC until use.

2.3.5 Preparation of GST and His tagged fusion proteins

The bacterial pellet was thawed on ice and resuspended in a total volume of 40 ml STE buffer for GST-tagged proteins and 40 ml of buffer A for His-tagged proteins. After resuspension, PMSF (a serine protease inhibitor final concentration 1mM) and either a protease inhibitor cocktail tablet or 50 µl of protease inhibitor cocktail solution were added. Lysozyme (final concentration 5.7 µM) and DTT (final concentration 5 µM for GST tagged proteins only) were added and the suspension was mixed and incubated on ice for 30 minutes. During this incubation, Glutathione beads (Amersham or Genscript) for GST-tagged proteins or Ni-NTA agarose beads (Qiagen), for His-tagged proteins, were prepared by washing with PBS for glutathione beads and buffer A for the Ni-NTA beads, and centrifuging at 64 g for 5 minutes. The supernatant was removed by aspiration and washing was repeated 3 times. Beads were then stored at 4 ºC until use. After 30 minutes, Triton X-100 was added to the bacterial suspension to a final concentration of 1 % (Triton X-100 will solubilise the membrane lipids). The suspension was sonicated on ice 6 times at 10 MHz for 30 seconds with 30 seconds interval between the sonications to prevent overheating (sonication will break down the bacteria). The suspension (bacterial lysate) was centrifuged at 17400 g for 30 minutes at 4 ºC. The supernatant was then added to the prepared beads and incubated at 4 ºC for 1-2 hours with rotation. The beads were pelleted by centrifuging at 64 g for 5 minutes, and the supernatant was removed by aspiration. Beads were washed 5 times in 30 ml PBS then once with

PBS + 1.2 M NaCl, and finally the PBS wash was repeated 2 more times (for Ni-NTA beads Buffer A was used instead of PBS). The beads were then resuspended in PBS (buffer A for Ni beads) to make a 50 % slurry. The 50 % slurry was then resuspended in 7 ml PBS (buffer A for Ni beads) and 7 ml of glycerol was added and mixed well. The suspension was then stored at -20 ºC until use.

2.3.6 Eluting GST-tagged and His-tagged proteins from beads

For binding assays, GST-tagged and His-tagged recombinant proteins had to be separated from beads. To perform this, 1 ml of the 50% bead slurry was put in a 1.5 ml eppendorf tube, centrifuged at 28 g at 4 ºC for 5 minutes, after which the supernatant was aspirated. The beads were resuspended in 500 µl glutathione elution buffer (section 2.1.5) for GST-tagged proteins and His-tagged elution buffer for His-tagged proteins. After 2 minutes the beads were centrifuged again and the supernatant removed and collected. This procedure was repeated 4 more times, and each time the supernatant was stored separately. All the supernatants were tested for eluted protein by mixing 10 µl of each elution with 5 µl 3X sample buffer and then 10 µl of this was run on SDS-PAGE gel and then coomassie stained and visualized for their protein contents.

Elution was also performed in Spin-X centrifuge tube filter instead of eppendorf tubes, where the 1 ml of 50% beads slurry was applied into a Spin-X centrifuge tube filter and centrifuged at 870 g at 4 ºC for 5 minutes. The flow through was discarded and the beads were resuspended in 500 ml glutathione elution buffer for GST-tagged proteins and His-tagged elution buffer for His-tagged proteins, kept for 2 minutes and then centrifuged again at 870 g at 4 ºC for 5 minutes. The flow through samples

were collected and the same procedure was repeated again 4 more times and each flow through was stored separately. All the flow through were tested for eluted protein on SDS PAGE gels.

2.4 Protein Assay

Protein concentrations were estimated using a Bradford assay (Bradford, 1976) where 0 – 10 µl of 1 mg/ml BSA (bovine serum albumin) were added into 1 ml of Bradford reagent and 1, 2 and 5 µl of the test protein were also added to into 1 ml of Bradford reagent, mixed and left for 5 min to set. The samples were read on spectrophotometer at 595 nm. The BSA samples were used to generate a concentration curve and draw a line of best fit, which was used to determine the concentration of the test protein. The other way to estimate protein concentration is by running the proteins against different concentrations of BSA on SDS-PAGE. If the protein samples are impure due to breakdown or other proteins such as chaperons are co-purified with the protein samples, a Bradford assay will give missleading results since it will calculate the total protein concentration including the contamination. In this case running the proteins against different concentrations of BSA will produce more acurate results.

2.4.1 Pull down assay using synaptosomal lysate

Pull-down assays involve coupling a protein of interest to beads to serve as an affinity resin. Proteins that bind to this protein can then be isolated from the lysate. Synaptosome lysates (prepared from rat forebrain by colleagues) were used for pull-down assays. Typically, 50 µg of protein on beads was used in a pull down assay.

Beads were applied to pre-washed G25 spin columns, spun at 870 g for 1 minute, and washed with lysis buffer (see 2.1.5) twice. 175 µl of rat brain synaptosomal lysate were applied to the G25 spin columns with beads and then incubated with rotation for 60 minutes at 4 °C. Columns were then placed in 2 ml Eppendorf tubes and spun at 870 g for 1 minute with the flow through being discarded. Beads were then washed with lysis buffer 3 times, lysis buffer with 500 mM NaCl once, lysis buffer twice, and finally Tris buffer (20 mM Tris pH 7.4) to remove any non-specific binding. After washes, samples were eluted from the beads using a 50 µl Laemmli stop solution (see 2.1.5) followed by boiling for 5 minutes and finally centrifugation for 10 minutes at 18890 g. Samples were then analysed by SDS PAGE.

2.4.2 Pull down Assay using bacterial lysate

This assay is very similar to the pull down assay for synaptosomal lysate; it tests the binding affinity of recombinant proteins in bacterial lysate. His-tagged proteins were expressed in BL21 bacteria, and the bacteria were lysed as described previously (section 2.3.4 and 2.3.5) using binding buffer B (section 2.1.5). Equal amounts of GST-tagged proteins on beads in glycerol were added to 15 ml falcon tubes containing 10 ml binding buffer B. The tubes were spun at 64 g for 5 minutes and the supernatant carefully aspirated. The beads were washed 2 more times and then the bacterial lysate containing His-tagged proteins was divided equally between the 15 ml falcon tubes containing the GST-tagged protein beads. Tubes were then incubated for 1-2 hours at 4 ºC with rotation. After the incubation, the tubes were spun at 64 g for 5 minutes and the supernatant aspirated and discarded. The beads with the remaining bacterial lysate were transferred to pre-washed G25 spin columns. The

columns were spun at 870 g in a chilled centrifuge for 1 minute and the flow through discarded. The beads in the columns were washed 3 times with binding buffer B, once with binding buffer B + 500 mM NaCl, and 2 times with binding buffer B. After washes, 40 µl of Laemmli stop solution was added to the column, incubated for 5 minutes, and then spun at 18890 g for 10 minutes collecting the flow through.

2.4.3 Binding assay

This assay was used to compare the affinity of GST-tagged recombinant proteins with His-tagged recombinant proteins. The main difference between this assay and the pull down from bacterial lysate is that the exact amounts of both the GST-tagged and His-tagged proteins are known. GST-tagged proteins 20 µl of 50 % slurry of glutathione beads, and His tagged protein were mixed in 1.5 ml eppendorf tube and binding buffer A was added to make a total volume of 200 µl (the final concentrations of the GST-tagged and the His-tagged proteins were 200 nM). The tubes were incubated at 4 ºC with rotation for 60 min. The tubes were then spun at 870 g for 30 seconds and their contents transferred to pre-washed G25 spin columns and centrifuged at 870 g for 30 seconds. The columns were then washed 3 times with binding buffer A, once with binding buffer A + 500 mM NaCl and twice with binding buffer A. GST tagged proteins (and associated His tagged proteins) were eluted from the beads using 40 µl of Laemmli buffer, incubated for 5 minutes and centrifuged for 10 minutes at 18890 g. The flow through was collected in 1.5 ml eppendorf tubes.

2.4.4 Kinase assay

Kinase assay was performed using commercial N2-src (Sigma) to define the extent of tyrosine phosphorylation of proteins. The kinase reaction was performed in either the presence or absence of substrate and also in the presence or absence of ATP to have two independent negative controls. The ATP solution (final concentration of 250 µM) was added last to start the reaction. The components were warmed to 30 ºC in a water bath before adding the ATP, and the tubes were incubated in the water bath at 30 ºC for one hour. For time course of 0, 10, 30 and 60 min were used. Reactions were stoped by addition of laemmli stop solution. Protein tyrosine phosphorylation was detected using western blot with phosphotyrosine antibody.

The chart below summarises the amounts added from each component of the reaction in different conditions

	No substrate reaction	**No ATP reaction**	**Normal reaction**
Kinase reaction buffer	29 µl	29 µl	19 µl
N2-src kinase	1 µl	1 µl	1 µl
ATP solution	10 µl	-	10 µl
Substrate	-	10 µl	10 µl
total	40 µl	40 µl	40 µl

When the src splice variants that were produced in our lap or from G. Evans were used, 10 µl of src kinases were used in each reaction and the kinase reaction buffer was reduced to 10 µl to have a total volume of 40 µl.

Chapter 3

Producing different constructs of src splice variants

3. Producing different constructs of src splice variants

3.1 Introduction

Producing the tools needed for research experiments is a necessary part of any research, and in this chapter making src proteins for identification of new protein interactions and substrates will be discussed. Recombinant protein over-expression is widely used by scientists to study the structure and function of proteins, to produce antibodies or to prepare proteins for other uses. Protein over-expression is usually performed either in eukaryotic cells such as insect cells (Geisse et al., 1996; Karlsson et al., 2000; Wang et al., 2008), or prokaryotic cells such as *E. coli* bacteria (Schumann and Ferreira, 2004; Hou et al., 2006; Liu et al., 2007). Protein over-expression in *E. coli* is usually preferred for it is easy to perform and regulate, and also because the resulting proteins are easily purified and harvested in good quantities. In this project, all the recombinant proteins were expressed in an *E. coli* bacterial system and purified as described in the methods section.

In this study constructs for src splice variant SH3 domains, full length, and truncated proteins needed to be made. The flow chart in figure 3.1 describes the strategy that was adopted for producing C-, N1-, and N2-src splice variant SH3 domains or full length proteins.

3.2 SH3 domains of src splice variants

All three splice variants of src (C-src, N1-src, and N2-src) share the same structure and the same amino acid composition with the exception of extra amino acids inserted in the SH3 domain of both of the neuronal forms of src (N1-src and

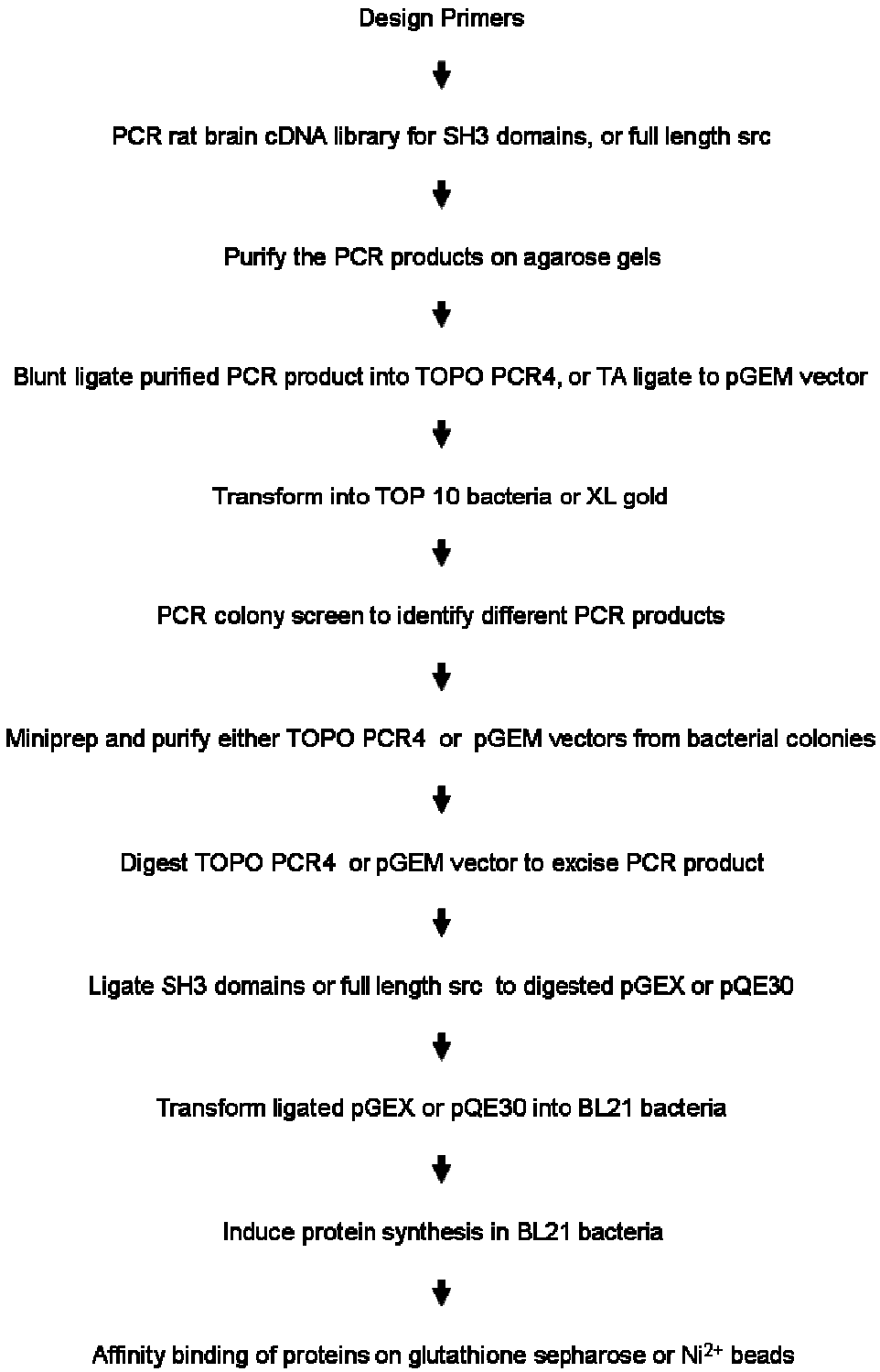


Figure 3.1- ***Strategy for production of src SH3 domains and full length src.***

Flow chart shows the strategy that was adopted for producing GST-tagged or His-tagged src splice variant SH3 domains, or His-tagged full length src from rat brain cDNA library.

N2-src). The C-src SH3 domain has 67 amino acids (81-147), N1-src SH3 domain has 6 extra amino acids inserted after residue T116 of the full length protein (36 of SH3 domain), and N2-src SH3 domain has a further 11 amino acids inserted after residue V122 of full length N1-src as shown in figure 3.2. Since the differences between the three splice variants of src are found only in the SH3 domains, and to reduce the binding interference of the other src domains, the SH3 domains were first selected for expression studies to investigate their binding partners.

3.2.1 Cloning splice variant SH3 domains DNA

To amplify C-, N1-, N2-src splice variant SH3 domains, primers were designed. Since the splice variants vary in the central region, one set of primers that complemented the ends of the C-src SH3 domain is adequate to perform PCR for all the SH3 domains. PCR was performed using Pfu polymerase enzyme and a rat brain cDNA library. All three splice variants were amplified in the same PCR reaction (Figure 3.3 A). The PCR product bands showed different intensities, with N1-src SH3 domain having the highest intensity and C-src SH3 domain the lowest intensity (Figure 3.3 A). In multiple PCR reactions, there was a fourth band repeatedly observed of approximate 300 bases (which we called N3?), which had the same relative abundance as the C-src SH3 band.

3.2.2 Ligation of SH3 domain DNA into TOPO vector

Ligation of PCR products into plasmids is an essential procedure to transform the PCR products into bacteria to express the DNA for purification. TOPO vectors are

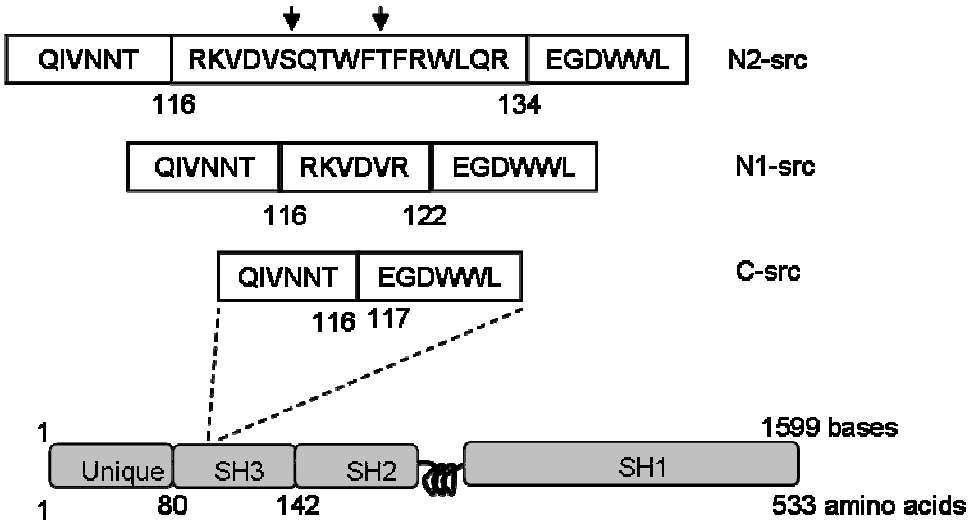


Figure 3.2- ***Splice variants of src.***

Diagram shows the extra amino acids inserted between 167 and 177 of (C-src) in the SH3 domains of N1-src and N2-src compared to C-src.

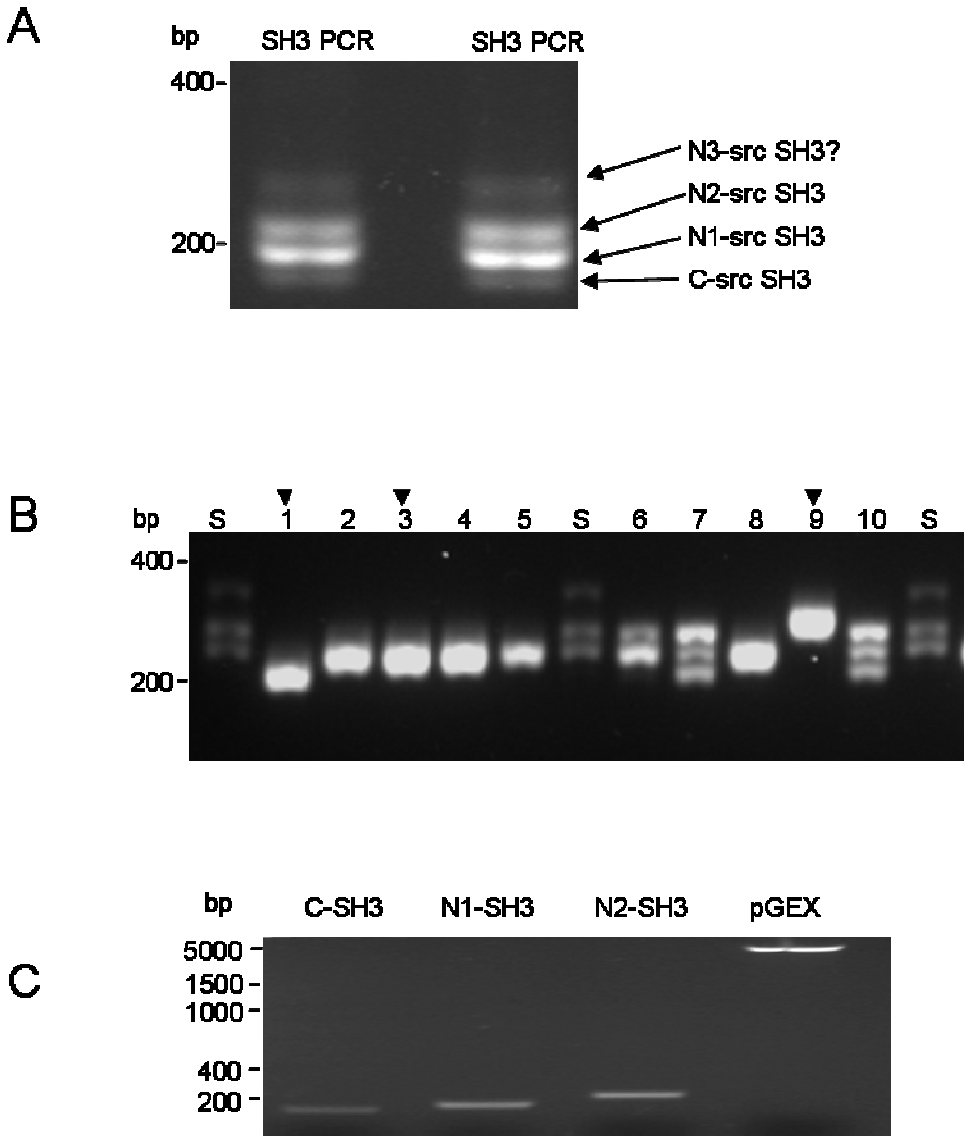


Figure 3.3- ***Production of DNA for SH3 domains of src splice variants.***

(A) Agarose gel of PCR products from rat cDNA library for SH3 domains of src splice variants. There were four PCR products (annotated with arrows C-, N1-, N2-, N3-src).

(B) Agarose gel of PCR products from a colony screen of top10 bacteria containing TOPO vector with SH3 domains of the src splice variants. The letter (S) refers to the standard (original) PCR product from (3.3 A) that was used for ligation. The arrows refer to the colonies that were picked for different SH3 domains. Colony 1 was picked for C-src SH3 domain, Colony 3 was picked for N1-src SH3 domain, and Colony 9 was picked for N2-src SH3 domain.

(C) Agarose gel of gel purified SH3 splice variant DNA, and pGEX vector double digested using BamH1 and Sal1.

one of the vectors that are used for this purpose. All three splice variants were ligated into TOPO vector (Figure 2.1) and then transformed into top 10 bacteria. Colony screening was then performed to determine which vector had which SH3 domain. More than 50% of the screened colonies contained the N1-src SH3 domain while less colonies contained the C-src or N2-src SH3 domains. Some colonies showed multiple bands. Colonies containing only a single band of one of the three src splice variant SH3 domains were re-cultured (marked with arrows in figure 3.3 B). This method allowed us to isolate and purify DNA encoding the three src SH3 domains. None of the colonies had the fourth band which we named N3. In an attempt to isolate it, the fourth band was gel purified and ligated to TOPO vector, but colony screens only showed multiple bands in all selected colonies similar to those in figure 3.3.B. The isolated src SH3 domains were sequenced and were identical to the SH3 domains of rat src.

3.2.3 Insertion of SH3 domain DNA into pGEX vectors

The pGEX-4T-1 vector (see 2.1.4 and also Figure 2.1) is used for GST-tagging of proteins to facilitate protein expression and purification. The vector has an inducible promoter for protein expression and cloning sites for different restriction enzymes, and also ampicillin antibiotic. The three src SH3 domains in TOPO vector were miniprepped (see 2.2.5) from TOP 10 bacteria, and double digested using the restriction enzymes BamH1 and Sal1, the restriction enzyme recognition sequence were introduced into the SH3 domain PCR product sequence when the primers were designed. The pGEX vector was also double digested using the same restriction enzymes and all the four restriction products were gel purified (Figure 3.3 C). The

SH3 domains were ligated into pGEX vector and transformed into TOP 10 bacteria. A colony was selected from each splice variant and used to miniprep the pGEX vector with the SH3 domain insert. The miniprepped DNA was tested for incorporation by double digesting (see 2.2.4) with BamH1 and Sal1 restriction enzymes (Figure 3.4 A).

3.2.4 Protein expression for GST-tagged SH3 domains

GST tagging is used to purify expressed proteins from bacteria, since GST can be bound to glutathione beads and then purified from the bacterial lysate via centrifugation of the beads. The pGEX vectors containing SH3 domains were transformed into BL21 bacteria for protein expression. Expression of GST-tagged src splice variant SH3 domains was then induced by addition of IPTG followed by their purification on glutathione beads. GST-tagged C-src and N1-src SH3 domains had a similar expression whereas the N2-src SH3 domain had a much lower expression compared to them (figure 3.4 B) under the following conditions (500 ml of super media 1 (see 2.1.2) with 3 hours incubation at 37 °C.

3.2.5 Insertion of src SH3 domains into the pQE30 vector

The src SH3 domains were also tagged with His to allow binding assays or pull down assays to be performed in conjunction with GST-tagged proteins. The pQE30 vector (see 2.1.4 and also figure 2.1) adds 6 histidine amino acid residues to either the N terminus or C terminus of a protein, which allows purification of the protein on Ni^{+2} beads. Since His-tagging only adds 10 amino acids (MRGSHHHHHH), it makes

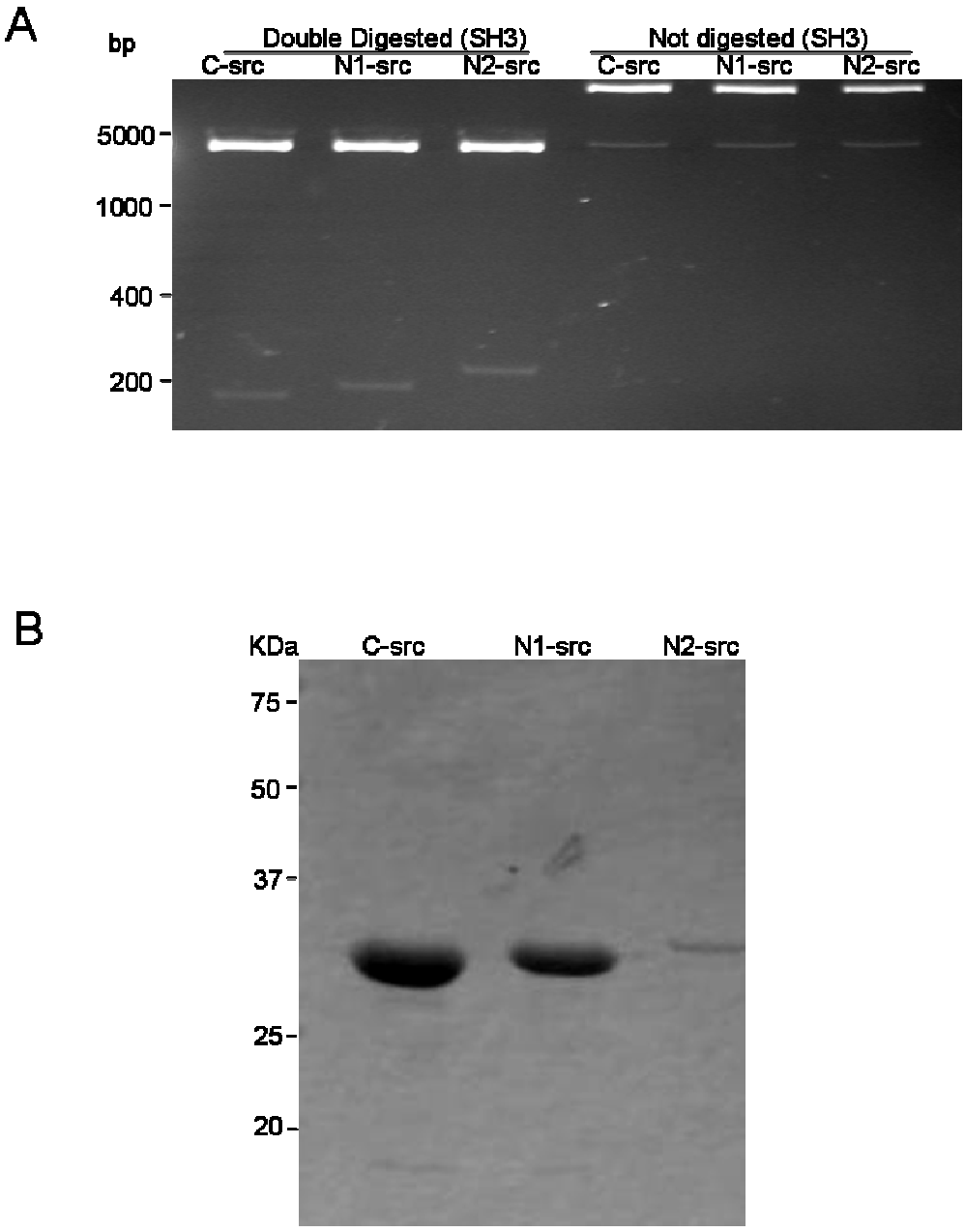


Figure 3.4- ***Expression of GST-SH3 domains of src splice variants.***
(A) Agarose gel of C-src, N1-src, N2-src SH3 domain DNA in pGEX vector miniprepped from Top10 bacteria, vectors were either not digested or double digested using BamH1 and Sal1.
(B) Coomassie stained gel for C-src, N1-src, and N2-src SH3 domain proteins expressed in BL21 bacteria and then purified on glutathione beads.

only a very small addition in the size of the SH3 domains compared to GST (about 220 amino acids). pGEX plasmids containing SH3 domain inserts were double digested using BamH1 and Sal1 restriction enzymes to release the src splice variant SH3 domain DNA (Figure 3.5 A). The SH3 inserts were then ligated to either pGEM or pQE30 vectors and transformed into XL 10 gold bacteria. Both pGEM and pQE30 vectors were miniprepped and tested for the presence of the insert by double digest using BamH1 and Sal1 restriction enzymes (figure 3.5 B). The pQE30 vectors containing the src splice variant SH3 inserts were then transformed into BL21 bacteria for protein expression. His-tagged SH3 domains were then expressed using super media 2 (see 2.1.2) with a 16 °C overnight induction with IPTG and purified on Ni^{+2} beads (figure 3.5 C). N2- src SH3 domain was expressed using double the volume and purified using half the amount of beads. Good expression was observed for all three His-tagged splice variants.

3.3 Full length src splice variants

Investigating protein interactions of isolated domains can produce misleading results since other regions of the protein may either enhance or inhibit binding. For this reason full length src splice variants were cloned so they could be used for further studies.

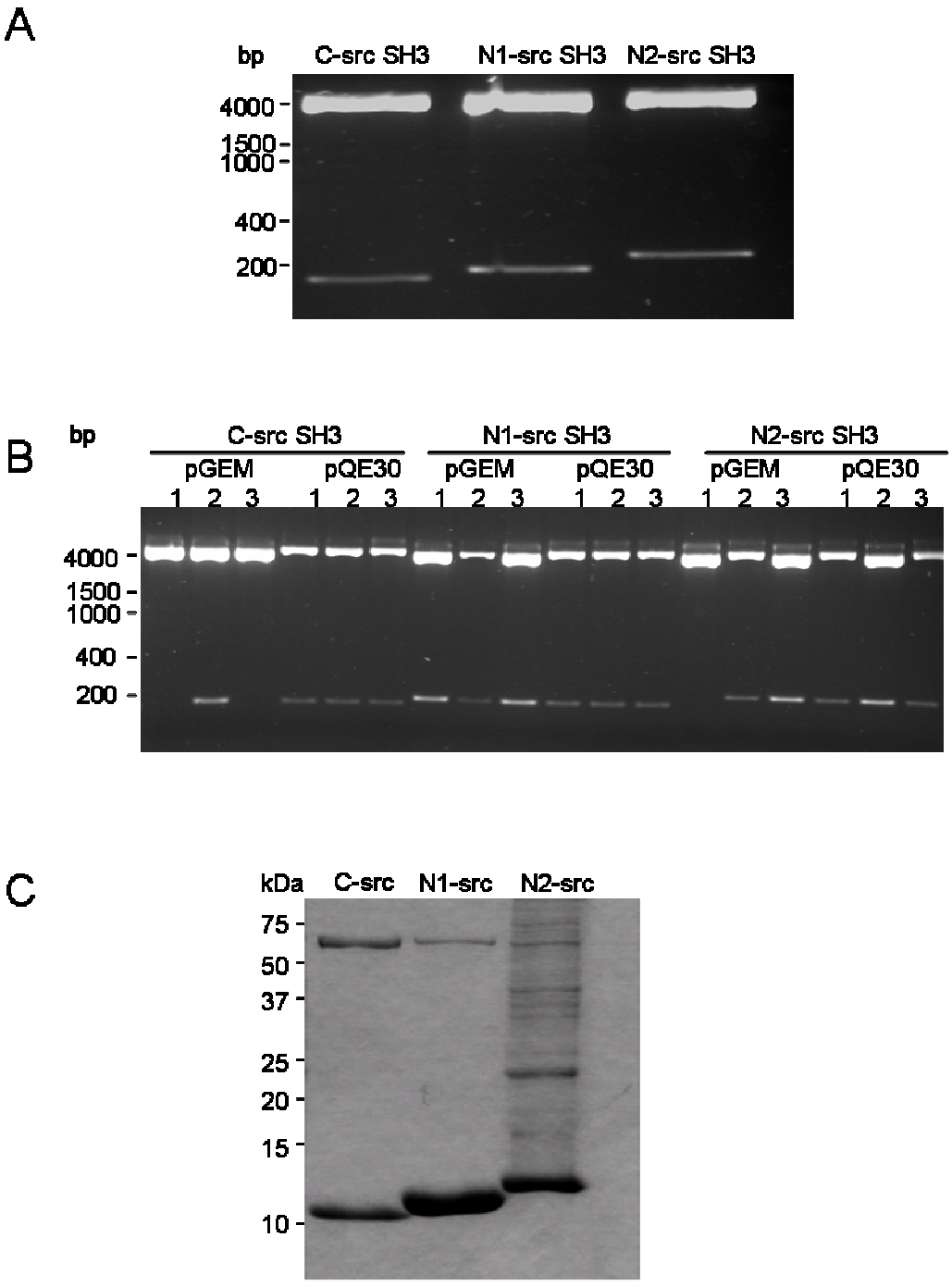


Figure 3.5- ***Production of His-tagged SH3 domains***

(A) Agarose gel of C-src, N1-src, and N2-src DNA for SH3 domains in pGEX vector double digested with BamH1 and Sal1.

(B) Agarose gel of miniprepped DNA from XL 10 gold overnight cultures of C-src, N1-src, and N2-src SH3 domains in pGEM and pQE30, double digested with BamH1 and Sal1. Three colonies for each splice variant were screened.

(C) Coomassie stained gel for His-tagged C-src, N1-src, and N2-src SH3 domains expressed in BL21 bacteria and purified using Ni^{2+} beads.

3.3.1 Cloning full length src splice variant DNA

To amplify full length C-, N1-, N2-src splice variants, the same set of primers were designed for all three splice variants. Cloning the three src splice variants was attempted from rat brain cDNA library. PCR was performed using either Pfu or Taq polymerase enzymes, however, no PCR products were detected. The PCR reaction was performed again with alterations (figure 3.6 A). Doubling the amount of rat brain cDNA library (the substrate) and doubling both the amount of rat brain cDNA library and taq polymerase (the enzyme) in the PCR reaction produced a detectable PCR product. Doubling the amount of rat cDNA library, the Taq enzyme, and reaction buffer did not produce any detectable amount. However, since the src PCR reactions produced a small amount of PCR product, the PCR products from these PCR reactions (PCR1 and PCR2 from figure 3.6 A) were used as a template for another round of PCR using taq polymerase enzyme. This approach gave a good amount of PCR product (figure 3.6 B). A similar approach using PCR products from PCR1 and PCR2 (figure 3.6 A) as templates but using Pfu turbo polymerase enzyme, or PCR2 as a template using expand polymerase enzyme were also performed (figure 3.6 C). In this case only when PCR2 was used as a template with Pfu turbo polymerase was a PCR product observed. It was impossible to determine whether all 3 src splice variants had been amplified due to the small difference in base number compared to the whole DNA product.

3.3.2 Ligation of FL src DNA into the pGEM vector

Once the PCR product (PCR2b in figure 3.6 C) had been generated, it was TA ligated into a pGEM vector (see 2.2.2 and figure 2.1) and then transfected into XL 10

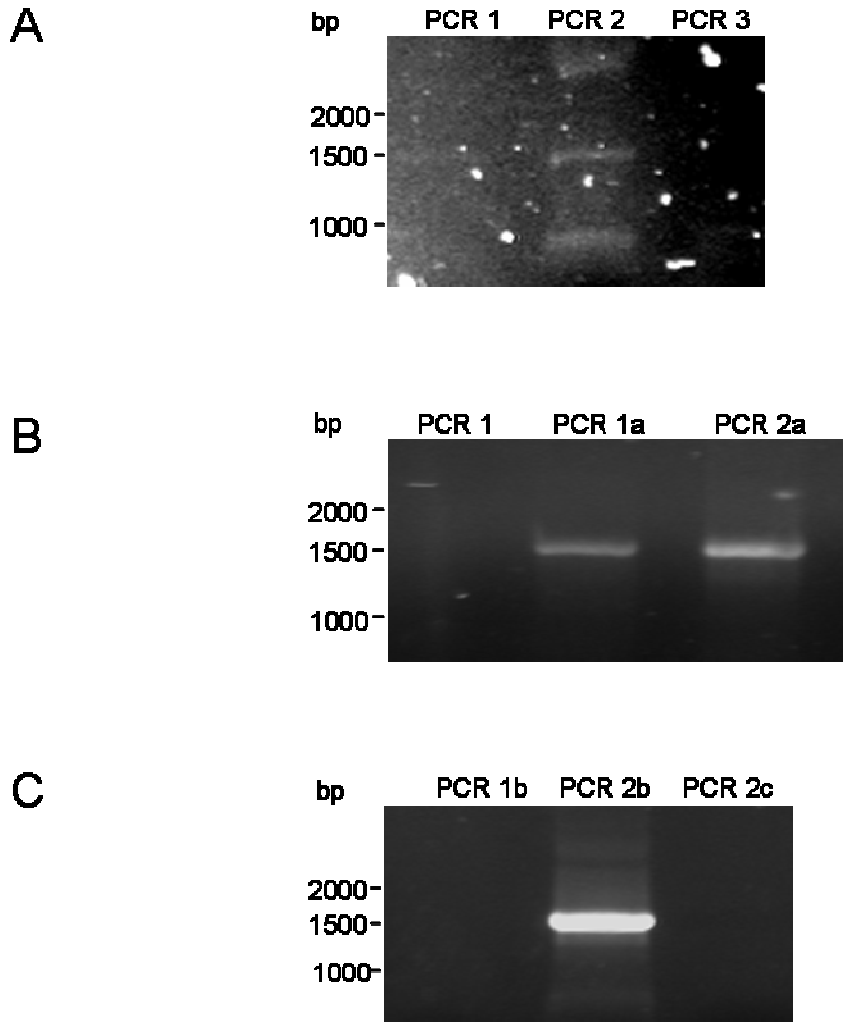


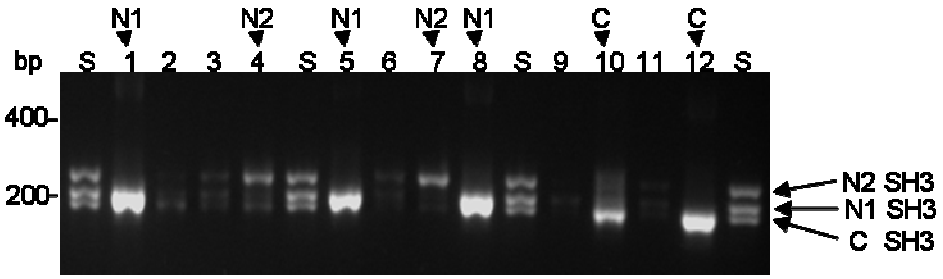
Figure 3.6- ***Full length src DNA production***

(A) Agarose gel of PCR products from rat cDNA library for full length src splice variants. (PCR 1 = the amount of cDNA library and Taq were doubled, PCR 2 = the cDNA library was doubled, PCR 3 = the buffer, the cDNA library, and Taq were doubled). There was only a detectable src band in PCR number 2.

(B) Agarose gel of PCR products for full length src splice variants from rat cDNA library (PCR 1) and from the previous PCR products (PCR 1 and PCR 2 from A corresponds to PCR 1a and PCR 2a respectively) using Taq. PCR from previous PCR products only produced a PCR product.

(C) Agarose gel of PCR products from the previous PCR products (PCR 1 and PCR 2 from A corresponds to PCR 1b and PCR 2b) using Pfu turbo, and PCR products from the previous PCR product (PCR 2 from A corresponds to PCR 2c) using expand. Only when the previous PCR product (PCR 2 from A) was used with pfu turbo a PCR product was produced.

A



B

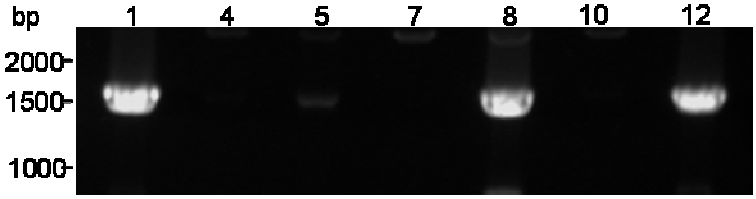


Figure 3.7- ***Full length src DNA testing***

(A) Agarose gel of PCR products from a colony screen of XL 10 Gold bacteria containing full length src splice variant DNA ligated into pGEM vector. Colonies marked with arrows were selected for mini-prep. For N1-src colonies 1, 5, and 8 were selected, for N2-src colonies 4 and 7 were selected, and for C-src colonies 10 and 12 were selected. SH3 primers were used for the PCR and a mix of C, N1, and N2 src SH3 domains was used as a standard (S).

(B) Agarose gel of PCR products of mini-prep testing of the colonies from (A). Only colonies 1 and 8 from N1, and colony 12 from C expressed full length src DNA.

Gold bacteria (see 2.2.3). Colony screening was performed using SH3 domain primers to determine which splice variants were in which colony. Colony screening showed that only 7 out of 12 colonies had a possible src insert (figure 3.7 A). The colonies that were expected to have the C-src insert were 10 and 12, the colonies that were expected to have the N1-src insert were 1, 5, and 8, and the colonies that were expected to have the N2-src insert were 4 and 7. All 7 colonies were miniprepped, and the miniprep was used as a template for a PCR reaction using full length src primers. PCR products were only seen from colonies 1 and 8 (with N1-src insert) and from colony 12 (with C-src insert) (Figure 3.7 B). When the DNA from these colonies were compared to the src splice variant sequences several mutations were found in full length C-src and two point mutations were found in full length N1-src at base-pair 1224 (C to T) and 1228 (T to C).

3.3.3 Producing the full length C-src and N2-src

The presence of multiple mutations in full length C- and N1-src presented a problem, however this could be solved using SH3 domain DNA. A strategy was formed to ligate the SH3 domains of C- and N2-src into the full length N1-src pGEM vector. This would produce full length versions of all 3 src splice variants. The strategy that was formulated to achieve this is described in figure 3.8. First, two restriction enzymes were needed that could cut src SH3 DNA at points flanking the N1-src and N2-src insertions. These enzymes also could not cut the full length src or pGEM vector. After database searching, several enzymes were found that could cut the SH3 domain after the N1-src and N2-src insertions, but no enzymes were found that could cut SH3 domain before the N1-src and N2-src insertions. Another option was to

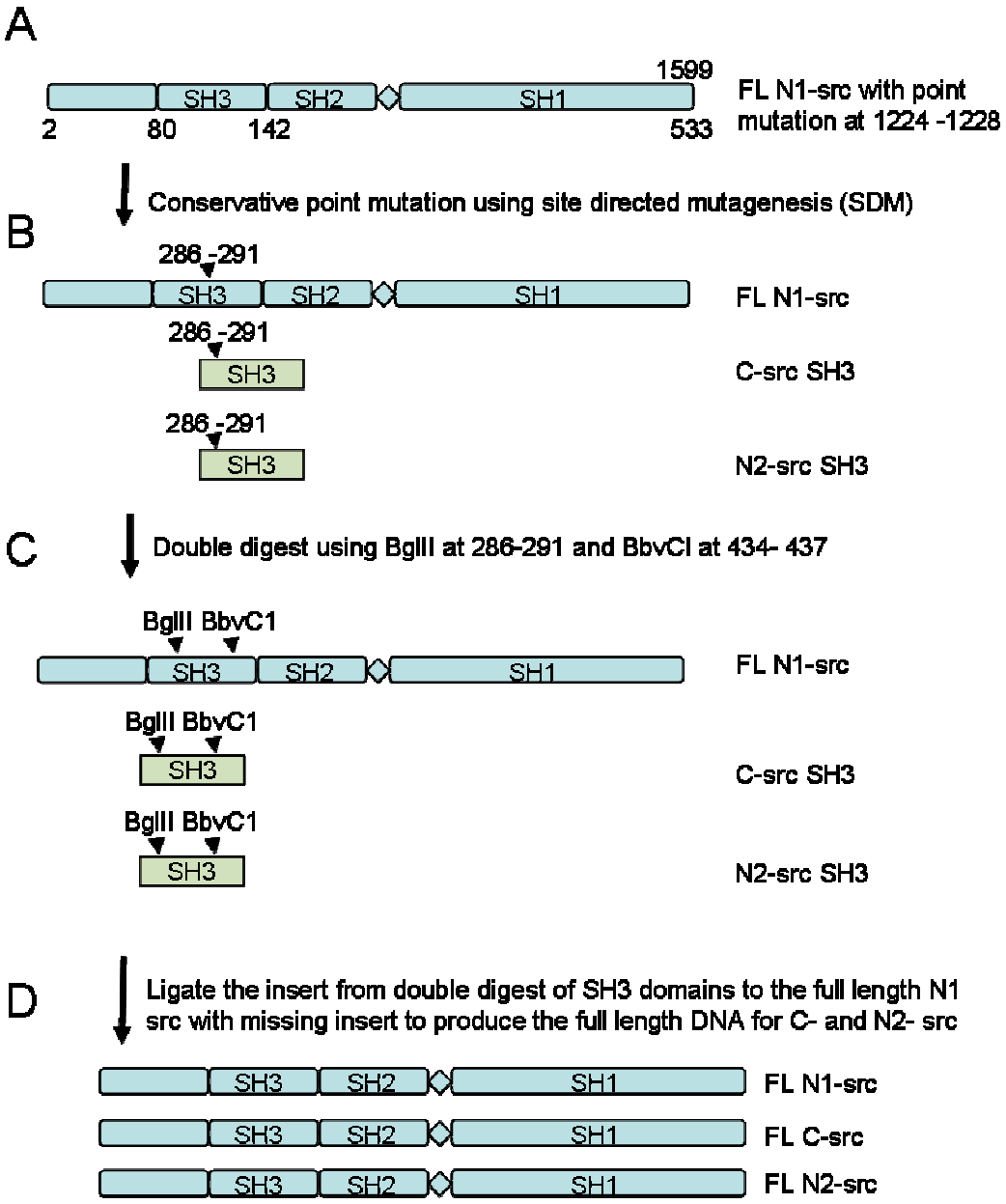


Figure 3.8- ***Strategy to produce full length src splice variants.***

Diagram shows the strategy that was used to produce all three full length src splice variants using point mutated N1-src and the SH3 domains of N2-src and C-src.

A) The full length N1-src with a point mutation at bases 1224 and 1228.

B) Site directed mutagenesis to insert a conservative mutation to create a restriction site for BglII restriction enzyme at bases 286-291.

C) Double digest of full length N1-src and C- and N2-src SH3 domains using BglII (286-291) and BbvCI (434-437).

D) Production of full length C-src and N2-src by replacing the C- and N2-src SH3 insert in full length N1-src.

create a restriction site in the area before N1- and N2-src. This strategy was adopted and the restriction enzyme BglII selected. The sequence from 286-291 (TGACCT) was selected for mutagenesis (AGATCT) (figure 3.8 B). The other enzyme that was selected to cut after the N1-src and N2-src insertion was BbvCI which cuts the sequence (CCTCAGC (432-439)). The cut sequence has about 150 base pairs, which makes it large enough to be separated on a 2 % agarose gel and purified, but also small enough to show differences between the three splice variants (figure 3.8 C). SDM was performed for both C-src and N2-src SH3 domains in pGEX, and also for full length N1-src in pGEM. All SDM PCR products were transformed into XL 10 Gold bacteria (see 2.2.3) and miniprepped. The vectors were then digested using both BglII and BbvCI restriction enzymes (see 2.2.4), and the inserts separated on agarose gels (figure 3.9 A). Both of the inserts from C-src and N2-src SH3 domains were purified and ligated to the purified FL N1-src digested pGEM vector, and then transformed into XL 10 Gold bacteria. A colony screen was performed using SH3 domain primers for 10 colonies from each ligation as shown in figure 3.9 B. Purified C-src, N1-src, and N2-src SH3 domains were used as controls. Most of the colonies contained N1-src which may have been due to the full length N1-src plasmid having only partially digested and resealing without taking the C-src or N2 src inserts. Two colonies from C-src and two colonies from N2-src were selected and were miniprepped (marked with arrows in figure 3.9 B).

3.3.4 Ligation of full length src DNA to pQE30 vector

After generation of the three full length src splice variants DNA, the pGEM vectors were double digested using BamH1 and Sal1 enzymes (these sites were inserted

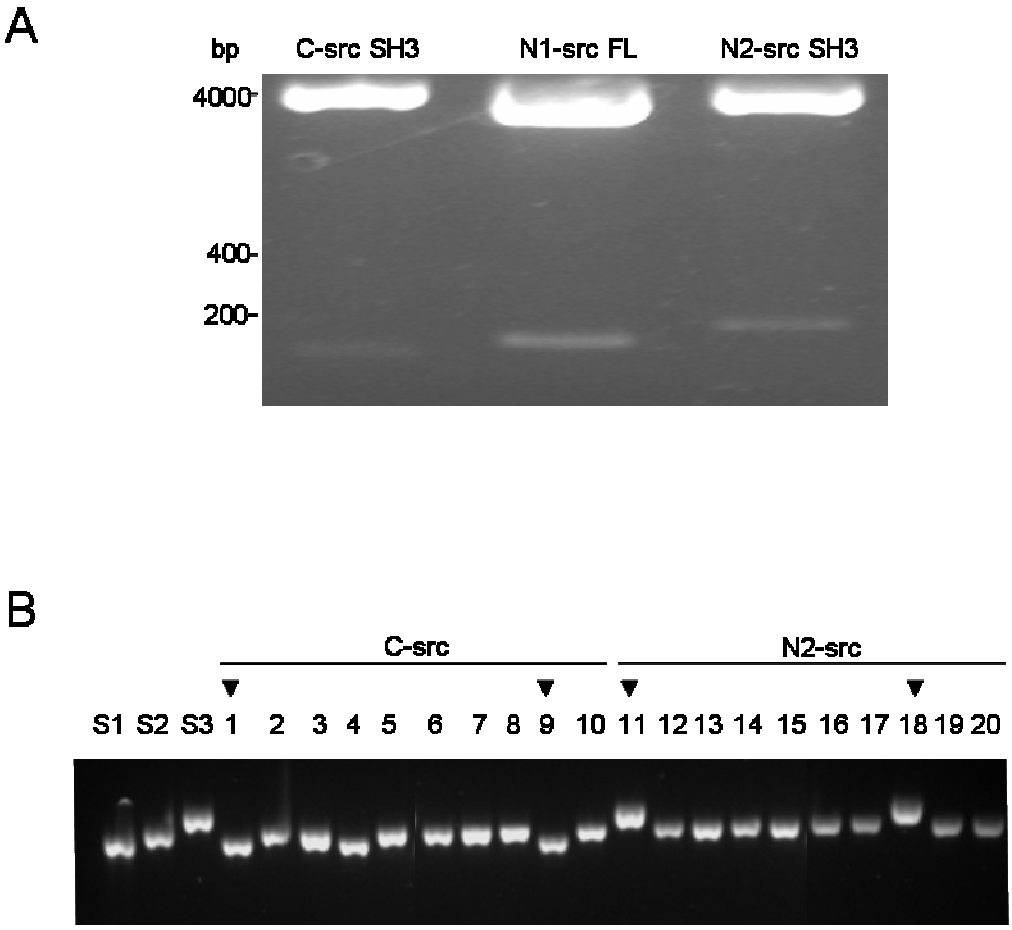


Figure 3.9- ***Production of full length src splice variants.***

(A) Agarose gel of a double digest using BglII and BbvCI to release the C- and N2-SH3 domain insert from pGEX, and the N1-SH3 domain insert from pQE30.

(B) Agarose gel of a colony screen of ligated C-src and N2-src inserts to FL N1-src in pGEM vector using SH3 primers (S1, S2, S3 = C, N1, N2). SH3 domains were used as standards. C-src colonies were from 1 to 10 while N2-src colonies were from 11 to 20. Colonies 1 and 9 were picked for C-src and colones 11 and 18 were picked for N2-src.

when the primers were designed (see 2.1.6)), along with the pQE30 vector (Figure 3.10 A). Two colonies from C-src and N2-src and one colony from the original N1-src vector were selected for overnight culture, miniprep, and then double digestion using BamHI and SalI restriction enzymes. All three full length src splice variant inserts were then ligated to pQE30 vector and transformed into XL 10 Gold bacteria. Two colonies from each splice variant were miniprepped and tested for the insert using double digestion with BamH1 and Sal1 enzymes (Figure 3.10 B). The full length src splice variants in pQE30 were sequenced to insure that there were no extra mutations inserted during the SDM.

3.3.5 Expression of His-tagged full length src splice variants

The three full length splice variants in pQE30 vectors were then transformed into BL21 bacteria for protein expression. Several attempts were made before finding the best expression conditions including using super media 1 and 2, varying expression temperatures and the time of induction. Finally a Gro-ESL chaperone in a separate vector (pREP4-Gro-ES-Gro-EL) was co-expressed with the src splice variants in the same bacteria in order to help protein folding and enhance expression (Lee et al., 2002b; Yanase et al., 2002). All three splice variants of src were co-expressed with Gro-ESL using two litres of super media 2 (see 2.1.2) at 16 °C overnight (see 2.3.4). The proteins were then purified on Ni^{+2} beads, and separated on SDS PAGE gels (figure 3.11 A). The Gro-ESL chaperone was also purified with src splice variants on Ni^{+2} beads, resulting in the presence of several bands and thus it was not possible to confirm the presence of the src splice variants by Coomassie staining alone. To confirm that src proteins were expressed, the purified proteins were subjected to

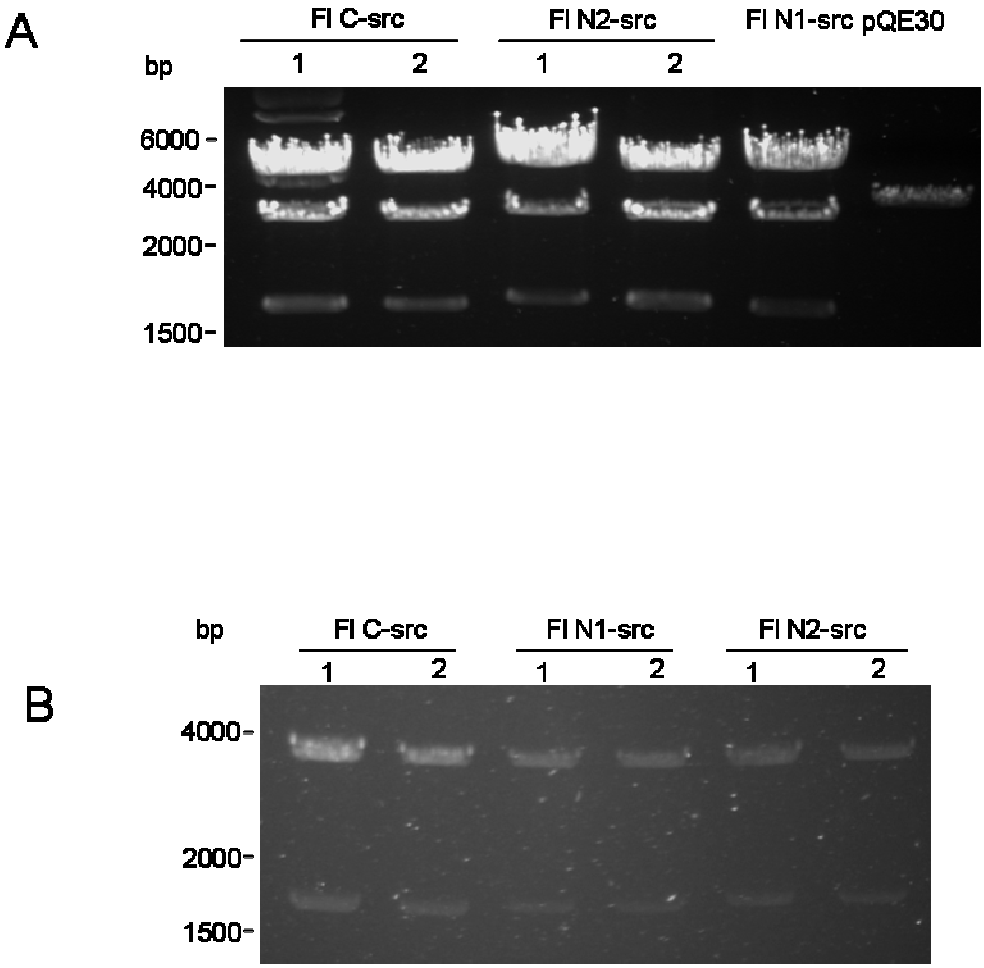


Figure 3.10- ***Ligation of full length src splice variants into pQE30.***

(A) Agarose gel of double digest using BamH1 and Sal1 of full length C-src (1 and 2), N2-src (1 and 2), and N1-src in pGEM and pQE30 vector. Inserts were ligated to pQE30 vectors to produce His-tagged proteins.

(B) Agarose gel of double digest using BamH1 and Sal1of full length C-src (1 and 2), N1-src (1 and 2), and N2-src (1 and 2) in pQE30 vector.

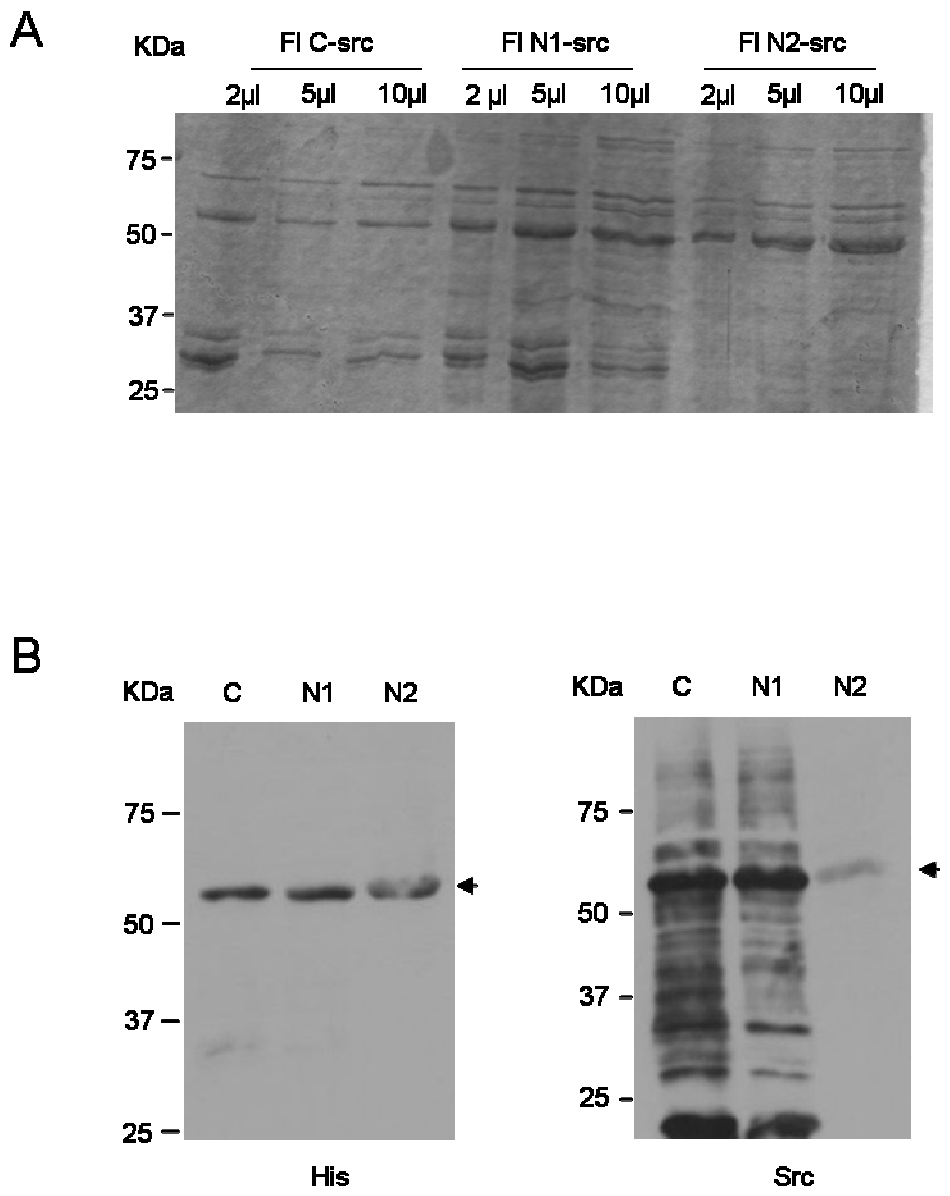


Figure 3.11- ***Expression of full length src proteins.***

(A) Coomassie stained gel of 2, 5, and 10 µl of full length src splice variants co-expressed in BL21 bacteria with Gro-ESL chaperone and purified on Ni^{2+} beads.

(B) Western blot for His and Src using full length src splice variants on Ni^{2+} beads. Arrows indicate His-tagged src. No bands were seen above 75 kDa in the blot.

western blot analysis (Figure 3.11 B). A His-tag antibody blot showed that all the src splice variants had similar expression levels. However a src antibody blot displayed a very faint N2-src band sized compared to C-src and N1-src splice variants. This could be due to N2-src SH3 domain contains insertion that might reduce the antibody recognition since the antibody was produced against C-src SH3 domain.

3.4 The Δ80 src splice variants

3.4.1 Cloning Δ80 src splice variant DNA

Multiple studies have observed increased expression of src levels if the N-terminal 80 amino acids are removed. Therefore src splice variants were produced lacking this region (Δ80). To amplify Δ80 src from full length src splice variant templates, the forward primer of the SH3 domain and reverse primer of full length src were used. All three splice variants were amplified using pfu turbo polymerase enzyme (Figure 3.12 B). The PCR products were separated from template DNA on agarose gels and gel purified. The purified Δ80 src splice variant DNA were ligated into pGEM and transformed into XL 10 Gold bacteria. Colony screening of five colonies for each splice variant was performed (Figure 3.12 C). Two colonies (marked with arrows) were picked from each splice variant to be miniprepped; each miniprep was tested by double digestion using BamH1 and Sal1 restriction enzymes and compared to the uncut vector (Figure 3.12 D).

3.4.2 Ligation of Δ80 src splice variant DNA into pQE30 and protein expression

Δ80 src splice variants in pGEM were double digested using BamH1 and Sal1 restriction enzymes, the src inserts purified (Figure 3.13 A), ligated into the pQE30

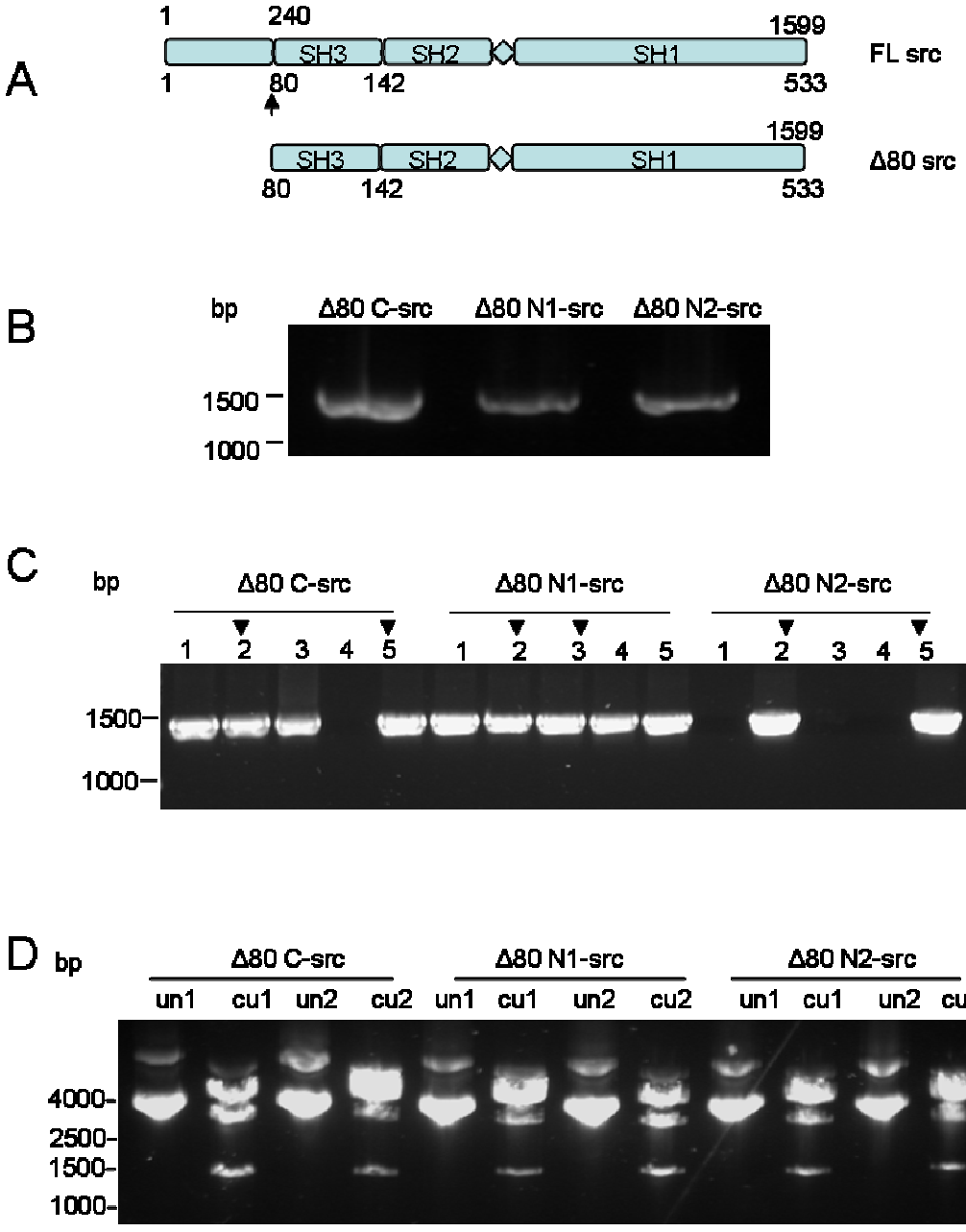


Figure 3.12- ***Production of Δ80 src DNA.***

A) A diagram showing the difference between full length src and Δ80 src, where the first 80 amino acids (240 BP) of Δ80 src are missing.

B) Agarose gel of PCR product of Δ80 src splice variants obtained from full length src in pGEM as a template (bases 241-1599).

C) Agarose gel for a colony screen of XL 10 Gold bacteria transformed with pGEM vector ligated with Δ80 C-, N1-, or N2-src. 5 colonies from each ligation were screened. The colonies that were selected are marked with arrows.

D) Agarose gel of double digest products using BamHI and SalI restriction enzymes to cut the pGEM vector containing Δ80 N1-src, C-src and N2-src. Each miniprep was either uncut (un) or double digested (cu).

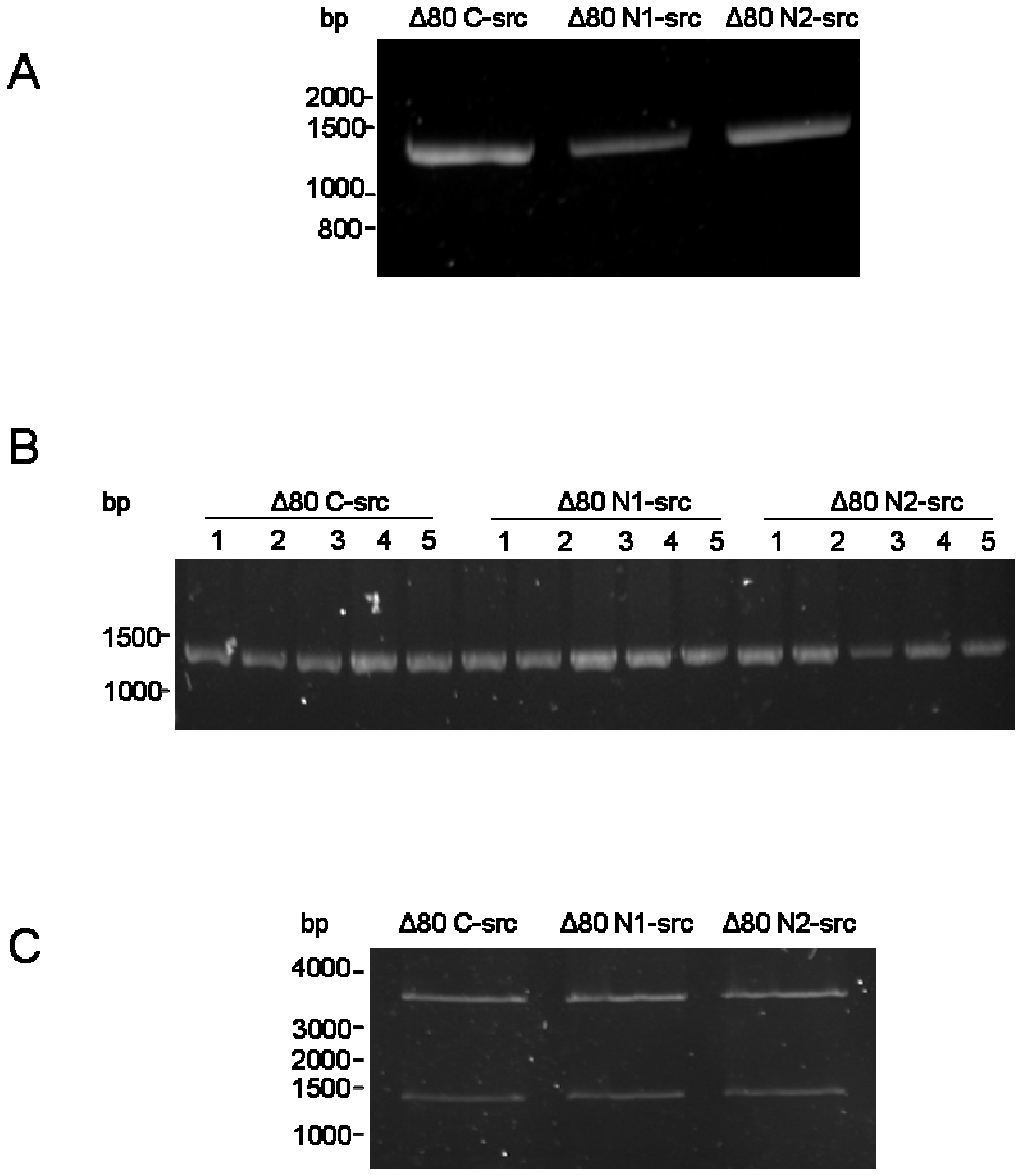


Figure 3.13- ***Production of Δ80 src splice variant DNA.***

A) Agarose gel of gel purified products for Δ80 C-src, N1-src, and N2-src.

B) Agarose gel of a colony screen of XL 10 Gold bacteria transformed with pQE30 vector ligated with Δ80C-, N1-, or N2-src. 5 colonies from each ligation were screened.

C) Agarose gel of double digest products using BamHI and SalI restriction enzymes to cut pQE30 vector containing the Δ80 C-src, N1-src, and N2-src.

vector and transformed into XL 10 Gold bacteria. Five colonies from each splice variant were screened and all showed positive results (figure 3.13 B). One colony from each splice variant was miniprepped and tested for inserts using BamH1 and Sal1 restriction enzymes (Figure 3.13 C). The Δ80 src splice variants in pQE30 were then transformed into BL21 bacteria for protein expression. A Gro-ESL chaperone was also transformed into the same bacteria. All three splice variants of src were co-expressed with Gro-ESL using two litres of super media 2 (see 2.1.2) at 16 °C overnight induction (see 2.3.4). The proteins were then purified on Ni^{+2} beads, and separated on an SDS PAGE gel (Figure 3.14 A). The Gro-ESL chaperone was purified with src splice variants on Ni^{+2} beads, resulting in the presence of several bands of approximately 50 kDa. To confirm that src proteins were expressed, a series of western blots were performed using both src and His antibodies (figure 3.14 B). All the three splice variants were expressed, and there seem to be some break down products.

3.5 Producing the active kinases of Δ80 src splice variant

The full length and the Δ80 src splice variants that were produced were tested and shown to be inactive kinases due to a mutation in the catalytic domain (using Taq to perform the first PCR generated two point mutations and one of them is not conservative where Lysine was mutated to Glutamic acid (amino acid 410) for full length N1 src at BP 1228 (see 3.3.2)). Therefore, a strategy that was adopted to produce active forms of Δ80 src splice variants by correcting their mutations (Figure 3.15). The first step was to correct the mutation in full length N1-src (Figure 3.15 A and B). The second step was to replace the mutated region in Δ80 src with fixed full

length N1-src. The enzymes selected were BstX1 (which will cut the DNA at (1185-1189)) and Sal1 which will cut at the end of the coding reagion (this restriction site was induced with the primers) (Figure 3.15 C and D).

3.5.1 Production of active Δ80 src splice variant DNA

The mutations in full length N1-src in pGEM were corrected. All Δ80 src splice variants in pQE30 and full length N1-src in pGEM were double digested using BstX1 and Sal1 restriction enzymes (figure 3.16 A). The insert from full length N1-src and the cut vectors from Δ80 src splice variants were separated on an agarose gel and purified (figure 3.16 A). Some of the Δ80 src splice variant vectors were only partially digested with one enzyme, resulting in two bands on the agarose gel; therefore the two bands were separated using a low percentage agarose gel and longer separation time. The lower band represents the double digested fragment (Figure 3.16 B). The Δ80 src splice variant vectors and the insert from the corrected full length N1-src were tested on agarose gels (Figure 3.16 C) and ligated to produce the active Δ80 src splice variants in pQE30.

3.5.2 Production of active Δ80 src splice variant protein

The corrected Δ80 src splice variants in pQE30 were then transformed into BL21 bacteria for protein expression. A Gro-ESL chaperone in a separate vector was inserted into the same bacteria as the Δ80 src splice variants. All three splice variants of src were co-expressed with Gro-ESL using two litres of super media 2 (see 2.1.2) at 16 °C overnight induction (see 2.3.4). The proteins were then purified on Ni^{+2} beads, and separated on an SDS-PAGE gel (Figure 3.17 A). To confirm that src

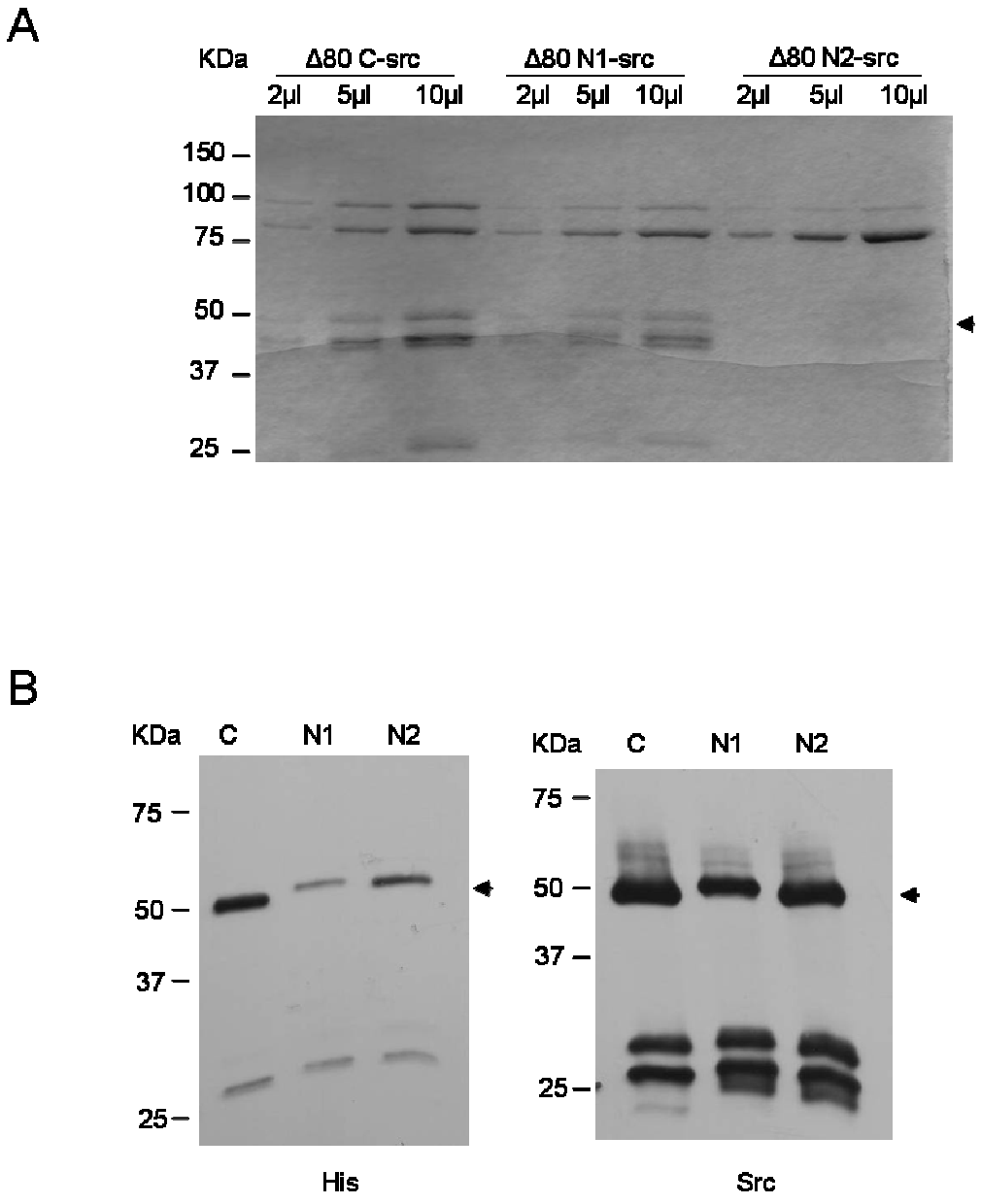


Figure 3.14- ***Expression of Δ80 src splice variant proteins.***

(A) Coomassie stained gel of 2, 5, and 10 μl of expressed Δ80 src splice variants bound to Ni^{2+} beads. Arrow indicates the position of src.

(B) Western blot for His and Src of Δ80 src splice variants on Ni^{2+} beads. Antibodies did not detect any bands above 75 kDa. Arrows indicate Δ80 src.

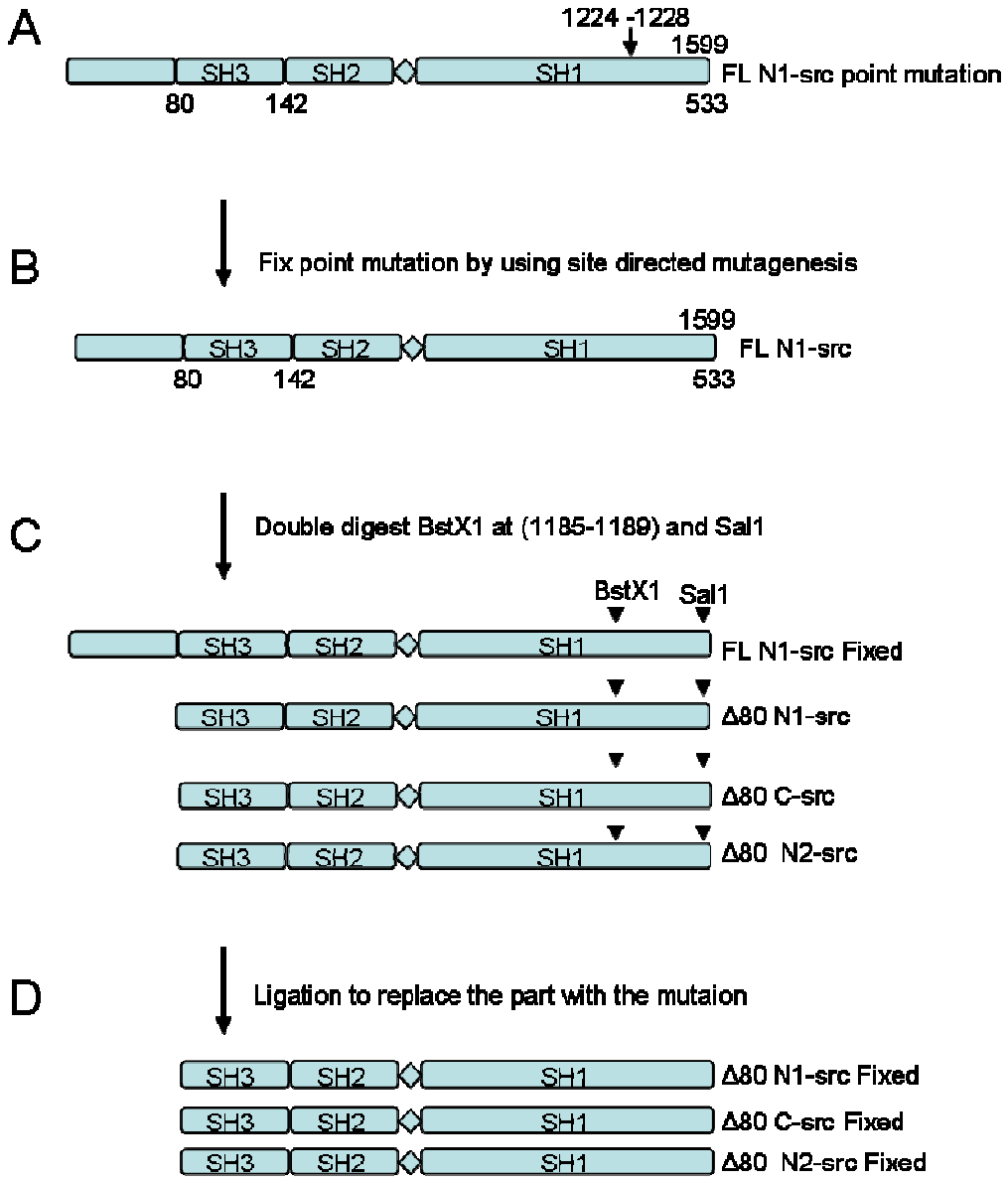


Figure 3.15- ***Strategy to produce Δ80 src splice variant active kinases.***

Diagram shows the strategy that was used to produce all three of the Δ80 src splice variants using the corrected full length N1-src and the Δ80 of N1-src, N2-src and C-src

A) Full length N1-src with a point mutation at bases 1224 and 1228.

B) The fixed full length N1-src using site directed mutagenesis.

C)Double digested full length N1-src and Δ80 N1-src, N2-src, and C-src.

D)Production of active Δ80 N1-src, N2-src, and C-src by replacement of the SH1 domain that contains the mutation with the equivalent area from corrected full length N1-src.

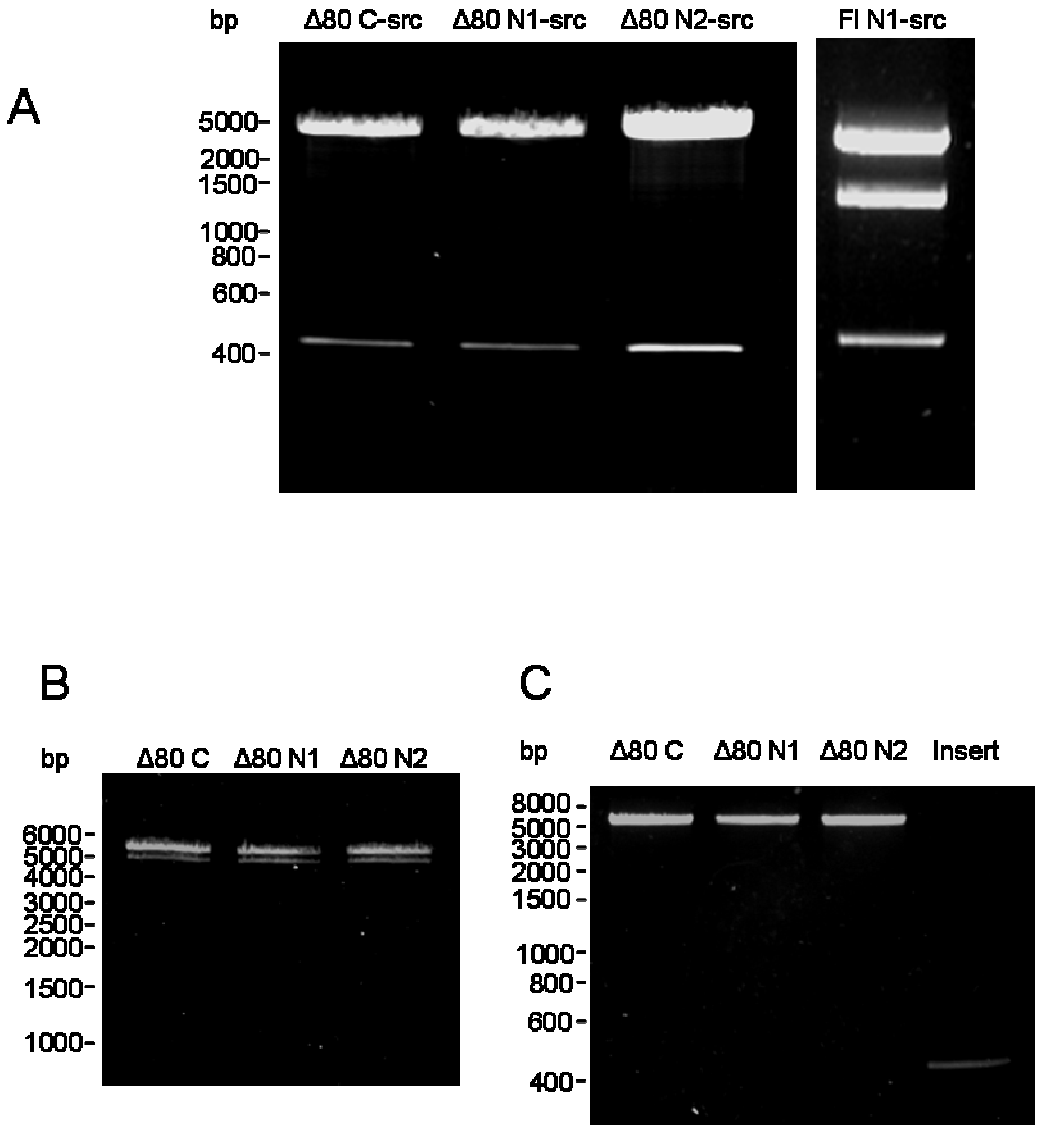


Figure 3.16- ***Production of active Δ80 src splice variant DNA.***

A) Agarose gels of Δ80 C-src, N1-src, and N2-src in pQE30 vector and SDM fixed full length N1-src in pGEM, double digested with BstX1and Sal1.

B) Agarose gel of Δ80 C-src, N1-src, and N2-src in pQE30 vector double digested with BstX1and Sal1restriction enzymes. Plasmids were partially digested with one enzyme and only the lower bands were used for fixing the splice variants.

C) Agarose gel of gel extracted double digest products using BstX1and Sal1 restriction enzymes of Δ80 C-src, N1-src, and N2-src in pQE30 vector and the insert from fixed full length N1-src in pGEM.

proteins were expressed, proteins were subjected to western blot with a His antibody (Figure 3.17 B). All three of the corrected Δ80 src splice variants were expressed under these conditions.

3.5.3 Testing the activity of Δ80 src splice variant protein

To test the activity of the Δ80 src, a kinase assay was performed (see 2.4.4). The amount of kinase was estimated according to a His blot (figure 3.17 B), and GST-synaptophysin C-terminus was used as a substrate (Synaptophysin is tyrosine phosphorylated by C-src (Barnekow et al., 1990)). The reaction was performed at 37 °C for 60 minutes. Kinase assays were tested by western blotting for His to determine the amount of kinase used, PY20 phospho-tyrosine antibody was used to determine the extent of tyrosine phosphorylated synaptophysin, and synaptophysin antibody was used to determine the amount of substrate (figure 3.17 C). This experiment confirmed that all three Δ80 src splice variant proteins were active and able to phosphorylate synaptophysin, a known src substrate.

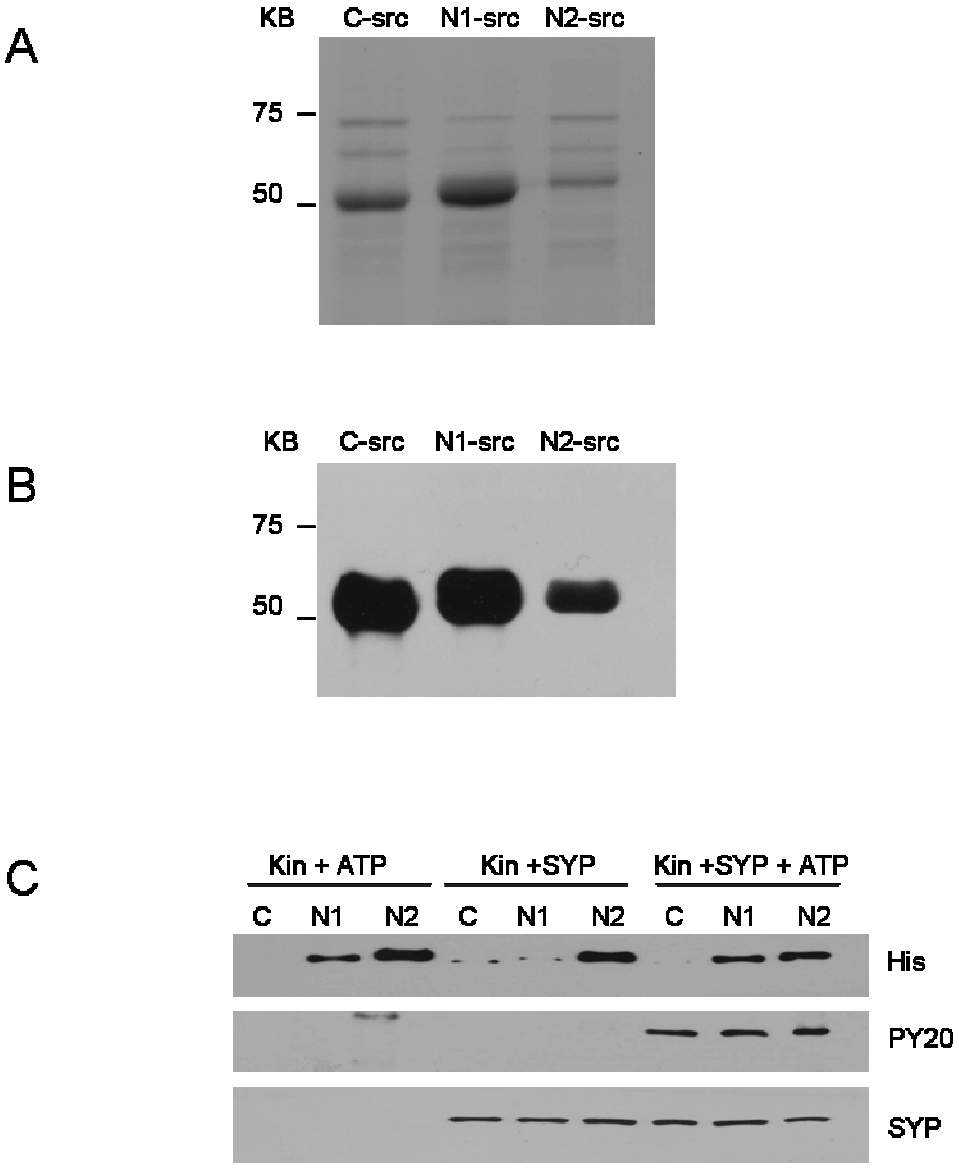


Figure 3.17- ***Production of active Δ80 src splice variants.***

A) Coomassie stained gel of His tagged Δ80 C-src, N1-src, and N2-src proteins co-expressed with Gro ESL chaperone in BL21 bacteria and purified using Ni^{2+} beads.

B) Western blot for His using His-tagged Δ80 C-src, N1-src, and N2-src splice variants on Ni^{2+} beads.

C) Kinase assay using Δ80 C-src, N1-src, and N2-src. GST-tagged C-terminus of synaptophysin was used as a substrate, the reaction was performed at 37 °C for 60 minutes (Kin = kinase, SYP = synaptophysin) and the kinase amounts were calculated according to 3.17 B (same amounts of beads from both C- and N1-src and 4 time more from N2-src). Blots show the amount of kinase (His), the phosphorylation (PY20), and the amount of synaptophysin (SYP).

3.6 Discussion

In this chapter, all the methods and strategies used for cloning and expressing the src splice variants were summarized. Firstly all the three src splice variant SH3 domains were cloned and amplified from a rat brain cDNA library. The amplified SH3 domains were ligated to different vectors for different reasons. All three src splice variant SH3 domains were expressed either as GST-tagged or as His-tagged recombinant proteins in *E. coli* expression systems. Full length src splice variants were also cloned from a rat brain cDNA library. Site directed mutagenesis was used to produce both C-src and N2-src splice variants and all the full length src splice variants were produced as His-tagged proteins with the help of Gro-ESL chaperone co-expression. Truncated versions of src splice variants (Δ80 src) were produced to enhance their expression by removing the first 80 amino acids. These truncated forms were co-expressed with a Gro-ESL chaperone as His-tagged proteins. Mutations in the kinase domain resulted in producing inactive forms of src splice variants but improved the expression at the same time. The active forms of truncated src were produced using site directed mutagenesis and restriction enzymes. These corrected truncated forms were co-expressed with a Gro-ESL chaperone as His-tagged recombinant proteins and proven to be active kinases.

3.6.1 SH3 domains

The SH3 domains of all three src splice variants were cloned successfully from rat brain cDNA library. The cloning of src SH3 domain DNA from the rat brain cDNA library resulted in four PCR products. A similar result was observed by Pyper and Bolen when they cloned src SH3 domains from adult human brain DNA (Pyper and

Bolen, 1990). Several attempts were made to clone the fourth band (N3) including gel purification and ligation, but colony screening always showed multiple bands even when single colonies were picked. This is abnormal since bacteria should only accept one plasmid. After multiple attempts, no further investigation was performed leaving the identity of the N3 band still undetermined.

When the GST-tagged SH3 domains were expressed, C-src and N1-src SH3 domains expressed well but there was poor expression of N2-src SH3 domain. The expression conditions were altered in many ways including using all three types of media described in 2.1.2, using different expression temperatures including (37°C, 19°C, and 16°C), using different expression times including (3 hrs, 5 hrs, and 14 hrs (overnight)), and using larger volumes of media. The optimal conditions (larger volumes at 16°C overnight) were adopted and adequate amounts of protein were produced as shown in the next chapter. A larger bacterial pellet was observed when expression of the N2-src SH3 domain was induced compared against the other splice variants. This suggested that the N2-src SH3 domain may be toxic to the bacteria.

All His-tagged SH3 domains expressed well, several higher molecular weight bands were also purified on the Ni^{+2} beads with one protein band running at about 60 KDa (figure 3.5 C). These bands may be bacterial chaperones which are often seen during recombinant protein expression in bacteria. Attempts to purify the His-tagged src SH3 domains from the contaminating proteins failed, even using a 30 KDa cut off membrane. One noticeable feature was that His-tagging enhanced N2-src SH3 domain expression compared to GST-tagging (even though twice the bacterial volume was used compared to C- and N1-src splice variant SH3 domains).

3.6.2 Full length src

Full length srcs were much more difficult to clone from the rat brain cDNA library. Several attempts were made using different polymerases and different annealing temperatures, but the result was always negative. A PCR product was obtained when taq polymerase enzyme was used in addition to more rat brain cDNA library (figure 3.6 A). The downside was that taq polymerase enzyme does not have proof reading, which makes the possibility of cloning errors higher. Since the amount of PCR product was low, another PCR reaction was performed using the previous PCR product as a template (figure 3.6 C). The absence of a full length N2-src clone was a surprise, since the previous amplification of SH3 domains showed that N2-was abundant (figure 3.3 A). All PCR products had errors in their sequences which was no surprise since taq DNA polymerase was used for the initial cloning step. Since it was so difficult to obtain PCR products from rat brain cDNA library, an alternative strategy was employed to generate full length C- and N2-src from N1-src. Full length N1-src had only two mutations at bases 1224 (C to T) and 1228 (T to C), The first mutation was a conservative mutation for valine (amino acid 408) but the second one was not conservative and it mutated Lysine to Glutamic acid (amino acid 410).

Since PCR from rat cDNA library was performed several times before and it was not easy to have successful cloning of src splice variants, the alternative method was to replace the area that is different from N1-src with its equivalents from C-src and N2-src. The only difference among the three splice variants is in the SH3 domains which were cloned previously, and swapping the equivalent areas only required finding the suitable digestion enzymes that are able to singularly cut SH3 domains in the right place (one enzyme before the insertion of N1-src and N2-src and another enzyme

after). These enzymes should not be able to cut the pGEX vector (which has the SH3 domains inserted) or pQE30 vector (which has the full length N1-src inserted). This mutant swaped the DNA sequences from both C- and N2-src into the full length full length N1-src. SDM was required to introduce a conservative mutation that created a restriction site for the BglII enzyme. After double digestion with BglII and BbvCI which flanked the SH3 domain inserts, the ligation with full length N1-src was successful resulting in the three full length src splice variants in pQE30 vector.

The full length src splice variants could not be expressed in bacteria until the Gro-ESL chaperone was also inserted into the same bacteria. The ability of full length src to be expressed at all may result from the point mutation (K410 to E) in the original N1-src in the kinase domain. Such a dramatic substitution (basic to acidic) may have severe consequence for kinase activity and induced these kinase were found to have no activity. The inactivity of src and thus relationship to their expression has been examined previously. Kemble and colleagues co-expressed src (83-533) with Gro-ES-EL chaperone and compared two mutants with the wild type. They found that the more the src activity is impaired the better the expression. While the wild type had a very poor expression and extensive degradation, src carrying a mutation that inhibits its kinase activity (K295M) is expressed nicely (Kemble et al., 2006). On the other hand a different src mutant (N391D) expressed to almost the same level as the kinase dead mutant still had a very low kinase activity (about 1:1000 compared to the wild type). In conclusion they suggested that the activity of the kinase is inversely related to its expression (Kemble et al., 2006).

3.6.3 Δ80 src

A truncated form of src was also produced since several groups had shown this enhances expression. However deletion of amino acids after residue 89 severely affects src kinase activity (Nemeth et al., 1989). Saya and colleagues ligated truncated C-src, where amino acids 15-225 were deleted, to *Staphylococcus* protein A **(***Staphylococcus* protein A is a 40-60 kDa surface protein originally found in the cell wall of the bacteria *Staphylococcus aureus*). This ligation resulted in enhanced expression of active C-src, which is able to phosphorylate other substrates and also auto-phosphorylate (Saya et al., 1993). Seeliger and collegues co-expressed truncated C-src from amino acid 251-533 with YopH phosphatase in two different plasmids in bacteria and they found that when they expressed src alone most of the product was insoluble, but with the phosphatase co-expression they were able to express and purify active src in a bacterial system (Seeliger et al., 2005). Weijland and colleagues expressed truncated C-src starting from amino acid 81 until the end of the protein. They co-expressed it with CSK in a yeast expression system in order to cause phosphorylation of Y527, which will result in binding of this residue to the SH2 domain and keep the kinase in a closed conformation form, reducing its kinase activity (kinase activity of src phosphorylated at Y527 is 20 times less than the non-phosphorylated). They found that co-expressing src with CSK resulted in producing good amounts of the kinase (Weijland et al., 1996; Weijland et al., 1997). Wang and colleagues co-expressed truncated inactive C-src (K295M) from amino acid 83-533 with GroSL/EL chaperones in separate plasmids in an *E. coli* bacterial system, or from 86 to 533 co-expressed with PTPIB phosphatase in the same plasmid, and they had nice expression in both cases (Wang et al., 2006). Xu and colleagues expressed human src residues 86-536 (equivalent to residues 83-533 in chicken src) in insect cells and used this construct for crystallisation (Xu et al., 1997).

3.6.4 Active Δ80 src

All the Δ80 src splice variants were dead kinases (same as the full length src) when they were tested for their kinase activity using synaptophysin C-terminus as a

substrate. For this reason active Δ80 src proteins were made. Only one splice variant was mutated using SDM to correct the point mutations at bases 1224 (C to T) and 1228 (T to C). The original full length N1-src was used for this and then the mutated areas from Δ80 src splice variants were replaced with the corrected equivalent region from full length N1-src. Expressing the active form was much harder than expressing the mutant form. Even using two litres of bacteria for each src splice variant at the optimized expression conditions only a small amount of His-tagged active Δ80 src splice variants was produced, however this was sufficient for kinase assay. The activity of these active src splice variant constructs was tested using synaptophysin C-terminus as a substrate; synaptophysin C-terminus is known to be tyrosine phosphorylated by src (Evans and Cousin, 2005). The results showed that all three src splice variants are able to phosphorylate synaptophysin C-terminus (figure 3.17 C). Because active src is so hard to express, different strategies had to be employed. Weijland and colleagues found that co-expressing the PTP-PEST tyrosine phosphatase with the src kinase domain alone (258-533) overcame the toxicity of src in a yeast expression system; this resulted in the production of a significant amount of the kinase domain. However they also found that expressing wild type src in the same expression system did not produce any significant amounts of protein (Weijland et al., 1996).

Chapter 4

Novel binding partners of N2-src SH3 domain

4. Novel binding partners of N2-src SH3 domain

4.1 Introduction

There is a huge range of SH3 containing proteins. SH3 domains consist of 50-70 amino acids and they are used for protein-protein interactions, where they bind to proline rich domains (Kay et al., 2000; Kolafa et al., 2000; Macias et al., 2002; Camara-Artigas et al., 2009). The typical structure of the binding site for SH3 domains is PxxP which will adopt a left hand helix with an arginine residue adjacent to the PxxP motif (Feng et al., 1994; Rickles et al., 1994; Alexandropoulos et al., 1995; Weng et al., 1995; Bedford et al., 2000; Macias et al., 2002). The C-src SH3 domain has been reported to bind to dynamin I (Gout et al., 1993; Okamoto et al., 1997; Foster-Barber and Bishop, 1998; Onofri et al., 2000; Solomaha et al., 2005). Dynamin I is also tyrosine phosphorylated by C-src both *in vivo* and *in vitro* (Ahn et al., 1999; Ahn et al., 2002). C-src is also known to bind synapsin I (Onofri et al., 1997; Foster-Barber and Bishop, 1998; Onofri et al., 2000; Onofri et al., 2007). Synapsin I is tyrosine phosphorylated by C-src and this interaction results in further activation of C-src kinase activity (Onofri et al., 1997; Onofri et al., 2007). C-src is also known to bind synaptojanin (Onofri et al., 1997; Onofri et al., 2000).

There is not much known about the interactions of the neuron specific splice variants, N1- and N2-src, apart from that N1-src does not bind to dynamin I and synapsin I (Gout et al., 1993; Foster-Barber and Bishop, 1998). However, since N1- and N2-src splice variants are expressed only in neurons, they are expected to have a role in neuronal function. Since the only difference between these splice variants is in their SH3 domains (Figure 3.2), an obvious hypothesis is that these SH3 domains would have different binding partners that determine their neuron-specific functions.

The aim of this chapter is therefore to investigate the binding partners for all the src splice variant SH3 domains in nerve terminals with particular emphasis on N1- and N2-src SH3 domains. The hypothesis is that by identifying novel interacting partners, new functions for both N1- and N2-src will be revealed.

To achieve this aim a general strategy was adopted (Figure 4.1). GST-pull downs from rat nerve terminal lysates were performed using GST-tagged src splice variant SH3 domains. The specific interacting proteins were then identified by using MALDI-tof mass spectrometry. Proteins that are known to bind to C-src, such as dynamin I, were not included in this identification process since they were expected to bind. Novel binding partners were then confirmed by western blotting. *In vitro* interaction assays were used to determine whether the binding is direct. At this stage the binding partners were examined for their ability to act as src substrates by using kinase assays and the src binding site on these proteins identified using sequence analysis and site directed mutagenesis.

4.2. SH3 domain interactions with proteins from synaptosomal lysates

GST, and GST-tagged C-, N1-, N2-src SH3 domains were used to perform pull downs from rat brain synaptosomal lysates (basal and stimulated). Basal and stimulated lysates were both used to investigate if any src splice variant displays stimulation-dependent binding. Proteins that were bound to these GST fusion proteins were separated on a 7.5%-15% gradient acrylamide gel and stained with

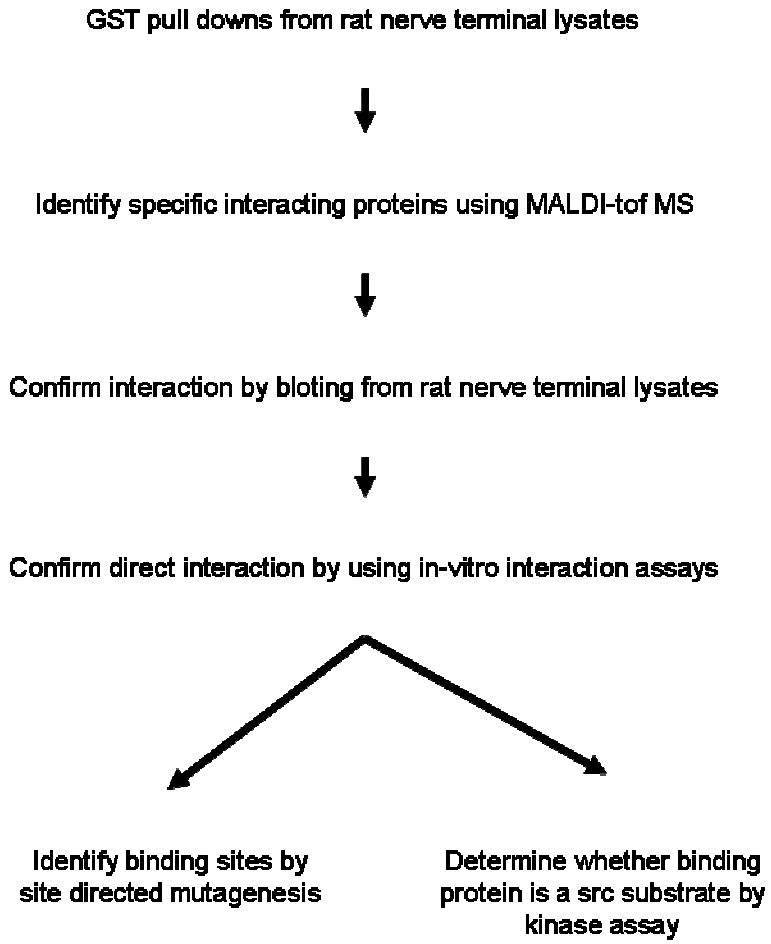


Figure 4.1- ***Strategy for src splice variant interactions with proteins.***

Flow chart shows the strategy that was adopted for identifying binding partners of src splice variants in rat nerve terminal lysates.

colloidal Coomassie stain (Figure 4.2). A number of proteins were extracted from nerve terminal lysates by the C-src SH3 domain (major bands seen at 145 kDa, 96 kDa, 85 kDa, and 55 kDa). No stimulation-dependency was seen for these interactions. N1-src SH3 domain exhibited much less binding compared to C-src, with very few bands observed with N1-src either at basal conditions or after stimulation. One noticeable difference was an increase in the binding of N1-src to a protein at approximately 100 kDa in stimulated lysates compared to basal (Figure 4.2 marked with stars). The N2-src SH3 domain displayed a completely different set of interacting partners, with major bands observed at 80 kDa, 67 kDa and 50 kDa.

4.2.1. Identification of proteins bound to SH3 domains

To identify which proteins from the GST-pull downs were extracted by the different SH3 domains, MALDI-tof MS analysis was performed. Since the majority of bands from the two splice variants were from N2-src SH3 domain pull downs compared to the N1-src SH3 domain, most of the bands sent for identification were selected from N2-src pull downs (Figure 4.2). Known binding partners for C-src such as synaptojanin (145 kDa), dynamin (96 kDa), and synapsin (85 kDa) were not selected for MALDI-tof MS analysis. N2-src SH3 domain binding partners that were identified included dynamin I (96 kDa), N-ethylmaleimide sensitive fusion protein (NSF) (80 kDa), and Munc18 (67 kDa). Synapsin 2 (50 kDa) was identified as a C-src pull down binding partner (Table 4.1).

4.2.2. Confirmation of SH3 domain interactions with nerve terminal proteins

The data from the colloidal Coomassie stained gel and MALDI-tof MS analysis

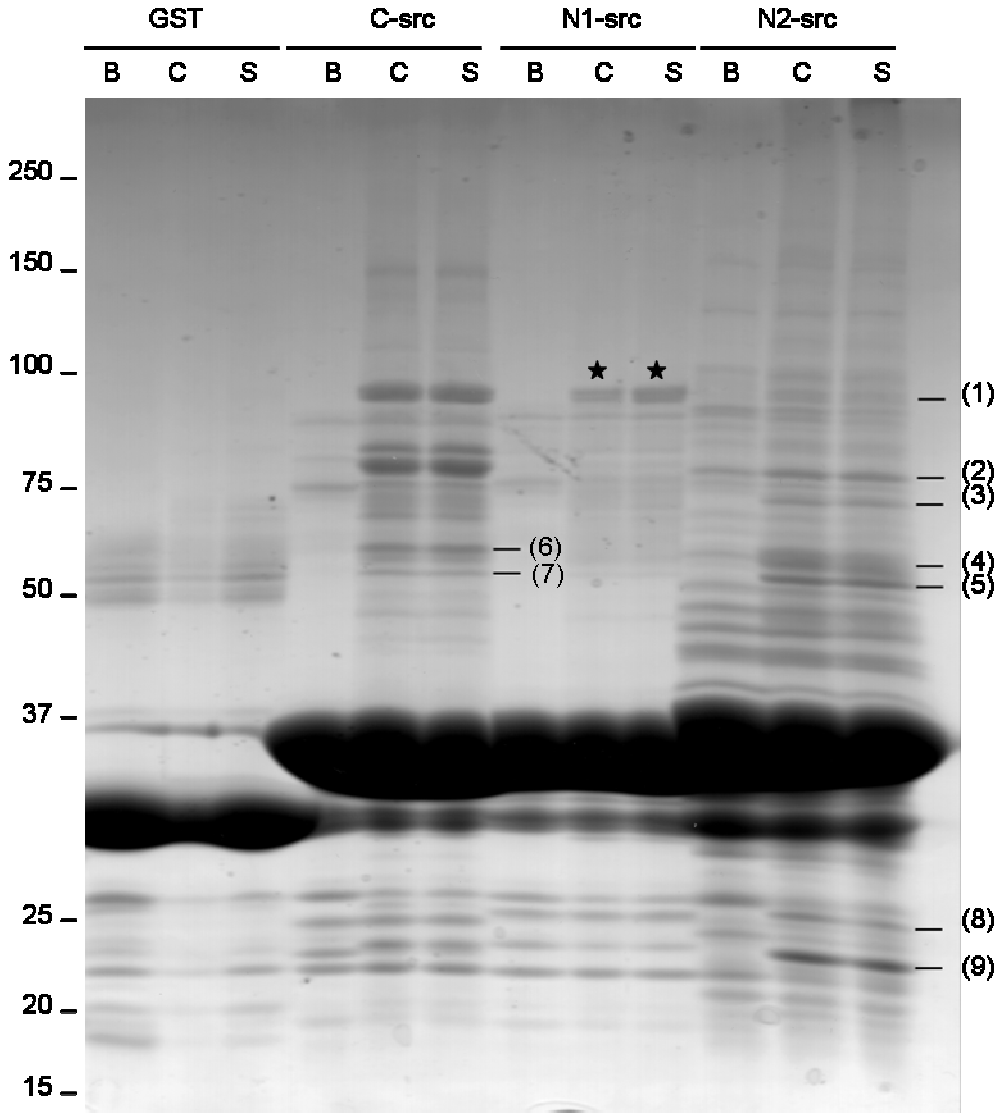


Figure 4.2- ***Identification of proteins bound to src splice variant SH3 domains from rat nerve terminal lysates.***

GST pull downs from rat nerve terminal lysates were performed using GST-SH3 domains C-, N1-, and N2-src splice variants (B = proteins on beads alone, C = pull downs using resting nerve terminal lysate, S = pull downs using stimulated (30 mM KCl) nerve terminal lysate). Colloidal coomassie stained gradient gel (7.5 % to 15 %) shows the binding of nerve terminal proteins to GST alone or GST tagged C-src, N1-src, and N2-src SH3 domains. Proteins that bound to specific SH3 domains were cut and identified by MALDI-tof MS.

A star highlights differential association of a 96 KDa protein to N1-src SH3 domain dependent on stimulation.

Band	MWt	Mowse Score	Protein
1	95.867 kDa	122 x 10^{10}	Dynamin I
2	82.6 kDa	127 x 10^{10}	Vesicular fusion protein (NSF)
3	67.526 kDa	136 x 10^{10}	Munc18
4	49.769 kDa	90 x 10^{10}	β -Tubulin
5	50.132 kDa	77 x 10^{10}	α –Tubulin
6	52.421 kDa	130 x 10^{10}	Synapsin II
7	59.419 kDa	170 x 10^{10}	ATP Synthase
8	18.458 kDa	99 x 10^{10}	Myelin basic protein
9	17.197 kDa	93 x 10^{10}	Myelin basic protein

Table 4.1- ***MALDI-tof MS identification of some bands.***

This table summarises the results of band identification using MALDI-tof MS identification. MALDI-tof MS analysis performed by V. Valova (CMRI). Proteins with Mowse scores greater than 69 x 10^{10} are significant

suggested which proteins bound to src splice variant SH3 domains. To confirm the identity of these proteins, pull downs from resting rat brain synaptosomal lysates were repeated using GST and GST-tagged C-, N1-, and N2-src splice variant SH3 domains. Western blot analysis was then used to confirm the binding partners of different src splice variant SH3 domains.

4.2.2.1. SH3 domain binding to dynamin, synapsin I, and synaptojanin

C-src SH3 domain has a well identified interaction with synaptojanin, dynamin I, and synapsin I (Gout et al., 1993; Okamoto et al., 1997; Onofri et al., 1997; Foster-Barber and Bishop, 1998; Onofri et al., 2000; Solomaha et al., 2005; Onofri et al., 2007). To confirm these proteins were the major bands observed in the GST-pull downs, western blot analysis was performed (Figure 4.3). These blots show that dynamin I bound to the C-src SH3 domain with little binding observed with both N1- and N2-src, in agreement with previous studies showing that C-src is the main binding splice variant for dynamin I (Gout et al., 1993; Onofri et al., 1997; Foster-Barber and Bishop, 1998; Onofri et al., 2000). Both synapsin I and synaptojanin bound only to C-src, and no binding was detected with N1- or N2-src, again confirming C-src is the major interacting partner for these proteins out of the splice variants (Onofri et al., 1997; Foster-Barber and Bishop, 1998; Onofri et al., 2000).

4.2.2.2 N2-src SH3 domain binding to NSF, synaptophysin, and Munc 18

MALDI-tof MS analysis suggests a specific interaction of both NSF and Munc 18 to N2-src. To confirm this, western blot analysis was performed on GST pull down

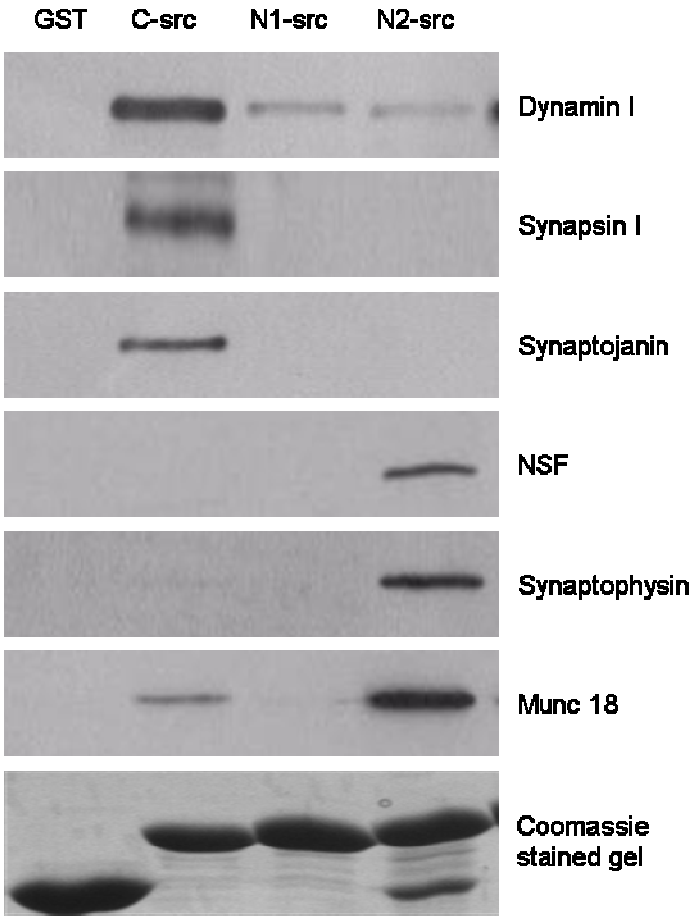


Figure 4.3- ***Verification of nerve terminal proteins binding to src splice variant SH3 domains.***

Similar pull down samples to figure 4.2 were subjected to western blot analysis. Pull downs were probed for dynamin I, synapsin I, synaptojanin, NSF, synaptophysin and Munc 18. Selective interactions were observed confirming the binding of these proteins to SH3 domains. Coomassie stained gel shows the amount of GST fusion proteins that were used for these pull down assays. Blots are representative of n = 3 experiments.

samples from resting nerve terminal lysates (Figure 4.3). When NSF binding was examined, it was found that NSF bound only to N2-src SH3 domain, but not C-src and N1-src. A similar pattern of binding was also seen for Munc 18, however some binding was also observed to the C-src SH3 domain. Synaptophysin is the major tyrosine phospho-protein in nerve terminals (Onofri et al., 1997; Evans and Cousin, 2005; Onofri et al., 2007), and is known to interact with src in nerve terminals (Barnekow et al., 1990; Linstedt et al., 1992; Zhao et al., 2000). Therefore we also determined the binding specificity of synaptophysin for the different SH3 domains. There was a selective interaction of N2-src with synaptophysin, with no binding observed to N1-src and C-src. This suggests that N2-src is the main binding splice variant for NSF, synaptophysin and Munc 18, all proteins with postulated roles in the synaptic vesicle life cycle.

4.2.2.3 Different SH3 domain binding to dynamin I, Munc 18, NSF, and synaptophysin

To determine whether the binding of Munc 18, NSF, and synaptophysin to N2-src SH3 domain was specific, or was shared across other SH3 domains, binding to other GST-SH3 domains from different proteins were screened. GST pull downs from synaptosomal lysates were performed using GST and GST-tagged C-, N1-, and N2-src splice variants, plus syndapin I, P85, endophilin I and II, and amphiphysin I and II SH3 domains (Figure 4.4). As expected dynamin I bound to all SH3 domains with the exception of N1- and N2-src. In contrast Munc 18, NSF and synaptophysin binding displayed a high degree of variation between different SH3 domains. Munc 18 bound to C-src, N2-src, syndapin I, P85, and amphiphysin I and II, with little

binding to endophillin I and II, and N1-src (Figure 4.4). A similar pattern of binding was observed for synaptophysin, the only difference being a lack of interaction with the C-src SH3 domain. In contrast NSF bound to N2-src, P85, and endophillin I and II, while no binding was observed to the other SH3 domains. Thus binding of these proteins to N2-src SH3 domain is not specific and the SH3 domains bound may provide clues to identify binding sites.

4.2.2.4 SH3 domain binding to SNARE proteins

N2-src SH3 domain bound to both Munc 18 and NSF, proteins that also interact with the SNARE proteins. To test whether NSF and Munc 18 bind N2-src indirectly via either syntaxin, VAMP, or SNAP 25, GST alone and GST-tagged src splice variant SH3 domains plus amphiphysin SH3 domain were used to perform pull downs from resting rat brain synaptosomal lysates (Figure 4.5). Munc 18 bound to N2-src SH3 domain, with some binding to C-src and amphiphysin I SH3 domains as seen previously (Figure 4.4). In contrast neither syntaxin, VAMP, or SNAP25 bound to any of these SH3 domains. This suggests the SNARE proteins do not bind any src splice variants and that Munc 18 and NSF do not bind N2-src via these proteins.

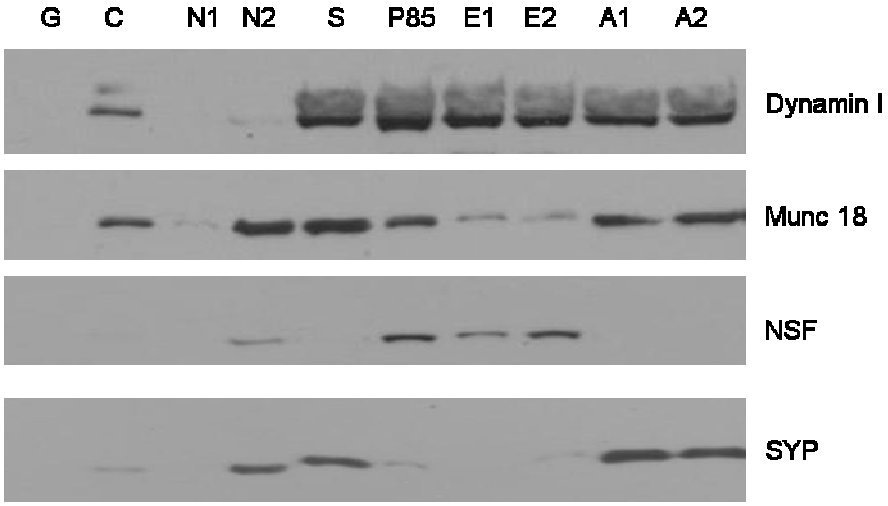


Figure 4.4- ***Determination of the specificity of the N2-src SH3 domain interaction with nerve terminal proteins.***
GST pull downs were performed from rat nerve terminal lysates using a range of SH3 domains (C-, N1-, N2-src splice variants, syndapin, P85, amphiphysin 1 and 2, and endophillin 1 and 2).G = GST, C = C-src,N1 = N1-src, N2 = N2-src, S = syndapin, E1 = endophilin I, E2 = endophilin II, A1 = amphiphysin I, A2 = amphiphysin II, L = synaptosomal lysates. Pull downs were probed by western blotting for the presence of either dynamin I, Munc 18, NSF, or synaptophysin (SYP).

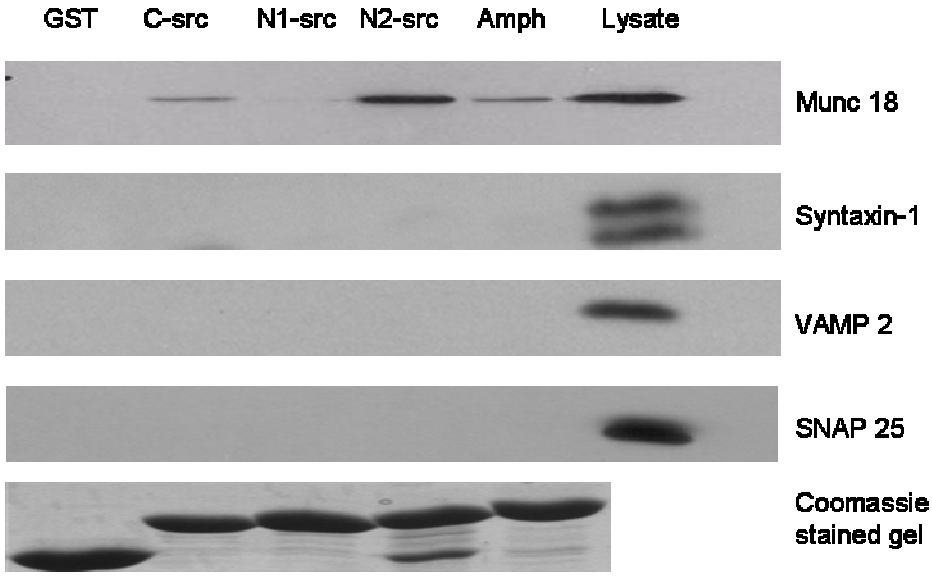


Figure 4.5- ***Binding of SNARE proteins to src splice variant SH3 domains.*** GST pull downs were performed from rat nerve terminal lysates using SH3 domains (C-, N1-, N2-src splice variants, and amphiphysin 1). Pull downs were probed by western blotting for the presence of either Munc 18, syntaxin-1, VAMP 2 or SNAP 25. Selective interactions were observed with Munc 18, confirming that binding to Munc 18 is not mediated by these SNARE proteins. Coomassie stained gel shows the amount of GST fusion proteins that were used for these pull down assays. Blots are representative of n = 3 experiments.

4.3 SH3 domain binding to recombinant proteins

4.3.1 SH3 domain binding to recombinant Munc 18

To determine whether Munc 18 interacts directly with N2-src, *in vitro* interaction assays were performed. In these experiments His-tagged full length Munc 18 was expressed in bacteria and either the bacterial lysates were used directly for pull downs using GST-tagged src splice variant SH3 domains, or purified Munc 18 was used for binding assays with GST-tagged src splice variant SH3 domains. GST-tagged syntaxin, which is the major Munc 18 binding partner (Dulubova et al., 1999; Ciufo et al., 2005b; Rickman et al., 2007), was used as a positive control.

GST and GST-tagged C-src, N1-src, N2-src, and syntaxin were used to perform pull downs from bacterial lysates expressing His-tagged Munc 18 (Figure 4.6 A). Munc 18 bound only to syntaxin, whereas no binding was detected with any of the src splice variant SH3 domains. This suggests that recombinant Munc 18 expressed in bacteria does not bind to src splice variant SH3 domains.

Next, GST and GST-tagged C-src, N1-src, N2-src, and syntaxin were used to perform binding assays with Munc 18 purified from bacterial lysates (Figure 4.6 B). Once again no specific binding was observed with any src SH3 domain. Therefore bacterially expressed Munc 18 does not bind to any src SH3 domain, questioning whether the interaction observed in nerve terminals is direct.

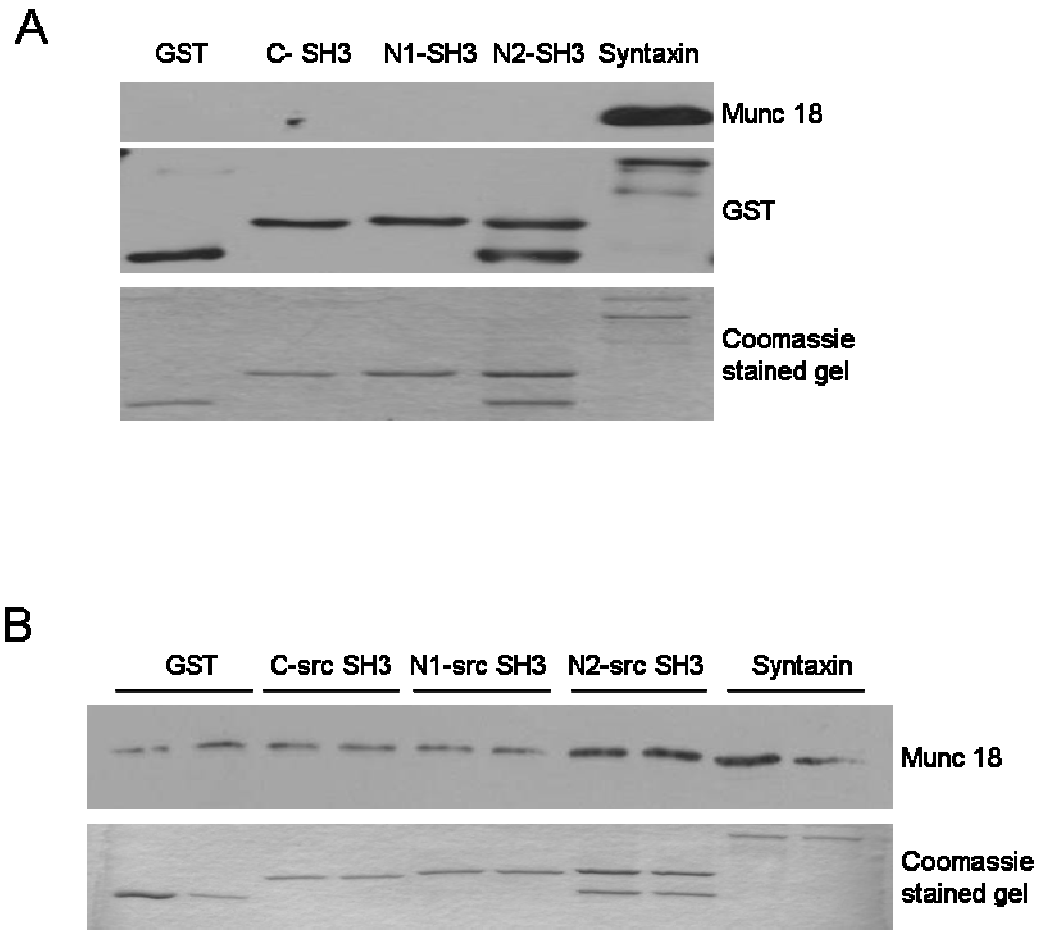


Figure 4.6- ***Binding affinity of Munc 18 and src splice variant SH3 domains.***

A) Western blot for Munc 18 and GST from pull downs using Munc 18 bacterial lysate and glutathione beads of GST alone and GST-tagged C-, N1-, and N2-src SH3 domains, and syntaxin. Coomassie stained gel shows the amounts of GST tagged proteins that were used for the pull down assay. No specific binding to N2-src SH3 domain was detected, while syntaxin bound Munc 18 (n = 10).

B) Western blot for Munc 18 from binding assay using recombinant Munc 18 and glutathione beads of GST alone and GST-tagged C-, N1-, and N2-src SH3 domains, and syntaxin. Coomassie stained gel shows the amounts of GST tagged proteins that were used for the pull down assay. Experiment was performed in duplicate (n = 10).

4.3.2 SH3 domain binding to recombinant synaptophysin C-terminus

To determine whether synaptophysin binding is direct, the cytoplasmic synaptophysin C-terminus (His-tagged) was expressed in bacteria and purified for binding assays or used for pull downs from bacterial lysates.

GST and GST-tagged C-src, N1-src, and N2-src SH3 domains were used to perform pull downs from bacterial lysates expressing His-tagged synaptophysin C-terminus (Figure 4.7 A). As with Munc 18, no binding was detected with any of the src splice variant SH3 domains, suggesting that recombinant synaptophysin C-terminus expressed in bacteria does not bind to src splice variant SH3 domains.

Next, GST and GST-tagged C-src, N1-src, and N2-src SH3 domains were used to perform binding assays with His-tagged synaptophysin C-terminus purified from bacteria (Figure 4.7 B). No binding was detected with any of the src splice variant SH3 domains, confirming the suggestion that recombinant synaptophysin C-terminus expressed in bacteria does not bind to src splice variant SH3 domains. Therefore as with Munc 18, this raises a doubt as to whether these interactions are direct in nerve terminals.

4.3.2 Phosphorylation of Munc 18 and synaptophysin C-terminus

Since both Munc 18 and synaptophysin bound to N2-src in nerve terminal lysates, it is possible that they could be substrates for its kinase activity. To test this possibility recombinant full length N2-src was used to perform kinase assays using both purified full length His-tagged Munc 18 and GST-tagged synaptophysin C-terminus as substrates (Figure 4.8). The synaptophysin C-terminus was phosphorylated, in agreement with previous studies showing that synaptophysin is a src substrate

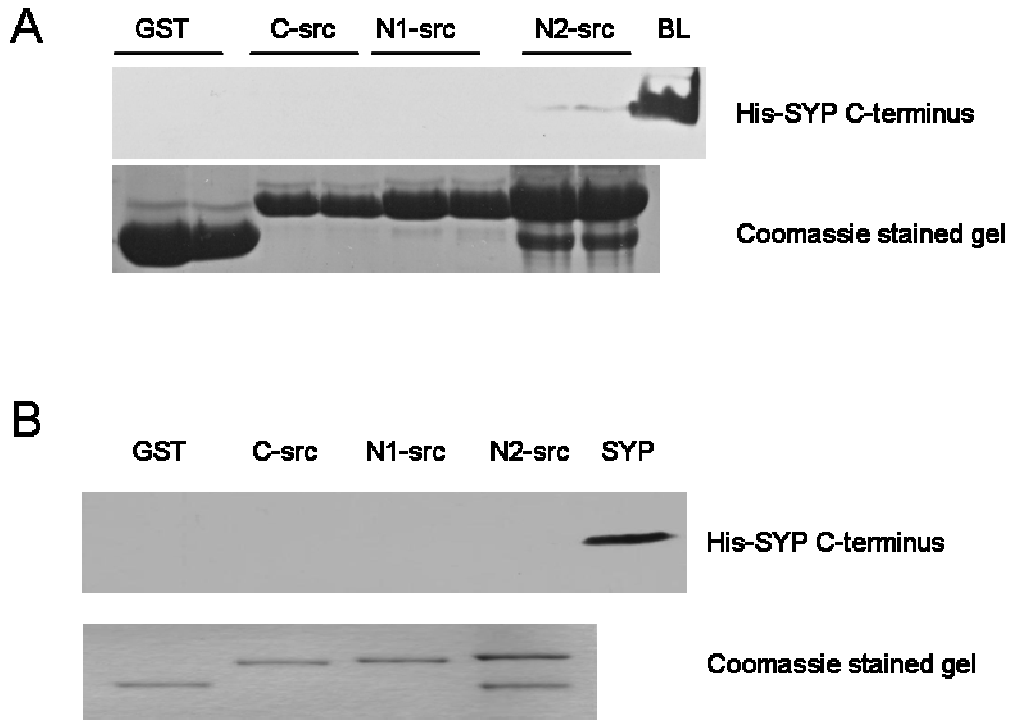


Figure 4.7- ***Binding affinity of synaptophysin and src splice variant SH3 domains.***

A) Western blot for synaptophysin from pull downs using His-synaptophysin C-terminus bacterial lysates and glutathione beads of GST alone and GST-tagged C-, N1-, and N2-src SH3 domains (BL = bacterial lysate). Coomassie stained gel shows the amounts of GST tagged proteins that were used for the pull down assay (n = 6).

B) Western blot for synaptophysin binding assay using recombinant His-tagged synaptophysin C-terminus (SYP) and GST alone and GST-tagged C-, N1-, and N2-src SH3 domains. Coomassie stained gel shows the amounts of GST tagged proteins that were used for the pull down assay. SYP refers to a positive control of synaptophysin C-terminus. No specific binding to N2-src SH3 domain was observed (n = 6).

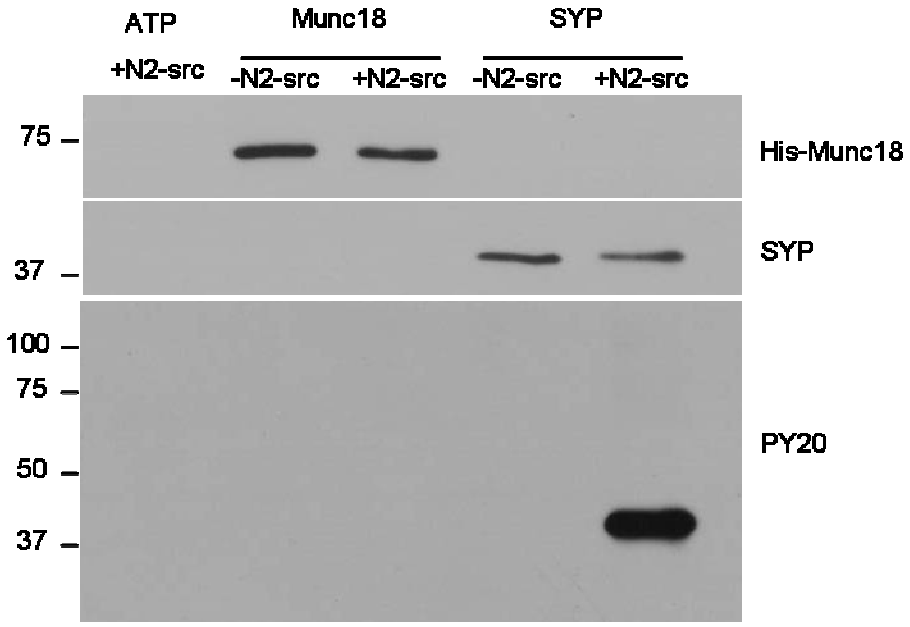


Figure 4.8- ***Testing tyrosine phosphorylation of munc18 and synaptophysin by N2-src.***

Kinase assay using recombinant N2-src. His-tagged Munc 18 and GST-tagged C-terminus of synaptophysin were used as substrates for recombinant N2-src, the reaction was performed at 37°C for 60 minutes. Blots show the amount of munc18 (His), the amount of synaptophysin (SYP) and tyrosine phosphorylation (PY20).

(Barnekow et al., 1990; Linstedt et al., 1992; Onofri et al., 1997; Zhao et al., 2000; Evans and Cousin, 2005; Onofri et al., 2007). However no phosphorylation was observed with Munc 18. This suggests that Munc 18 cannot bind to N2-src directly or be phosphorylated by it, therefore its identification as a possible N2-src interaction partner is still open to question.

4.4 Discussion

The aim of this chapter was to identify novel interacting partners for the different src splice variant SH3 domains to identify proteins that might indicate a possible role for N1- and N2-src in neuronal function. It was found that the N2-src splice variant had binding partners different from the C-src splice variant. C-src, the most common splice variant, bound to dynamin I, synaptojanin, and synapsin I. N1-src had little binding to any nerve terminal protein but may have a stimulation-dependent interaction with dynamin I. In contrast N2-src specifically bound three proteins, which are NSF, Munc 18 and synaptophysin, all proteins involved in the synaptic vesicle life cycle.

4.4.1 C-src binding partners

Both N1- and N2-src splice variants are expressed only in neurons, whereas C-src is expressed in all cell types (Brugge et al., 1987; Levy et al., 1987; Martinez et al., 1987; Levy and Brugge, 1989; Pyper and Bolen, 1989; Raulf et al., 1989; Pyper and Bolen, 1990; Dorai et al., 1991). In this study, the investigation was focused on proteins that are found in the nerve terminals and their specific interactions to the SH3 domains of src splice variants. Thus C-src SH3 domain was treated as the reference for the neuronal specific splice variants, N1- and N2- src SH3 domains. Binding was compared in nerve terminal lysates plus and minus stimulation, which initiates synaptic vesicle turnover. Stimulation also causes the phosphorylation or dephosphorylation of proteins which can alter protein-protein interactions. For example, dephosphins such as dynamin I and synaptojanin are dephosphorylated

after stimulation (Cousin and Robinson, 2001; Cousin et al., 2001; Clayton et al., 2007; Evans and Cousin, 2007).

Synapsin I was observed to bind C-src but not N1- or N2-src splice variant SH3 domains, in agreement with previous studies (Onofri et al., 1997; Foster-Barber and Bishop, 1998; Onofri et al., 2000; Zhao et al., 2000; Onofri et al., 2007). Synaptojanin also bound only to C-src. It has been reported that synaptojanin binds to several other SH3 containing proteins including amphiphysin I and II, endophillin I and II, syndapin, and Grb2 (Bauerfeind et al., 1997; de Heuvel et al., 1997; Micheva et al., 1997; Onofri et al., 1997; Cestra et al., 1999; Hill et al., 2001; Zucconi et al., 2001; Schuske et al., 2003; Verstreken et al., 2003). This shows that even though synaptojanin has several binding partners containing SH3 domains, it binds specifically to C-src but not the neuronal splice variants suggesting that both N1-src and N2-src are not involved in synaptojanin interactions.

Dynamin I binds specifically to the C-src SH3 domain with little binding to N1- and N2-src SH3 domains, in agreement with previous studies showing that C-src interacts with dynamin I (Gout et al., 1993; Grabs et al., 1997; Okamoto et al., 1997; Foster-Barber and Bishop, 1998; Solomaha et al., 2005). This is also supported by previous findings where both synapsin I and dynamin I were shown to bind to C-src but not N1-src (Onofri et al., 1997; Foster-Barber and Bishop, 1998).

The N1-src splice variant SH3 domain bound very few nerve terminal proteins. It did bind to dynamin I, although binding was much lower when compared to the C-src SH3 domain. Interestingly an increase in dynamin I binding to N1-src SH3 domain was seen after stimulation (marked with stars in Figure 4.2) suggesting a possible

phosphorylation-dependent interaction. This stimulation dependent dynamin- N1-src interaction will be investigated in the next chapter.

4.4.1.1 N2-src binding partners

The N2-src splice variant displayed a completely different pattern of binding compared to C- and N1-src SH3 domains with interactions identified for Munc 18, NSF and synaptophysin. This is the first identification of N2-specific binding partners. In an effort to determine whether binding was specific for the N2-src SH3 domain, binding was compared across SH3 domains. When this was performed, Munc18 and synaptophysin displayed a very similar binding pattern across different SH3 domains, suggesting similarity in their binding sites. This might suggest that Munc 18 and synaptophysin have similar functions and they may compete for binding partners in nerve terminals.

4.4.1.2 Synaptophysin

In previous studies, synaptophysin was shown to co-precipitate with C-src using immunoprecipitation, co-localize with C-src using immunofluoresce and, interact with C-src SH2 domain using FRET (Barnekow et al., 1990; Linstedt et al., 1992; Zhao et al., 2000; Felkl and Leube, 2008). However, none of these studies showed a direct interaction between C-src SH3 domain.

Synaptophysin bound to the SH3 domain of the N2-src splice variant, while C-src had little binding to synaptophysin, suggesting the putative synaptophysin and C-src interaction is not via the C-src SH3 domain. N2-src SH3 domain interaction with synaptophysin may be direct, since it contains a classical type I PxxP motif

($_{228}$RAPPGAP$_{235}$) which is accessible for binding since it is in the cytoplasmic C-terminus, not the trans-membrane region (Figure 4.9 A).

However, *in vitro* experiments failed to show a direct interaction between synaptophysin C-terminus and N2-src in pull downs from bacterial lysates or recombinant proteins, casting doubt on a direct interaction. There are several possibilities explaining the lack of binding. The first possibility is that binding of N2-src might also require amino acid residues of the cytosolic loop of the trans-membrane domain of synaptophysin, with the C-terminus alone not sufficient for binding to the N2-src SH3 domain. To test this possibility several attempts were made to produce full length synaptophysin in bacteria and use it for pull downs. However, it was not easy to express probably due to synaptophysin being a trans-membrane protein. The great majority of protein was trapped in detergent-insoluble structures and was therefore unavailable for binding. Different expression systems such as eukaryotic cells like yeast or insect cells might improve expression and allow the investigation of binding to full length synaptophysin.

The other possibility is that the synaptophysin C-terminus needs to be phosphorylated to bind to N2-src. Synaptophysin is phosphorylated in a Ca^{2+}-dependent manner by both CaM kinase II (Ca^{2+}/ calmodulin-dependent protein kinase II) at two serine residues (Pang et al., 1988; Greengard et al., 1993; Rubenstein et al., 1993; Braun and Schulman, 1995; Evans and Cousin, 2005) and by src on multiple residues (Barnekow et al., 1990; Onofri et al., 1997; Zhao et al., 2000; Evans and Cousin, 2005; Onofri et al., 2007). If the synaptophysin C-terminus was subjected to phosphorylation by either CaM kinase II or src, and then used in binding assays it might show binding to N2-src, since serine and tyrosine

A Synaptophysin

```
  1 MDVVNQLVAG GQFRVVKEPL GFVKVLQWVF AIFAFATCGS YTGELELSVE CANKTESALN
 61 IEVEFKYPFR LRQVYFDAPS CVKGGTTKIF LVGDYSSSAE FFVTVAVFAF LYSMGALATY
121 IFLQNKYREN NKGPMMDFLA TAVFAFMGLV SSSAHAKGLS DVKNATDPEN IIKEMFNCRQ
181 TGNTCKELED PVTSGLNTSV VFGFLNLVLW VGNLWFVFKE TGWAAFTMRA PPGAPEKQPA
241 PGDAYGDAGY GQGPGGYGPQ DSYGPQGGYQ PDYGQPASGG GGYGPQGDYG QGGYGQQGAP
301 TSFSNQM
```

B Munc 18

```
  1 MAPIGLKAVV GEKIMHDVIK KVKKKGEHKV LVVDQLSMHM LSSCCKMTDI METGSITIVED
 61 INKRREPLPS LEAVYLITPS EKSVHSLISD FKDPPTAKYR AAEVFFTDSC PDALFMELVK
121 SHAAKVIKTL TEINIAFLPY ESQVYSLDSA DSFQSFYSPH KAQMKNPILE KLAEQIATLC
181 ATLKEYPAVR YRGEYKDNAL LAQLIQDKLD AYKADDPTNG EGPDKARSQL LILDRGFDPS
241 SPVLHELTPQ AMSYDLLPIE NDVYKYEISG IGEARVKEVL LDEDDDLWIA LRHKHIAEVS
301 GEVTRSLKDF SSSKEMNTGE KTTMEDLSQM LKKMPQYQKE LSKYSTHLHL AEDCMKHYQG
361 TVDKLCKVEQ DLAMGTDAEG EKIKDPMRAI VPILLDANVS TYDKIRIILL YIFLKNGITE
421 EMLNKLIQHA QIPPEDSEII TNMAHLGVPI VTDSTLRRRS KPEEKERISE QTYQLSRETP
481 IIKDIMEDTI EDKLDTKHYP YISTRSSASF STTAVSARYG HWEKNKAPGE YRSGPRLIIF
541 ILGGVSLNEM RCAYEVTQAN GKWEVLIGST ENLTPQKLLD TLKKLNKTDE EISS
```

C NSF

```
  1 MAGKTMQAAR CPTDELSLSN CAVVNEKDYQ SGQHVMVRTS PHHKYIPTLR THPSVVPGCI
 61 AFSLPQRHSA GLSIGQDIEV ALYSFDKAKQ CIGTMTIEID FLQKKNIDSN PYDTDKMAAE
121 FIGGFNFGAF SVGQGLVFSF NDKLPGLLVK DIEAMDPSIL KGEPASGKRQ KIEVGLVVGN
181 SQVAFEKAEN SSLNLIGKAK TKENRQSIIN PDNNFEKMGI GGLDKEFSDI FRRAFASRVF
241 PPEIVEQMGC KHVKGILLYG PPGCGKTLLA RQIGKMLNAR EPKVVNGPEI LNKYVGESEA
301 NIRKLFADAE EEQRRLGANS GLHIIIFDEI DAICKQRGSM AGSTGVHDTV VNQLLSKIDG
361 VEQLNNILVI GMTNRPDLID EALLRPGRLE VKMEIGLPDE KGRLQILEIH TARMRGHQLL
421 SADVDIKELA VETKNFSGAE LEGLVKAAQS TAMNRHIKAS TKVEVDMEKA ESLQVTRGDF
481 LASLENDIKP AFGTNQEDYA SYIMNSILKM GDFVTKVLDD GELLVQQTKN SDRTPLVSVL
541 LEGPPHSGKT ALAAKIAEES NFPFIKICSP DKMIGFSETA KCQAMKKIFD DAYKSQLSCV
601 VVDDIERLLD YVPIGPRFSN LVLQALLVLL KKAPPQGRKL LIIGTTSRKD VLQEMEMLNA
661 FSTTIHVPNI ATGEQLLEAL ELLGNFKDKE RTTIAQQVKG KKVWIGIKKL LMLIEMSLQM
721 DPEYRVRKFL ALMREESASP LDFD
```

Figure 4.9- ***synaptophysin, NSF and Munc 18 amino acid sequences.***

Sequences of synaptophysin, Munc 18 and NSF. The PxxP motifs that are expected to be N2-src SH3 domain binding sites are coloured with red.

phosphorylation can modulate protein interactions (Chung et al., 1997; Viollet et al., 1997; Pitcher et al., 1999; Mazurek et al., 2000; Ma et al., 2001).

Another possibility is that the synaptophysin/N2-src binding is indirect and occurs via other proteins. The major synaptophysin interacting partner in nerve terminal is VAMP 2 (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995; Khvotchev and Sudhof, 2004; Felkl and Leube, 2008) suggesting that VAMP 2 may mediate the interaction. However, our data showed that none of the SNARE proteins including VAMP 2 bound to the N2-src SH3 domain, suggesting that the synaptophysin-N2-src interaction is not through this protein. Further investigation is needed to identify the protein, if any, that is mediating synaptophysin-N2-src binding.

Synaptophysin is phosphorylated at multiple tyrosine residues on its C-terminus (Evans and Cousin, 2005) with previous studies showing that C-src can phosphorylate synaptophysin *in vivo* and *in vitro* (Barnekow et al., 1990; Linstedt et al., 1992; Onofri et al., 1997; Zhao et al., 2000; Evans and Cousin, 2005; Onofri et al., 2007). Our data showed that synaptophysin C-terminus was phosphorylated *in vitro* by all src splice variants as seen in figure 3.17 C and figure 4.8.

Since synaptophysin binds more to N2-src SH3 domain than C-src SH3 domain (as it was seen in the pulldowns from synaptosomal lysates figure 4.3), it is expected that N2-src would have a lower K_m than C- and N1-src and thus be more efficient in synaptophysin phosphorylation if tests were performed. This hypothesis would fit with the fact that N2-src is expressed only in neuronal cells where faster or specific responses might be essential. Since SH2 domains in all src splice variants are identical and they would bind to the phospho-tyrosine, there might be a

compensatory role for the SH2 domain in maintaining the interaction of C- and N1-src with synaptophysin, even if the initial binding between the SH3 domain and synaptophysin is not strong.

4.4.1.3 Munc 18

Munc 18 is known to be involved in regulating exocytosis through its binding to syntaxin, which is not an SH3 domain containing protein (Dulubova et al., 1999; Ciufo et al., 2005a; Schutz et al., 2005; Yerrapureddy et al., 2005; Dulubova et al., 2007; Rickman et al., 2007). Munc 18 had three modes of binding to syntaxin 1) when syntaxin is in the closed conformation 2) to the N-terminus of syntaxin when it is in an open conformation and 3) to syntaxin in the SNARE complex (Dulubova et al., 1999; Dulubova et al., 2007; Rickman et al., 2007; Shen et al., 2007; Burgoyne et al., 2009).

Munc 18 bound specifically to the N2-src SH3 domain with little binding to C-src and no binding to N1-src. The lack of binding to C- and N1-src is surprising since Munc 18 bound to most of the other screened SH3 domains. Thus Munc 18 interacts with a wide range of the SH3 domains, which might either suggest multiple roles of Munc 18 in the nerve terminal or that many proteins can regulate or modulate Munc 18 function.

Munc 18a, the neuronal isoform, contains only one PxxP motif ($_{239}$PSSP$_{242}$) which is a prospective binding site for N2-src SH3 domain (Figure 4.9 B). However, this PxxP motif may not be accessible when the crystal structure is examined, suggesting N2-src may not bind at this motif. Other possibilities exist such as a conformational

change when Munc 18 is phosphorylated, resulting in exposure of the PxxP motif to N2-src binding. In support of this, Munc 18 binding to syntaxin is regulated by phosphorylation. For example, Munc 18 serine phosphorylation by protein kinase C resulted in decreased binding affinity to syntaxin (de Vries et al., 2000; Barclay et al., 2003). Furthermore, in glucose-stimulated insulin secretion, stimulation resulted in a change in the Munc18c structure and tyrosine phosphorylation (Y219) of Munc 18c. This reduced binding to syntaxin 4 in pancreatic β cells, and increased binding to Doc2 β instead (Oh and Thurmond, 2006; Jewell et al., 2008). Munc 18c, a non-neuronal form, does not have the PxxP motif while both Munc 18a and b contain a PxxP motif (Oh and Thurmond, 2006). Since Munc 18 binding to syntaxin is regulated by tyrosine phosphorylation in other systems, it suggests a possible role for N2-src binding to Munc 18, whereby it might regulate Munc 18 binding to syntaxin. On the other hand, tyrosine phosphorylation might result in N2-src binding to Munc 18 and thus induce further phosphorylation for Munc 18 since Munc 18 has four possible tyrosine phosphorylation sites (Oh and Thurmond, 2006). Since N2-src did not bind to any of the SNARE proteins, there is a possibility that N2-src binds to free Munc 18 only and modulates its function via binding or phosphorylation or both.

The *in vitro* experiments failed to show any direct interaction between Munc 18 and N2-src in pull downs from bacterial lysates or as recombinant proteins in binding assays. This might be due to the fact that Munc 18 aggregated when purified. This aggregation caused false results in the binding assays since the aggregated Munc 18 could not pass through the spin columns during sample washes, resulting in apparent binding to all GST fusion proteins. This problem was overcome by performing pull downs direct from bacterial lysates, however no binding was observed. This leaves

the possibility that there might be phosphorylation needed in order to expose the N2-src SH3 domain binding site and facilitate binding. Another possibility is that Munc 18 binding is mediated by syntaxin or the other SNARE complex. However none of the SNARE complex proteins bound to N2-src, suggesting that Munc 18 is not binding to N2-src via these proteins.

Munc 18 aggregation in fact might explain the lack of specific binding as well as the lack of phosphorylation in the kinase assay. The same kinase assay showed tyrosine phosphorylation for synaptophysin C-terminus it failed to show any phosphorylation for Munc 18. The question of whether Munc 18 is phosphorylated by N2-src is still not answered, and if Munc 18 is immunoprecipitated from nerve terminal (if it does not aggregate) and then used as a substrate for N2-src it might be phosphorylated. Expressing Munc 18 in a baculovirus expression system might reduce the aggregation problem too (Hu et al., 2003). Thus the ability of Munc 18 to either bind or be a substrate for N2-src is still open to question.

4.4.1.4 NSF

NSF bound to N2-src SH3 domain but not to C- or N1-src SH3 domains. Among the SH3 domains that were screened, only N2-src, P85, and endophillin I and II SH3 domains bound NSF, suggesting a more specific interaction. NSF contains one PxxP motif ($_{613}$PIGP$_{616}$), which is a prospective N2-src SH3 domain binding site (Figure 4.9 C). There is no crystal structure of NSF to confirm if this motif is available for binding, similar to synaptophysin, or whether a conformation change is needed to make it available (similar to Munc 18). N2-src did not bind to the SNARE proteins

suggesting that it might have a direct binding to NSF rather than indirect interaction through SNARE proteins.

NSF has an ATPase activity and its role in disassembling SNARE complex allows SNARE proteins to recycle (Matveeva and Whiteheart, 1998; Matveeva et al., 2002; Whiteheart and Matveeva, 2004). NSF binding to the SNARE complex stimulates its ATPase activity (Haynes et al., 1998; Matveeva and Whiteheart, 1998). NSF is phosphorylated at S237 by protein kinase C, and this phosphorylation prevents NSF binding to SNARE complexes (Matveeva et al., 2001). NSF is also phosphorylated at S569 by Pctaire I, and this phosphorylation regulates NSF oligomerization (Liu et al., 2006). In support of a direct role for src binding, NSF is also tyrosine phosphorylated at Y83 and is dephosphorylated by the tyrosine phosphatase PTP-MEG2, resulting in an increase in its ATPase activity (Huynh et al., 2004). Thus, there is a possibility that N2-src phosphorylates NSF and regulates its ATPase activity. Whether the N2-src SH3 domain binds to NSF directly or NSF serine phosphorylation is required before N2-src binds is still to be investigated. In addition, further investigations still have to be performed in order to confirm the role of N2-src binding and phosphorylation in regulating NSF activity.

4.4.2 Limitations of this approach

There are advantages and disadvantages of the strategy that were used. The advantages of this strategy include 1) Proteins are taken from brain directly, not from cell lines, which would reflect the same structure and status that the proteins have in neurons, and 2) these are only from the synaptic region highlighting possible interactions in synaptic vesicle recycling. Other advantages include 3) protein

binding can be tested in basal or stimulated conditions to reflect the effect of the mechanism that controls synaptic vesicle release on protein interactions, and 4) Proteins are phosphorylated endogenously. Another advantage is 5) the interference of other src domains was eliminated by using only SH3 domains and any difference in binding would be related to the splicing difference of the SH3 domains alone. The flip side of this advantage however is that these SH3 domains might not act the same way if the whole protein was used, where the other src domains might facilitate or inhibit the SH3 domain interactions with other proteins.

Other disadvantages include 1) N2-src might not be found in the synaptic region and thus it might not encounter these proteins in live neurones. It is not clear whether N2-src is expressed in all neuronal cells or it is specific for some cell types or specific regions. There is no N2-src specific antibody available and C-src SH3 domain antibodies do not recognize all the splice variants equally. We twice attempted to produce N2-src specific antibodies, however these studies met with no success.

Further disadvantages also include the fact that 2) using lysates would destroy the structure of the nerve terminal causing proteins to be disorganised. This means proteins that bound to N2-src may not normally encounter each other in intact neurones. 3) the *in vitro* assay does not reflect the real interaction in nerve terminals where other proteins might interfere by inhibiting or facilitating interactions. Other disadvantages of this method are 4) that some proteins are hard to express and purify, especially if these proteins are toxic to the host system (such as src and full length synaptophysin), and also 5) some proteins are degraded rapidly or they aggregate (such as Munc 18) which will make them unusable for further experiments.

Chapter 5

Phosphorylation-dependent dynamin I interaction with N1-src

5. Phosphorylation-dependent dynamin I interaction with N1-src

5.1 Introduction

Dynamin I is an important protein in vesicle recycling where it causes the internalization of the vesicle by wrapping itself around the vesicle's neck and causing it to be separated from the membrane (Takei et al., 1995; Takei et al., 1996; Lu et al., 2008). Many SH3 domain containing proteins are known to bind to the dynamin I PRD including syndapin I, endophilin I & II, amphiphysin I & II, Grb2, PLCγ (Phospholipase Cγ), LYN, P85, and intersectin (Gout et al., 1993; Seedorf et al., 1994; Grabs et al., 1997; Okamoto et al., 1997; Solomaha et al., 2005; Anggono et al., 2006; Elhamdani et al., 2006; Anggono and Robinson, 2007). Several proteins seem to modulate dynamin I function either by direct binding such as amphiphysin which results in stimulation of dynamin I GTPase activity (Yoshida et al., 2004), or by phosphorylation such as CDK5 which phosphorylates dynamin on multiple sites and reduces dynamin I recruitment to the membrane (Tan et al., 2003; Smillie and Cousin, 2005).

The protein tyrosine kinase, C-src, has been identified as a binding partner for dynamin I and II in neurones and other non neuronal cells (Foster-Barber and Bishop, 1998; Bruzzaniti et al., 2005), and can tyrosine phosphorylate dynamin I in non neuronal cells (Ahn et al., 1999; Ahn et al., 2002; Shajahan et al., 2004). The src SH3 domain and dynamin I PRD domain were the main interaction points between these two proteins (Gout et al., 1993; Solomaha et al., 2005). However, N1-src did not show any interaction with dynamin I (Gout et al., 1993; Foster-Barber and Bishop, 1998).

The aim of these experiments is to compare serine phosphorylation of dynamin I PRD on both C- and N1-src binding, and also to determine whether C- and N1-src bind to the dynamin I PRD. All experiments involving full length src constructs within this chapter were performed using kinase dead variants, the merits of which are discussed in the general discussion. The previous chapter demonstrated that dynamin I binds to N1-src in a stimulation dependent manner, therefore the hypothesis is that N1-src but not C-src binds to dynamin I in a phosphorylation dependent manner, and that they would have distinct binding sites with N1-src binding being regulated by serine phosphorylation.

5.2 Effect of dynamin I phosphorylation on its interaction with N1-src

Protein phosphorylation regulates a number of protein-protein interactions, including those of dynamin I. It was therefore tested whether the previously observed lack of N1-src binding was due to dynamin I phosphorylation. To test this hypothesis, pull downs from resting rat brain synaptosomal lysates were performed using GST-tagged C-, N1-, N2-src SH3 domains. Dynamin I was observed to bind to the C-src SH3 domain (Figure 5.1 A). This finding agrees with the previous finding from chapter 4 (Figure 4.2) and others (Gout et al., 1993; Foster-Barber and Bishop, 1998). Phosphorylated dynamin I was bound to C-src, but not N1-src or N2-src (Figure 5.1 A), suggesting dynamin I phosphorylation may play a role in regulating N1-src and N2-src interactions. Since dynamin I phosphorylation is controlled by stimulation, its binding to C-src and N1-src SH3 domains was examined in resting and stimulated nerve terminal lysates. Synaptosomal lysates were treated with 10 µM cyclosporin A which prevents dynamin I dephosphorylation through its inhibition of

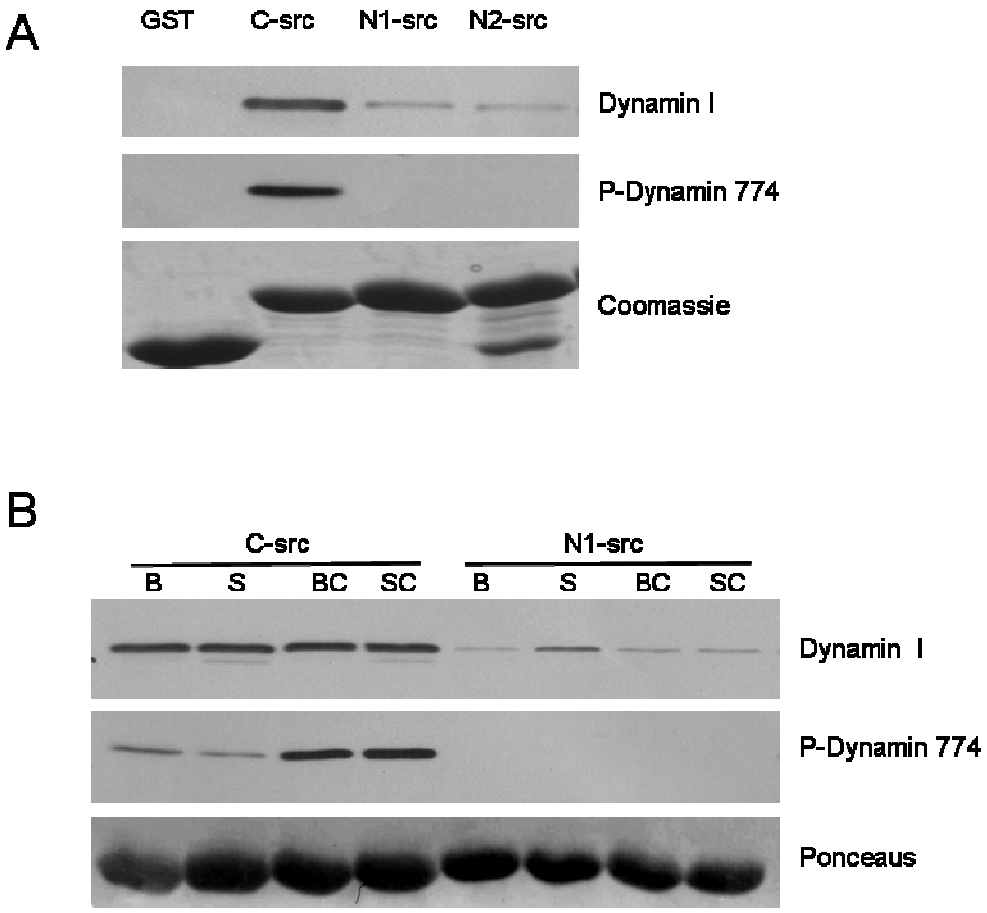


Figure 5.1- ***The effect of dynamin I phosphorylation on C-src and N1-src SH3 domain binding.***

A) GST pull downs were performed using C-, N1-, N2-src SH3 domains from rat nerve terminal lysates. Western blot shows dynamin I and phospho-dynamin (P-774) binding to the different src splice variants. Ponceaus stain shows the amount of GST fusion protein in the assay. Blots are representative of n=3 experiments.

B) GST pull downs were performed using either C-src or N1-src SH3 domains from rat nerve terminal lysates. Lysates were taken from resting nerve terminals (B), stimulated (30 mM KCl for 10 seconds S) or resting or stimulated in the presence of 10 μM cyclosporine A (BC, SC). Western blot shows binding of dynamin I and phospho-dynamin (P774) to C-src and N1-src SH3 domains. Blots are representative of n=3 experiments.

calcineurin (Faulds et al., 1993; Jorgensen et al., 2003). C-src SH3 domain showed no noticeable difference in total dynamin I binding between the stimulated and non-stimulated conditions either in the presence or absence of cyclosporin A (Figure 5.1B). This suggests that C-src binds to dynamin I in a phosphorylation-independent manner. However, more dynamin I bound to N1-src after stimulation (when dynamin I is dephosphorylated) supporting the hypothesis that N1-src binds to dynamin I in a phosphorylation-dependent manner (Figure 5.1B). No increase in dynamin I binding was observed in stimulated synaptosomal lysates with cyclosporin A. To confirm the phosphorylation dependency of this interaction, a phospho-dynamin I 774 antibody was used on the same samples. The amount of phospho-dynamin I bound to C-src in stimulated conditions was increased in samples treated with cyclosporin A, in contrast to the similar extent of total dynamin I binding. This confirms that C-src binding to dynamin I is phosphorylation-independent. No phospho-dynamin I was detected bound to N1-src in any condition. These findings suggest that C-src binding to dynamin I is phosphorylation-independent, while N1-src only binds to dynamin I in its dephosphorylated state.

5.3.1 Effect of dynamin I PRD mutations at S774/778

Dynamin I is phosphorylated at two major sites in nerve terminals, S774 and S778 (Tan et al., 2003; Smillie and Cousin, 2005; Graham et al., 2007). To determine whether these sites are important in the regulation of N1-src binding, two mutations (Figure 5.2) of the PRD were used. These mutations were a double phospho-null mutation, where both serine residues were mutated to alanine (DynI^{dmA}-PRD), and a

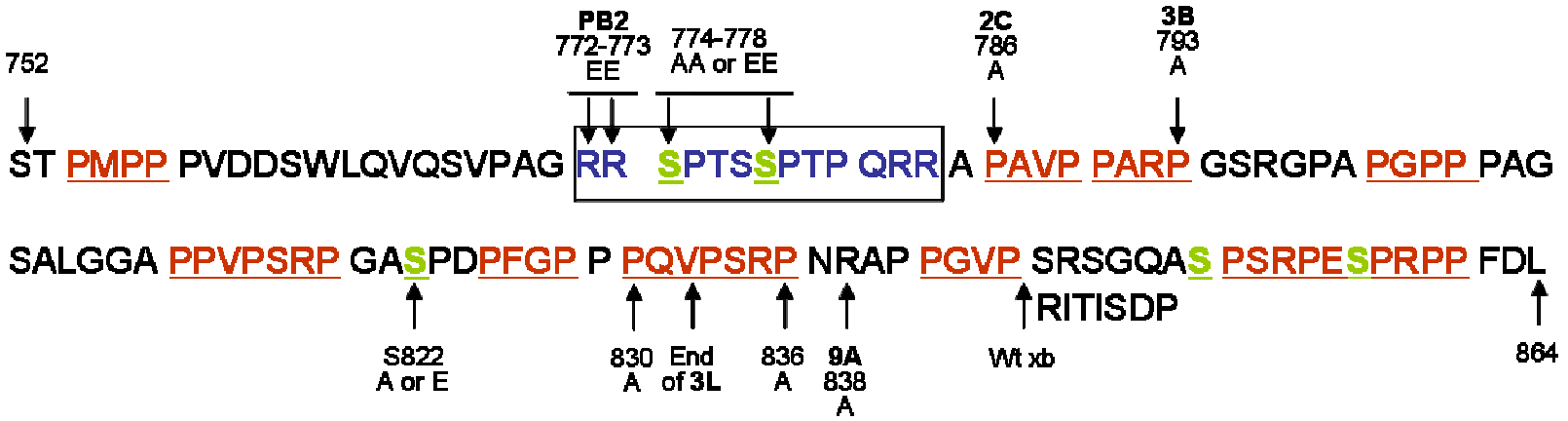


Figure 5.2- ***The amino acid sequence of the dynamin I proline rich domain (PRD) with all mutations.***

This figure shows the amino acid sequence of the dynamin I PRD, all the PxxP motifs are underlined and marked red, the phosphobox marked blue and surrounded by a rectangle and other serine residues known to be phosphorylated marked green. The mutations that are used in these experiments are shown with the respective changes that were made. PB2 is a mutation in the N-terminal end of the phosphobox where both of the argenine residues RR772/773 were mutated to glutamate, the serine residues SS 774)778 were mutated to either alanine (phosphonull) or glutamate (phosphomemetic), the mutant 2C is a mutation in the PxxP motif 2, the mutant 3B is a mutation in PxxP motif 3, the serine residue S822 was mutated to either alanine or glutamate, the mutant P830A is a mutation in PxP motif 8, the mutant 3L is a truncation where PxxP motifs 8, 9, 10, 11, 12 and 13 were removed, The mutant P836A is a mutation in PxxP motif 9 and the mutant 9A is a mutaion in the argenine residue flanking PxxP motif 9. WT xb has an alternatively spliced c-terminus of the PRD after PxxP motif 10

double phosphomimetic mutation where both serine residues were mutated to glutamate (DynI^{dmE}-PRD). Previous studies have shown that phosphomimetic, but not phospho-null mutants, inhibit the binding of the endocytosis protein syndapin I (Anggono et al., 2006).

To investigate the effect of these phosphorylation sites on C- and N1-src interactions GST-tagged PRD wild type, GST-tagged Dyn^{dmA}-PRD, and GST-tagged Dyn^{dmE}-PRD were used to pull down His-tagged C- and N1-src SH3 domains from bacterial lysates (Figure 5.3). These experiments failed to show any significant effect of mutagenesis of the S774/778 sites on the dynamin I PRD interaction with C- or N1-src SH3 domains, suggesting that phosphorylation of S774 and S778 do not play a role in the regulation of binding to either C- or N1-src SH3 domains.

To confirm the lack of effect of either S774 or S778 in regulating src binding, these experiments were repeated using kinase dead full length His-tagged src. This was because it has previously been observed that syndapin I SH3 domain itself showed no phosphorylation dependency in its binding to dynamin I, whereas the whole protein bound in a phosphorylation dependent manner (Anggono et al., 2006). Therefore, to determine whether full length C- and N1-src splice variant binding could be regulated by mutagenesis of S774 and S778, a new series of pull downs were performed (Figure 5.4). Neither mutation of the dynamin I PRD had any effect on full length C-src binding, confirming the lack of role for these sites. Similarly there was no significant effect of both mutations on full length N1-src binding. Thus the phosphorylation-dependent regulation of the dynamin I-N1-src interaction is not due to sites S774 and S778.

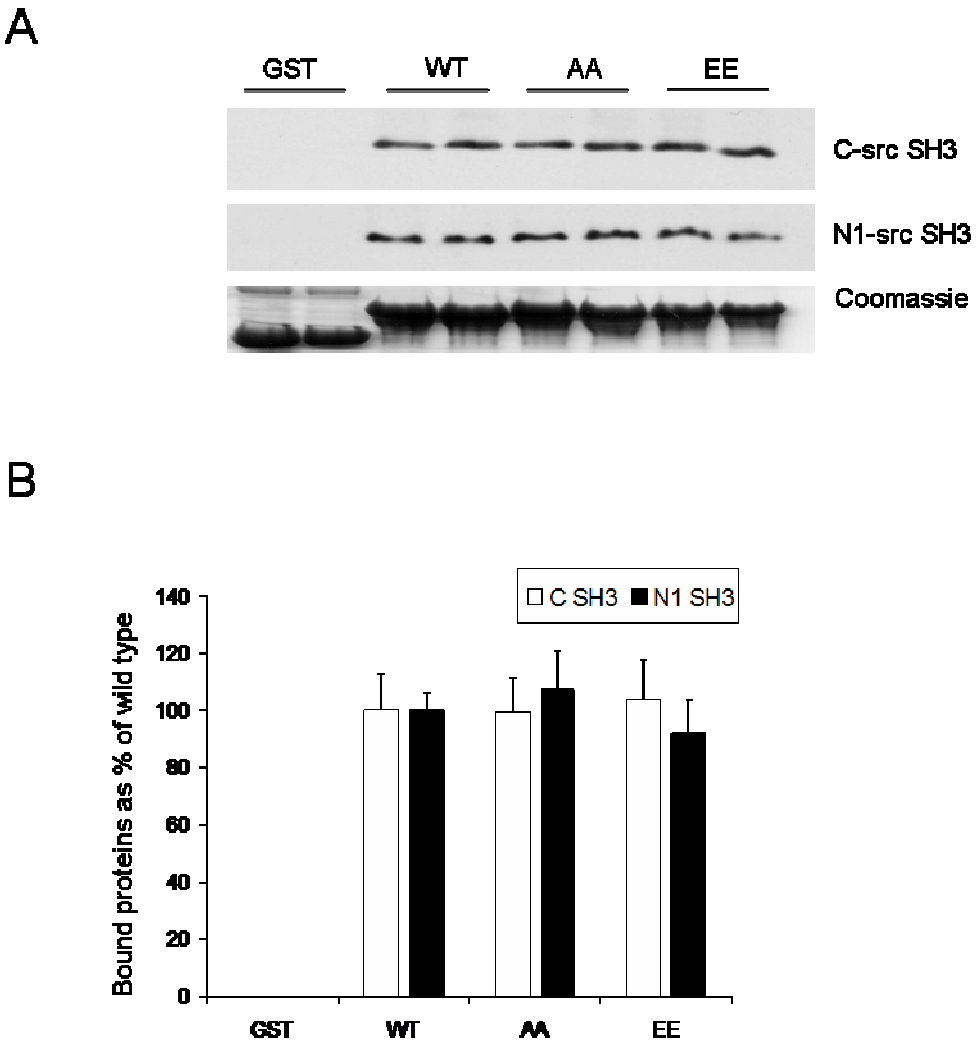


Figure 5.3- ***The effect of phosphomimetic or phospho-null mutations in the phosphobox of dynamin I on binding to the SH3 domains of N1- and C-src.***

A) GST pull downs were performed using GST-PRD mutants of dynamin I (Wt = wild type, AA = S774, 778 A, EE = S774, 778 E (see figure 5.2). Pull downs were performed using bacterial lysates expressing either C-, or N1-src SH3 domains tagged with His. Western blot for His showing the binding of His-C-src SH3 and His-N1-src SH3 to different PRD mutants. Coomassie gel shows GST-PRD mutants that were used for the pull downs. Experiment was performed in duplicate.

B) Comparison of SH3 domains of C-src (open bar) and N1-src (solid bar) bound to dynamin I PRD mutants. The values are normalized to PRD levels expressed as a percentage of wild type. One way ANOVA was performed and there were no significant differences. Experiments were performed n=4. Data presented as mean ± SEM

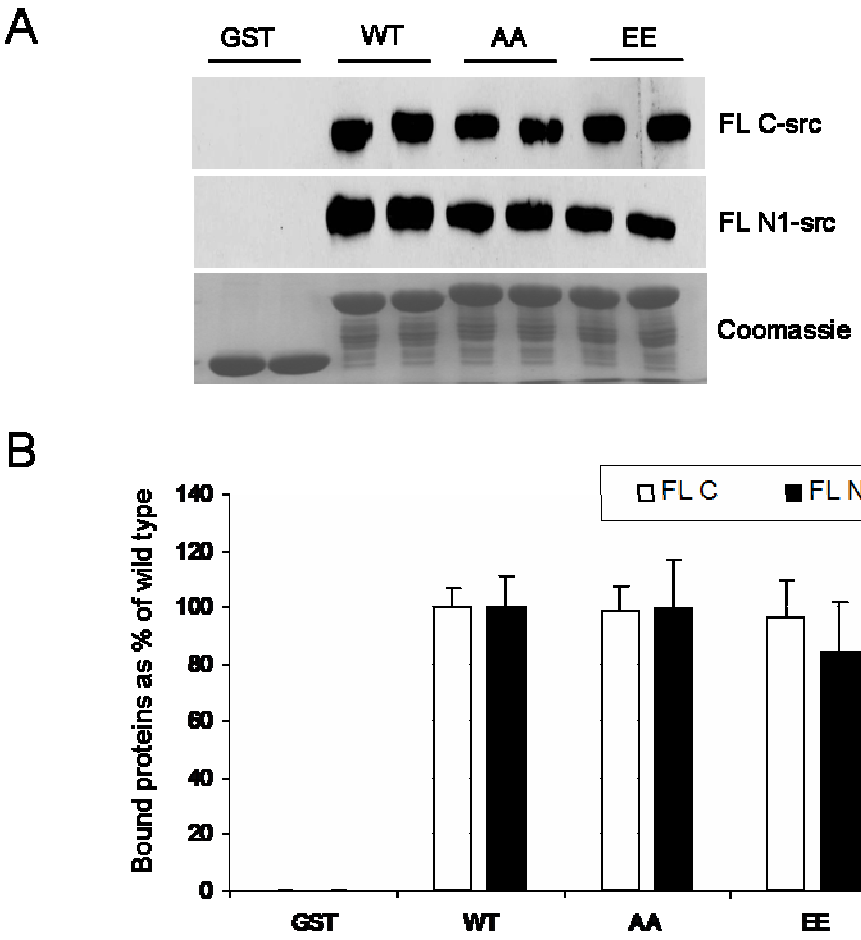


Figure 5.4- ***The effect of phosphomimetic or phospho-null mutations in the phosphobox of dynamin I on binding to full length C- and N1-src.***

A) GST pull downs were performed using GST-PRD mutants of dynamin I (WT = wild type, AA = S774, 778 A, EE = S774, 778 E (see figure 5.2). Pull downs were performed using bacterial lysates expressing either full length C-, or N1-src tagged with His. Western blot for His showing the binding of His –full length C-src and His-full length N1-src to different PRD mutants. Coomassie gel shows GST-PRD mutants that were used for the pull downs. Experiment was performed in duplicate.

B) Comparison of full length C-src (open bar) and N1-src (solid bar) bound to dynamin I PRD mutants. The values are normalized to PRD levels expressed as a percentage of wild type. One way ANOVA was performed and there were no significant differences. Experiments were performed n=4. Data presented as mean ± SEM

5.3.2 Effect of dynamin I PRD mutations at S774/778 on syndapin I, endophilin, and amphiphysin binding

To confirm that the DynI^{dmA}-PRD and DynI^{dmE}-PRD mutations were acting in a similar manner to previous publications, syndapin I, endophilin, and amphiphysin binding was examined. Previous experiments showed that the DynI^{dmE}-PRD mutant inhibited binding of syndapin I and endophilin, while amphiphysin binding was unaffected (Anggono et al., 2006).

The experiments were performed using GST-tagged dynamin I PRD, Dyn^{dmA}-PRD, Dyn^{dmE}-PRD to pull down binding proteins from rat brain synaptosomal lysates (Figures 5.5 and 5.6). Western blots showed that endophilin binding to both Dyn^{dmA}-PRD and Dyn^{dmE}-PRD was reduced by more than 50 %, while syndapin I and amphiphysin binding was unaffected. These results are contrary to those observed by Anggono and coleagues where they observed inhibition of syndapin I binding in addition to endophilin (Anggono et al., 2006).

5.3.3 Dynamin I PRD mutations (2C, 3B, 9A, and PB2)

The lack of effect of Dyn^{dmA}-PRD and Dyn^{dmE}-PRD mutations, suggested that neither C- or N1-src bound near the phosphobox region of dynamin I, which includes S774 and S778 (Figure 5.2). To confirm this, dynamin I PRD mutants were employed that disrupt the binding of syndapin I, endophilin and amphiphysin (Anggono and Robinson, 2007). Both endophilin and syndapin I bind to PxxP motifs 2 and 3 of dynamin I PRD. In addition syndapin I interacts with the whole phosphobox, while endophilin needs only the two arginine residues that are adjacent

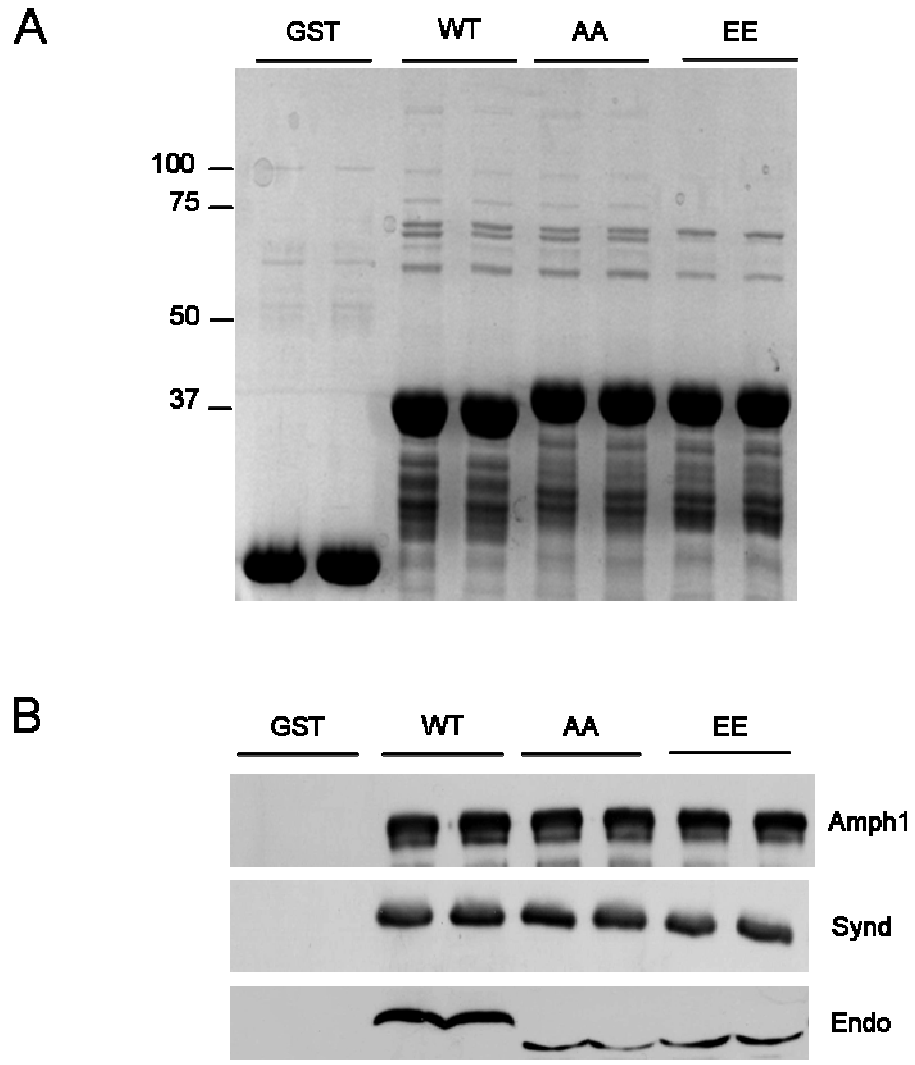


Figure 5.5- ***The effect of phosphomimetic or phospho-null mutations in the phosphobox of dynamin I on binding to amphiphysin I, syndapin, and endophillin.***

A) GST pull downs were performed using GST-PRD mutants of dynamin I (WT= wild type, AA = S774, 778 A, EE = S774, 778 E (see figure 4.2). Pull downs were performed using rat nerve terminal lysates. Coomassie stained gel shows the binding of PRD mutants to different binding partners. Experiment was performed in duplicates.

B) Western blot shows the effect of phosphomimetic and phospho-null mutations of dynamin I on binding to amphiphysin I (amph I),syndapin (Synd), and endophillin (endo).

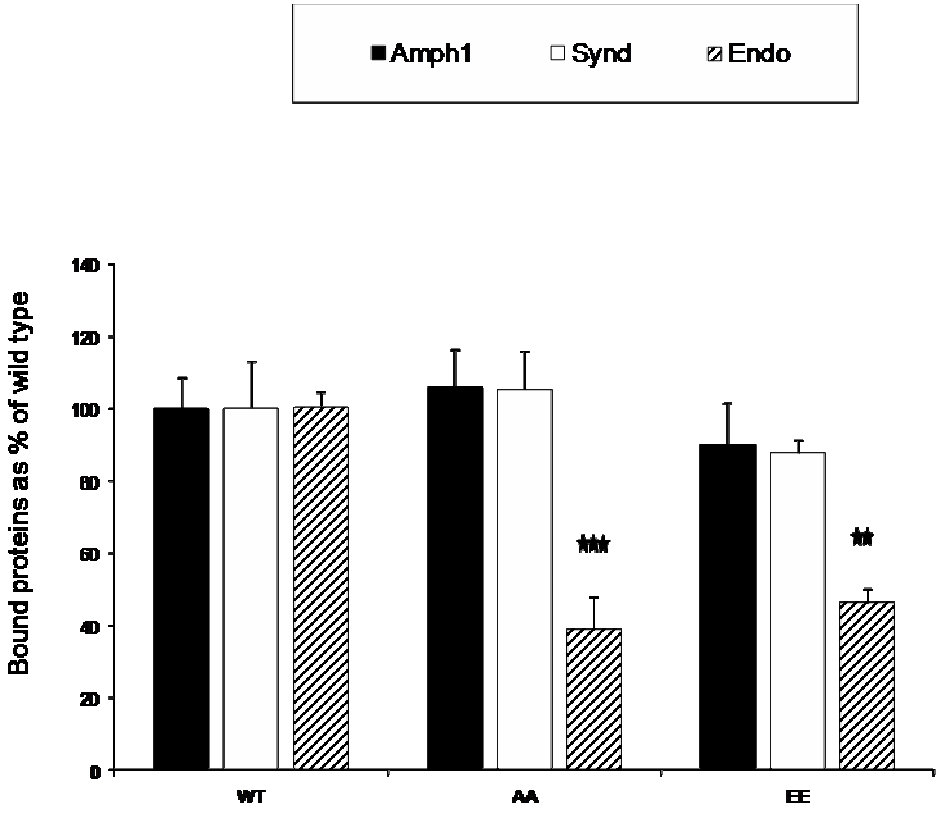


Figure 5.6- ***Comparison of the effect of phosphomimetic or phospho-null mutations in the phosphobox of dynamin I on binding to amphiphysin I syndapin, and endophillin.***

Comparison of amphiphysin I, syndapin, and endophillin bound to dynamin I PRD mutants (W t= wild type, AA = S774, 778 A, EE = S774, 778 E (see figure 5.2). The values are normalized to PRD levels expressed as a percentage of wild type Endophillin = open bar, Syndapin = hatched bar, Amphiphysin = solid bar. One way ANOVA was performed and significant differences as follows (** = P<0.01, *** = P<0.001). Experiments were performed n=4. Data presented as mean ± SEM

to PxxP motif 2 (Figure 5.2). Amphiphysin binds to a different site, PxxP 9, near the C-terminus (Grabs et al., 1997; Anggono and Robinson, 2007).

GST-tagged PRD (2C, 3B, 9A, and PB2) binding site mutants were used to perform pull downs for full length C- and N1-src splice variants from bacterial lysates (see figure 5.2 for details). Full length C-src binding to the dynamin I PRD was not affected by any of these mutants, suggesting C-src did not bind to any of these sites. N1-src binding to the dynamin I PRD was not affected by the 2C and 3B mutations, but binding to the 9A and PB2 mutants was greatly affected, with an approximate 80 % decrease in binding to the 9A mutant and more than 90 % decrease in binding to the PB2 mutant (figure 5.7). The results suggest that neither PxxP motifs 2 and 3 are binding sites for N1-src. In contrast, N1-src binding is affected by the PB2 mutation, suggesting that arginine residues R772, R773 may form part of the N1-src binding site. The ablation of N1-src binding with the 9A mutant suggests that N1-src may have more than one interaction site on the dynamin I PRD.

5.3.4 Effect of dynamin I PRD mutations (2C, 3B, 9A, and PB2) on endophilin, syndapin I, and amphiphysin binding

To confirm that the dynamin I PRD mutants were acting as previously published, these experiments were repeated using rat brain synaptosomal lysates (Figures 5.8 and 5.9). None of the mutants had any effect on syndapin I binding. Endophilin binding to the 2C mutant was unaffected, but its binding to the other mutants was reduced by at least 50 % (Figure 5.9). Amphiphysin binding was only affected by the 9A mutation. These results were not expected for a number of reasons. Syndapin I was expected to be affected by 2C, and PB2 mutants since these mutants showed

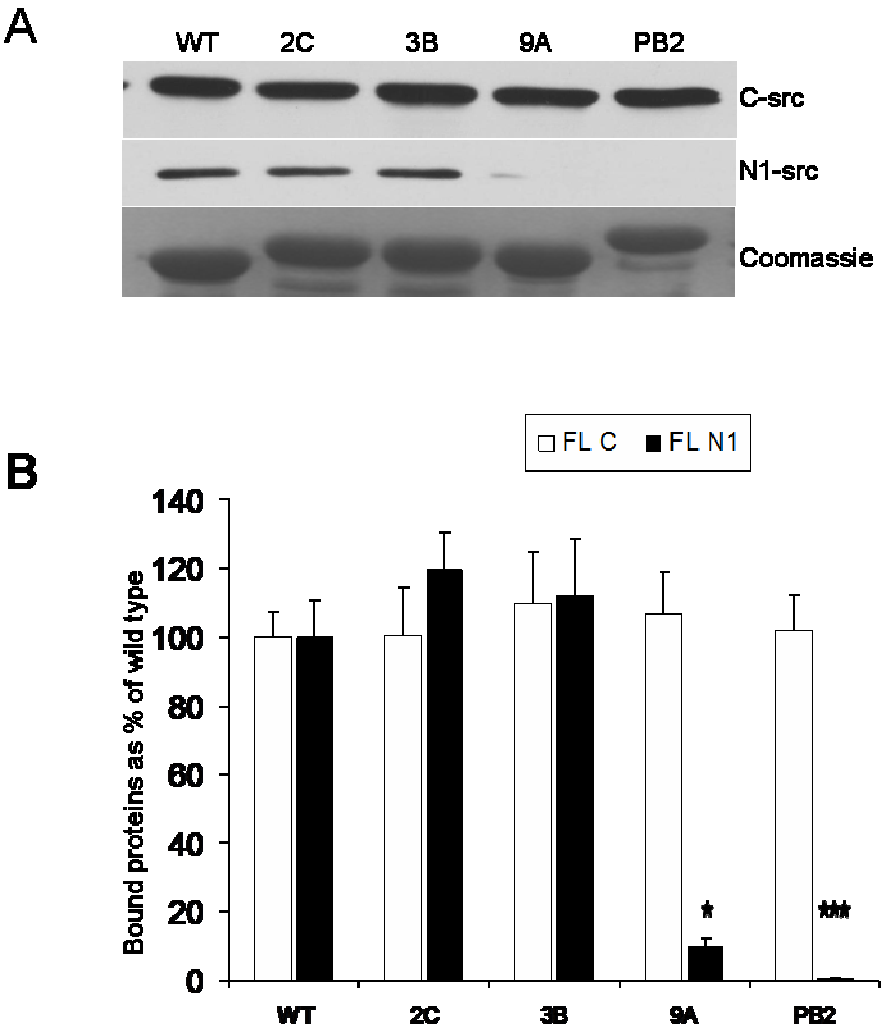


Figure 5.7- *The effect of mutations in the phosphobox and PXXP sites 2, 3, 9 on full length C- and N1-src binding to dynamin PRD.*

A) GST pull downs were performed using GST-PRD mutants of dynamin I (WT = wild type, 2C = P786A, 3B = P793A, 9A = R938A, PB2 = R772, 773 E (see figure 5.2). Pull downs were performed using bacterial lysates expressing either full length C-, or N1-src tagged with His. Western blot of His showing the binding of C-src and N1-src to different PRD mutants. Coomassie gel shows GST-PRD mutants that were used for the pull down.

B) Comparison of C-src (open bar) and N1-src (solid bar) bound to dynamin I PRD mutants. The values are normalized to PRD levels expressed as a percentage of wild type. One way ANOVA was performed and significant differences as follows (* = $P<0.05$, *** =$P<0.001$). Experiments were performed n=4. Data presented as mean ± SEM.

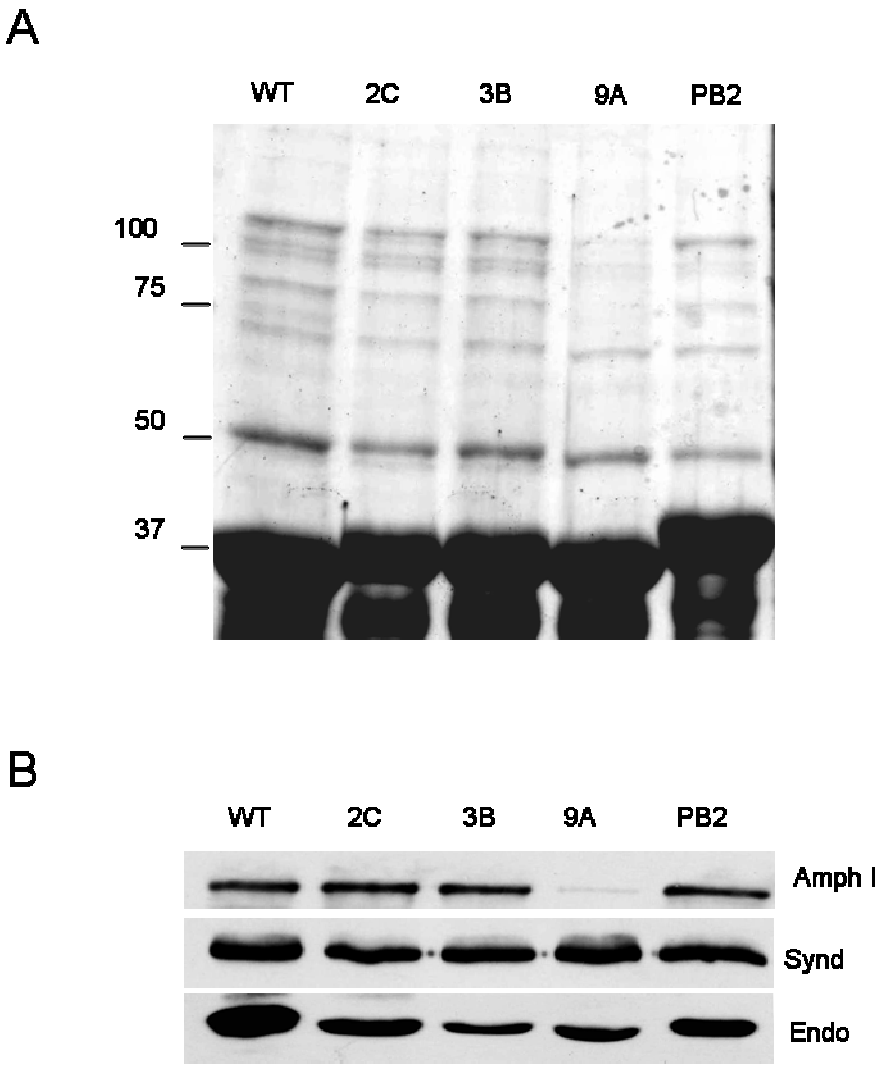


Figure 5.8- ***The effect of mutations in the phosphobox and PXXP sites 2, 3, 9 on amphiphysin I, syndapin, and endophillin binding to dynamin I PRD.***

A) GST pull downs were performed using GST-PRD mutants of dynamin I (WT = wild type, 2C = P786A, 3B = P793A, 9A = R938E, PB2 = R772, 773 E (see figure 5.2). Pull downs were performed using rat nerve terminal lysates. Coomassie stain gel shows the binding affinity of PRD mutants to different binding partners.

B) Western blot shows the binding of syndapin (Synd), endophillin (Endo), and amphiphysin I (Amph I) to different PRD mutants.

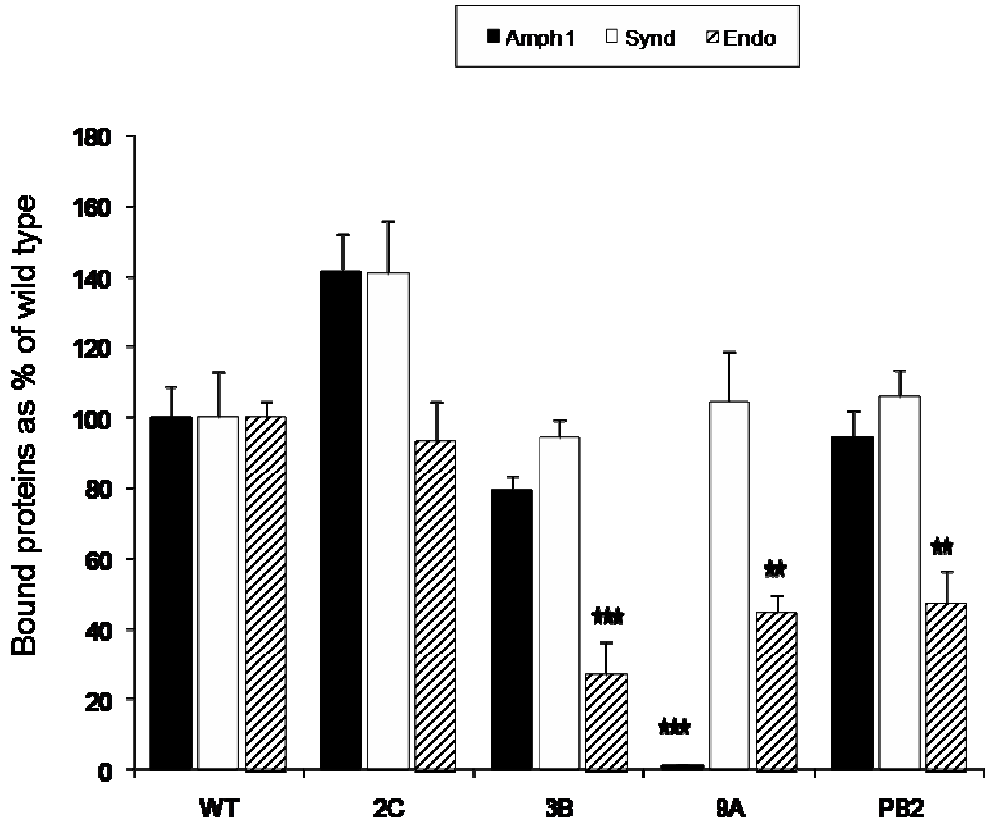


Figure 5.9- ***Comparison of the effect of mutations in the phosphobox and PXXP sites 2, 3, 9 on amphiphysin I, syndapin and endophillin binding to dynamin I PRD.***

Comparison of amphiphysin I (solid bar), syndapin (open bar), and endophillin (hached bar) bound to dynamin I PRD mutants (WT = wild type, 2C = P786A, 3B = P793A, 9A = R938A, PB2 = R772, 773 E (see figure 5.2). The values are normalized to PRD levels expressed as a percentage of the wild type. One way ANOVA was performed and significant differences as follows (** = P<0.01, *** = P<0.001). Experiments were performed n=4. Data presented as mean ± SEM

significant decrease in syndapin I binding to the dynamin I PRD when used previously (Anggono and Robinson, 2007). Endophilin showed the expected results in binding to 3B and 9A, however the PB2 mutation was not expected to affect its binding to the PRD, while the 2C mutant was expected to reduce it's binding to the PRD. Amphiphysin binding occurred as expected and was only affected by the 9A mutation.

5.3.5 Dynamin I PRD mutations (S822A and S822E)

Since mutations at either S774, S778 or sites 2 and 3 had no effect on the N1-src interaction, the role of another phosphorylation site was investigated. The binding of N1-src at sites 8 and 9 was also investigated. These sites are adjacent to S822 which is a minor phosphorylation site on the dynamin I PRD (Graham et al., 2007). The 3L mutant was used to determine whether the C-terminal region of dynamin I PRD starting from PxxP motif 8 was essential for N1-src binding to dynamin I. Since the deletion of the C-terminal region of the dynamin I PRD after V832 blocked amphiphysin binding (Grabs et al., 1997), it may also affect N1-src binding to dynamin I, since N1-src binding to the dynamin I PRD was affected by the 9A mutation (Figure 5.7). To test this hypothesis, this mutant was used with the wild type PRD to perform pull downs for full length C- and N1-src splice variants from bacterial lysates. C-src binding to the dynamin I PRD was not affected by this 3L mutant. On the other hand, N1-src binding to the dynamin I PRD was greatly affected, with an approximate 90 % decrease in binding observed (figure 5.10). This suggests that the C-src binding site might be before PxxP motif 8, and the N1-src binding after this motif. To narrow down the region which N1-src could be

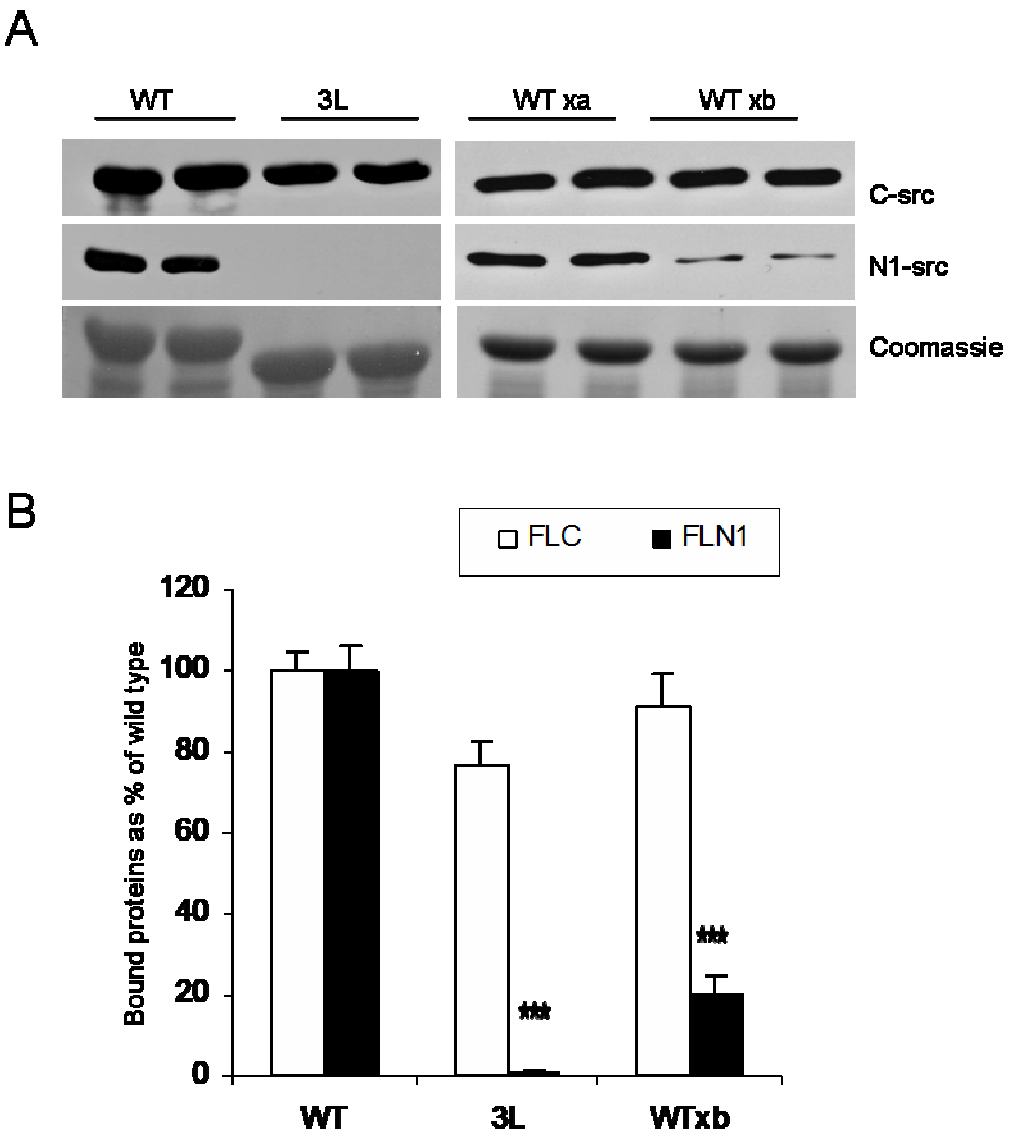


Figure 5.10- ***The effect of truncating the dynamin I PRD and splice variant WTxb on full length N1- and C-src binding.***

A) GST pull downs were performed using GST-PRD mutants of dynamin I (WT= wild type, 3L= truncated at V832, WTxb= splice variant xb (see figure 5.2). Pull downs were performed using bacterial lysates expressing either full length C-, or N1-src tagged with His. Western blot for His showing the binding of C-and N1-src to different PRD mutants. Coomassie gel shows GST-PRD mutants that were used for the pull downs. Experiment was performed in duplicate.

B) Comparison of C-src (open bar) and N1-src (solid bar) bound to dynamin I PRD mutants. The values are normalized to PRD levels expressed as a percentage of wild type. One way ANOVA was performed and significant differences as follows (*** = P<0.001). Experiments were performed n=4. Data presented as ± SEM

binding to, a dynamin I PRD splice variant (WT xb) was used. Dynamin I xb PRD is similar to the dynamin I xa PRD up to PxxP motif 10, but has no PxxP motifs after this point (Figure 5.2). C-src binding to the xb PRD was not different compared to the xa PRD (Figure 5.10). On the other hand, N1-src binding to this splice variant was reduced by approximately 75 % (Figure 5.10). This decrease could mean that N1-src needs the C-terminal PxxP motifs 11, 12, and 13 for its binding to dynamin I PRD, suggesting differential binding of N1-src to xa and xb dynamin I.

Serine residue 822 of dynamin I is phosphorylated during basal conditions and dephosphorylated during stimulation (Graham et al., 2007). S822 is adjacent to site 8 and 9 suggesting potential regulation of N1-src binding by phosphorylation. To test this hypothesis phospho-null (S822A) and phosphomimetic (S822E) mutations were used with wild type xa dynamin I PRD to perform pull downs for full length C- and N1-src splice variants from bacterial lysates. C-src binding to the xa PRD was not affect by these mutants. On the other hand N1-src binding to the PRD was decreased about 50 % by the phospho-null mutant S822A and about 60 % by the phosphomimetic mutant S822E (Figure 5.11). These findings suggest that S822 phosphorylation might regulate N1-src binding to dynamin I. Furthermore, this serine residue might be part of the N1-src binding site since even the phospho-null mutation resulted in binding inhibition.

5.3.6 Effect of dynamin I PRD mutants (S822A, S822E and 3L) and wt xb on syndapinI, endophilin, and amphiphysin binding

The effect of the S822A, S822E, and 3L mutants, and WT xb were also investigated in pull downs from synaptosomal lysates to test their effects on known interaction

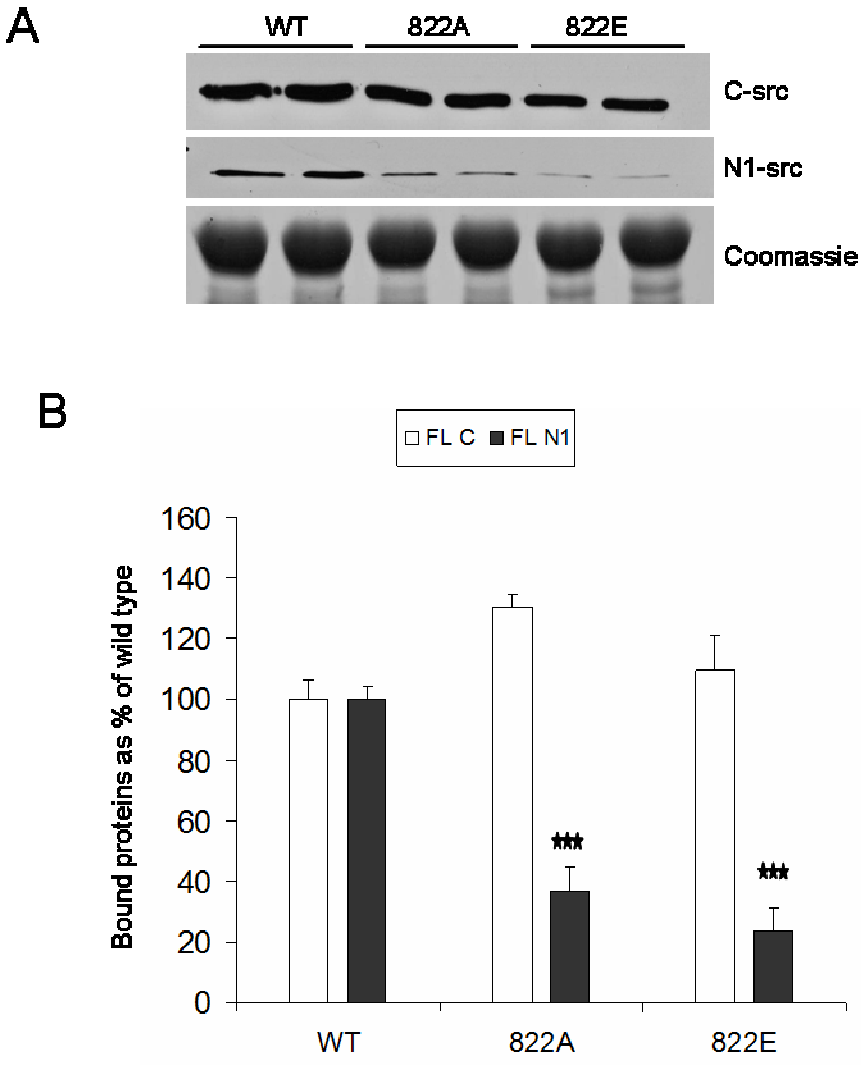


Figure 5.11- ***The effect of mutations S822A and S822E in dynamin I PRD on full length C-, and N1-src binding.***

A) GST pull downs were performed using GST-PRD mutants of dynamin I (WT = wild type, 822A = S822A, 822E = S822E (see figure 5.2). Pull downs were performed using bacterial lysates expressing either C-, or N1-src tagged with His. Western blot for His shows the binding of C-src and N1-src to different PRD mutants. Coomassie gel shows GST-PRD mutants that were used for the pull downs. Experiments were performed in duplicate.

B) Comparison of full length C-src (open bar) and N1-src (solid bar) bound to dynamin I PRD mutants. The values are normalized to PRD levels expressed as a percentage of wild type. One way ANOVA was performed and significant differences as follows (*** = P<0.001). Experiments were performed n = 4. Data presented as mean ± SEM

partners. When syndapin I binding was investigated, no change in binding was observed for any PRD protein (Figures 5.12 and 5.13). Endophilin binding to the S822A mutant, the S822E mutant and the WT xb splice variant was greatly reduced, while the 3L mutant had no effect. Amphiphysin showed a huge decrease in binding to the 3L mutant while with other proteins no significant decrease was observed (Figure 5.12 and 5.13). When coomassie gels were studied an extra band was identified at about 60 kDa in WT xb pull downs that was not found with the WT xa or other mutants (Figure 5.12A). Further investigation is needed in order to identify this band and why it binds only with the WT xb splice variant.

5.3.7 Dynamin I PRD mutations P830A and P836A

Since the S822 mutation beside PxxP motifs 8 and 9 affected N1-src binding to dynamin I PRD, it is possible that one of these motifs is the main site for N1-src binding. To investigate which motif is more important for N1-src binding or if both of these motifs are required, these PxxP motifs were mutated. Two mutants were made, a P830A mutation which will only affect PxxP motif 8, and P836A which should only affect PxxP motif 9 (Figure 5.2). These mutants were used to perform pull downs for full length C- and N1-src splice variants from bacterial lysates. There was a small non-significant reduction in binding of C-src to the P830A mutant while N1-src was not affected. The P836A mutant also had no significant effect on either C- or N1-src binding, (althogh was decreased by about 35 %) (figure 5.14), this suggests that either site 8 or 9 may not be part of the N1-src binding site.

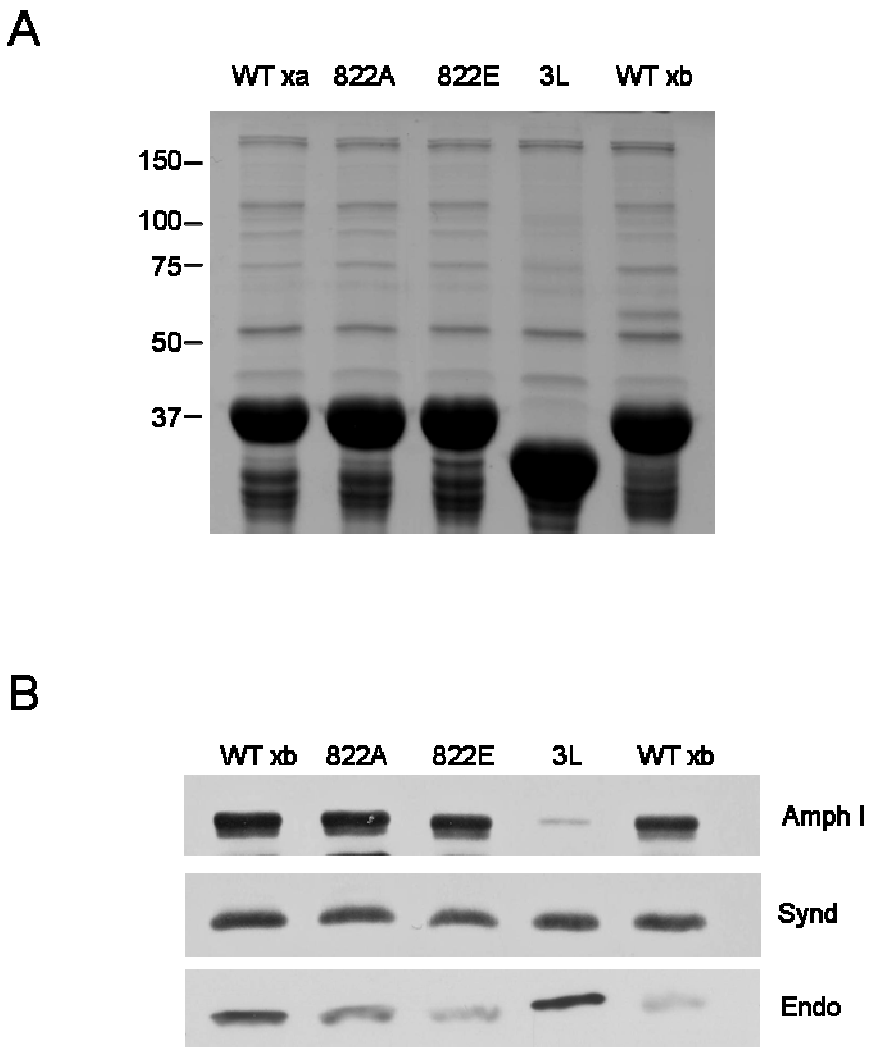


Figure 5.12- ***The effect of mutations S822A, S822E, 3L, and splice variant xb on amphiphysin I, syndapin, and endophillin binding to dynamin I PRD.***

A) GST pull downs were performed using GST-PRD mutants of dynamin I (WT = wild type, 822A = S 822A, 822E = S 822E, 3L = truncated at 832, Wt xb = splice variant xb (see figure 5.2). Pull downs were performed using rat nerve terminal lysates. Coomassie stained gel shows the binding affinity of PRD mutants to different binding partners.

B) Western blot shows the binding of syndapin (Synd), endophillin (Endo), and amphiphysin I (Amph I) to different PRD mutants. Experiments were performed n = 4.

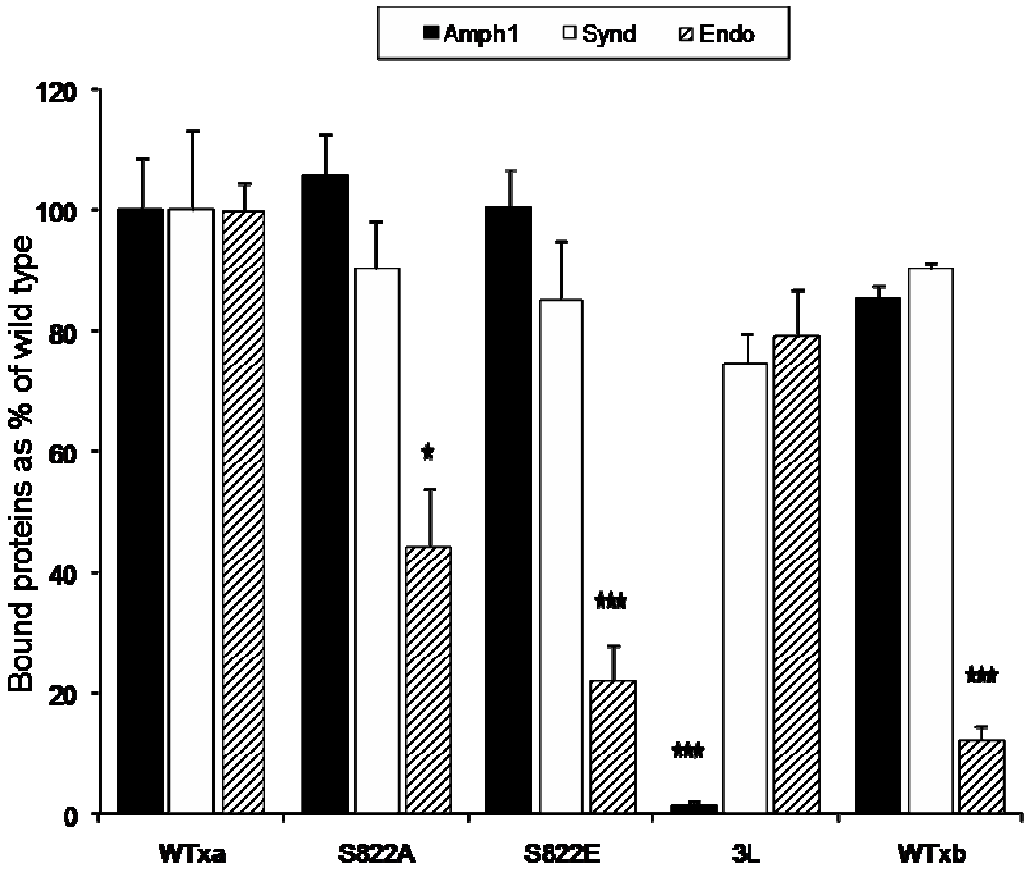


Figure 5.13- *Comparison of the effect of mutations S822A, S822E, 3L, and splice variant xb on amphiphysin I, syndapin, and endophillin binding to dynamin I PRD.*

Comparison of amphiphysin I (solid bar), syndapin (open bar), and endophillin (hatched bar) bound to dynamin I PRD mutants (WTxa = wild type, 822A = S 822A, 822E = S 822E, 3L = truncated at 832, WT xb = splice variant xb (see figure 4.2). The values are normalized to PRD levels expressed as a percentage to the wild type. One way ANOVA was performed and significant differences as following (* = P<0.05, *** =P<0.001). Experiments were performed n = 4. Data presented as mean ± SEM

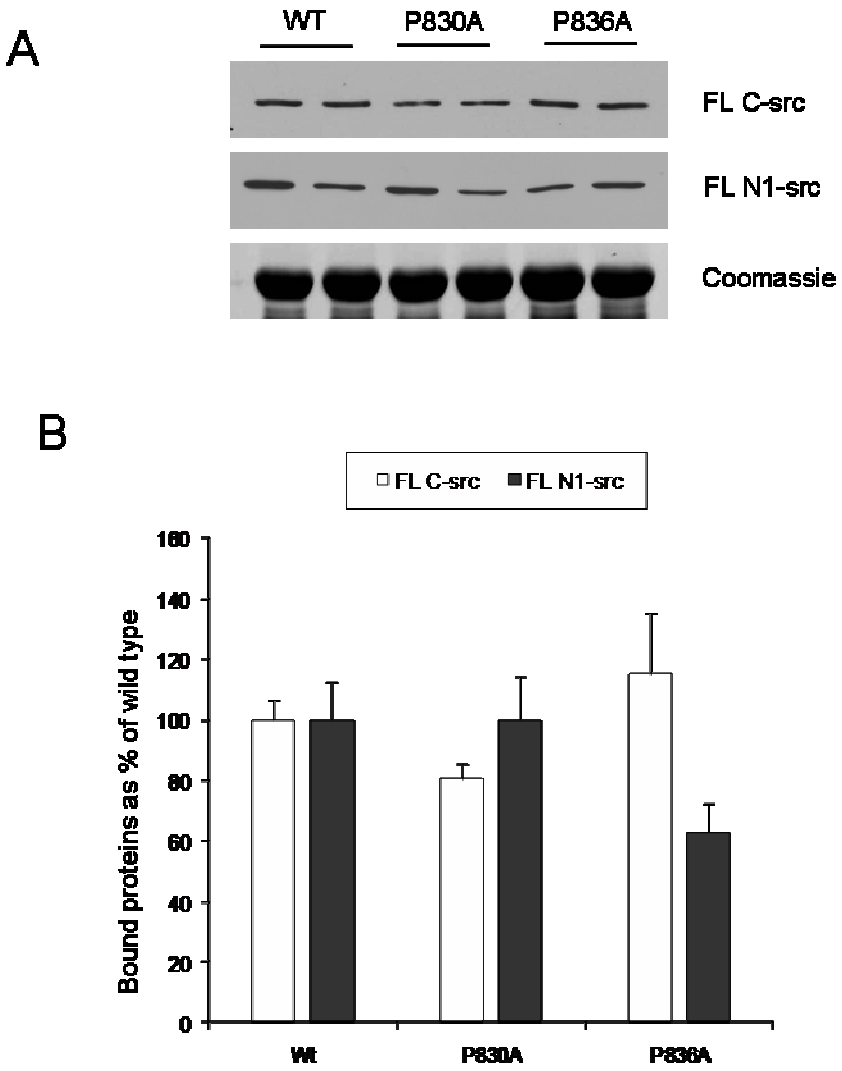


Figure 5.14- ***The effect of mutations P830A and P836A*** **in dynamin I PRD** ***on full length C-, and N1-src binding.***

A) GST pull downs were performed using GST-PRD mutants of dynamin I (Wt = wild type, P830A, P836A (see figure 5.2). Pull downs were performed using bacterial lysates expressing either C-, or N1-src tagged with His. Western blot for His shows the binding of C-src and N1-src to different PRD mutants. Coomassie gel shows GST-PRD mutants that were used for the pull downs. Experiments were performed in duplicate.

B) Comparison of full length C-src (open bar) and N1-src (solid bar) bound to dynamin I PRD mutants. The values are normalized to PRD levels expressed as a percentage of wild type. One way ANOVA was performed and there were no significant differences. Experiments were performed n = 4. Data represented as mean ± SEM.

5.3.8 Effect of dynamin I PRD mutations P830A and P836A on syndapin I, endophilin, amphiphysin and Grb2 binding

Sites 8 and 9 have been shown to be the major sites for both amphiphysin and Grb2 binding (Grabs et al., 1997; Anggono and Robinson, 2007). The binding to these mutants plus other nerve terminal proteins was therefore investigated using synaptosomal lysates. Syndapin I binding was unaffected by either PRD mutant (Figures 5.15 and 5.16). Endophilin binding to P830A was not affected, however its binding to the P836A mutant was reduced by about 40 %. Amphiphysin on the other hand showed a decrease of about 60 % binding to the P830A mutant while there was no effect on binding to the P836A mutant, suggesting site 8 is the major amphiphysin binding site, contrary to published data (Grabs et al., 1997).

Since Grb2 binding was reported to bind in the area of sites 8 and 9, its binding to P830A, P836A, 3L, and 9A was investigated (Figures 5.17 and 5.18). The S830A and 3L mutants reduced Grb2 binding by almost 60 % compared to the wild type; however, the S836A and 9A mutants had no significant effect. The data suggests that PxxP motif 8 might be an important binding site for Grb2 as previously reported (Grabs et al., 1997), but it is not the only binding site.

5.4 Effect of dynamin I PRD presence on the kinase activity of the Δ80 C- and N1-src splice variants

One unaddressed question for the N1-src dynamin I interaction is what function does it perform? One possibility is that dynamin I binding may regulate src activity, possibly by disrupting its clamp position (Young et al., 2001). In agreement, synapsin binds to src through its SH3 domain and enhances its kinase

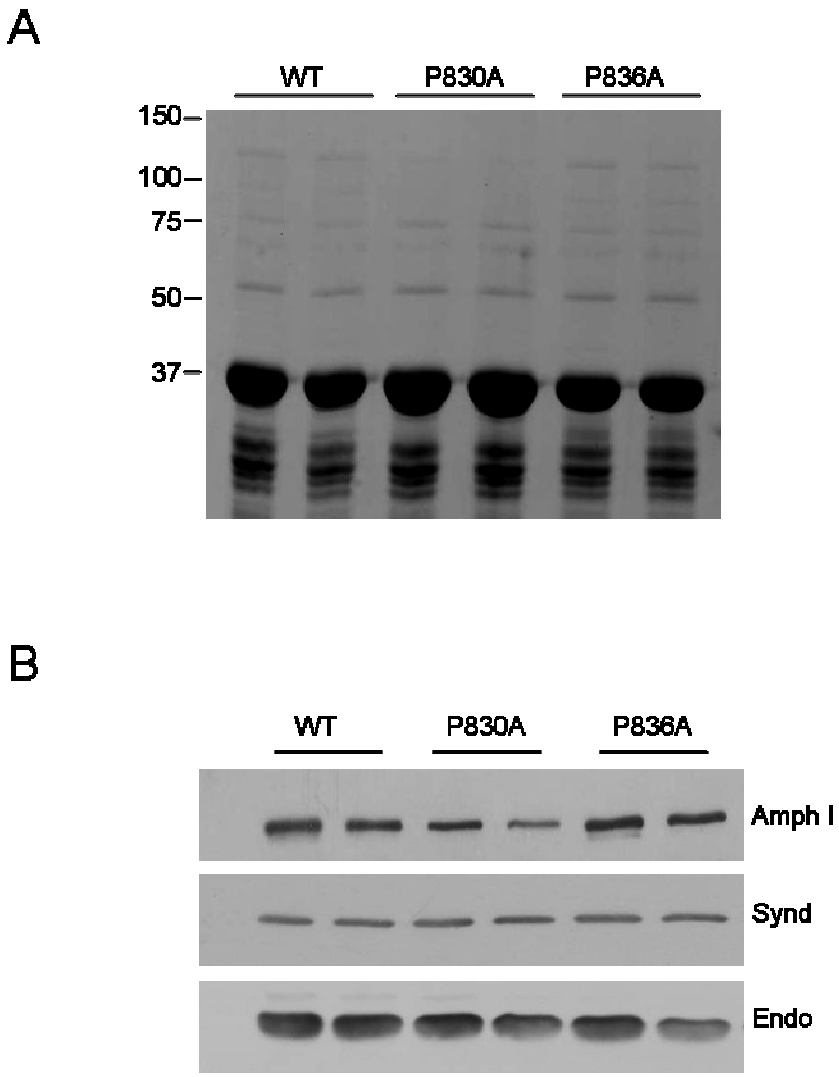


Figure 5.15- ***The effect of mutations P830A and P836A on syndapin, endophillin and amphiphysin I binding to dynamin I PRD.***

A) GST pull downs were performed using GST-PRD mutants of dynamin I (WT = wild type, P830A and P836A (see figure 4.2)). Pull downs were performed using rat nerve terminal lysates. Coomassie stained gel shows the binding affinity of PRD mutants to different binding partners.

B) Western blot shows the binding of syndapin (Synd), endophillin (Endo), and amphiphysin I (Amph I) to different PRD mutants. Experiments were performed in duplicates.

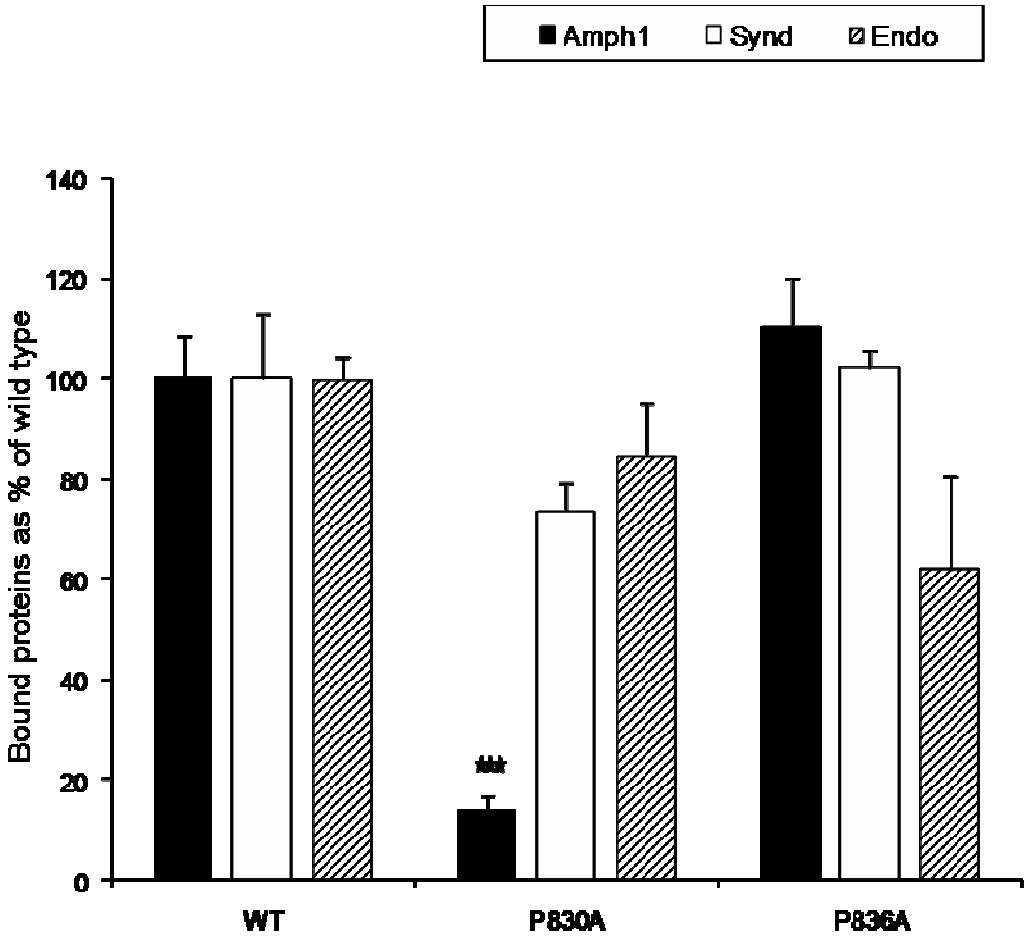


Figure 5.16- *Comparison of the effect of mutations P830A and P836A on syndapin, endophillin, and amphiphysin I binding to dynamin I PRD.*

Comparison of syndapin (open bar), endophillin (hatched bar), and amphiphysin I (solid bar) bound to dynamin I PRD mutants (WT = wild type, *P830A and P836A* (see figure 5.2). The values are normalized to PRD levels expressed as a percentage to the wild type One way ANOVA was performed and significant differences as following (*** =P<0.001). Experiments were performed n=4. Data presented as mean ± SEM

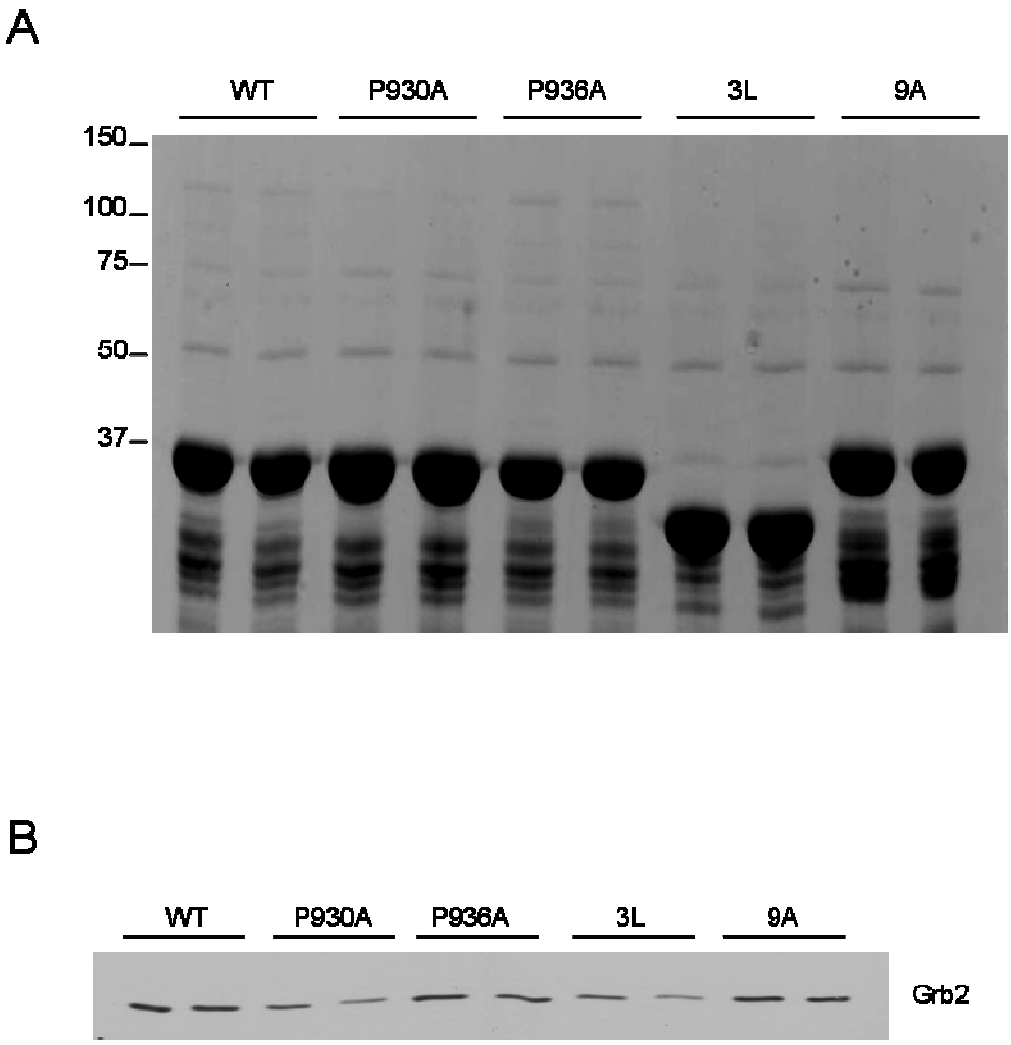


Figure 5.17- ***The effect of mutations P930A, P936E, 3L, and 9A on Grb2 binding to dynamin I PRD.***

A) GST pull downs were performed using GST-PRD mutants of dynamin I (Wt = wild type, P930A, P936A, 3L, and 9A (see figure 4.2)). Pull downs were performed using rat brain nerve terminal lysates. Coomassie stained gel shows the binding affinity of PRD mutants to different binding partners.

B) Western blot shows the binding of Grb2 to different PRD mutants. Experiments were performed in duplicates.

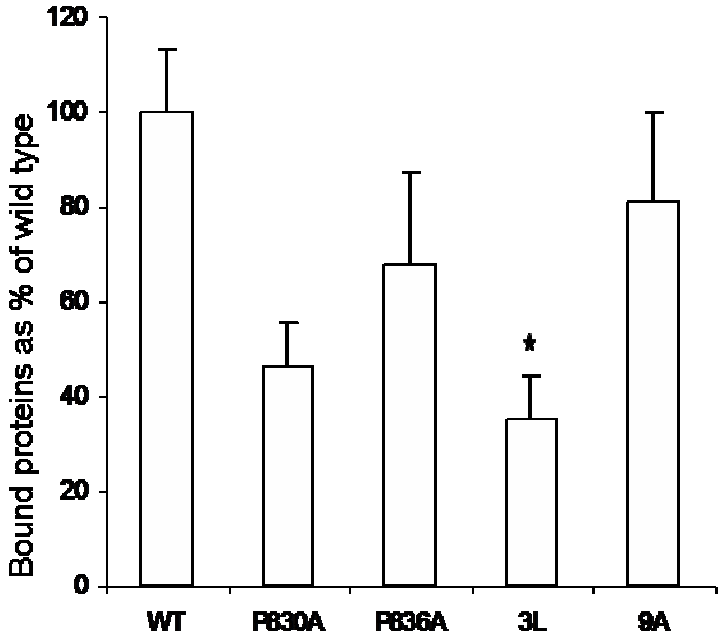


Figure 5.18- ***The effect of mutations P830A, P836A, 3L, and 9A on*** **Grb2** ***binding to dynamin I PRD.***

Grb2 bound to dynamin I PRD mutants (WT = wild type, *P830A, P836A, 3L, and 9A* (see figure 4.2)). The values are normalized to PRD levels expressed as a percentage of the wild type. One way ANOVA was performed and significant differences as follows (* = $P<0.05$). Experiments were performed n = 4. Data presented as mean ± SEM

activity (Onofri et al., 1997; Onofri et al., 2007). To test the effect of dynamin I PRD binding on Δ80 C- and N1-src kinase activity, a kinase assay was performed (see chapter 2.4.4). A time course of synaptophysin C-terminus phosphorylation was performed to establish a time point where phosphorylation is strong enough for detection on a western blot but not saturated, enabling further substrate phosphorylation (Figure 5.19 A). A 30 minute time point was selected to be optimal for this kinase reaction. The WT dynamin I PRD was added to the kinase reaction for both Δ80 C- and N1-src with synaptophysin C-terminus as a substrate (figure 5.19B). In the absence of synaptophysin C-terminus, no dynamin I PRD phosphorylation was observed, confirming that the dynamin I PRD is not a src substrate. However, there were also no obvious differences in the phosphorylation of the synaptophysin C-terminus in the presence of dynamin PRD either when the Δ80 C-src or N1-src was used for the kinase reaction. This experiment suggests that dynamin I PRD binding does not have any effect on Δ80 C- and N1-src activity.

5.5 Tyrosine phosphorylation of dynamin I in neurons

Another possible role for the dynamin I/src interaction is to phosphorylate another part of dynamin I. Previous studies have produced conflicting data on whether dynamin I is tyrosine phosphorylated in nerve terminals (Graham et al., 2007; Ballif et al., 2008). To address this and to determine whether there is a stimulation-dependent tyrosine phosphorylation of dynamin I; we examined its phosphorylation status in nerve terminals. When pull downs from stimulated and resting

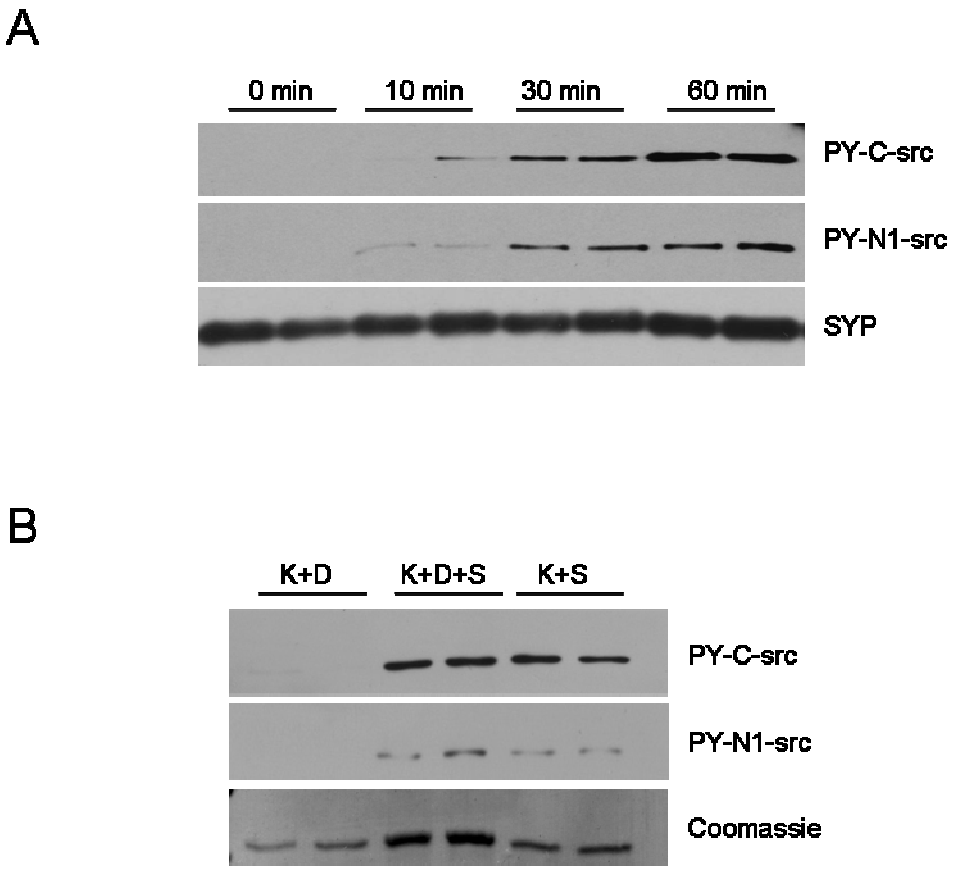


Figure 5.19- ***Effect of dynamin I PRD presence on synaptophysin C-terminus phosphorylation by C- and N1-src splice variants.***

A) Kinase assay time course using Δ80 C-src and N1-src. GST-tagged C-terminus of synaptophysin was used as a substrate, the reaction was performed at 37 °C for 0, 10, 30, and 60 minutes (PY-C-src = phospho-tyrosine when C-src was used, PY-N1-src = phospho-tyrosine when N1-src was used, SYP = synaptophysin). Blots show the phosphorylation time By C-src and N1-src, and synaptophysin blot shows the total amount of synaptophysin C-terminus.

B) Kinase assay using Δ80 C-src and N1-src. GST-tagged C-terminus of synaptophysin was used as a substrate, the reaction was performed at 37 °C for 30 minutes (K = kinase, D = dynamin PRD, S = synaptophysin, PY-C-src = phospho-tyrosine when C-src was used, PY-N1-src = phospho-tyrosine when N1-src was used). Blots show the phosphorylation By C-src and N1-src, and coomassie stained gel shows the amount of Dynamin I PRD and synaptophysin C-terminus.

synaptosomal lysates were performed using GST-tagged C-src SH3 domain, no dynamin I tyrosine phosphorylation was observed either in the presence or absence of sodium vanadate (Figure 5.20 A). In addition, no phosphorylation of dynamin I was also observed following dynamin I immunoprecipitation from stimulated and resting synaptosomal lysates in the absence of sodium vanadate. However, dynamin I was tyrosine phosphorylated in both basal and stimulated conditions in the presence of sodium vanadate, but there was no obvious difference in the level of dynamin I tyrosine phosphorylation between these conditions (Figure 5.20 B). Since sodium vanadate was added before stimulation in the stimulated samples, one would expect that if dynamin I is tyrosine phosphorylated during basal conditions and dephosphorylated after stimulation, it would not be seen since dephosphorylation is blocked by sodium vanadate. The other possibility is that dynamin I phosphorylation is not stimulation-dependent or other signals such as Ca^{2+} influx are required for its tyrosine phosphorylation.

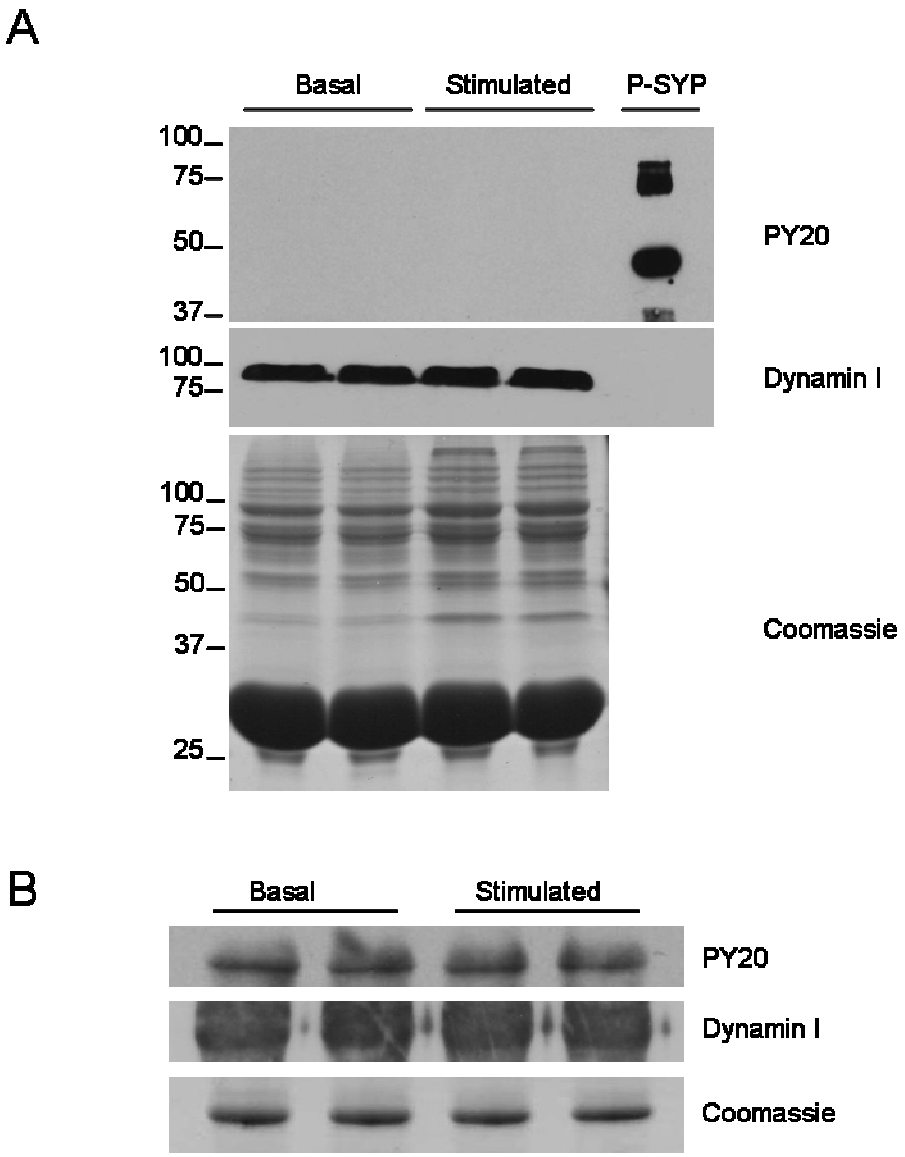


Figure 5.20- ***Dynamin I tyrosine phosphorylation in synapses.***

A) GST pull downs were performed using GST-C-src SH3 domain FROM basal and stimulated rat nerve terminal lysates. Blots show the tyrosine phosphorylation (PY20 antibody), tyrosine phosphorylated GST-synaptophysin C-terminus (P-SYP) was used as a positive control, total dynamin (dynamin antibody, and coomassie stained gel shows the binding of C-src SH3 domain to different binding partners. Experiment was performed in duplicate. .

B) Dynamin immuno-precipitation (IP) from rat brain synaptosomal lysates. Dynamin antibody was used to bind dynamin from rat brain synaptosomal lysates, and then was separated using protein G beads. Samples were separated on SDS-PAGE gels and coomassie stained or prepared for western blot. Blots show the dynamin tyrosine phosphorylation (PY20 antibody), total dynamin I (dynamin I antibody, and coomassie stained gel shows the amount of dynamin I.

5.6 Discussion

In summary, I have shown N1- src binding to dynamin I PRD is phosphorylation dependent, and S822 is a key regulator in this dependency. The binding site for N1-src is likely to be at PxxP motifs 8 and 9 and the regions C-terminal to this site. C-src binding to dynamin I is not phosphorylation dependent and the major binding site is still to be determined.

5.6.1 Dynamin I PRD mutations used in these experiments

In this study 10 mutations were used in order to investigate their effect on N1-src binding to dynamin I. These mutants can be divided into two major regions. The first region is at PxxP motifs 2 and 3, while the second region is at PxxP motifs 8 and 9. For each region, the two PxxP motifs were mutated (2C and 3B for the first region, and P930A and P836A for the second region). Serine phosphorylation sites flanking these sites were also tested (S774/778 for first region and S822 for second region) in addition to arginine residues (PB2 for first region and 9A for second region). Finally two shorter C-terminal versions were used, the 3L mutant starting from PxxP motif 8 and the xb splice variant that has no PxxP motifs after 10.

The reason for choosing these two regions is that many SH3 domain containing proteins have been mapped to bind to one of these regions. Syndapin I and endophillin I were mapped to bind to the first region, where as amphiphysin I and Grb2 were mapped to bind to the second region (Anggono and Robinson, 2007). In addition these were flanked by serine residues S774/778 in the first region and S822 in the second region which have been reported to be phosphorylated (Graham et al., 2007).

5.6.2 N1-src and C-src binding to dynamin I

5.6.2.1 N1-src binding to dynamin I is phosphorylation dependent

Dynamin I is one of the dephosphins that are dephosphorylated upon stimulation by calcineurin in a Ca^{2+} dependent manner (Liu et al., 1994; Cousin and Robinson, 2001; Cousin et al., 2001). N1-src was observed to bind to dynamin I in stimulated synaptosomal lysates, suggesting dephosphorylation of dynamin I is central for the interaction. This was confirmed by blocking the stimulation-dependent interaction using the calcineurin inhibitor cyclosporin A. In contrast, the C-src dynamin I interaction is not affected by phosphorylation, which suggests that C-src association with dynaminI is not related to stimulation.

Previous data has shown that the C-src, but not the N1-src SH3 domain, binds to dynamin I (Gout et al., 1993; Foster-Barber and Bishop, 1998). Gout and colleagues (1993) used the dynamin I WT xb splice variant which did not bind much to N1-src in this study suggesting why they did not observe any binding (Gout et al., 1993). The other reason for the lack of binding is that they used resting bovine brain lysates where dynamin I is most likely to be phosphorylated while N1-src showed binding to dephosphorylated dynamin I.

5.6.2.2 S822 is the phosphorylation site that affects N1-src

Serine residues 774 and 778 are the main serine phosphorylation sites for dynamin I, being phosphorylated by CDK5 both in nerve terminals and *in vitro* (Tan et al., 2003; Graham et al., 2007). Anggono and colleagues (2006) showed that syndapin I binding to dynamin I is regulated by phosphorylation of these serines (Anggono et al., 2006). Both C-src and N1-src SH3 domains did not show any difference in

binding to these mutants. This is consistent with previous studies showing that full length syndapin I but not the SH3 domain shows phosphorylation dependent binding to dynamin I (Anggono et al., 2006). However, in contrast to syndapin I neither full length N1-src or C-src binding was affected by mutations of these sites.

S822 is a minor phosphorylation site in dynamin I with less than 5 % of the total phosphorylation (Graham et al., 2007). Phosphomimetic and phospho-null mutations of this site significantly reduced binding between N1-src and the dynamin I PRD. The fact that even the phospho-null mutant caused a significant decrease in N1-src binding suggests that the presence of a serine residue itself is important for N1-src binding and not just phosphorylation status of this residue. Two other phosphorylation sites are present in the dynamin I PRD: S851 and S857. These account for 12 % of the total phosphorylation (Graham et al., 2007). These are the likely sites regulating N1-src binding since the 3L mutant and WT xb (which do not have them) abolish binding. In support, mutation of S857 regulates amphiphysin binding (Huang et al., 2004). Since N1-src binding is similar to amphiphysin, this suggests that S857 may be the key phospho-regulated site. Further experiments using phospho-null and phosphomimetic mutants should confirm this hypothesis.

5.6.2.3 Binding site for N1-src starts from PxxP motif 8.

It was expected that the N1-src binding site would be adjacent to its phosphorylation regulating site, therefore as expected neither the 2C or 3B mutants affected N1-src binding, since the S774 and S778 mutants had no effect on the interaction. The lack of binding of the PRD 3L mutant indicated that the N1-src binding site was from PxxP site 8 onwards.

Furthermore, the majority of N1-src binding is still retained with the PxxP 8 and 9 mutations, suggesting that the major binding site is further C-terminal to these sites. This is supported further by the xb splice variant, which has no PxxP motifs past the splice site at PxxP motif 10. Dynamin I xa (WT xa) has 684 amino acids (considered as the wild type in these experiments) and dynamin I xb (WT xb) has 851 amino acids (Robinson et al., 1993; Urrutia et al., 1997). Dynamin I xa seems to have a higher affinity than dynamin I xb for several SH3 domain containing proteins including Grb2, fyn, lyn, and P85, while they have the same affinity for PLCγ (Okamoto et al., 1997). The WT xb splice variant of dynamin I does not contain PxxP motifs 11, 12, and 13 and lacks the phosphorylated serines 851 and 857 instead it has 7 additional amino acids (Figure 5.2). Since the WT xb splice variant exhibited little binding to N1-src, it suggests PxxP motifs 11-13 are the major binding site. Further experiments mutating this region will need to be performed to confirm this.

5.6.2.5 Arginine residues are essential for N1-src binding

Arginine residues form part of the SH3 domain binding sites, and are adjacent to the PxxP motifs. The arginine residues are essential for SH3 domains to bind to PxxP motifs (Alexandropoulos et al., 1995). The PB2 (RR772/3EE) mutation affected syndapin I binding to the dynamin I PRD, where as the 9A (R838A) mutant affected amphiphysin and endophilin binding to the dynamin I PRD (Anggono and Robinson, 2007). Both of these mutations showed a huge effect on N1-src, but not C-src, binding to the dynamin I PRD, suggesting that either arginine residues in both sites are important for N1-src binding or that changing the positive charge of the arginine to a negative charge (PB2) or no charge (9A) would affect the ability of the SH3

domains to bind to the PxxP motifs. The fact that C-src binding to the dynamin I PRD was not affected with these mutations supports the first possibility.

5.6.2.6 C-src binding to dynamin I

The C-src binding site is still unidentified, because none of the mutations tested had any significant effect on binding to the dynamin I PRD. There is a possibility that C-src has multiple binding sites and any one single mutation would not show a significant reduction on its binding. Previous studies suggested that the C-terminal region including PxxP motif 2 need to be truncated before any significant effect on binding can be observed; in fact the presence of PxxP motifs 2 and 3 was able to retain about 70 % of the binding (Okamoto et al., 1997). The multiple binding sites for C-src were suggested to be at PxxP motifs 2, 6, and 8 (Solomaha et al., 2005).

5.6.3 Syndapin I, endophilin, and amphiphysin binding to dynamin I

Syndapin I, endophilin I, and amphiphysin I are the major interacting proteins of dynamin I (Qualmann et al., 1999; Simpson et al., 1999; Takei et al., 1999; Hill et al., 2001; Solomaha et al., 2005; Anggono et al., 2006; Elhamdani et al., 2006; Anggono and Robinson, 2007; Graham et al., 2007; Andersson et al., 2008). Their binding to the dynamin I PRD mutants was used to corroborate effects seen with src binding. The data observed conflicted with some published results and as such the possible reasons are discussed below.

5.6.3.1 Effect of the PRD mutants on amphiphysin binding

Amphiphysin I is known to bind to dynamin I in a phosphorylation independent manner (Graham et al., 2007) and it is suggested to bind to PxxP motif 9 (Anggono et al., 2006; Anggono and Robinson, 2007). However, it was reported that mutation of serine residue 857 (S857E) resulted in inhibition of amphiphysin binding to the dynamin I PRD and thus phosphorylation of this site by the protein kinase Mnb/Dyrk1A inhibits amphiphysin binding (Huang et al., 2004). These findings are conflicting, but since phosphorylation of this site is about 6 % of the total phosphorylation, this could explain the reason why this phosphorylation effect on amphiphysin binding was not observed by other groups.

In the experiments in this thesis amphiphysin binding to the dynamin I PRD was significantly affected by mutations in the area of PxxP motifs 8 and 9 as expected. Deletion of this binding site (3L) and mutation of R838 (9A) both resulted in a huge reduction of amphiphysin binding, while using the xb PRD did not affect its binding confirming that the amphiphysin binding site lies within this region (PxxP motifs 8 and 9) agreeing with previous studies (Grabs et al., 1997; Okamoto et al., 1997; Solomaha et al., 2005; Anggono and Robinson, 2007). However, PxxP motif 9 has been suggested to be the main binding site for amphiphysin rather than PxxP motif 8 (Solomaha et al., 2005; Anggono and Robinson, 2007), which does not agree with the findings presented here. The fact that none of these previous studies used mutations in PxxP motif 8 or 9 makes this study a unique approach, since Anggono and Robinson made their suggestion about the binding site according to 9A mutation results (which is similar to results in this study) and not according to PxxP motifs mutations (Anggono and Robinson, 2007). The fact that mutation of site 8 did not

have a total block on amphiphysin binding (about 60 % reduction) while 3L abolished the binding (about 95 % reduction) suggests that site 9 might have a role in the binding of amphiphysin. This agrees with Grabs *et al* (1997) where they found that deleting the N-terminal region up to PxxP motif 8 resulted in loss of some of the binding while using the 3L mutant resulted in total loss of binding (Grabs et al., 1997). All other mutations did not affect amphiphysin binding, suggesting that the amphiphysin binding site is most likely to be at PxxP motifs 8 and 9.

5.6.3.2 Effect of the mutants on endophilin binding

Endophilin binding sites were suggested to be at PxxP motifs 2 and 3 and PxxP motifs 8 and 9 (Solomaha et al., 2005; Anggono and Robinson, 2007). In this study only PxxP motif 3 seemed to be the main binding site. However the effect of mutation of the phosphorylation sites S774, S778 and S822 suggested that these serine residues have a role in endophilin binding. Also, arginine residues 772, 773 and 838 seem to be essential parts of the binding site. The fact that the 3L mutant has no effect on endophilin binding suggests for sites 8 and 9 play little role in endophilin binding, where as the effect seen with the splice variant xb suggests that splicing might mediate differential binding binding of proteins between the two isoforms. Some of these findings conflict with Anggono and Robinson (2007), where they suggested that PxxP motif 2 is part of the endophilin binding site, while my findings suggested that this site is not important for endophilin binding. Furthermore, the two arginine residues in the phosphobox (RR772/3) were suggested not to be part of the endophilin binding site, while in this study these two residues seem to be part of endophilin binding site. The finding that PxxP motif 3 and R838 both are

important for endophilin binding agrees with their findings (Anggono and Robinson, 2007). The finding that the 3L mutation does not affect endophilin binding does not agree with Solomaha *et al* where they suggested that endophilin binds to both PxxP motifs 8 and 9 (Solomaha et al., 2005). The fact that the S774/8 and S822 mutations affected endophilin binding implies that these residues themselves are important for endophilin binding as well as phosphorylation effect. The phosphorylation effect on endophilin binding agrees with Anggono *et al* (2006) where they found that endophilin binding to dynamin I is significantly affected by S774/778 Dyn^{dmE}-PRD, suggesting phosphorylation dependent binding, Analysis of the single mutants showed that S778 may be the key phosphorylation site involved in endophillin binding (Anggono et al., 2006).

5.6.3.3 Effect of the mutants on syndapin I binding

Syndapin I was shown to bind in phosphorylation dependent manner to the PxxP motifs 2 and 3 on dynamin I (Anggono et al., 2006; Anggono and Robinson, 2007). In the experiments in this thesis syndapin I was not affected by any of the mutations tested or the splice variant WT xb. This was not expected since the 2C, 3B, and PB2 mutations are in this region. These findings do not agree with previous studies where syndapin I was shown to be affected by phosphorylation of S774/8 and phosphomimetic mutations of these sites. The mutation of RR772/3 and PxxP motifs 2 and 3 also affect its binding (Anggono et al., 2006; Anggono and Robinson, 2007). This difference observed could be due to using synaptosomal lysates (this thesis) which has different population of proteins from the whole brain lysates (Anggono *et al*), or they have the same proteins which may have different phosphorylation states.

5.6.3.4 Effect of the mutants on Grb2 binding

Grb2 was suggested to bind to dynamin I at PxxP motifs 8 and 9 (Okamoto et al., 1997). It was also suggested that there were three binding sites for Grb2 at PxxP motifs 2, 8, and 10 (Solomaha et al., 2005). In the experiments in this thesis Grb2 binding to dynamin I was examined only with mutations within the region of PxxP motifs 8 and 9. The only mutation that significantly affected Grb2 binding was the 3L mutation. The P830A mutant shows a trend towards a decrease in binding suggesting that PxxP motifs 8 and 9 might be a binding site for Grb2. It seems this is not the only binding site, which does not agree with previous studies which have suggested that truncation of the C-terminal area of the dynamin I PRD starting from PxxP motif 8 resulted in a total block of Grb2 binding (Grabs et al., 1997; Okamoto et al., 1997). However, other studies suggested that Grb2 binding might include two sites at PxxP motif 2 and 3 and at PxxP motifs 8 and 9 (Solomaha et al., 2005; Anggono and Robinson, 2007), agreeing with the findings in this chapter.

5.6.4 Effect of the dynamin I PRD presence on the kinase activity of the Δ80 C- and N1-src splice variants

C-src interaction with the PRD containing protein, synapsin I, resulted in an increase in src kinase activity, and this activation was antagonized by the presence of the src SH3 domain (Onofri et al., 1997; Onofri et al., 2007). The ability of the src-dynamin I interaction to increase src kinase activity was therefore investigated. Tyrosine phosphorylation of synapsin also activates src kinase activity, which might be related to src SH2 domain binding to phospho-tyrosine of synapsin (Onofri et al., 1997).

HIV-1 Nef is also able to activate src and other src family members which have been inactivated with C-terminal Src kinase **(**Csk) by direct interaction with their SH3 domain through its PxxP motif (Trible et al., 2006). All of the above findings suggested that src could be activated by direct binding to other proteins either through its SH2 or SH3 domains.

The dynamin I PRD is not a src substrate since it contains no tyrosine residues. The PRD had no effect on src activity in the kinase assay. There are several possibilities that might explain this. The first possibility is that dynamin I binding does not affect src kinase activity. The second possibility is that the whole dynamin I protein and not just the PRD domain is needed to achieve the required effect. Thirdly, the binding protein may need to be tyrosine phosphorylated, and thus both SH3 and SH2 domains need to bind to further activate src. The fourth possibility is that this effect can not be seen with synaptophysin as a substrate and it still can be achieved with other substrates. The fifth possibility is that src is already fully active and can not be activated further. In this case src might need to be deactivated by Csk before the experiment is performed. Finally it may have been auto-phosphorylated which resulted in its desensitization to further activation which has been suggested by Sun *et al* (2002). Further experiments are required in order to address these possibilities (Sun et al., 2002).

5.6.5 Tyrosine phosphorylation of dynamin I in neurons

One role of the N1-src dynamin I interaction could be to phosphorylate dynamin I; however tyrosine phosphorylation of dynamin I in neurones is controversial. Previous studies using ^{32}P to radioactively label dynamin I showed no incorporation

of radioactivity on tyrosine residues (Graham et al., 2007), but phosphorylation of tyrosine sites was found when using vanadate in the buffer (Ballif et al., 2008). In non-neuronal cells, exogenous dynamin I is phosphorylated by src at site Y231 *in vivo*, at site Y352 *in vitro* and at site Y597 both *in vivo* and *in vitro* (Ahn et al., 1999; Ahn et al., 2002). Dynamin I tyrosine phosphorylation seems to be important in dynamin I self assembly and ligand induced endocytosis for epidermal growth factor receptor in COS-7 cells transfected with dynamin I (Ahn et al., 2002), and β_2 adrenergic receptor internalization in HEK cells (Ahn et al., 1999). In endothelial cells, src phosphorylation activates dynamin II and increases its association with caveolin I, suggesting that tyrosine phosphorylation may activate dynamin II (Shajahan et al., 2004).

Dynamin I has been shown to be phosphorylated in mouse brain lysates at three sites, Y80 and Y125 in the GTPase domain and Y354 in the middle domain (Ballif et al., 2008), which are all different from the sites mapped previously. The fact that dynamin I is tyrosine phosphorylated in brain suggests a regulatory role for tyrosine phosphorylation similar to other tissues. A dynamin I IP in the presence of vanadate showed that dynamin I was tyrosine phosphorylated both under basal and stimulated conditions. There was no difference in the level of tyrosine phosphorylation between basal and stimulated conditions, suggesting dynamin I can be phosphorylated under both conditions.

Chapter 6

General discussion

6 General discussion

In this study, the neuronal specific splice variants of src were compared to the non neuronal form (C-src). Differences in src occur due to alternative splicing of the SH3 domain which is a protein- protein interaction domain. Therefore the hypothesis was that this splicing modulates src interactions with neuronal proteins. Since the three splice variants of src are expressed in the brain, it is expected that the neuronal splice variants (N1- and N2-src) will have some distinct functions from C-src that will result in more efficient or more specific function in neurones.

6.1 C-src interactions

The isolated SH3 domains were a useful starting point for the study since they eliminate the possibility that protein-protein interactions of src splice variants is through other src domains. This is especially true for the SH2 domain which is known to bind to phosphorylated tyrosine residues, or tyrosine residues such as Y416 and Y527, that might be binding sites for SH2 domain containing proteins. On the other hand, the down side is that there might be important modulatory effects of other src domains on SH3 domain interactions that can not be observed when only the SH3 domains were used.

Pull downs from rat brain synaptosomal lysates using GST tagged SH3 domains of src splice variants revealed different binding partners as confirmed by MALDI-tof analysis and western blot. The C-src SH3 domain main binding partners were dynamin I, synapsin I and synaptojanin, which had been described before (Onofri et al., 1997; Foster-Barber and Bishop, 1998). No stimulation dependency was

observed in C-src interactions with these proteins, suggesting that C-src binding to these three proteins is stimulation independent.

6.2 N2-src interactions

The N2-src SH3 domain seemed to have many binding partners in synaptosomal lysate. Novel N2-src SH3 domain binding partners were identified including Munc18, NSF, and synaptophysin suggesting a different role in neurones from C-src. The lack of stimulation dependency in N2-src interactions with these proteins suggests that N2-src function is constitutively active rather than being regulated by neuronal activity. There is a possibility that these proteins are not always available for N2-src binding such as Munc 18-1 which might bind to N2-src when it binds to syntaxin at the closed conformation or open conformation only. NSF also might only bind to N2-src when it is free or engaged in binding with the SNARE complex. However, this possibility is not supported by the pull down results and further investigation is needed to clarify these interactions. The fact that both Munc18 and NSF are binding to N2-src splice variant SH3 domain suggests that N2-src might play a role in exocytosis. This could occur either by having these proteins as substrates, since both of them are tyrosine phosphorylated (Huynh et al., 2004; Oh and Thurmond, 2006), or by providing access for N2-src to phosphorylate binding partners for these proteins. The fact that none of the SNARE proteins bound to N2-src proved that they do not interact directly with its SH3 domain, but whether N2-src gains access to these proteins through its binding to Munc18 or NSF is not clear. The same idea has been suggested for C-src phosphorylation to synaptic vesicle proteins via its binding to synapsin (Onofri et al., 1997; Onofri et al., 2007).

The *in vitro* binding experiments failed to prove direct interactions between N2-src and the synaptophysin C-terminus or Munc 18-1. For the synaptophysin interaction, there is a possibility that the trans-membrane loops play a role in its binding to the N2-src SH3 domain and since it was not possible to express and purify the full length synaptophysin in bacteria, this hypothesis was not tested any further. On the other hand, all the src splice variants were able to phosphorylate synaptophysin C-terminus *in vitro*, suggesting that binding was not necessary for its tyrosine phosphorylation.

In vitro binding experiments failed to prove a direct interaction between Munc 18-1 and the N2-src SH3 domain, suggesting that this interaction might not be direct, especially given that the 3D structure of Munc 18-1 suggested that the only PxxP motif in Munc 18-1 was not accessible for binding. This does not eliminate the possibility that other Munc 18-1 interactions or phosphorylation could result in conformational changes that make this binding site accessible and thus N2-src could be recruited to Munc 18. Since Munc 18-1 has several binding modes to syntaxin, there is a possibility that one or more of these binding modes results in allowing N2-src binding to Munc 18-1. This could result in syntaxin dissociation from Munc 18-1, since no syntaxin was found in the N2-src pull downs. No tyrosine phosphorylation of Munc 18 was observed for N2-src kinase activity, suggesting that either Munc18-1 is not a substrate for N2-src or N2-src may need to bind to Munc 18 prior to using it as a kinase substrate since the recombinant Munc 18-1 did not bind to N2-src in the *in vitro* experements.

The distribution of the src splice variants could be a key factor in understanding the function of src splice variants in neurones, with each splice variant being found in specific neuronal subtypes or intracellular locations. Two attempts were made to

produce an N2-src specific antibody in rabbit with no success. This might be due to the fact that the only difference between N2-src and other splice variants is the extra amino acids that are inserted in N2-src SH3 domain. These inserted amino acids are very hydrophobic, which might cause them to fold in a way that makes them inaccessible for antibodies. This might also be the reason for an N2-src specific antibody not being available commercially even though it has been known for almost 20 years (Pyper and Bolen, 1990). Since the production of N2-src specific antibody failed, N2-src distribution within the brain was not tested. Not knowing the N2-src distribution within neurons made it difficult to suggest a specific role for the N2-src splice variant in certain areas in the brain or specific locations.

6.3 N1-src interactions

The N1-src SH3 domain showed a very specific binding with pull downs from rat brain synaptosomal lysates revealing very few binding partners. The N1-src SH3 domain had a stimulation dependent binding to dynamin I that is related to dynamin I dephosphorylation, since cyclosporine A prevented its binding to dynamin I in nerve terminals. The phosphorylation dependent interaction was shown not to be regulated by the two main phosphorylation sites on dynamin (S774 and S778) which regulate syndapin binding (Anggono et al., 2006). Mutation at the S822 phosphorylation site affected N1-src binding where both phosphomimetic and phosphonull decreased N1-src binding to the dynamin PRD. Since this site represents only a small amount of the total phosphorylation in vivo (Graham et al., 2007), there is a possibility that phosphorylation sites S851 and S857 are the main sites that regulate N1-src binding

to dynamin. In support of this hypothesis, the binding of other proteins such as amphiphysin is regulated by phosphorylation of S857 (Huang et al., 2004).

The binding site for dynamin was also investigated. N1-src binding was not affected by mutations in PxxP motifs 2 and 3. However, a truncated version of dynamin I lacking the C-terminus up to PxxP 8 (3L) eliminated its binding, suggesting that the N1-src binding site is after the 3L truncation site (PxxP motifs 8-13). Mutations in PxxP motifs 8 and 9 decreased N1-src binding but did not totally block it, suggesting that even if these two sites are involved in N1-src binding there is another binding site. The lack of binding of N1-src to the short tailed splice variant of dynamin I (dynamin I xb) which contains up to PxxP motif ten suggested that the main binding site for N1-src is at PxxP motifs 11-13 at the end of the dynamin PRD, which supports the suggestion that the phosphorylation site controlling N1-src binding is most likely to be S851 and S857. The lack of binding of N1-src to dynamin PRD when the arginine residues RR772/3 or R838 were mutated suggested that these arginine residues play a role in N1-src binding. These mutations are preventing binding and not causing a conformational change in the dynamin PRD, since not all protein binding was affected by these mutations, especially C-src, which did not show any decrease in binding with any of the mutations that were used.

No data is available about the distribution or distinct functions of dynamin I with long C-terminus tail (xa) or and the short C-terminus tail (xb). Since N1-src is associated only with dynamin I xa, there is a possibility that this association has a unique function. There might be a regulatory role for N1-src in dynamin I xa function in a stimulation dependent manner where N1-src will have a very specific function either by phosphorylating dynamin I xa or gaining access to other proteins

such as endophillin. The first possibility is supported by the fact that dynamin I is tyrosine phosphorylated in the brain at multiple sites (Y80, Y125 and Y354) (Ballif et al., 2008). This tyrosine phosphorylation might be of importance in modulating dynamin activity, especially after repeated stimulation which will result in greater dynamin serine dephosphorylation (Clayton et al., 2009) allowing more N1-src interaction and activity. The second suggestion is supported by the fact that endophilin A2 phosphorylation at Y315 by src is mediated by the endophillin SH3 domain interaction with FAK PRD (Wu et al., 2005). The repeated stimulation also will result in more N1-src interaction with dynamin and thus being closer to its substrates. In this manner the phosphorylation dependent interaction of N1-src with dynamin might act as mechanism of activating an extra pathway for endocytosis after extensive stimulation whereas its function in normal conditions is minimal.

The fact that there are different distribution patterns for src splice variants suggests that the neuronal specific splice variants are not required in all neuronal cell types or locations, but instead they are expressed only where they are needed. Both C-src and N1-src are found in the synaptic region suggesting that both of these isoforms are needed there. C-src is enriched on synaptic vesicles which might be related to C-src binding to synapsin. N1-src is more specifically located to the plasma membrane suggesting different roles there (Onofri et al., 2007). These distinct locations and the distinct binding interactions could provide specificity for src splice variant to regulate different stages of the synaptic vesicle cycle.

6.4 Experimental issues

The first issue is the use of the kinase dead src splice variants in this project. The dynamin PRD is expected to interact only with the SH3 domain of src which has no mutations. However, there could be some effects from the other src domains on SH3 domain interactions, including the kinase domain. This could lead to a different effects from the mutated kinase domain compared to the active kinase domain on the SH3 domain interactions. However, the active kinase src is very hard to express in bacteria which make it very difficult to express in adequate amounts for pull down experiments. The kinase domain however is not generally considred to be involved in protein protein interactions, which makes this single mutation in this domain unlikely to affect the SH3 domain interactions. Furthermore, the effect of the kinase domain mutation would be equal on all PRD constructs including the wild type which is used in this project as a standard and all the mutants interactions were compared to it.

The other issue is coexpressing the Gro-ESL chaperone with the full length src splice variants. The presence of Gro-ESL chaperone in the bacterial lysates might affect the dynamin PRD interactions with the full length src splice variants. However, Gro-ESL chaperone is expected to bind only to unfolded protein and dissociates from the protein when it is folded properly (Wang et al 1999; Falke et al 2001). Furthermore, if the presence of Gro-ESL chaperone would affect the dynamin PRD interactions with src splice variants, its effected is expected to be equal on all PRD construct including the wild type. This means that its effect is elemintated since wild type PRD is used as a standard.

The other issue is using the phosphomemetic mutations. Phosphomimetic mutants mimic both the bulky structure of the phosphate group and the negative charge but it

is not exactly the same as real phosphorylation. In some cases, phosphomemetic mutations would have a similar effect as the true phosphorylation on the protein protein interactions (Barclay et al 2003). On the other hand, both phosphomimetic and phosphonull mutations might affect protein protein interactions as it was seen with S822A/E mutations where both affected the dynamin PRD intractions with N1-src leading to the expectation that this residue is important as is in this interaction instead of the effect of the charge and or the bulky structure. This however does not elemenate the possibility that phosphorylation of this residue would cause a decrease in the PRD interaction since phosphorylation would cause a change in charge and bulky structure that could affect its interaction.

The other issue is the protein concentrations that are used in these experiments. In these experiments high concentrations of proteins were used to make sure that any protein protein interactions will be picked up even if it is at a low affinity. Any protein protein interactions that are not seen mean either they have a very low affinity or need some other conditions such as the presence of some other proteins or phosphorylation conditions alterated. On the other hand, there might be some differences in affinity that can not be seen at high concentrations and lower concentrations of the protein would show these differences. May be a series of descending concentrations in the binding and pull down assays would help in assessing these differences that can not be seen in the high protein concentrations.

6.5 Model for src splice variant function

From this study a general model can be proposed for src splice variant function in nerve terminals. C-src functions as the major src in all non neuronal cells and its

expression is adequate for normal cellular functions. Neuronal cells are known for their high activity levels, stimulation and firing rates, which might require extra src roles to maintain their functions. Indeed there are several neuronal specific proteins and protein isoforms that have structures that only recognize the neuronal splice variants to accommodate these differences (Chih et al., 2006).

For the N2-src splice variant the extra amino acids in its SH3 domain cause a shift in it's binding from synapsin, synaptojanin and dynamin I (the classical binding partners for C-src) to a new group of proteins including Munc 18, NSF and synaptophysin. All of these proteins are tyrosine phosphorylated and they are involved in vesicle cycling at different stages. This suggests that N2-src might be involved in regulating exocytosis, where both Munc 18 and NSF play essential roles. The possible role for N2-src in the synaptic area includes regulation of these proteins function or changing the phosphorylation status during different patterns of neuronal activity (Figure 6.1).

N1-src function on the other hand might be very specific for synaptic vesicle endocytosis specifically in regulating dynamin I activity, which might include some vesicle recycling routes that require only the dynamin I xa splice variant but not the short version. These routes will also be activated after repeated stimulation since that will result in more dynamin I being serine dephosphorylated thus increasing binding to N1-src. This might be a key function for understanding activation of various different routes of synaptic vesicle recycling. Since dynamin PRD alone is not a src substrate, it is possible that N1-src function may be to phosphorylate both dynamin I xa and dynamin I xb since dynamin forms a ring around vesicles neck. Therefore, the dynamin ring may be a mix between xa and xb. It is also possible that N1-src binding

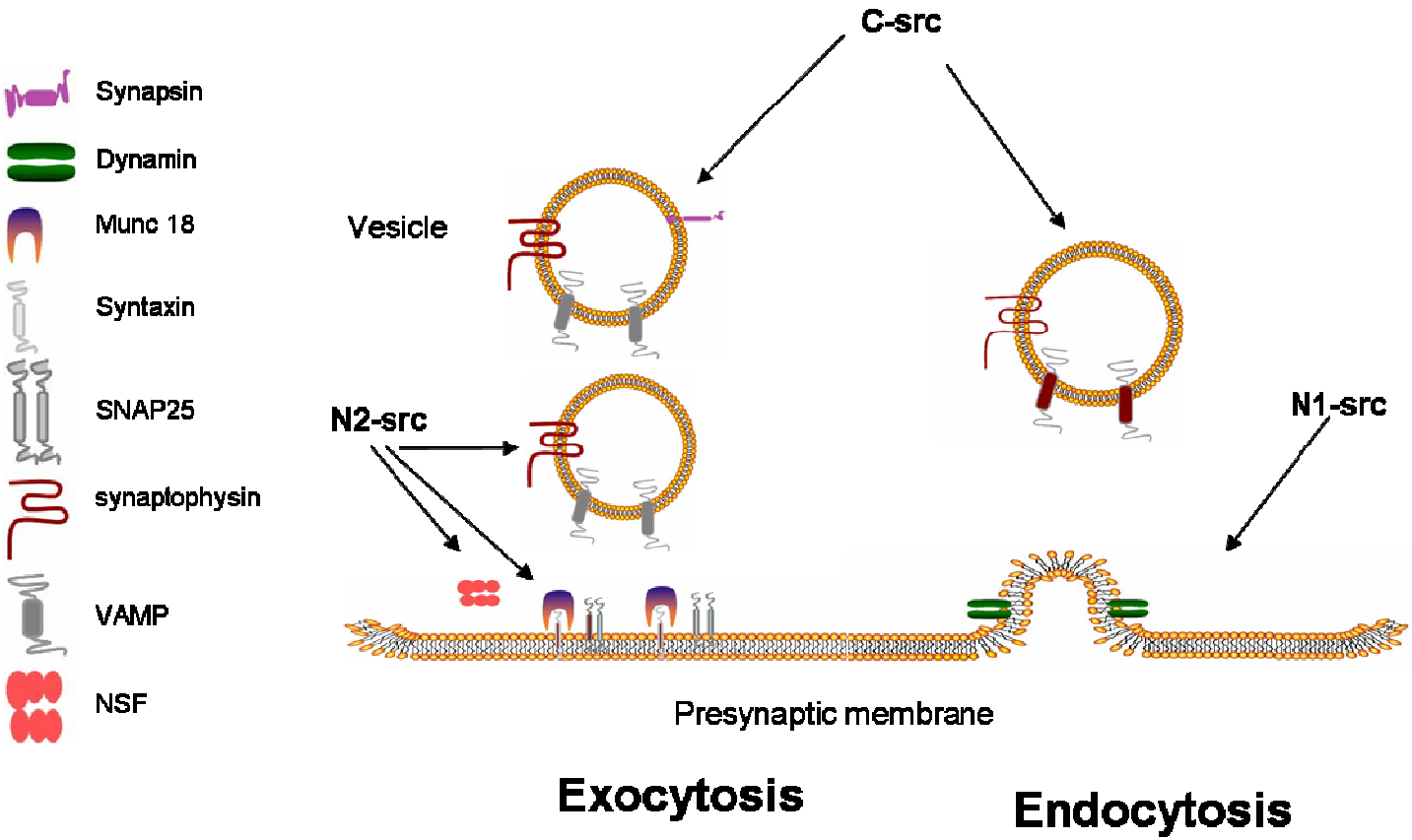


Figure 6.1- Possible roles for src splice variants in presynaptic terminal.

This figure illustrates the possible distinct functions of neuronal src splice variants in the nerve terminal; C-src has a general function while N2-src is more specific towards proteins involved in exocytosis and N1-src is more specific toward proteins involved in endocytosis

to dynamin acts as a bridge for N1-src to phosphorylate substrates that specifically interact with dynamin xa such as endophillin.

6.4 Further experiments

There are still many aspects of src splice variants that need to be inspected in more detail. These include using the full length or the truncated Δ80 src splice variants to perform pull down assays from both whole brain and synaptosomal lysates to compare differences in their bindings in the presence of other domains besides the SH3 domain. These splice variants could then be co expressed with these proteins in eukaryotic cells, such as PC12 cells or neurones to inspect their co localization and see whether there is a direct interaction between them as well as any changes in their phosphorylation status.

It would be useful to mutate the PxxP motifs in Munc 18, NSF and synaptophysin and co express them with N2-src to investigate if N2-src binding to these proteins is via the PxxP motif or if there is another motif that provides an alternative binding site for N2-src SH3 domain, which might help in understanding the effect of src splicing on its function. It would also be useful to mutate the extreme C-terminal PxxP motifs of dynamin I xa and inspect their binding to N1-src in order to map the exact binding site for N1-src. After mapping the exact binding site for N1-src, a mutant that does not bind to N1-src could be expressed in neurones to observe its effect on different types of vesicle endocytosis. It would also be intersting to inspect if only one phosphorylation site in dynamin PRD regulates N1-src binding or if multiple phosphorylation sites are needed to achieve the inhibitory effect. This can also be further investigated by expressing either a phosphonull or phosphomimetic

dynamin I in neurones to observe their effect on different types of vesicle endocytosis. Their effect on different types of endocytosis can be investigated by using morphological analysis of horse radish peroxidase (HRP) uptake for all endocytotic routes (Clayton et al., 2008), FM-1 43 uptake for endocytosis and dextran for bulk endocytosis (Clayton and Cousin, 2008), and by comparing the changes for the uptake of each one of them, the effect of N1-src interaction with dynamin can be studied. It is possible that N1-src is involved in bulk endocytosis, since bulk endocytosis is activated after extensive stimulation (Clayton et al., 2009) which result in more dynamin dephosphorylation (Clayton et al., 2009) and the N1-src interaction with dynamin also increases after dephosphorylation. It would also be useful to investigate whether some dynamin I binding partners will be src substrates in the presence and absence of either full dynamin I or just its PRD. This will show the extent of N1-src binding to dynamin I effect on proteins in the synaptic region and also provide a better understanding of synaptic function and regulation. Using shRNA to specifically silence either dynamin I xa or xb expression and observe its effect on different endocytotic routes and protein phosphorylation would also be useful in understanding the possible functional differences between src splice variants.

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