Investigating Binding Partners of the Neuronal Adaptor Protein ShcC Involved in Learning and Memory

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biochemistry

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INVESTIGATING BINDING PARTNERS OF THE NEURONAL ADAPTOR PROTEIN SHCC INVOLVED IN LEARNING AND MEMORY

(Spine title: ShcC Binding Partners)

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by

Kayla Rose Maxine Driver

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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The thesis by

Kayla Rose Maxine Driver

entitled:

Investigating Binding Partners of the Neuronal Adaptor Protein ShcC Involved in Learning and Memory

is accepted in partial fulfillment of the requirements for the degree of Master of Science

Date ________________________

Chair of the Thesis Examination Board
Abstract

Learning and memory are complex processes: many signaling pathways must come together to start the process of long-term potentiation (LTP), an improvement in the connection between two neurons as a result of a previous signal. LTP involves both the TrkB and NMDA receptor signaling cascades, and there is evidence to support cross-talk between TrkB and the NMDA receptor subunit NR2B using adaptor proteins. ShcC is thought to be involved in learning and memory as ShcC knockout mice have increased hippocampal LTP and better performance in Spatial Memory tasks (Miyamoto et al., 2005). Our results indicate that ShcC binds to both Fyn and Src, likely through CH2 and SH2 domain interactions, and is facilitated by phosphorylation of three tyrosines in the internal CH1 domain.

Keywords

LTP, TrkB, Neurotrophins, BDNF, ShcC, MAP Kinase, NMDA, Glutamate, Tyrosine Phosphorylation.
Dedication

First, and foremost, to Jill Driver: There are no words to express how grateful I am to be your daughter. You always encouraged me to aim high and to never hold a question back. You always let me believe I could do anything I set my mind to. Here is your proof.

Secondly, to my father, Robert Driver: You always made sure I knew how proud you were of me and that I was loved. Thank you for always supporting me.

And third, to Shirley and Jack Titus, proud grandparents: Thank you so very much for your love and praise my whole life - and for putting me on the school bus my first day.

Love always,

Kayla.
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Chapter 1. Introduction

Human intelligence can be attributed to, at a molecular level, the transmission of an electrical signal from one neuron to the next; much like the feeling of pain is transferred along a synapse from its site of origin to the brain. The mammalian brain, in addition to receiving such electrical signals, is capable of storing and retrieving them as memories; a phenomenon which is, to date, not well understood. The human brain is unique in many regards, most notably speech, emotion, and higher learning, but the underlying mechanisms are remarkably similar to our mammalian counterparts. This allows researchers to use animal models to study the learning process. With the technological advancements made in the century, the steps of the learning process are finally coming to light. Science is coming close to understanding how learning and memory are achieved at a molecular level and, hopefully, with time, we will understand the molecular basis of learning disorders and develop new technologies for their treatment.

1.1 The Phenomenon of Learning and Memory.

Learning and memory arise from synaptic plasticity in the brain - either strengthened or diminished connection between two neurons. There are two types of memory: long-term and short-term. The latter arises from changes in protein activity; while the former is maintained by synthesis of new proteins (Xia et al., 1996). The two main forms of synaptic plasticity are long-term potentiation and long-term depression. Long-term potentiation (LTP), i.e. the improved communication between two neurons as a result of a previous signal, is commonly regarded as the key mechanism behind learning and memory. Long-term depression (LTD), i.e. a weakened connection between
two neurons, is also involved in learning and memory, but will not be elaborated further. Long-term potentiation can be further divided into early, or classical, and long-lasting LTP (E-LTP and L-LTP, respectively). Classical, or early, LTP proceeds without synthesis of new proteins, and thus requires only changes in the activity of existing proteins (short-term), whereas long-lasting LTP, which can continue for days, requires transcription and synthesis of new proteins (Xia et al., 1996). Long-term memory (LTM) formation and storage is not as well understood, but is thought to involve persistent changes in neuronal functionality and repeated activation of signaling pathways over long periods of time (Eckel-Mahan et al., 2008; Mozzachiodi et al., 2008).

It is commonly known that neurons have two main functional sites: in addition to the cell body (containing the nucleus), neurons have both dendrites, which serve to receive signals from other stimuli, such as other neurons; and one central axon, which serves in turn to transmit signals from one neuron to the next. In a synaptic chain, the neuron which transmits a signal, from the axon, is termed the pre-synaptic neuron. Alternatively, the neuron which receives a signal in the dendrites is called the post-synaptic neuron. More specifically, small protrusions on the dendrites, called dendritic spines, contain all the proteins involved in learning and the induction of LTP, in a small protein-rich structure called the post-synaptic density (PSD), which will be elaborated further in section 1.2. In addition, dendritic spines have been shown to expand in size during the induction of LTP (Lang et al., 2004; Matsuzaki et al., 2004). Moreover, LTP can also be thought of as having two components, the traditional post-synaptic component, as well as the slower, pre-synaptic component where there are also modifications taking place (Bayazitov et al., 2007).
There are many kinds of synaptic plasticity, but the most commonly studied form with respect to learning is in response to the neurotransmitter glutamate, termed glutaminergic signaling. The hippocampus is also the most commonly studied site of learning (Minichiello et al., 2002). There are many pathways involved in this kind of LTP which include, but are not limited to the Trk receptor family and downstream signaling, including the Shc family of adaptor proteins, AMPA and NMDA receptor signaling, and their subsequent regulation by Fyn (Src family kinases) and Striatal-Enriched Protein Tyrosine Phosphatase (STEP). The Trk family of receptors facilitate a number of signaling pathways, which will be discussed in further detail in section 1.3, but the most important pathway with respect to its role in learning and memory is the phospholipase $\text{C}_\gamma$ ($\text{PLC}_\gamma$) pathway. It has been shown that point mutations in the TrkB receptor at the binding site for $\text{PLC}_\gamma$ (Tyrosine 816) result in an impairment of hippocampal LTP, whereas point mutations in the Shc binding site (Tyrosine 515) do not (Minichiello et al., 2002). It is also known that $\text{PLC}_\gamma$ signaling through TrkB activates cyclic-AMP responsive element-binding protein (CREB), a transcription factor responsible for induction of many LTP-dependent genes (Minichiello et al., 2002). In addition, CREB can also be activated, via phosphorylation at Serine 133, in response to Erk/MAPK signaling and also to glutamate, a key neurotransmitter in synaptic plasticity and learning (Xia et al, 1996; Eckel-Mahan et al., 2008). Glutamate, when released during synaptic transmission activates two key receptors: $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and N-methyl-D-aspartate (NMDA), ionotropic glutamate receptors which generally serve as ion channels to sodium and potassium, and calcium ions, respectively (Hollmann et al., 1991; Xia et al., 1996). In fact, calcium
influx via the NMDA receptor is often viewed as a critical step in the induction of LTP (Xia et al., 1996). AMPA and NMDA receptor functions in LTP will be further described in section 1.5.

Calcium influx via the NMDA receptor induces a number of cellular changes, whether direct or indirect, including alterations in tyrosine phosphorylation of proteins involved in learning, such as Src (Lu et al., 1998). In fact, blockade of Src activity prevented LTP in rat hippocampal neurons (Lu et al., 1998). NMDA receptors have also been shown to induce transcription of LTP-dependent genes, namely *c-fos*, via the MAPK/Erk signaling pathway (Xia et al., 1996). Lastly, the expansion of dendritic spines is also shown to be dependent on NMDA receptors (Matsuzaki et al., 2004).

Neurotrophins, namely brain-derived neurotrophic factor (BDNF), have also been shown to play a role in LTP, through independent pre-synaptic and post-synaptic elements (Alder et al., 2005). BDNF-induced synaptic plasticity is regulated by cyclic AMP (cAMP), a messenger required for many functions of BDNF (Ji et al., 2005). In addition, both pre-synaptic and post-synaptic components of TrkB/BDNF-mediated LTP are regulated by the PLCγ signaling pathway (Gartner et al., 2006).

In addition to structural and functional remodeling upon LTP induction, synapses can also be functionally remodeled in a more permanent capacity. Drug abuse can significantly impair neuronal excitability and consequently impairment in synaptic plasticity is thought to be the transitioning factor to addiction (Kasanetz et al., 2010). Alternatively, there is evidence to support that conditioning can positively alter the basal excitability of a neuron such that memory persists, providing a possible mechanism for long-term memory storage in *Aplysia* (Mozzachiodi et al., 2008). Recently, it was also
discovered that many proteins involved in long-term memory are subject to oscillations with respect to circadian rhythm. In particular, adenylyl cyclase activity and subsequent cAMP levels, Erk and MAPK phosphorylation, and CREB Serine 133 phosphorylation were all significantly higher during the day (Eckel-Mahan et al., 2008). It has also been shown that circadian rhythm is important in memory persistence, as there was a deficit in fear memory in mice that were subject to all-dark conditions (Eckel-Mahan et al., 2008).

1.2 The Post-Synaptic Density: A Site for Learning.

Learning and memory processes are initiated at the cell surface; the synapse of neurons located at dendritic spines receives signals from the pre-synaptic neuron via its axon, and transmits the signal further down the chain. More specifically, the site of synaptic transmission is a protein-dense region called the post-synaptic density (PSD), marked by an enrichment of the scaffolding protein post-synaptic density 95 (PSD-95; Cho et al., 1992). PSD-95 contains two PDZ domains and an SH3 domain, and the PDZ domains are known to bind receptors such as the NMDA receptor through its subunit NR2B and serve to anchor kinases such as Fyn or Src to the PSD, and mediate regulatory processes such as tyrosine phosphorylation of NR2A and NR2B by Fyn kinase (Tezuka et al., 1999).

Over the decades, there have been several key articles with regards to isolating the post-synaptic density by subcellular fractionation and to further characterize its constituents. First and foremost, it was found that synaptic membranes separate with the mitochondria upon centrifugation, making the technique a useful tool for isolating synaptic membranes (De Robertis, 1967). Next, researchers began to optimize conditions to remove the synaptosomal plasma membrane from mitochondrial contamination,
including the addition of an osmotic shock, and the removal of alkaline phosphatase via addition of Triton X-100 (Cotman and Matthews, 1971). It was also concluded that polypeptides were the major component at this time (Cotman and Taylor, 1972). In fact, the PSD, which could be isolated from the brain and even from the synaptic membrane, was set up as a polypeptide matrix potentially formed by a single polypeptide and small amounts of other proteins of varying sizes (Cotman et al., 1974; Banker et al., 1974). Afterwards, researchers began to characterize proteins present in the post-synaptic density by size, and believed that actin and neurofilaments were present, but little to no tubulin (Cohen et al., 1977; Blomberg et al., 1977). Later, many proteins, some receptors, were added to the list of proteins in the PSD such as AMPA receptors, Calmodulin, CaMKII, NMDA receptors, TrkB, and the scaffolding protein PSD-95 (Wu et al., 1996; Ji et al., 2005; Swulius et al., 2010). The levels of each protein present in the PSD vary at different developmental stages, with structural data also showing that the PSD changes drastically throughout development (Swulius et al., 2010). In turn, each protein is regulated and receptors are turned over in an activity-dependent manner, utilizing the ubiquitin proteasome system (Ehlers, 2003).

1.3 Trk Receptor Signaling and Neurotrophins.

The Trk family of Receptor Tyrosine Kinases serve a pivotal role in the growth and survival of terminally differentiated, post-mitotic neurons. There are three Trk family members: TrkA, B, and C which are expressed in the brain and bind specific neurotrophins. TrkA binds specifically to NGF, TrkB to BDNF and also to neurotrophin-4/5 (NT-4/5), and TrkC to NT-3 (Kaplan et al., 1991; Klein et al., 1993; Lamballe et al., 1993). TrkB, which is expressed solely in the nervous system, is regarded as the key Trk
receptor in learning and memory (Klein et al., 1989; Klein et al., 1990) and will be the subject of further discussion in this section.

Throughout the brain, BDNF and TrkB have been shown to be involved in spatial memory, associative learning, fear learning, and object recognition memory (Mizuno et al., 2003; Gruart et al., 2007; Musmeci et al., 2009; Hopkins and Bucci, 2010), and have involvement in cortical development, neurite outgrowth, dendritic development, dendritic spine density, and synaptic plasticity (Bartkowska et al., 2007; Meakin et al., 1999; Alonso et al., 2004; Finsterwald et al., 2010; Crozier et al., 1999; Alder et al., 2005). Furthermore, BDNF and TrkB have been implicated in both Limbic Epileptogenesis and Huntington Disease (He et al., 2010; Ginés et al., 2010). The biological significance of TrkB (and BDNF) was shown when Klein et al (1993) attempted to engineer TrkB mutant mice, lacking a functional tyrosine kinase domain, which died shortly after birth due to large nervous system defects and poor motor control and breathing (Klein et al., 1993). A conditional knock-out of \textit{TrkB} in the postnatal forebrain produced mice with impairment in hippocampal LTP and complex learning tasks such as Morris Water Maze, which tests the hippocampal-dependent spatial learning, while succeeding at less difficult tasks, such as passive avoidance (Minichiello et al., 1999). This led researchers to believe the mice had a problem adapting to stress (Minichiello et al., 1999).

As with all receptors, TrkB activates a number of downstream signaling pathways which lead to many cellular functions such as growth, survival, and gene transcription. For simplicity, only a set group of proteins that are shown to bind will be discussed. The TrkB receptor itself is a dimer, with two identical subunits containing both intra- and extra-cellular regions, as well as a transmembrane region (Klein et al., 1989). The
intracellular region contains a tyrosine kinase domain which contains two main sites for downstream signaling cascades: Tyrosine 515 (Y515), for Shc or FRS2 competitively, and Tyrosine 816, for PLCγ (Klein et al., 1989; Meakin et al., 1999; Minichiello et al., 2002). In addition, the Plekstrin Homology domain (PH1) of Ras-Guanine releasing factor 1 (RasGRF1), a nucleotide exchange factor for the small GTPase Ras and Rac, has been shown to bind to TrkB through its HIKE domain, whereby Trk phosphorylates RasGRF1 and potentiates neurite outgrowth in response to both NGF (Robinson et al., 2005) and BDNF (Talebian et al., 2012). The Shc site, at Tyrosine 515, leads to activation of MAP kinase, while the PLCγ site, at Tyrosine 816, leads to activation of downstream CREB signaling (Minichiello et al., 2002). TrkB, through interaction with Shc, can give rise to neuronal growth and survival through either PI3-Kinase or MAPK/Erk (Atwal et al., 2000). Through PLCγ and related signaling, TrkB can give rise to LTP (Minichiello et al., 2002). The PLCγ site is also essential for hippocampal LTP: mice lacking a Tyrosine at residue 816 had a significant reduction in LTP (Minichiello et al., 2002). In fact, PLCγ signaling supports both pre- and post-synaptic hippocampal LTP (Gartner et al., 2006). Though not indicated by this experiment, ShcC may also play an alternative role in LTP and learning. ShcC knockout mice have an enhancement of LTP in the hippocampus, and better performance in many hippocampal-dependent tasks, such as Morris Water Maze (Miyamoto et al., 2005).

Upon BDNF binding, active TrkB undergoes tyrosine phosphorylation, leading to the binding of PLCγ, Shc and/or FRS2 (Klein et al., 1989; Kaplan et al., 1991; Minichiello et al., 2002; Meakin et al., 1999). The PLCγ signaling cascade leads to downstream Calcium/Calmodulin kinase activation, CamKII and CaMKIV in particular,
and CREB activation, leading to gene transcription and synaptic plasticity, namely LTP (Minichiello et al., 2002). Upon Shc binding, there is recruitment of Grb2 and downstream transient activation of the MAP kinase signaling cascade leading to growth and survival (Rozakis-Adcock et al., 1992; Minichiello et al., 2002). ShcB and C are also capable of binding to TrkB at this site and eliciting the same response (Liu and Meakin, 2002). Upon FRS2 binding to Tyrosine 515, FRS2 also binds to Grb2, but subsequently binds to Crk/p38, Sh-PTP-2/Shp2, and Src leading to differentiation, or neurite outgrowth in vitro, though it may also activate or prolong MAPK signaling (Meakin et al., 1999). For a schematic representation of TrkB signaling, see Figure 1.

Brain-derived neurotrophic factor also plays a role in modulating synaptic plasticity in the hippocampus. BDNF administration, opposed to NGF, was shown to enhance NMDA receptor activity, indicating some specificity among the neurotrophins (Levine et al., 1998). Similarly BDNF, but not NT-3, was shown to increase the tyrosine phosphorylation of the NMDA receptor subunits NR1 and NR2B indicating there is some cross-talk between TrkB receptor signaling and NMDA receptors (Suen et al., 1997; Levine et al., 1998; Lin et al., 1998). Interestingly, interference of NR2B-containing NMDA receptors with ifenprodil, an NR2B inhibitor, prevents the effects of BDNF on synaptic plasticity, providing further evidence for cross-talk between TrkB and NMDA in LTP (Crozier et al., 1999). BDNF has also been shown to increase the tyrosine phosphorylation of the AMPA receptor subunit GluR1, though this effect is dependent on NMDA receptor activity as well (Wu et al., 2004). This effect is not only seen in the hippocampus; BDNF has also been shown to influence NMDA-mediated LTP in the spinal cord, where it may contribute to inflammatory pain responses (Kerr et al., 1999).
Figure 1. Schematic representation of TrkB signalling that leads to differentiation, neurite outgrowth, LTP, and neuronal survival. Briefly, activation of TrkB by BDNF leads to phosphorylation of Tyrosines 515 and 816, which serve as recruitment sites for Shc/FRS2 and PLC\(\gamma\), respectively (Minichiello et al., 2002). This leads to activation of many downstream signalling pathways including MAPK/Erk, and CREB (Rozakis-Adcock et al., 2002, Minichiello et al., 2002). Activation of MAP kinase through Shc has been shown to mediate neuronal growth and survival (Rozakis-Adcock et al., 1992; Minichiello et al., 2002); whereas FRS2 binding is thought to lead to neurite outgrowth and differentiation, and potentially additional or prolonged MAPK activation (Meakin et al., 1999). Alternatively, binding of PLC\(\gamma\) to TrkB has been shown to activate downstream CREB signaling and subsequently lead to LTP (Minichiello et al., 2002). Image adapted from Minichiello (2009).
1.4 The Shc Family of Adaptor Proteins.

To date, there are four identified Shc family members: ShcA (abbreviated Shc), ShcB, ShcC, and recently characterized, ShcD. They are structurally similar but vary in sequence (Sakai et al., 2000). While ShcA is expressed ubiquitously through the body, its expression is tightly controlled in the brain during development, and reduced to very low levels after birth (Conti et al., 1997). ShcB is found to be expressed widely throughout the developing nervous system, particularly strongly in the dorsal root ganglion and superior cervical ganglion, involved in sensory and sympathetic systems, respectively (Sakai et al., 2000). ShcC is expressed only in the nervous system, as shown by mRNA and protein studies, where protein expression is low during development and rises at birth (Nakamura et al., 1998; Sakai et al., 2000). There is evidence that ShcB and C govern the maintenance of different types of neurons, based on their differential expression patterns across the brain (Ponti et al., 2005). For example, ShcB is greatly enriched in primary sensory neurons and olfactory nerve fibers, whereas ShcC is enriched in the striatum and motor fibers (Ponti et al., 2005). In addition, ShcB mRNA is detectable in the adult peripheral nervous system, where ShcC mRNA is not (Nakamura et al., 1998). ShcD, the most recently identified Shc family member, is expressed in both the adult brain and skeletal muscle, in particular at the neuromuscular junction, and has the most similarity to ShcA (Jones et al., 2007). During development, ShcD is expressed, widely throughout the body but also in the brain (Hawley et al., 2011).
Figure 2. Schematic representation of the adaptor protein, ShcC, expressed in the brain. There are two isoforms, 69 and 55 kDa, differing in the presence of an N-terminal Collagen-Homology 2 (CH2) domain (O’Bryan et al., 1996). Both isoforms of ShcC contain two phosphotyrosine-binding domains, PTB (N-terminus) and SH2 (C-terminus), with an internal, Collagen Homology 1 (CH1) domain containing three tyrosines which, when phosphorylated, serve as recruitment sites for Grb2 as well as polyproline residues (PP), which serve as binding sites for SH3 domain-containing proteins (van der Geer et al., 1995; Liu and Meakin, 2002; Rozakis-Adcock et al., 1992; McGlade et al., 1992; Sato et al., 1997; van der Geer et al., 1996) It has also been postulated that tyrosine phosphorylation in the CH1 domain serves as a “gate” for SH2 domain activity due to the fact that the SH2 domain is only active when the three key tyrosines are phosphorylated (George et al., 2008). Amino acid nominations are shown below for each domain according to Sakai et al. (2000).
Shc family members contain two domains for phosphotyrosine binding (Figure 2): N-Terminal Phosphotyrosine Binding (PTB) and C-Terminal Src Homology 2 (SH2; van der Geer et al., 1995). They also contain an internal, collagen-homology (CH1) domain, which contains three target sites for tyrosine phosphorylation by Src and Trk, among other tyrosine kinases (Y239, 240, and 317 on ShcA); and polyproline motifs which serve as docking sites for SH3 domain-containing proteins (van der Geer et al., 1995; Rozakis-Adcock et al., 1992; McGlade et al., 1992; Liu and Meakin, 2002; Sato et al., 1997; van der Geer et al., 1996). Phosphorylation of the three tyrosine sites upon Trk activation results in the activation of Ras and MAP kinase signaling through recruitment of Grb2 (Rozakis-Adcock et al., 1992; Figure 1). It has also been shown that phosphorylation of these three tyrosines is crucial for SH2 domain function (George et al., 2008). Larger isoforms also contain an N-terminal CH2 domain, containing serines in addition to polyproline residues, which may be targets for serine phosphorylation, but to date this has only been investigated in p66ShcA (Xi et al., 2010a). There is also evidence that the longer isoforms of Shc family members, containing CH2 domains, have opposing roles in downstream receptor signaling, to their shorter family members. For example, p52 and p66 ShcA bind competitively to the activated Insulin-Growth Factor Receptor (IGF-1) and when p66 ShcA is over-expressed we see a reduction in Grb2 recruitment and downstream signaling resulting in a reduction in cell proliferation (Xi et al., 2008). ShcC specifically has two isoforms as well, which are 55 and 69 kDa (O’Bryan et al., 1996). As previously described in section 1.3, the ShcC PTB domain binds to activated TrkB (at Tyrosine 515), resulting in the phosphorylation of Tyrosine 221, 222, and 304 in the collagen homology domain and a conformational change in the SH2 domain allowing for
activity (Liu and Meakin, 2002; George et al., 2008). ShcD, much like other Shc family members, binds to TrkB and regulates MAPK singling, the exception being that both PTB and SH2 domains can bind to TrkB (You et al., 2010). George et al. (2008) generated a non-phosphorylatable mutant (3YF) of ShcA, and showed that its SH2-binding capabilities were severely reduced. In addition, a phospho-mimic ShcA was generated where these same tyrosines were mutated to aspartic acids, and no SH2 binding was detected, indicating further importance for tyrosine phosphorylation of the CH1 domain in SH2 domain activity. However, mimicking the active state by glutamic acid substitution may be more beneficial as it contains one extra carbon compared to aspartic acid, and thus glutamic acid substitution of salient tyrosine residues in ShcC where the PTB domain is not present (ShcC CH1SH2 3YE) may be useful in studying SH2 domain function.

ShcC, being an adaptor protein, is expected to have several binding partners. Upon NGF stimulation in pheochromocytoma (PC12) cells, which express endogenous TrkA, ShcC is capable of binding to F-actin (Thomas et al., 1995). Through its PTB domain we see interaction with TrkB (Liu and Meakin, 2002), its CH1 domain with Grb2 (Rozakis-Adcock et al., 1992) and its SH2 domain with epidermal-growth factor receptor (van der Geer et al., 1995). There is also evidence for ShcC interaction with the NR2B subunit of the NMDA receptor and Src family kinases upon glutamate stimulation (Miyamoto et al., 2005). However, interaction with Src family kinases is not clear as this experiment did not use antibodies specific to any Src family member, and therefore did not distinguish which protein ShcC was binding to. In addition, they did not include a negative control (i.e. IgG), nor did they include a Western blot indicating the ShcC
antibody was correctly immunoprecipitating. Recently, there has been much evidence to support p66 ShcA binding to Src through its CH2 and SH2 domains in smooth muscle cells in response to insulin, resulting in an impairment of Src activity and downstream activation of MAP Kinase and PI3-Kinase (Xi et al., 2010a; Xi et al., 2010b). Taken together, these studies demonstrate an importance for distinguishing among the Src family members with respect to ShcC binding, and to elucidate domains of ShcC which bind to the Src family kinases.

1.5 Glutaminergic Signaling through AMPA and NMDA Receptors.

Synaptic transmission is initiated at the PSD, through neurotransmitter release at the pre-synaptic terminal, and a resultant electrical impulse in the post-synaptic neuron. For the purpose of simplicity, only glutaminergic synapses, i.e. those that respond to the neurotransmitter glutamate, will be discussed. When glutamate is released into the synaptic cleft, it binds to one of two ionotropic receptors at the PSD: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and N-methyl-D-aspartate (NMDA), which are permeable to Na+/K2+, and Ca2+ ions, respectively (Hollmann et al., 1991; Matsubara et al., 1996; Xia et al., 1996). AMPA receptors are also permeable to Ca2+, depending on the subunit composition (Hollmann et al., 1991). The receptors both exist as heterotetramers, i.e. four non-identical subunits. AMPA receptors may be composed of different subunit types: GluR1, GluR2, GluR3, and GluR4, with the presence of GluR2 limiting permeability to Ca2+ (Hollmann et al., 1991; Matsubara et al., 1996; Figure 3). NMDA receptors, alternatively, consist of GluN1/NR1, GluN2/NR2 or the lesser-known GluN3/NR3 (Al-Hallaq et al., 2007; Henson et al., 2008; Figure 3). Though receptors can consist of any combination, the presence of NR2A or NR2B is required to produce
functional receptors (Saito et al., 2003). The subunit composition of these receptors is not static, and in fact there is a switch in preference from NR2B to NR2A during postnatal development, likely through an increase in NR2A expression and incorporation into new synapses (Liu et al., 2004). In addition, NR2A- and B-containing receptors may associate with different proteins in the PSD (Al-Hallaq et al., 2007). Though not necessarily as prominent with age, NR2B, but not NR2A receptors are required for LTP: Loss of NR2B results in learning deficits (Brigman et al., 2010; Foster et al., 2010).

Figure 3. The structure of AMPA and NMDA receptors. Both receptors generally exist as heterotetramers, composed of either GluR1 and GluR2, or GluN1 (NR1) or GluN2 (NR2) subunits, respectively, shown by convention here as two of each (Hollmann et al., 1991; Matsubara et al., 1996; Al-Hallaq et al., 2007; Henson et al., 2008). The functionally relevant subunit on AMPA receptors is GluR1, which determines $\text{Ca}^{2+}$ permeability (Hollmann et al., 1991). The NMDA receptor subunit NR2B is responsible for regulating internalization of the receptor; when tyrosine phosphorylated, the internalization process is uncoupled (Roche et al., 2001; Lavezarri et al., 2003).
The process of LTP is initiated at the post-synaptic density, by activity changes in receptors, namely the AMPA and NMDA receptors. NMDA receptors have been implicated in a number of diseases, including Alzheimer’s disease (Snyder et al., 2005). NMDA receptor activity is limited by Magnesium; in order for NMDA receptors to initiate LTP, an Mg$^{2+}$ block must first be removed (Coan and Collingridge, 1985), though this process is not very well understood. After NMDA receptors are activated, AMPA receptors are trafficked to dendritic spines, which may account for an increase in AMPA receptor activity during LTP (Shi et al., 1999). This is mediated by the GluR1 subunit binding with PDZ-domain containing proteins (Hayashi et al., 2000), potentially with PSD-95.

There are many regulators of NMDA receptor activity, but the most prominent is tyrosine phosphorylation. NMDA receptor activity is modified by both protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs), one such tyrosine kinase implicated in activating the NMDA receptor is c-Src (Wang and Salter, 1994). As previously stated, there is a marked increase in NR2B tyrosine phosphorylation upon LTP induction (Rostas et al., 1996), indicating further importance of tyrosine phosphorylation in learning. In addition, AMPA receptors are also serine/threonine phosphorylated upon LTP induction, by the calcium-dependent kinase, CaMKII, implicating both PLC$\gamma$ and calcium-dependent signaling in LTP (Barria et al., 1997). BDNF has been shown to increase NMDA receptor activity by indirectly increasing tyrosine phosphorylation of both NR1 and NR2B subunits of the NMDA receptor (Suen et al., 1997; Levine et al., 1998; Lin et al., 1998). The BDNF-dependent increase in NMDA receptor activity can be prevented by blocking the activity of CaMKII (Crozier et
Src family kinases, Src and Fyn, are also implicated in tyrosine phosphorylation of both NR2A and NR2B at many sites, in particular Y1472 on NR2B (Cheung and Gurd, 2001; Nakazawa et al., 2001). Fyn is in fact regarded as the key kinase in NR2B Tyrosine 1472 phosphorylation (Nakazawa et al., 2001).

NMDA receptors are rapidly turned over by clathrin-mediated endocytosis, (Roche et al., 2001). In the C-terminal domain of NR2B, there is a YEKL motif that serves as a binding site for AP-2, an adaptor protein involved in clathrin internalization, and binding of the PDZ domain of PSD-95 to an adjacent motif effectively blocks NMDA receptor internalization (Lavezzari et al., 2003; Roche et al., 2001). Tyrosine 1472, in the YEKL motif, is critical for AP-2 binding, as no internalization occurs when it is changed to an alanine or phenylalanine residue (Lavezzari et al., 2003). It has also been shown that tyrosine phosphorylation by Fyn at Y1472 increases the number of NMDA receptors at the cell surface, implicating Fyn in the uncoupling of NMDA receptor internalization (Prybylowski et al., 2005). Alternatively, NMDA receptors can be internalized when Y1472 is de-phosphorylated by Striatal-Enriched Protein Tyrosine Phosphatase (STEP), for example, in response to β-Amyloid (Snyder et al., 2005). STEP is the key phosphatase in the regulation of NMDA receptor internalization (Braithwaite et al., 2006). STEP is also responsible for inactivating Fyn, by de-phosphorylating Tyrosine 420 (Nguyen et al., 2002). This leads to the notion that Fyn and STEP have opposing roles in NMDA receptor regulation (Figure 4).

The Src family of tyrosine kinases consists of many members, three of which are expressed ubiquitously (Src, Yes, and Fyn), but most important in learning and memory are Src and Fyn, both of which have been implicated in NMDA receptor regulation.
(Wang and Salter, 1994; Sun et al., 1998; Prybylowski et al., 2005). However, the key player in NMDA receptor phosphorylation is regarded as Fyn: Fyn mutant mice, but not Src mutant mice, have impairment in NMDA-dependent LTP and spatial learning (Grant et al., 1992). Fyn contains both an SH2 and SH3 domain, as well as the kinase (KIN) or SH1 domain, containing Tyrosine 420 (Superti-Furga et al., 1993; Nguyen et al., 2002). Fyn, when inactive, is phosphorylated at the C-terminus, Tyrosine 531 in human Fyn, and is in a folded conformation bound to the SH2 domain (Superti-Furga et al., 1993; Nguyen et al., 2002). When unphosphorylated at the C-terminus, Src family kinases can be tyrosine phosphorylated in the kinase domain (Y420 on Fyn), thought to be by autophosphorylation (Sun et al., 1998). When the activating tyrosine is phosphorylated, phosphorylation at the C-terminus does not inactivate the protein (Sun et al., 1998). Fyn is dephosphorylated at Y420, but not 531, by STEP (Nguyen et al., 2002), which would render it inactive. Fyn is also responsible for mediating BDNF-mediated increase in NMDA receptor activity, and is also shown to regulate the localization of TrkB receptors in response to BDNF (Xu et al., 2006; Pereira and Chao, 2007).

Striatal-Enriched Protein Tyrosine Phosphatase (STEP) is, as its name implies, expressed specifically in the brain and enriched in the striatum (Lombroso et al., 1991). There are multiple isoforms, arisen by alternative splicing, but the 46 kDa isoform is the most prominent, with other identified isoforms of 20 and 61 kDa (Lombroso et al., 1991; Sharma et al., 1995). In addition to the protein tyrosine phosphatase (PTP) domain shared with STEP_{46}, STEP_{61} contains two PEST sequences, which may serve as sites for proteolytic cleavage, two polyproline-rich regions, and two transmembrane domains, which serve to anchor it to the membrane of the endoplasmic reticulum (Bult et al.,
Both STEP$_{46}$ and STEP$_{61}$, ER-bound, contain a kinase interacting motif (KIM), necessary for recognizing targets (Nguyen et al., 2002; Paul et al., 2003). STEP$_{61}$ may be regulated in some manner by cleavage: there is evidence to support STEP$_{61}$ being cleaved by calpain, in response to calcium influx, to a 33 kDa product (Nguyen et al., 1999). The main function of STEP appears to be regulation of LTP. STEP is a part of the NMDA receptor complex, and reduces NMDA receptor activity to block or reduce LTP induction (Pelkey et al., 2002). Upon NMDA receptor calcium-influx, STEP is activated by calcineurin via dephosphorylation of Serine 221 in the KIM domain (Paul et al., 2003). This activated form of STEP also serves to regulate the duration of Erk signaling (Paul et al., 2003). STEP knockout mice are viable and show, not surprisingly, an increase in Erk phosphorylation (Venkitaramani et al., 2009).

**Figure 4.** Schematic representation of the opposing roles of STEP and Fyn on NMDA receptor regulation. Phosphorylation of NR2B Y1472 uncouples internalization of NMDA receptors by facilitating binding with the PDZ domain of PSD-95 (Roche et al., 2001; Lavezarri et al., 2003). Fyn, and to a lesser extent Src, are responsible for phosphorylating NR2B at this site (Prybylowski et al., 2005). Alternatively, STEP is responsible for dephosphorylating NR2B at this site, as well as dephosphorylating Fyn kinase at its active tyrosine, 416 (Prybylowski et al., 2005; Braithwaite et al., 2006), allowing for two-tiered regulation of NMDA receptor activity. Furthering this notion, STEP is responsible for dephosphorylating Fyn at tyrosine 420, rendering it inactive (Nguyen et al., 2002).
1.6 The ShcC Knockout Mouse.

In hopes of understanding the function of a protein, scientists often return to the genetic level and knock out the gene of interest in order to study the consequences. With many cases, the knockout is not viable, or has a very visible phenotype that gives insight into the protein’s role. The Shc family members are no exception to this rule. First, scientists attempted to generate a Shc(A) null mouse to no avail, and it was realized that this scenario is embryonically lethal, with embryos dying at E11.5 due to large cardiovascular defects (Lai and Pawson, 2000). A conditional knockout of ShcA in the subventricular zone (SVZ), the location of neural stem cells, showed irregularities in SVZ organization, and a reduction in proliferation in the adult brain, which is required to replenish olfactory bulb interneurons (Ponti et al., 2010). Secondly, ShcB and ShcC knockouts were generated, as well as a ShcB/C double knockout. ShcB knockout mice are viable but at 8-9 weeks of age are shown to experience, among other phenotypes, a ~32% reduction in myelinated axons and substantial sensory neuronal loss (~38%) in the dorsal root ganglion, including a >50% reduction in TrkA+ neurons (Sakai et al., 2000). The combination ShcB/C knockout mouse demonstrates similar losses to ShcB alone, indicating a lack of compensation for loss of ShcB by ShcC, while ShcC knockout mice do not demonstrate any of the aforementioned phenotypes. One population of neurons affected by the loss of ShcB and ShcC, but not either loss alone, is the sympathetic neurons of the superior cervical ganglion, where there is a ~33% reduction in the number of neurons at 8 weeks of age (Sakai et al., 2000).

Surprisingly, ShcC knockout mice have no brain abnormalities or visible neuronal loss (Sakai et al., 2000). ShcC is expressed highly in the hippocampus, yet loss of ShcC
does not seem to affect hippocampal morphology (Miyamoto et al., 2005). This led researchers to examine changes in neural functions, i.e. behaviour, of these mice. In this respect, the loss of ShcC had a significant impact on the learning and memory performances of these mice compared to their wild-type litter mates. ShcC knockout mice have enhanced spatial and temporal memory as shown through the Morris Water Maze test, improved contextual fear conditioning, and a better visual recognition of novel objects, all of which are hippocampus-dependent modes of learning (Miyamoto et al., 2005). Physiologically, ShcC knockout mice demonstrate enhanced hippocampal LTP. At the molecular level, ShcC knockout mice display an increase in the levels of tyrosine phosphorylation of the NR2A and NR2B subunits of the NMDA receptor, which positively correlates with receptor activation, and subsequent LTP (Miyamoto et al., 2005). It is important to note that antibodies used to detect tyrosine phosphorylation were not residue-specific, and thus could not identify if NR2B tyrosine 1472 specifically had been affected. However, there were also no changes in phosphorylation of the Src family kinases Src or Fyn, at the active site, and an in vitro Src activation assay showed no changes in Src kinase activity (Miyamoto et al., 2005).
1.7 Hypothesis

ShcC binds to Fyn through its CH2 and SH2 domains and prevents its activity, allowing for regulation of NR2B Tyrosine 1472 phosphorylation. ShcC knockout mice would thus have an increase in NR2B tyrosine phosphorylation because of increased availability of Fyn kinase.

1.8 Objectives

a. To determine if ShcC can bind to Fyn and/or Src.

b. To determine the domains of ShcC involved in interaction with Fyn or Src \textit{in vitro}.

c. To determine if tyrosine phosphorylation of the ShcC CH1 domain is important for Fyn and Src binding \textit{in vitro}.
Chapter 2. Materials and Methods

Note: For a full listing of all solutions named below, please see Appendix I.

2.1 DNA Cloning, Extraction and Purification

a. Site-directed mutagenesis.

The reference protocol for site-directed mutagenesis was obtained in the QuickChange-XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The intention was to introduce three tyrosine to glutamic acid substitutions at the three tyrosine sites in mouse ShcC (Y221, 222, and 304) that are involved in downstream signaling through Grb2 (Figure 5). The rationale for changing three tyrosine sites in the CH1 domain to glutamic acids was to create a mutant that carries a negative charge at these sites, and is more likely to adopt the correct conformation and potentially allow for constitutive binding in the absence of activation by Trk. These mutations were introduced in a ShcC GST fusion construct containing only the CH1 and SH2 domains (pGEX ShcC CH1SH2), to investigate SH2 binding partners in the absence of the PTB domain to confound data. Briefly, circular DNA was amplified using a primer set that was complementary and contained the mutation of interest in the middle (Table 1) and PfuTurbo, a high-fidelity Taq polymerase enzyme supplied with the kit. Briefly, 1-5µL of template DNA and 125ng of forward and reverse primer were incubated with 200µM dNTPs, 1X concentration of PfuTurbo buffer, and 2.5U PfuTurbo in the conditions outlined in Table 2. Following PCR amplification, parental DNA was digested with DpnI, a methylation-specific frequent cutting restriction enzyme by addition of 1µL (10U) to the PCR product and a 1 hour incubation at 37°C. Following digestion, 1µL of the amplified DNA was transformed into 100µL XL10-Gold, a cloning strain of E. coli.
provided in the kit, using 100µg/mL Ampicillin selection as described below (b). Colonies were selected; DNA was prepared and sequenced as described below (c) to screen colonies for the mutation of interest. In addition, the native vector (pGEX ShcC CH1SH2) was co-expressed with pET28a V-Src to allow for tyrosine phosphorylation at these sites.

**Table 1.** List of primers used to introduce Y221E, Y222E, and Y304E mutations into ShcC.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sequence (5’-3’)</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;</th>
</tr>
</thead>
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<tr>
<td>Y221E</td>
<td>Forward ATGGCCCTGATCACCCAGAAATACAACACAGCTTCCAC</td>
<td>83.9</td>
</tr>
<tr>
<td></td>
<td>Reverse GTGGGAACGCTGTTGTATTTCTGGGTGATCACGGGCCAT</td>
<td>83.9</td>
</tr>
<tr>
<td>Y222E*</td>
<td>Forward ATGGCCCTGATCACCCAGAAACACACAGCTTCCAC</td>
<td>84.4</td>
</tr>
<tr>
<td></td>
<td>Reverse GTGGAACGCTGTTGTATTTCTGGGTGATCACGGGCCAT</td>
<td>82.7</td>
</tr>
<tr>
<td>Y304E</td>
<td>Forward GTAGGAGAAACACACACCGAGTGCAACACCCAGCCAGTCCC</td>
<td>85.7</td>
</tr>
<tr>
<td></td>
<td>Reverse GGGACTGGCTGGGTGTTGACCTCGGTTGGTGTTCCTCCTAC</td>
<td>85.7</td>
</tr>
</tbody>
</table>

Note: Nucleotides designed to introduce mutations are underlined. Y222E Primers (*) were designed for a template with the Y221E mutation completed. T<sub>m</sub> values are given in °C.

**Table 2.** PCR conditions for site-directed mutagenesis.

<table>
<thead>
<tr>
<th>30 Cycles</th>
<th>95°C 1:00</th>
<th>95°C</th>
<th>55°C</th>
<th>68°C 20:00</th>
<th>Hold 4°C</th>
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<td>1:00</td>
<td>0:30</td>
<td>1:00</td>
<td>20:00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Extension time was meant to be at least 1 minute per kb of plasmid DNA. Extension temperature of 68°C was indicated by PfuTurbo (Stratagene).
b.  **Making Competent Cells and Transformation**

Rubidium Chloride competent *E. coli* were prepared using a protocol adapted from the McManus Lab (University of California San Francisco, San Francisco, CA). A 3mL LB culture inoculated with the BL21-DE3 strain under 25µg/mL chloramphenicol selection was grown overnight at 37°C with constant agitation. The following day, the culture was transferred over to a 50mL LB flask and grown under the same selection to an OD$_{600}$ of approximately 0.6. Then, cells were centrifuged at 5000xg for 10 minutes in a Beckman JA-20 rotor, and the pellet rinsed with a small amount of TFBI to remove excess media. Then, the cells were re-suspended in 20mL transformation buffer I (TFBI; 100mM Rubidium chloride, 50mM Manganese chloride, 30mM Potassium acetate, 10mM Calcium chloride, and 15% glycerol) by repeated pipetting and placed on ice for 5 minutes. Next, the cell suspension was centrifuged at 5000xg for 5 minutes in a Beckman JA-20 rotor. The resultant pellet was re-suspended in 2mL of TFBII (10mM MOPS, 10mM Rubidium chloride, 75mM Calcium chloride, and 15% glycerol) and placed on ice for 15 minutes. The resultant suspension of competent cells was separated into 200µL aliquots, which were flash frozen in liquid N$_2$ and stored at -80°C prior to use.

On the day of transformation, the desired amount of DNA (typically 1-5µL) was mixed with 100-200µL of competent cells prepared as described above. This reaction was mixed by pipetting and allowed to incubate on ice for 30 minutes. Next, transformations were given a 45 second heat shock at 42°C, and placed briefly back on ice. For recovery, 0.5-1mL of LB was added to the transformation and tubes were placed at 37°C with constant agitation for 1 hour. Transformations were then centrifuged at 1520xg in the IEC Micromax centrifuge for 1 minute, to sediment cells, media was decanted minus
approximately 100µL and the remainder was re-suspended, plated on LB agar plates containing the appropriate selection pressure (100µg/mL Ampicillin and/or Kanamycin) and allowed to incubate overnight at 37°C. Individual colonies were selected the following day for further experimentation.

c. DNA Extraction and Purification

Bacteria containing the vector of interest were first grown overnight in 3mL or 50mL LB media, under the appropriate selection measure (100µg/mL Ampicillin for pGEX and pEBG vectors, or Kanamycin for pET28a vectors), and centrifuged at 3901xg in the Beckman coulter Allegra X-22R centrifuge for 10 minutes to pellet cells. For a small-scale DNA extraction, pellets were placed on ice and re-suspended in 100µL Solution I (2.5mM Tris-Cl pH 8.0, 10mM EDTA, 50mM glucose) by pipetting and transferred to a 1.5mL microcentrifuge tube. Next, 200µL of Solution II (1% SDS, 200mM NaOH) was added and samples were inverted 3-5 times. In the same manner, 175µL of Solution III (3M Potassium acetate, 1.18M Acetic Acid) was added. Next, 500µL buffer-saturated phenol:chloroform (BioShop) was added, and samples were vortexed until opaque. Samples were centrifuged at 10,621-20,817xg in the Eppendorf 5417R centrifuge for 15 minutes to separate the aqueous and organic phases. The upper, aqueous phase was transferred to a fresh tube and washed with 24:1 Choloroform:Isoamyl alcohol and centrifuged again as described above to separate phases. To precipitate DNA, 1mL of 100% ethanol was added and samples were left at room temperature until a DNA precipitate was visible, approximately 10-30 minutes. The DNA was pelleted by centrifugation at 10,621-20,817xg in the Eppendorf 5417R centrifuge for 15 minutes, washed once with 70% ethanol, and once with 100% ethanol,
centrifuging for only 5 minutes. The DNA samples were left to dry, and re-suspended in 50µL TE. If sequencing, samples were treated with 1µL RNAse and incubated at 37°C overnight. The following day, DNA was re-extracted by the addition of 150µL H₂O and 200µL Phenol:Chloroform and centrifuged as described above. The aqueous phase was transferred to a fresh tube and washed with 200µL Chloroform:Isoamyl as described above. DNA was precipitated by the addition of 20µL 3M Sodium Acetate (pH5-6) and 450µL 100% ethanol, and was placed on ice for 0.5h. Samples were centrifuged at 10,621-20,817xg as previously described and the DNA pellet was washed with 70% and 100% ethanol again. Dried pellets were re-suspended in 50µL TE and 5µL was run on an agarose gel to verify purity and approximate concentration. To be sequenced, 10µL DNA, or DNA made up to 10µL with TE, was mixed with 5µL sequencing primer (GST Sequencing primer, indicated in pGEX vector map, was used for pGEX and pEBG; pET28a was sequenced using the T7 primers provided by the facility) and sent to the Robarts Sequencing Facility to be sequenced. Electropherograms were obtained and analyzed afterwards.

For a large-scale DNA extraction, bacterial cultures grown in 125mL Erlenmeyer flasks were transferred to 50mL conical tubes prior to centrifugation. Pellets were placed on ice and re-suspended in 10mL Solution I by repeated pipetting. Next, 10mL of Solution II was added and samples were inverted 3-5 times. In the same manner, 5mL of Solution III was added. Next, 10mL buffer-saturated phenol:chloroform (BioShop) was added, and samples were vortexed until opaque. Samples were centrifuged at 3901xg in the Beckman Coulter Allegra X-22R centrifuge for 15 minutes to separate the aqueous and organic phases. The upper, aqueous phase was transferred to a fresh tube and DNA
was precipitated using, 15mL of isopropanol. Successfully extracted DNA was visible as large, cotton-like white strings, and was subsequently centrifuged at 3901xg in the Beckman Coulter Allegra X-22R centrifuge to pellet DNA. Pellets were left to dry and re-suspended in 1mL TE. Exactly 1.08g of CsCl₂ was weighed out and dissolved in the DNA:TE mixture. DNA was visualized by the addition of 20µL Ethidium Bromide (10mg/mL). Samples were spun at 20,817xg in the Eppendorf 5417R centrifuge for 3 minutes to remove any residual protein which appears very dark. Next, the DNA:CsCl₂ mixture was transferred to a plastic ultracentrifuge tube (Beckman Polyallomer Quick-Seal Centrifuge tubes, 8x35mm) sealed using a tube topper, while checking for air bubbles and leaks. Tubes were placed in a Beckman TLN120 rotor and centrifuged at 448804xg for 1.5h. The CsCl₂ forms a gradient, and circular plasmid DNA presents as a large pink band in the centre of the gradient under ambient light, which is then extracted using a syringe and transferred to a fresh 1.5mL microcentrifuge tube. Ethidium bromide was removed by washing with an equal volume of H₂O-saturated butanol and centrifuged at 20,817xg as previously described for 3 minutes. DNA went into the aqueous phase while ethidium bromide went into the butanol-rich phase (less dense, upper phase). The upper phase was removed and the DNA was washed repeatedly until both the top and bottom phases were clear. DNA was re-precipitated in 2.5 volumes 70% ethanol at room temperature. Samples were washed once with 70% and once with 100% ethanol, allowed to dry, and re-suspended in 100µL TE. DNA was then assessed by spectrophotometry using a NanoDrop (Thermo Scientific) hosted by the Cregan Lab at Robarts Research Institute. Both the concentration (in µg/mL) and the A₂₆₀/₂₈₀ ratio were noted, and then DNA was diluted to desired concentration (typically 1µg/µL) for use.
For cloning of new vectors, insert DNA was made by PCR amplification from full-length mouse ShcC obtained from Dr. Tony Pawson’s laboratory (Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto; pcDNA ShcC Full Length) and both the amplicon and vector DNA were digested with the same two restriction enzymes, allowing for ligation in the correct orientation. All ShcA and ShcC domains (Figure 5) were cloned consistent with the domain breakdown done by Sakai et al (2000). The ShcA CH2 domain was amplified from full-length ShcA cDNA prepared from a small-scale DNA extraction using 40ng of the primers 5’-GGTT GGATCC ATG AAC AAG CTG AGT GG-3’ (BamHI site in bold, T\_m=74.4\(^\circ\)C) and 5’- CCTG GCGGCCGC CTA TTT GTC GTT GGG ATG CAG -3’ (NotI site in bold, stop codon underlined, T\_m=87.3\(^\circ\)C), incubated with 200\(\mu\)M dNTPs, 1X concentration of PCR buffer and 1mM MgCl\_2 provided with Taq, and 2.5U Taq (Invitrogen) and the thermocycler conditions outlined in Table 3. The ShcC CH2 domain was cloned similarly using the primers 5’- TGGATT GGATCC ATGAGTGCCACCAGGAAGAGCGGGCC -3’ and 5’- CAGA GCGGCCGC TCA CAC CTG ATC GCT GGT GTG CGG CGC -3’ (T\_m=90.2 and 95.4\(^\circ\)C, respectively), and ShcC CH1 was cloned using 5’- GGTT GGATCC CAG TGC CCT TCC AAG GTT CC -3’ and 5’- CCTG GCGGCCGC CTA AGC GTT AAG CTC CTC CAG -3’(T\_m=81.0 and 85.2\(^\circ\)C, respectively). It is important to note that the predicted size of ShcC CH2 (approx. 3 kDa) does not account for the change in protein size between p55 and p69 ShcC (14 kDa), likely due to upstream DNA sequence that had yet to be identified when the cDNA was cloned out of mouse. In addition, V-Src was PCR-amplified using the primers 5’- TT GCGGCCGC C ATGGGGAGTAGCAAG -3’
(NotI site in bold, $T_m=82.7^\circ C$) and 5’- TTA CTCAGG CTA CTAGCGACCTCC -3’
(XhoI site in bold, stop codon underlined, $T_m=69.5^\circ C$). The desired amount of insert DNA
amplified by PCR (typically 3-5µL) and vector backbone DNA (pEBG and pGEX 4T-1
for ShcC vectors, pET28a for V-Src, typically 5µL) was combined with 3µL of the
correct restriction enzyme buffer, 3µL of 1mg/mL BSA (Fermentas; 0.1mg/mL final
concentration) if not already in the buffer, and 1µL (5U) total restriction enzyme
(BamHI, NotI, or XhoI; Fermentas) in a final volume of 30µL (with H$_2$O). For digestion
with two enzymes, the most compatible restriction enzyme buffer was used and 0.5µL
(2.5U) of each restriction enzyme. Digestions were allowed to proceed overnight at 37°C.

**Table 3.** PCR conditions for DNA cloning.

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<th>30 Cycles</th>
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<td>72°C</td>
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<tr>
<td>Hold 4°C</td>
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Note: Extension temperature of 72°C was indicated by Taq enzymes (Invitrogen).

The following day, digestions were run out on an agarose gel and desired bands of the
correct size were excised and purified using the QiaQuick Gel Extraction Kit taking
precautions to increase DNA yield. Briefly, bands were excised into 3 volumes buffer
QG and heated in a 50°C water bath until no gel slice was visible, and one volume of
isopropanol was added. The samples were transferred to a spin column and allowed to
incubate for 5 minutes at room temperature, allowing DNA to bind to the membrane, and
then centrifuged at 15,700xg in the Eppendorf 5415D centrifuge for 1 minute. Next, the
membranes were washed with 0.5mL buffer QG to remove excess agarose, and spun
again for 1 minute at 15,700xg in the Eppendorf 5415D centrifuge. The DNA bound to
the membrane was then washed with 0.75mL buffer PE, and allowed to incubate at room temperature prior to centrifugation at 15,700xg in the Eppendorf 5415D centrifuge for 1 minute. This wash was repeated to remove the extra salt. Once the second wash was completed, the spin column was centrifuged at 15,700xg as previously described for 1 minute, rotated 180°, then centrifuged again to remove any liquid from the membrane. Then, DNA was eluted by adding 30µL of elution buffer provided with the kit (buffer EB) which had been warmed to 37°C and a 5 minute incubation at room temperature. DNA was removed from the membrane by placing the upper portion of the spin column in a fresh 1.5mL microcentrifuge tube and centrifuging this at 15,700xg for 1 minute, allowing it to incubate for one minute, and repeating the spin. Purified DNA was then used in ligation reactions.

To ligate compatible end fragments, 1µL of plasmid DNA was combined with an excess of insert DNA (typically 4µL), 1.5µL 10mM ATP, 1.5µL ligation buffer, and 1µL T4 DNA ligase in a final volume of 15µL with H2O. A control ligation was also set up, using 4µL H2O in place of insert DNA. The reaction was allowed to proceed at 4°C overnight and ligations were directly transformed into XL-10 gold strain of *E. coli*. Colonies were selected, DNA was extracted, and insert was verified by DNA sequencing as described above.
Figure 5. Schematic representation of all ShcC vectors manipulated in 2.1.a (A) and 2.1.d (B), and used for GST pulldown experiments either in HEK 293T cells (2.2) or expressed and purified from bacteria and incubated with mouse brain lysate (2.5), with protein sizes shown in brackets. Domain breakdowns were setup according to Sakai et al. (2010) and cloned from a full-length ShcC cDNA obtained from Dr. Tony Pawson’s laboratory (Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto). Note: Constructs denoted with an asterisk were previously cloned by former lab members Ian Grant (pEBG and pGEX ShcC CH1SH2 and ShcC SH2, and pEBG ShcC PTB) or Hui-Yu Liu (pGEX ShcC PTB).
2.2 Cell Culture and DNA Transfection

Note: For a full listing of all vectors named below, please see Appendix I, Table I.

All cell cultures were incubated in a 37°C incubator with 5% CO₂ and constant humidity. All passages, transfections, and media changes were completed in a HEPA-filtered biological cabinet. Primarily, the HEK 293T cell line was used, which required passing every 3-4 days to avoid overgrowth and senescence. Standard HEK 293T media was used for preparation: Dulbecco’s Modified Eagle Medium (DMEM; Wisent), 10% Fetal Bovine Serum (FBS; Wisent), and 50µg/mL Gentamycin sulfate (Wisent).

Transfections were originally carried out through the calcium phosphate method. Briefly, cells were plated at approximately 500,000-750,000 cells per 10cm dish in order to have cells at 50-60% confluency the day of transfection. Cell media was changed 1-2 hours prior to transfection. Desired amount of DNA was dissolved in 450µL water: 2.5µg pcDNA 4/T.O. Fyn(1-536)-GFP (Naoto Yamaguchi; Kasahara et al., 2007) and 5µg pEBG(-), ShcA CH2, ShcC CH2, PTB, CH1, or SH2, and 50 µL 2.5mM CaCl₂ was added drop-wise to the mixture. Next, 450 µL of 2XHBSS (See Appendix I) was added drop-wise and the reagents were mixed by pipetting up and down, passing air through the mixture. The transfection mixture was added drop-wise to the plates and allowed to incubate overnight, and media was replaced the next day. Cells were lysed 48 hours later and subject to further experiments. To reduce cytotoxicity with over-expression of Fyn-GFP, the amount was reduced to 1µg, and after optimization for low transfection efficiency a switch to Lipofectamine- 2000™ (Invitrogen) transfection was used.

For Lipofectamine-2000™ transfection, cells were also plated to reach 50-60% confluency for use. Approximately 1 hour prior to transfection, media was changed and
reduced to a volume of 4mL to increase the concentration of reagent. For each plate, two 1.5mL microcentrifuge tubes were aliquoted with 0.5mL Opti-MEM (Invitrogen) transfection media. To one tube for each plate, 1µg of pcDNA 4/T.O.Fyn(1-536)-GFP, pcDNA 4/T.O. Src(1-532)-GFP (Naoto Yamaguchi, Kasahara et al. 2007), or pCMV5 C-Src (Joan Brugge via Addgene) and 5µg of one of pEBG (-), ShcA CH2, ShcC CH2, PTB, CH1, or SH2 were added and allowed to incubate. Note: there was more success transfecting pEBG ShcC CH1 when reduced to 2.5µg. To the second tube, 10µL of Lipofectamine was added and allowed to incubate for 5 minutes. One tube of each DNA and Lipofectamine were combined and allowed to incubate for 20 minutes. The DNA:Lipofectamine mixture was added drop-wise to plates and allowed to incubate overnight. Media was replaced the following day, to 10mL, and plates were lysed for experiments 24-48 hours after transfection, dependent upon cell density and transfection efficiency.

2.3 Cell Lysate Preparation

a. From CD-1 Mouse Brain Lysates

Standard NP-40 lysis buffer (see Appendix I) was prepared, containing 1mM Sodium orthovanadate (Sigma-Aldrich), 1mM PMSF (Sigma-Aldrich) and 10µg/mL each Aprotinin and Leupeptin (Bioshop). Mice were euthanized according to the procedures outlined in animal protocol #2009-048 according to standard operating procedures: Mice under post natal day 21 (P21) were euthanized by decapitation, mice P21 and older were euthanized by CO2 asphyxiation. Mouse brains were dissected into the aforementioned NP-40 lysis buffer and lysed by needle shearing, with needles increasing in gauge (18-22-25). Samples were incubated on ice, and centrifuged at 20,817xg in the Eppendorf 5417R
centrifuge 10 minutes to remove insoluble materials. A second spin was added if necessary. The protein-containing supernatant was saved, and subject to protein determination for experiment, using the BioRad DC Protein Assay.

b. **From HEK 293T Cell Lysates**

Lysis buffer was prepared using Interaction Buffer (IB, see Appendix I) and 1mM Sodium orthovanadate, 1mM PMSF and 10µg/mL Aprotinin and Leupeptin. Prior to lysis, dishes were washed on ice with cold PBS to remove all traces of media. Next, cells were scraped off into 500µL IB into a 1.5mL microcentrifuge tube and incubated on ice for 5-15 minutes, vortexing intermittently until all chunks of tissue disappeared. Any plates where cells had begun to detach were lysed similarly, but media was also collected from the dish and spun at 3901xg in the Beckman coulter Allegra X-22R centrifuge to sediment cells for lysis. Cells were then centrifuged at 20,817xg in the Eppendorf 5417R centrifuge 10 minutes to remove all insoluble components. Supernatants were saved in a fresh tube and subject to protein determination and further experiments. If the insoluble pellet was also saved for experiment, it was re-suspended in 200µL of the same buffer and further lysed by sonication. Samples were quantified as described above.

### 2.4 GST Fusion Protein Preparation

*Note: For a full listing of all vectors named below, please see Appendix I, Table I.*

For pGEX 4T vector expression (GST, GST-ShcA CH2, GST-CH2, GST-PTB, GST-CH1, GST-SH2, and GST-p55 ShcC) vectors were left in the original, cloning strain (DH5α, XL-10 Gold). When pGEX 4T vectors (GST, GST-CH1, GST-CH1SH2, and GST-CH1SH2 3YE) were co-expressed with pET28a or pET28a V-Src, vectors were co-transformed into the BL21-DE3 strain to allow for proper expression. Bacterial
cultures were inoculated and grown to an OD$_{600}$ of 0.8-0.9, and induced with 1-2mM IPTG for 2h. Following induction, cells were pelleted at 3901xg in the Beckman coulter Allegra X-22R centrifuge for 15 minutes. Cell pellets were stored at -80°C until purification. Pellets were re-suspended in PBS containing 2mM EDTA, 1mM PMSF and Sodium orthovanadate, and 10μg/mL Aprotinin and Leupeptin (PBS Lysis I) and lysed by sonication. Triton X-100 was added to the lysate, at 1% final v/v, and samples were incubated at 4°C with constant rotation of 0.5-1h. The lysate was centrifuged at 26832xg for 15 minutes in a Beckman JA-20 rotor and supernatant saved to a fresh tube. Fusion proteins were purified by the addition of washed (with PBS) glutathione-agarose beads, incubated overnight. If fusions were purified alongside pET28a, they were incubated for 2h, to avoid phosphorylation decay. GST fusion proteins were then washed 7-10 times with PBS containing 1% Triton X-100, 2mM EDTA, 1mM PMSF and Sodium orthovanadate, and 10μg/mL Aprotinin and Leupeptin (PBS Lysis II) and stored at 0°C bound to glutathione-agarose. Protein concentration was estimated by running 10, 20, and 40μL of the fusion protein alongside 1, 2, and 4μg of BSA on a polyacrylamide gel and staining with Coomassie Blue. The advantage of visualizing GST fusion proteins was also being able to assess purity and any possible degradation.

2.5 **GST Pulldowns**

Once protein quantity and quality assessment of purified GST fusion proteins were completed, GST fusion proteins were incubated with quantified mouse brain lysate (see above) at a ratio of 2μg fusion protein to 3-5mg protein, preferably 4, overnight at 4°C with constant rotation. The following day, pulldowns were washed 5-8 times at
3901xg in the Beckman Coulter Allegra X-22R centrifuge, for 3-5 minutes and stored at -20ºC in sample buffer prior to SDS-PAGE.

2.6 Immunoprecipitation

Note: For a full list of antibodies used in immunoprecipitations, see Appendix I, Table II.

Immunoprecipitation (IP) was carried out in a similar manner to GST pulldowns. Protein-G-Sepharose (Gammabind; GE Life Sciences) was washed in NP-40 lysis buffer and 25µL of 50% Gammabind:NP-40 was incubated with 3-5mg quantified mouse brain lysate (see above) and 1-2µg primary antibody overnight at 4ºC with constant rotation. Antibody concentrations were determined empirically in addition to product information sheets provided by suppliers and are listed in Appendix I. The following day, IPs were washed 3-5 times at 2655-10,621xg in the Eppendorf 5417R centrifuge for 3-5 minutes and stored at -20ºC in sample buffer prior to SDS-PAGE.

2.7 SDS-PAGE and Western Blotting

Note: For a full list of all antibodies used in Western Blotting, see Appendix I, Table II.

Polyacrylamide gels were prepared in two parts according to previously outlined methods in our laboratory (Grant, 2009): a lower gel for sample resolution, and an upper, stacking gel for well creation. The correct volume of 30% Acrylamide/Bisacrylamide for the desired percentage (3.3mL for 10%, 2mL for 7.5%) was combined with 2.5mL Lower Tris buffer, H₂O to 10mL, 50µL 10% Ammonium Persulfate (APS) and 7.5µL TEMED (BioShop). To straighten the edge of the gel, 1mL of 100% ethanol was over-layed. When polymerized, the ethanol was washed away with water and area was blotted dry with blotting paper. The upper gel was prepared using 2.5mL Upper Tris, 1.3 mL 30% Acrylamide/Bisacrylamide (4% gel), 6.2mL dH₂O, 50µL 10% APS and 7.5µL TEMED,
and over-layed on the lower gel, and a 10-well or 15-well comb was inserted. When set, the gel was loaded into the rig and flooded with SDS-PAGE buffer.

Prior to sample loading, all samples were boiled at 65°C for 10 minutes and placed on ice. Pulldowns and IPs were centrifuged at 20,817xg in the Eppendorf 5417R centrifuge for 3 minutes to pellet beads. Samples were loaded into the wells, with lane 1 typically containing 10µL PrecisionPlus Dual Colour Protein Molecular Weight Standards (BioRad) as a size indicator. Gels were resolved at 200V for 30-40 minutes, until the dye front approached the bottom of the gel. Afterwards, gels were either stained with Coomassie Blue, or proteins were transferred onto PVDF membranes using a semi-dry transfer apparatus. Briefly, a labelled 0.2µm PVDF membrane (Bio Rad) was wet with methanol and soaked in transfer buffer, along with two pieces of blotting paper. The gel was popped out of the glass slides and placed on top of blotting paper in the semi-dry apparatus, smoothing out any air bubbles. Next, the PVDF membrane was placed on top of the gel, smoothing out any bubbles, and the second piece of blotting paper. The apparatus was run at 0.5A for 1-2h, depending on the gel percentage and target protein size.

When removed, protein molecular weight marker was visible on the membrane as pink and blue bands on the left hand side. Membranes were blocked with either 10% Milk-TBS-Tween, or BSA-TBS-Tween with 100µM Sodium orthovanadate, for 1h at room temperature and primary antibody was added at the correct dilution, typically 1:2000, determined empirically, and allowed to incubate at 4°C overnight. Note: For a full list of all antibody dilutions, please see Appendix I. The next day, blots were washed 4-6 times with TBS-Tween and secondary antibody (1:10,000 HRP-coupled Goat anti-
Mouse or Goat anti-Rabbit; Jackson Laboratories) was added in new blocking buffer for 1h at room temperature. Secondary antibody was washed in the same manner as primary, and blots were visualized using the ImmunStar WesternC Kit (BioRad) on a Chemi-Doc using the ImageLab software version 3.0 (Beta) provided with the Gel Doc. Signal quantifications were carried out by determining the relative band intensity of each lane to a selected reference band (designated 1.00).
Chapter 3. Results

3.1 *ShcC and STEP are expressed at varying levels in development, while Fyn Remains Constant*

First, I sought to assess the developmental expression levels of ShcC, STEP, and either Src or Fyn, both of which are ubiquitously expressed proteins (Sun et al., 1998). STEP was also considered a potential binding partner for ShcC at this time. Since Fyn is the primary player in NMDA receptor regulation (Nakazawa et al., 2001), I selected Fyn rather than Src for this experiment. The purpose was not only to verify literature on protein expression in different mouse strains to CD-1 mice, but to select an appropriate age for co-immunoprecipitation studies. To elucidate the protein levels of ShcC and STEP during postnatal development, and the age at which protein levels are most abundant, cortices were taken at different ages of CD-1 mice and 50µg of whole cell lysate was run on a 10% polyacrylamide gel and blotted either for mouse-anti ShcC (BD); mouse-anti STEP (Santa Cruz Biotechnology); or mouse-anti β-actin (Sigma) as a protein loading control. Fyn levels during development were similarly determined in whole mouse brain with mouse-anti Fyn (Abcam). As shown in Figure 6, ShcC protein levels were not static. Both isoforms seem to rise after birth, are clearly detectable at P7 and peak at around P21 (Figure 6). STEP, similarly, seems to be more abundant postnatally, being detectable at P7 and peaking around P21 (Figure 6). Thus, mice at the ages of P7 and P21 were used for further studies. Fyn levels, as previously reported (Sun et al., 1998), remain relatively constant (Figure 7A). It is important to note, however, that with several Western Blots, I was not able to generate data where β-actin levels were constant. Next, I compared the relative band intensity of Fyn to actin at each age, and
found that the intensity of the Fyn signal ranged from 0.76 to 1.38 times that of actin (Figure 7B). Though several attempts were made, with fresh samples, it was concluded that, as Fyn levels remained comparable to actin at each age, that Fyn levels were likely constant. This was performed only to verify what was published in the literature (Sun et al., 1998) and was not attempted any further.

Figure 6. Developmental expression analyses of ShcC and STEP in CD-1 cortices, using 50 µg whole-cell lysate (WCL). ShcC presents as two isoforms, of 55 and 69 kDa; while STEP presents as two isoforms of 46 and 61 kDa. Western Blots were carried out with 1:5000 mouse-anti ShcC, 1:2000 mouse-anti STEP, and 1:10,000 mouse-anti β-actin. N=2.
Figure 7. Developmental expression analysis of Fyn in CD-1 whole brain lysates. A) Western Blots were carried out with 1:5000 mouse-anti Fyn (59 kDa) and 1:10,000 mouse-anti β-actin. N=2. B) Relative band intensity of Fyn to the corresponding β-actin band for each mouse age was determined utilizing the relative intensity function of Image lab software. N=1.
3.2 **ShcC Co-Precipitates with Fyn and Src, but not with STEP**

Previously in our lab, ShcC was shown to weakly co-immunoprecipitate with STEP from mouse brain lysates (Grant, 2009) though this result was not consistent. This indicated a potential interaction between ShcC and STEP. Here, the association of ShcC and STEP was re-investigated using 2-4 mg of brain lysate from CD-1 mice. In addition, association of ShcC with two Src family kinases, Src and Fyn, was similarly investigated. Co-immunoprecipitations (co-IPs) were carried out using mouse-anti ShcC, mouse-anti Fyn, rabbit-anti ShcC (Santa Cruz Biotechnology), rabbit-anti Src (Cell Signaling) and rabbit-anti STEP antibodies (Cell Signaling), with normal mouse IgG (Millipore) and rabbit IgG’s (Santa Cruz Biotechnology) serving as negative controls. As shown in Figure 8 (A,B), immunoprecipitation of Fyn indicates a interaction with the lower (p55) but not the upper (p69) isoform of ShcC at both P7 and P21. This co-IP is not considered background as the relative band intensity is higher than that of mouse IgG (Figure 8D). Similarly, as shown in Figure 9A, the p55 isoform of ShcC was shown to co-IP with Src, yet a co-IP was also observed with p69 ShcC and Src (Figure 9A). This co-IP is also considered above background as the relative band intensity is higher than that of mouse IgG (Figure 9D). In addition, both Fyn and Src antibodies were found to successfully precipitate their target proteins (Figure 8C, and 9A). Two replicates of each co-IP were shown to circumvent minor imperfections in each blot which, after several repetitions, I was not able to remove. Most importantly, I was not able to rid Western Blots of the IgG heavy chain band (50 kDa) when I immunoprecipitated and blotted with mouse antibodies (Figure 8). I attempted to change both primary and secondary antibodies to different species, but doing so destroyed the co-IP signal. The interaction between ShcC
and Fyn or Src was albeit weak, however the results did support my hypothesis that ShcC could bind to Fyn. The absence of interaction between p69 ShcC and Fyn or Src could be due to the fact that p69 ShcC is much less abundant (Figure 6). However, this warrants further investigation as published literature has demonstrated that full-length ShcA can bind to Src (Xi et al., 2010a). To further address this, a subdomain approach was taken in section 3.3 to determine if the CH2 domain, present only in p69 ShcC, is capable of pulling down Src or Fyn, with the notion that if the CH2 domain is capable of binding, the full-length 69kDa protein has the potential to bind. In addition, after several attempts, I was unable to replicate any data to suggest that ShcC co-immunoprecipitated with STEP, and therefore concluded that ShcC interaction with STEP was unlikely (Figure 9B). However, I was able to verify the STEP antibody was immunoprecipitating its target (Figure 9C).
Figure 8. ShcC co-immunoprecipitation studies with Fyn in both P7 and P21 whole brain lysates. A) and B) P7 and P21 mouse whole brains were used to precipitate with both mouse-anti Fyn and mouse-anti ShcC. As a positive control, 50µg of the same whole brain (cell) lysate (WCL) was loaded in the first lane. Both Mouse IgG and mouse-anti Fyn lanes were loaded with relative fractions representative of 1mg IP from brain lysate, mouse-anti ShcC lanes were loaded with half of that value (0.5mg) to prevent antibody saturation during Western Blotting. Western Blots were carried out with 1:2000 mouse-anti ShcC. Note: An asterisk (*) is placed to the left of a band indicating a successful co-IP. N>3. C) Fyn was immunoprecipitated in the same manner as (A) and (B), and 1mg representative fraction was loaded with Western Blots being carried out with 1:2000 mouse-anti Fyn. N=2. D) Quantification of relative band intensity of p55 ShcC, for (A) and (B) using Mouse IgG as a reference, was carried out using Image lab software.
Figure 9. ShcC co-immunoprecipitation studies with Src and STEP in P7 and P21 whole brain lysates. A) P21 mouse whole brain lysates were used to precipitate with rabbit-anti ShcC, rabbit-anti Src and/or rabbit-anti STEP. As a positive control, 50µg of the same brain lysate was loaded. Samples were loaded with volumes representative of 1mg IP from brain lysate. Western Blots were carried out with 1:2000 mouse-anti ShcC and 1:2000 rabbit-anti Src. Western Blotting for Src yields a band of 60 kDa. Note: An asterisk (*) is placed to the left of a band indicating a successful co-IP. N=3. B) P7 mouse whole brains were used to precipitate with rabbit-anti ShcC and rabbit-anti STEP, with Western Blots were carried out as described in (A). N>3. C) Immunoprecipitation of STEP, in the same manner as (B). Western Blotting was carried out with 1:2000 mouse-anti STEP. N=1. D) Quantification of relative band intensity of p69 ShcC (A) and p55 ShcC(A and B), using Rabbit IgG as a reference, was carried out using Image lab software.
3.3 *GST Pulldowns of Transfected HEK 293T Cells Do Not Show an Interaction between ShcC and GFP-Tagged Src*

To determine whether individual ShcC domains could bind directly to Fyn or Src, mammalian expression vectors containing GST-tagged ShcC domains were co-transfected with either EGFP-tagged Fyn or Src expression vectors into HEK 293T cells using Lipofectamine 2000. Cells were lysed in Interaction buffer (Appendix I) due to success in our lab using this buffer for GST pulldowns. Each of the individual CH2, PTB, CH1, and SH2 domains of ShcC were included (refer to Figure 5), as well as the CH2 domain of ShcA as a positive control. In addition, GST alone was used as a negative control, while Src was immunoprecipitated using the GFP tag and GFP-Agarose conjugated antibody (Santa Cruz Biotechnology) as a positive control. Each Western blot contained 50 µg of each whole cell lysate and a 1 mg representative fraction of GST pulldown. Western blotting was carried out with mouse-anti Src (1:2000; Calbiochem) and rabbit anti-GST (1:2000, Santa Cruz Biotechnology), with a positive and negative control (GFP-IP and GST empty vector, respectively). Results indicate there is no interaction between Src-GFP and any of the GST fusion proteins, though all are present in the whole cell lysate and GST pulldown lanes (Figure 10). Substituting Fyn-GFP for Src-GFP also did not yield any interaction (data not shown). This led me to believe that there could be technical difficulties preventing any GST pulldowns: 1) The solubility of GST fusion proteins in lysis buffer could be limiting their availability to bind; 2) The GFP tag on the C-terminus of Fyn and Src could be affecting the ability of ShcC to bind; or 3) The domain is not functional outside of the full-length protein. The best way to investigate these possibilities further was to try different lysis buffers and look for the
relative abundance of these GST fusion proteins in the supernatant and pellet of HEK 293 cell lysates. In addition, it would be wise to obtain an untagged Src construct and transfect it similarly alongside these GST fusions.

Figure 10. GST-ShcC subdomain fusions and Src-GFP co-transfection studies in HEK 293T cells. GST pulldowns with GST-ShcC CH2 (29 kDa), ShcC PTB (44 kDa), ShcC CH1 (47 kDa), ShcC SH2 (37 kDa) and ShcA CH2 (31 kDa) were carried out on cell lysates and relative fractions representative of 1mg of lysate were loaded in each lane in the right panel. GST presents as one band of 26kDa. The left panel, alternatively, contains 50µg of each lysate corresponding to the pulldowns in the right panel. N=3.
3.4  **Lysis Buffer has Little to No Effect on GST Fusion Protein Solubility**

I investigated the solubility of each GST fusion protein in HEK 293T cells in order to determine if lysis buffer could be affecting any subsequent GST pulldowns. First, I transfected each mammalian expression vectors encoding the GST fusion proteins into HEK cells The following day, plates were split into three 6 cm dishes, and the day after, each plate was lysed in either standard NP-40 lysis buffer plus inhibitors as described above, NP-40 lysis buffer +0.1% SDS, or Interaction Buffer. Lysates were centrifuged at 20,817xg in the Eppendorf 5417R centrifuge for 15 mins and pellets were broken down by sonication and re-suspended in 200 µL of the same buffer. Next, 50 µg of each supernatant and pellet were run on a 10% gel under reducing conditions and subject to Western blotting with rabbit anti-GST. Results indicate that, despite choice of lysis buffer, the majority of each GST fusion is found in the supernatant (Figure 11). There are, however, some notable discrepancies. Though interaction buffer demonstrated the most protein in the supernatant fraction, the reduction in both NP-40 and NP-40 +0.1% SDS protein levels did not correspond with an increase in the pellet, indicating that there was some loss during the lysis process as all three samples came from the same original transfection, and thus had the same efficiency. It is also important to note here that the ShcC CH2 domain, in all three samples, demonstrated a band large in size (40-45 kDa) in the pellet, not seen in the supernatant, and could potentially be an aggregate of GST-CH2 that was not broken down by SDS in the sample buffer. This was not investigated further because there was still a considerable amount of GST-CH2 in the supernatant available for GST-pulldown experiments. After assessing solubility of these
GST fusions in each lysis buffer, I concluded that Interaction Buffer was the most appropriate buffer to perform further experiments.

**Figure 11.** The effect of lysis buffer on GST fusion protein solubility following overexpression in HEK 293T cells. The GST-tagged domains of ShcA CH2 (31 kDa), ShcC CH2 (29 kDa), PTB (44 kDa), CH1 (47 kDa), and SH2 (37 kDa) were present primarily in the supernatant fraction of HEK 293T cell lysates. A 50µg aliquote of both pellet (left) and supernatant (right) of each GST fusion protein was loaded. N=1.
3.5 **GST Pulldowns of Transfected HEK 293T Cells Do Not Show an Interaction between ShcC and Untagged Src**

To determine whether the GFP tag of Src-GFP was limiting the interaction between ShcC and Src *in vitro*, the GST fusion protein experiment shown in Figure 10 was repeated using instead a Src vector which did not have a tag on the C-terminal end. Each of the CH2, PTB, CH1, and SH2 domains of ShcC were included, as well as ShcA CH2 as a positive control and GST alone as a negative control. Results indicate there is still no interaction between Src and any of the GST subdomain fusion proteins, though all are present in both the whole cell lysate and GST pulldown lanes (Figure 12). Having tested both the solubility of GST fusion proteins and the effect of the GST fusion protein tag, it was determined that neither affected the ability of Src to come down with the GST-ShcC subdomain vectors. Therefore, there is no direct interaction between ShcC subdomains and Src in HEK 293 cells, in contrast to co-IP studies in mouse brain. This experiment was then attempted in parallel using bacterially-purified GST-ShcC subdomain constructs (pGEX vector system) and mouse brain lysate as the source of Fyn and/or Src in section 3.6.
Figure 12. GST-ShcC subdomain fusions and untagged Src co-transfection studies in HEK 293T cells. GST pulldowns with GST-ShcC CH2 (29 kDa), ShcC PTB (44 kDa), ShcC CH1 (47 kDa), ShcC SH2 (37 kDa) and ShcA CH2 (31 kDa) were carried out on cell lysates and relative fractions representative of 1mg of lysate were loaded in each lane in the right panel. The left panel, alternatively, contains 50µg of each lysate corresponding to the pulldowns in the right panel. An immunoprecipitation for Src was included as a positive control, in substitute of an IP to GFP. Western Blotting was carried out with 1:2000 mouse-anti V-Src and 1:2000 rabbit-anti GST. N=3.
3.6 *ShcC Binds Endogenous Fyn Through its CH2 and SH2 Domains*

As an alternate approach to examine ShcC interactions with Fyn or Src, each domain of ShcC was cloned into pGEX for expression of a GST-fusion protein with an IPTG-inducible promoter (GST-PTB and GST-SH2 were previously cloned by Ian Grant). GST fusion proteins were quantified and assessed for purity and used for GST pulldowns with 2 µg of bacterially purified GST fusion proteins (pGEX-, ShcA CH2, ShcC CH2, PTB, CH1, and SH2) and 2-4 mg of fresh CD-1 mouse brain lysate prepared in NP-40 as described above. The resultant pulldowns were electrophoresed on 10% polyacrylamide gels and blotted first for mouse anti-Fyn (Abcam), or rabbit anti-Src, and second for rabbit anti-GST (Santa Cruz Biotechnology). Results indicate an interaction between the CH2 and SH2 domains of ShcC with Fyn in lysates prepared from both P8 and P21 brains (Figure 13A and B). As there is some background at both P8 and P21, the relative band intensities were compared to the GST negative control, and both ShcC CH2 and ShcC SH2 signal intensities were above background at P7 and P21 (Figure 13C). There were some technical difficulties with this experiment, mainly GST fusion protein degradation resulting in unequal protein loading. After several attempts, and adaptations (i.e., quantifying GST fusion proteins the day of the pulldown), I was not able to obtain a replicate where GST fusion protein levels were relatively equal in each lane. Though the results might not be 100% convincing, they do indicate that ShcC is capable of binding to Fyn through its CH2 and SH2 domains, further supporting my hypothesis. This also demonstrates that, though not observed in mouse co-IPS (Figures 8 and 9), p69 ShcC should be able to bind to Fyn and/or Src.
Figure 13. GST pulldown studies using bacterially-purified GST fusion ShcC subdomains with Fyn at P8 (A) and P21 (B). GST pulldowns from (26kDa) and GST-ShcC CH2 (29 kDa), ShcC PTB (44 kDa), ShcC CH1 (47 kDa), ShcC SH2 (37 kDa) and ShcA CH2 (31 kDa) were carried out on whole brain lysates and relative fractions representative of 1mg of lysate were loaded in each lane, with 50µg of the same mouse brain lysate as a positive control. N=1 for each (A) and (B). Successful GST pulldowns are denoted by an asterisk (*) to the left of the indicated band. C) Quantification of relative band intensity of Fyn, using GST as a reference, was carried out using Image lab software.
3.7 Tyrosine Phosphorylation of GST ShcC CH1SH2 when Co-Purified with V-Src

To determine the importance of tyrosine phosphorylation in SH2 domain binding properties I took a two-fold approach: I generated a phosphomimetic form of ShcC (pGEX ShcC CH1SH2 Y221, 222, and 304E; 3YE) that does not contain the PTB domain, as well as co-expressing the native form of this vector (pGEX ShcC CH1SH2) with bacterially-expressed viral Src (pET28a) to allow for tyrosine phosphorylation. These tyrosine residues were chosen as phosphorylation at these three sites were shown to be essential for SH2 domain activity in ShcA (George et al., 2008). This protein has an empirically-determined size of approximately 62 kDa (Figure 14). Following GST fusion protein purification, 0.5-1µg of each GST fusion protein was run beside Daoy-TrkA (NGF-responsive medulloblastoma cell line) cell lysate with 10 minutes of NGF stimulation, provided by a former lab member, Chunhui Li, and Western Blotting was carried out with 1:2000 Mouse anti-Phosphotyrosine (Cell Signaling). Preliminary results indicate some tyrosine phosphorylation in GST-CH1SH2 when expressed with V-Src (Figure 15). However, this strain of *E. coli* (XL10-gold) does not possess the necessary RNA polymerase (T7) to properly express from the pET28a (T7) promoter, and vectors were then re-transformed into a BL21-DE3+ strain. Tyrosine phosphorylation may still be witnessed because there was a low level of expression of pET28a V-Src, enough to phosphorylate some protein. I then transferred the pGEX (empty vector ShcC CH1, CH1SH2, and 3YE) vectors alongside pET28a (empty vector or V-Src) vectors into BL21-DE3+ strain of *E. coli*, and tyrosine phosphorylation is much more discernible (Figure 16). Both pGEX ShcC CH1SH2 and 3YE, when co-purified with V-Src, are tyrosine phosphorylated (Figure 16). This result indicated that ShcC can be tyrosine
phosphorylated at other sites than 221, 222, and 223, as the phosphomimetic (3YE) ShcC did not contain them, yet tyrosine phosphorylation was still visible. However, ShcC CH1 alone was not tyrosine phosphorylated by V-Src, indicating that V-Src showed specificity to ShcC CH1SH2 over ShcC CH1. In addition, though no tyrosine phosphorylation was observed in the GST (empty vector) lane, upon re-probe for GST no band of the correct size was present, indicating the the protein was not there and likely ran off the gel due to its small size (26 kDa) in this case.

**Figure 14.** Quantification of GST-ShcC CH1SH2 and CH1SH2 3YE (62 kDa) with respect to BSA standards (Bio-Rad; 66 kDa). Shown here is an example gel; samples were quantified by utilization of the ImageLab software and amounts of protein are shown below the appropriate gel bands.
Figure 15. GST-ShcC CH1SH2 (65kDa) in the presence of V-Src in the cloning strain of *E. coli*, XL10-Gold. GST-ShcC CH1 (47 kDa) was also included, in addition to GST-ShcC CH1SH2 3YE, which does not contain the three tyrosines in the CH1 domain. As a positive control, 50µg of NGF-treated Daoy-TrkA cell lysate was used. Note: potential bands of the appropriate size of 47 kDa (ShcC CH1) or ~62 kDa (ShcC CH1SH2) are denoted by an asterisk (*). N=1.

Figure 16. Tyrosine phosphorylation assessment of GST-ShcC CH1SH2 (65kDa) in the presence of pET28a empty vector (-) or pET28a V-Src (+) in the expression strain of *E. coli*, BL21-DE3. Western Blotting was first carried out with 1:2000 mouse-anti Phosphotyrosine, and then for rabbit-anti GST. As a positive control, 50µg of NGF-treated Daoy-TrkA cell lysate was used. For phosphotyrosine assessment, only the area of the blot with detected bands was shown. Note: Bands of appropriate size for GST fusion proteins of 47 kDa (ShcC CH1) or ~62 kDa (ShcC CH1SH2) are denoted below the band by an asterisk (*). N=1.
3.8 Tyrosine Phosphorylation is Important for SH2 Domain Binding

The phosphomimetic mutant of ShcC (3YE) described above, along with ShcC CH1SH2, were co-purified in the presence and absence of V-Src, and 2 µg of these fusion proteins were incubated with 2-4 mg CD-1 mouse brain as described above to pull down any potential SH2 binding partners. As a negative control, the CH1 domain of ShcC was also co-purified with V-Src. Results indicated an interaction between tyrosine phosphorylated ShcC with Fyn or Src at P8 (Figure 17A and B). As there is some background in Figure 17B, the relative band intensities were compared to GST. GST ShcC CH1SH2 + V-Src signal intensities were above background when probing for Fyn or Src (Figure 17C). An interaction between ShcC CH1SH2 3YE and Fyn was seen only once (data not shown), suggesting that using glutamic acid substitution did not correctly mimic tyrosine phosphorylated ShcC. Upon transformation of the aforementioned vectors into BL21-DE3+ E. coli, this experiment was repeated to assess whether or not results would change. However, to date this has only been attempted once and, with the change in strain, the optimization process needs to be repeated to eliminate the background now seen when blotting for Src (Figure 18). As with the GST-subdomain pulldown experiment (Figure 13), there were some technical difficulties. Apart from being unable to see tyrosine phosphorylation prior to transforming the vectors into BL21-DE3+ E. coli, there were also problems with GST fusion protein degradation, resulting in unequal amounts of GST fusion protein. In addition, once in BL21, there was signal background in the GST pulldowns when blotting for Fyn, such that signal was virtually indistinguishable from background. The inferences we can make from Figure 17, however, are that tyrosine phosphorylation is important for ShcC binding to Src or Fyn.
Figure 17. GST pulldown studies using bacterially-purified GST fusion ShcC in the absence of the PTB domain. A) and B) GST pulldowns were implemented with ShcC CH1SH2 in the presence or absence of V-Src, and phosphomimetic ShcC CH1SH2 3YE (all ~62 kDa). Successful GST Pulldowns are indicated by an asterisk (*). GST ShcC CH1 was co-purified in the presence of V-Src as a negative control in (B). Note: an asterisk (*) below a band in the lower panel of (B) denotes band of the correct size for GST-ShcC CH1. N=3. C) Quantification of relative band intensity of Fyn in (A), Src in (B), using GST as a reference, was carried out using Image lab software.
Figure 18. GST pulldown studies using bacterially-purified GST fusion ShcC CH1, CH1SH2, and CH1SH2 3YE in BL21-DE3+ cells. GST pulldowns were implemented in the presence or absence of V-Src. Western Blotting was carried out, as previously described, with 1:2000 rabbit-anti Src and 1:2000 rabbit-anti GST. The GST reprobe was separated here due to the presence of the Src band in the middle. N=1.
Chapter 4. Discussion

There is considerable evidence to support the working model that ShcC is involved in NMDA receptor activity during LTP. As ShcC is generally activated by TrkB (Rozakis-Adcock et al., 1992), this provides a potential mode for receptor cross-talk between TrkB and NMDA receptors. First and foremost, ShcC knockout mice demonstrate enhance hippocampal LTP and an increase in tyrosine phosphorylation of the NMDA receptor subunits NR2A and NR2B, but there were no differences in Src family kinase phosphorylation at the active site (Miyamoto et al., 2005). The authors also performed an in vitro Src kinase assay and demonstrated that there was no difference in Src family kinase activity (Miyamoto et al., 2005). However, there is new evidence to support that Shc(A) can regulate the activity of Src family kinases differentially through its two isoforms, p52 Shc and p66 Shc (Xi et al., 2010a), and therefore the association of ShcC with Src family kinases in the brain warrants further investigation.

There is also evidence to support the association of Shc family members with the Src family kinases. Both p52 and p66 ShcA were shown to bind to Src, whereas ShcC was shown to bind to one of the Src family kinases, but the specific kinase was not determined (Miyamoto et al., 2005; Xi et al., 2010a). I investigated the binding properties of ShcC with respect to Fyn and Src, and observed a co-immunoprecipitation between p55 ShcC and both Fyn and Src, and between p69 ShcC and Src (Figures 8 and 9). In addition, after many attempts I was unable to observe an interaction between either ShcC isoform and STEP (Figure 9b). The interaction between ShcC and Fyn or Src was albeit weak, and required more than 2mg of protein during the IP. If the IP was attempted with 2 mg or less, even though only a 1 mg representative fraction was loaded on each
Western Blot, a co-IP was not seen (data not shown). A weak co-IP could be an indicator of an activity-dependent or transient interaction. This interaction is likely activity-dependent, however, as Shc binding to Src in smooth muscle cells is dependent on IGF-1 stimulation, it is likely interaction is activity-dependent (Xi et al., 2010a).

The interaction of ShcC and Src family kinases is thus likely mediated by TrkB activation in this case. Knowing that Fyn kinase is the key player in NMDA receptor tyrosine phosphorylation (Grant et al., 1992), these results, taken together, lead me to believe the cross-talk between TrkB and NMDA is mediated by ShcC interaction with Fyn kinase, though it may also signal through Src kinase. There is also an explanation of why I did not observe an interaction between p69 ShcC and Fyn: at the overall level, p55 ShcC is much more abundant (Figure 6) and, since I only studied co-IPs in whole brain, not stimulated cells, it is likely the small amount of p69 ShcC was not visible. To test this idea, it would be beneficial to co-express either p55 or p69 ShcC with Fyn in PC12 cells, and stimulate with NGF. Alternatively, our lab has a mutagenized form of PC12 cells lacking TrkA and stably-expressing TrkB, termed TrkB-B5, which would respond to BDNF. I predict that, when cells are stimulated with either NGF or BDNF, I would see an increase in the interaction between p69 ShcC and Fyn.

Despite the lack of change in Src family kinase activity in ShcC knockout mice, the association of ShcC and Src family kinases is still worth investigating as there is promising evidence to support a role for Shc family members in regulating Src family kinase activity. It was demonstrated that p66 Shc presence, or over-expression of p66 Shc, interrupted p52 Shc activation by IGF-1 stimulation and prevented Grb2 association, resulting in impaired MAP kinase or PI3-kinase activation (Xi et al., 2008; Xi et al.,
In fact, p66 Shc inhibited the activation of p52 Shc via tyrosine phosphorylation by Src, indicating that the presence or over-expression of p66 Shc might also be binding to Src (Xi et al., 2008; Xi et al., 2010a). Following logically, association of p66 Shc and Src was investigated. It was then demonstrated that p66 Shc bound directly to Src through its CH2 (polyproline) and SH2 domains, to the SH3 and catalytic (SH1) domains of Src, respectively, and that binding of p66 Shc inhibited IGF-1-dependent Src kinase activation (Xi et al., 2010a). Src family kinases are folded in their inactive form and upon tyrosine phosphorylation in the kinase domain (416 on Src, 420 on Fyn) there is a conformational change resulting in activation (Superti-Furga et al., 1993; Sun et al., 1998; Nguyen et al., 2002). Expression of the CH2 domain alone could also attenuate Src kinase activity, but not to the extent of full-length p66 Shc (Xi et al., 2010a). Alternatively, p66Shc-SH2 deletion mutant expression did not affect Src family kinase activity, implicating the CH2-SH3 interaction as the inhibitor of Src kinase activation (Xi et al., 2010a). This led researchers to believe that p66 Shc binding to Src resulted in an either a prevention of Src unfolding, or an allosteric change, preventing its activation (Xi et al., 2010a). In support of this notion, using an inhibitory peptide to block the polyproline-SH3 interaction rescued Src kinase activation (Xi et al., 2010a).

In my research, I observed an interaction of both the CH2 and SH2 domains of ShcC with Fyn utilizing bacterially-purified GST fusion proteins to each (Figure 13), consistent with literature on ShcA and Src (Xi et al., 2010a). To further this notion, it would be interesting to break Fyn down into its subdomains (namely SH3, SH2, and SH1) to ascertain which could bind to full-length ShcC, or to individual ShcC domains. By convention, a different tag would be used (i.e. Myc) so both vector sets could be used
simultaneously. These experiments may also indicate the potential for p69 ShcC to bind to Fyn or Src, which was not witnessed in co-IP studies from mouse whole brain (Figures 8 and 9). To further investigate the potential for p69 ShcC to bind to Fyn and Src, it would be advantageous to co-express p55 or p69 ShcC with Fyn or Src in PC12 cells as described above. To ascertain whether or not p69 ShcC could inhibit Src family kinase activation, an in vitro kinase assay could be implemented as previously used on both ShcA and ShcC (Miyamoto et al., 2005; Xi et al., 2010a). I would co-express either p55 or p69 ShcC with Fyn in PC12 or TrkB-B5 cells and stimulate with NGF or BDNF, respectively, to measure kinase activity. As neurotrophin-mediated enhancement of NMDA receptor activity is often shown to be BDNF-specific, it would be beneficial to use both PC12 and TrkB-B5 cells, to evaluate whether interaction between ShcC isoforms and Fyn is also specific to TrkB-activation of ShcC upon BDNF administration (Suen et al., 1997; Levine et al., 1998; Lin et al., 1998; Crozier et al., 1999; Nakazawa et al., 2001).

I was unable to support the data suggesting both CH2 and SH2 domains of ShcC bind to Fyn by co-expressing GST-tagged ShcC domains with Src in HEK 293T cells (Figures 10 and 12). Through troubleshooting, I have shown that protein solubility in lysis buffer is not likely the cause (Figure 11), nor is the GFP tag on the N-terminus of Src (Figure 12) as using an untagged form did not influence the lack or Src interaction. This does not however take into account any potential confounding variables of using an artificial system to investigate binding capabilities. For example, it is possible that ShcC and Fyn/Src, though present as the part of the same complex in the mouse brain, do not actually bind to each other directly and require a third protein to mediate the interaction,
in contrast to the direct binding of ShcA and Src (Xi et al., 2010a). Absence of that third protein in this artificial system could result in a false negative result. Secondly, if the proteins are not properly solubilized when overexpressed in HEK 293T cells, or if aggregation occurs, constituents may not be available to bind to each other and therefore can also lead to a false negative. This insolubility is in fact seen with the ShcC CH2 domain, where I witnessed a band in the pellet fraction that was much too large (Figure 11). This must not be confused with protein insolubility in lysis buffers, as the use of several tested lysis buffers did not change these results (Figure 11). Third, it is also possible that subcellular localization plays a role in the inability of ShcC to bind to Fyn/Src. In the brain, ShcC and Fyn/Src are localized to the post-synaptic density, which is held together by a scaffolding protein, PSD-95. Without this regulation and co-localization binding may not occur. Fourth, using a subdomain approach to study binding has its disadvantages, in particular to those with multi-domain binding. If there is mutual attraction of both ShcC and Fyn during binding, this scenario may eliminate necessary elements for one protein to recognize and/or recruit the other. This could be addressed by running a similar experiment with full length ShcC as described above. In addition, using a subdomain approach may not allow for proper domain folding improper structure would influence results of the study. Lastly, it is also possible that if co-IPs from mouse brain are not reproducible by another means, there is no direct interaction. However, this is an unlikely scenario: although co-expressing GST subdomain vectors of ShcC with Src in HEK 293 cells did not indicate an interaction (Figures 10 and 12), this was successful using bacterially-purified GST fusions incubated with mouse brain (Figure 13).
There exists additional evidence to support the importance of tyrosine phosphorylation in the CH1 domain of ShcC at Y221, 222, and 304 in SH2 domain activity. George et al (2008) demonstrated that, when three critical tyrosines in the CH1 domain of ShcA were mutated to phenylalanine (3F), the SH2 domain was unable to bind its targets. In fact, SH2 domain binding was only witnessed when these three tyrosines were phosphorylated (George et al., 2008). A phosphomimetic, where these tyrosines were mutated to aspartic acids, did also not result in any SH2 domain binding. In turn, I generated a GST fusion ShcC construct lacking the PTB domain (pGEX CH1SH2) with glutamic acid substitutions at the three critical tyrosines (3YE) in an attempt to study SH2 domain binding partners. In parallel, I co-expressed the native form of ShcC CH1SH2 with V-Src (in pET28a). I used ShcC CH1 as a negative control to be more confident that any identified binding partners were interacting through the SH2 domain. I have demonstrated that a tyrosine-phosphorylated ShcC construct (pGEX ShcC CH1SH2) was capable of pulling down Fyn or Src whereas the non-tyrosine phosphorylated form was unable to bind (Figure 17). However, I did not consistently witness an interaction with the phosphomimetic form. It is also notable that GST-ShcC SH2 is capable of pulling down Fyn (Figure 13) whereas GST-ShcC CH1SH2 is not able to unless it is tyrosine phosphorylated (Figure 17), indicating a potential functional role of tyrosine phosphorylation when the CH1 and SH2 domains are together as they are in the endogenous protein. However, after re-transforming the vectors into the DE3+ BL21 strain of *E. coli*, I was able to observe tyrosine phosphorylation of both CH1SH2 and CH1SH2 3YE when co-purified with V-Src (Figures 15 and 16). This indicates that V-Src also phosphorylated tyrosine residues other than 221, 222, and 304 (not present in the
3YE mutant). Alternatively, ShcC CH1 alone did not show any tyrosine phosphorylation (Figure 16) indicating that V-Src did have some specificity for CH1SH2 over CH1 alone. However, repeating GST pulldowns with proteins expressed and purified from the BL21-DE3+ strain did not yield the same result (Figure 18) and this experiment requires further optimization. It is important to note that ShcC CH1SH2, when co-purified with V-Src in a cloning strain of *E. coli*, resulted in an interaction between ShcC CH1SH2 and Fyn or Src (Figure 17A and B, respectively), indicating that though V-Src expression was not constitutive when proteins were expressed and purified in XL10-gold cells, due to the lack of a T7 RNA polymerase to express from the pET28a T7 promoter, there was some baseline expression that was sufficient to phosphorylate some of the ShcC CH1SH2 protein (Figure 15). This experiment needs to be optimized in the BL21 strain though, however, to be more confident in the results.
In conclusion, I determined that ShcC binds to Fyn through its CH2 and SH2 domains. If this is truly the case, this could explain why ShcC knockout mice have increased NR2B tyrosine phosphorylation (Miyamoto et al., 2005). However, based on the literature, it is only p69 ShcC that has the potential to inhibit Src kinase activation (Xi et al., 2010a). Perhaps it is the ratio of p55 to p69 ShcC that governs the regulation of Src and Fyn kinases. Perhaps there are still other players that need to be identified. Nonetheless, ShcC or Fyn represent potential drug targets to treat learning disorders: By blocking the cross-talk between p69 ShcC and Fyn, but not p55 ShcC and Fyn, we could up-regulate NR2B tyrosine phosphorylation and resultant learning and memory. This could be studied by knocking-in p55 ShcC in a ShcC knockout mouse, or by knocking-in a non-splicable form of p69 ShcC, and studying their learning capabilities compared to both wild-type and total ShcC knockout mice. ShcC binding partners in the hippocampus have the potential to be the targets of treatment for learning disorders.
References


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## Appendix 1. Supplementary Materials and Methods

### Table I. List of all Vectors used, origin, expression type, and both predicted and empirical size.

<table>
<thead>
<tr>
<th>Vector Name</th>
<th>Source</th>
<th>Expression</th>
<th>Size (kDa)</th>
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<td>Mammalian</td>
<td>26</td>
</tr>
<tr>
<td>pEBG ShcA CH2</td>
<td>Kayla Driver</td>
<td>Mammalian</td>
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<td>Bacterial</td>
<td>N/A</td>
</tr>
<tr>
<td>pET28a V-Src</td>
<td>Kayla Driver</td>
<td>Bacterial</td>
<td>60</td>
</tr>
<tr>
<td>pGEX 4T-2</td>
<td>Pharmacia</td>
<td>Bacterial</td>
<td>26</td>
</tr>
<tr>
<td>pGEX ShcA CH2</td>
<td>Kayla Driver</td>
<td>Bacterial</td>
<td>31</td>
</tr>
<tr>
<td>pGEX ShcC CH2</td>
<td>Kayla Driver</td>
<td>Bacterial</td>
<td>29</td>
</tr>
<tr>
<td>pGEX ShcC PTB</td>
<td>Hui-Yu Liu</td>
<td>Bacterial</td>
<td>44</td>
</tr>
<tr>
<td>pGEX ShcC CH1</td>
<td>Kayla Driver</td>
<td>Bacterial</td>
<td>47</td>
</tr>
<tr>
<td>pGEX ShcC SH2</td>
<td>Ian Grant</td>
<td>Bacterial</td>
<td>37</td>
</tr>
<tr>
<td>pGEX ShcC CH1SH2</td>
<td>Kayla Driver</td>
<td>Bacterial</td>
<td>57/62</td>
</tr>
<tr>
<td>pGEX ShcC CH1SH2 3YE</td>
<td>Kayla Driver</td>
<td>Bacterial</td>
<td>57/62</td>
</tr>
<tr>
<td>pcDNA 4/T.O. Src(1-532)-GFP</td>
<td>Naoto Yamaguchi (Kasahara et al. 2007)</td>
<td>Mammalian</td>
<td>87</td>
</tr>
<tr>
<td>pcDNA 4/T.O. Fyn(1-536)-GFP</td>
<td>Naoto Yamaguchi (Kasahara et al. 2007)</td>
<td>Mammalian</td>
<td>86</td>
</tr>
<tr>
<td>pCMV5 C-Src</td>
<td>Joan Brugge (Addgene)</td>
<td>Mammalian</td>
<td>60</td>
</tr>
<tr>
<td>pEGFP-C1</td>
<td>Clontech</td>
<td>Mammalian</td>
<td>27</td>
</tr>
</tbody>
</table>

Note: Protein sizes indicated with two numbers (i.e. 57/62) indicate first predicted, then empirically determined protein sizes.
Table II. List of all Antibodies used for immunoprecipitation and/or Western Blotting, company purchased from, and all applicable dilutions.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species</th>
<th>Company</th>
<th>Immunoprecipitation</th>
<th>Western Blot</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>Mouse</td>
<td>Sigma-Aldrich</td>
<td>N/A</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Fyn</td>
<td>Mouse</td>
<td>Abcam</td>
<td>1µg</td>
<td>1:2000-5000</td>
</tr>
<tr>
<td>GFP-AC</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>5-10µg</td>
<td>N/A</td>
</tr>
<tr>
<td>GFP-FL</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>N/A</td>
<td>1:2000</td>
</tr>
<tr>
<td>GST-Z5</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>N/A</td>
<td>1:2000</td>
</tr>
<tr>
<td>Phosohotyrosine</td>
<td>Mouse</td>
<td>Cell Signaling</td>
<td>N/A</td>
<td>1:2000</td>
</tr>
<tr>
<td>ShcC</td>
<td>Mouse</td>
<td>BD Biosciences</td>
<td>1µg</td>
<td>1:2000-5000</td>
</tr>
<tr>
<td>ShcC</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>2µg</td>
<td>1:1000-2000</td>
</tr>
<tr>
<td>Src</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>5µL</td>
<td>1:2000</td>
</tr>
<tr>
<td>V-Src</td>
<td>Mouse</td>
<td>Calbiochem</td>
<td>N/A</td>
<td>1:2000</td>
</tr>
<tr>
<td>STEP</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>2µg</td>
<td>1:1000-2000</td>
</tr>
<tr>
<td>STEP</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>5µL</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

Note: Immunoprecipitation amounts are listed as either volume (in µL) or quantity (in µg) added to 1mL final volume of the IP; Western blot concentrations are listed as a ratio to 10mL final volume blocking buffer (i.e. 1:2000 represents 5µL in 10mL final volume).
### List of Buffers, Reagents, and Their Constituents, in Order of Appearance

#### TFB1
- 100mM Rubidium chloride
- 50mM Manganese chloride
- 30mM Potassium acetate
- 10mM Calcium chloride
- 15% Glycerol
  
  Adjusted to pH 5.8 with 1M Acetic Acid

#### TFBII
- 10mM MOPS
- 10mM Rubidium chloride
- 75mM Calcium chloride
- 15% Glycerol
  
  Adjusted to pH 6.5 with KOH.

#### LB Media and Plates
Note: LB was autoclaved in bottles or flasks, LB Agar plates were autoclaved and then poured into petri dishes and stored at 4°C. Per 500mL:
- 5g Bio-Tryptone (Bioshop)
- 2.5g Yeast Extract (Bioshop)
- 5g NaCl
- 7.5g Agar (for plates only)

#### Solution I
- 2.5mM Tris-Cl (pH 8.0)
- 10mM EDTA
- 50mM Glucose
  
  pH to 8.0, store at 4°C.

#### Solution II
- 1% SDS
- 200mM NaOH

#### Solution III
- 3M Potassium Acetate
- 1.18M Acetic Acid
  
  pH to 5.5, store at 4°C.

#### TE
- 1M Tris pH 8
- 500mM EDTA
HEK 293T Media
450 mL Dulbecco’s modified Eagle's medium (Wisent)
50 mL 10% Fetal Bovine Serum (Wisent)
500µL 50µg/mL Gentimycin Sulfate (Company)

2.5M CaCl₂ for Calcium Phosphate Transfection
183.7g CaCl₂·2H₂O
H₂O to 500mL. Filter sterilize through 0.45µM mesh, store 10mL aliquots at -20°C.

2X HBSS (HEPES-Buffered Saline Solution)
0.28M NaCl
0.05M HEPES
1.5MM Na₂HPO₄
Add 4/5 volume H₂O, triturate to pH 7.5 with 5M NaOH, top to final volume.

PBS (10X)
Note: To make 1X, 100mL was diluted in 900mL dH₂O.
40g NaCl
1g KCl
7.2g Na₂HPO₄
1.2g KH₂PO₄
H₂O to 500mL. Adjust pH to 7.4 with NaOH.

NP-40 Lysis Buffer
50mM Tris (pH 8.0)
137mM NaCl
1mM EDTA
1% NP-40
10% Glycerol

Interaction Buffer
20mM HEPES (pH 7.5)
150mM NaCl (5M)
0.1% NP-40
10% Glycerol

PBS Lysis I
PBS (1X)
2mM EDTA
1mM Sodium Vanadate, PMSF
10µg/mL Aprotinin, Leupeptin
**PBS Lysis II**

Note: PBS Lysis II was prepared using PBS (10X) and made up with dH2O.

- 1% Triton X-100 (20%)
- 2mM EDTA (0.5M)
- 1mM Sodium Vanadate, PMSF
- 10µg/mL Aprotinin, Leupeptin

**SDS-PAGE Sample Buffer (5X)**

- 1mL 20% SDS
- 0.6mL 1M Tris-Cl (pH 6.8)
- 2.4mL Glycerol
- 1mL 10% Bromophenol Blue

H2O to 10mL, aliquot 1mL each and store at -20°C. Prior to use, add 15.1mg DTT (Bioshop).

**Lower Tris**

- 36.34g Tris
- 8mL 10% SDS

H2O to 200mL, adjust pH to 8.8, filter sterilize, then add SDS.

**Upper Tris**

- 12.12g Tris
- 8mL 10% SDS

H2O to 200mL, adjust pH to 6.8, filter sterilize, then add SDS.

**30% Acrylamide/Bis-Acrylamide**

- 150g Acrylamide
- 4g Bisacrylamide

H2O to 500mL, filter sterilize.

**SDS-PAGE Buffer (10X)**

- 121.6g Tris
- 577.6g Glycine
- 40g SDS

H2O to 4L.

**Transfer Buffer (10X)**

Note: To make 1X, 100mL was diluted in 200mL methanol and 700mL dH2O.

- 29g Tris
- 144g Glycine

H2O to 1L.
TBS (10X)
24.2g Tris
80g NaCl
H₂O to 1L. Adjust pH to 7.6 with HCl.

TBS-Tween (1X)
100mL 10X TBS
900mL dH₂O
1mL Tween-20

BSA Blocking Buffer (10X)
20g BSA
200mM Tris (pH 7.2)
2M NaCl
0.1% Tween-20
H₂O to 100mL. Filter sterilize.
Curriculum Vitae

Name: Kayla Rose Maxine Driver

Post-secondary Education and Degrees:
University of Western Ontario – London, Ontario, Canada
2009-2012 M.Sc. in Biochemistry (in completion)

University of Western Ontario – London, Ontario, Canada
2005-2009 B.Sc. Honours Specialization in Genetics

Honours and Awards:
Dean’s Honour List
2008-2009
Western Scholarship of Excellence
2005

Publications:
An asterisk (*) Indicates the presenting author for posters.

Li, C., Driver, K.R.M., MacDonald, J.I.S.*, and Meakin, S.O. Reprogramming GTPase Networks to Drive Macropinocytotic Death of Brain Tumors. J. Allyn Taylor International Prize in Medicine Symposium, Robarts Research Institute, University of Western Ontario, London, Ontario, Canada. November 2011. (First place in Research Staff Category).

Chang, M.*, Driver, K.R.M., MacDonald, J.I.S., and Meakin, S.O. Novel molecular interaction between neuronal adapter Nesca with motor protein Kinesin-1 (Kif5B) and Syntaxin-1. Biochemistry Undergraduate Summer Research Program Poster Session, Department of Biochemistry, University of Western Ontario, London, Ontario, Canada, August 2011.

