Neuronal development relies on the coordination of various biological mechanisms, including the trafficking and function of neurotransmitter receptors and synaptic cell adhesion molecules (CAMs). In this dissertation, I investigated various distinct, yet related, mechanisms of neuronal development: the roles of synaptic adhesion-like molecules (SALMs) in neurite outgrowth and cell adhesion, and the transient expression of N-methyl D-aspartate receptors (NMDARs) at growth cones of young hippocampal neurons. First, I showed that the SALMs, a newly discovered family of CAMs, regulate changes in neurite outgrowth with distinct morphological characteristics. Through transfections of primary hippocampal neurons, I investigated the roles of each SALM in neurite outgrowth. In addition to neurite outgrowth, SALMs are involved in synapse formation. In a parallel study, I further investigated SALM function in development by examining the formation of SALM-mediated cell-cell contacts, and their implications on synaptogenesis. In my final study, I investigated the transient expression of NMDARs at axonal growth cones of young hippocampal neurons. While NMDAR function at synapses is well known, their roles earlier in development are less characterized. The data indicate that
NMDARs are present and functional at axonal growth cones of young hippocampal neurons. Somatic whole-cell recordings of young neurons reveal NMDAR-mediated currents in response to local application of NMDA at axonal growth cones, while calcium imaging experiments show that these NMDARs elicit localized calcium influx. Together, the studies in this dissertation give insights into the recurring phenomena of proteins and mechanisms that have dual/multiple roles throughout neuronal development. While a considerable amount of information is known about various biological events that occur at opposite ends of the developmental spectra, the mechanisms connecting them are often enigmatic, but can be elucidated through examining the proteins that they share in common.
MOLECULAR MECHANISMS OF NEURONAL DEVELOPMENT

by

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Dissertation submitted to the Faculty of the Graduate School of the University of Maryland, College Park, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

2009

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Preface

Work in this dissertation has resulted in the publication/preparation of five manuscripts (four journal articles and one book chapter), as listed below. Much of the text in this dissertation has been adapted from these manuscripts. The experiments presented in this dissertation were performed by Philip Wang, and directed by Dr. Robert Wenthold. Dr. Stephan Brenowitz co-directed the experiments in Chapter 4 (abstract #1, below). In Seabold et al. (2008), Philip contributed to the design and performance of the experiments that resulted in Figure 6 of the publication (presented as Figure 3.1 in this dissertation). Only those experiments that Philip participated in are presented in this dissertation. Dr. Ya-Xian Wang prepared the primary hippocampal cultures in these various studies. Though not presented in this dissertation, Sans et al. (2005) is a study that Philip participated in prior to entering the GPP/NACS Program, and subsequently continued during the first year of his graduate studies (abstract #5, below).

   Transient expression of NMDA receptors at axonal growth cones of young hippocampal neurons. *(manuscript in preparation)*

2. Wang PY & Wenthold RJ

3. Wang PY, Seabold GK, Wenthold RJ


Dedications

To Mom and Dad, thanks for all of your wonderful support. I couldn’t have done this without you.

To Dr. Edward and Mrs. Eileen Roccella, thank you for all of your help to make this day possible.

To the memories of my dear family and friends: Grandma Sheue Lin Wang, Grandpa Kim-long Wang, Grandma Mang Sun Huang, Grandpa Sun Wang Huang, Calvin Sunrise Wang, Charles Pencoff, and Andrew Michael Roccella.

Your lives inspired me, and your memories drive me forward.
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Abbreviations

AIS axon initial segment
AM acetoxyethyl
AMPA alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA analysis of variance
CAM cell adhesion molecule
CaMKII calcium/calmodulin-dependent protein kinase II
cDNA complementary DNA
CICR calcium induced calcium release
CNS central nervous system
CPA cyclopiazonic acid
CTX conotoxin
DIV days in vitro
E18 embryonic day 18
EM electron microscopy
EPSC excitatory postsynaptic current
ER endoplasmic reticulum
FN3 fibronectin type 3
GFP green fluorescent protein
GK guanylate kinase
HA hemagglutinin
IgC2 immunoglobulin C2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Lrfn</td>
<td>leucine-rich repeat and fibronectin III domain-containing</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine-rich repeat</td>
</tr>
<tr>
<td>LTD</td>
<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>MAGUK</td>
<td>membrane associated guanylate kinase</td>
</tr>
<tr>
<td>MASA</td>
<td>mental retardation, aphasia, shuffling gait, and adducted thumbs</td>
</tr>
<tr>
<td>mEPSC</td>
<td>miniature excitatory postsynaptic current</td>
</tr>
<tr>
<td>mPins</td>
<td>mammalian partner of inscuteable</td>
</tr>
<tr>
<td>NCAM</td>
<td>neural cell adhesion molecule</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl D-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl D-aspartate receptor</td>
</tr>
<tr>
<td>P(number)</td>
<td>postnatal day (number)</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95, discs-large, zonula occludens-1</td>
</tr>
<tr>
<td>PDZ-BD</td>
<td>PDZ binding domain</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PSD</td>
<td>postsynaptic density</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SALM</td>
<td>synaptic adhesion-like molecule</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SH3</td>
<td>src homology 3 domain</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>VSCC</td>
<td>voltage-sensitive calcium channel</td>
</tr>
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Chapter 1: Introduction

1.1 Overview

Neuronal development is fascinating. From the molecular cues that guide neurite outgrowth and synapse formation, to the environmental experiences that lead to learning and memory, how neurons develop relies on a myriad of biological processes studied at all levels of neuroscience research. Understanding these mechanisms is critical to revealing the extraordinary complexities of the brain, and has implications on a wide variety of neurological disorders. In this dissertation, I investigated mechanisms of neuronal development at the molecular level, focusing on growth cones, early events of neurite outgrowth and the subsequent cell-cell contacts that lead to synapse formation. Specifically, I focused on two classes of proteins, the synaptic adhesion-like molecules (SALMs) and the N-methyl D-aspartate receptor (NMDAR). The SALMs are a newly discovered family of cell adhesion molecules (CAMs) that has very interesting roles in development. First, I performed a detailed analysis on how the SALMs mediate neurite outgrowth in young hippocampal neurons (Chapter 2). Next, I investigated the roles of SALMs in cell-cell adhesions, and the implications on synapse formation and maintenance (Chapter 3). Finally, in a distinct, yet highly related line of experiments, I examined the transient expression, and function, of NMDARs at axonal growth cones and at the cell body of young hippocampal neurons (Chapter 4). Neurotransmitter receptors and CAMs are critical for various stages of neuronal development. While a considerable amount of information is known about biological phenomena that occur early and later in
neuronal development, the mechanisms connecting them are still enigmatic. The road from neurite outgrowth to synapse formation is one of the central themes of this thesis. One effective way to understand the intricacies of this process is to examine the molecules that are present at both the start and final destinations of the journey.

1.2 Neuronal polarity to synaptogenesis

Neuronal development involves a remarkable coordination of a variety of factors and biological mechanisms. At the early stages of development, neurons undergo a dramatic change in cellular morphology. Using embryonic day 18 (E18) rat hippocampal cultures, Dr. Gary Banker and colleagues have observed five distinct stages of neuronal polarity (Dotti et al., 1988; Craig and Banker, 1994). The first stage, occurring within hours of plating, is characterized by the extension of web-like lamellipodia around the cell body. In the second stage, which occurs within one day in vitro (DIV1), the cell body begins to extend 4-5 short, non-differentiable neurites (15-20 μm long), with growth cone structures at the leading edges of the neurites, guiding their outgrowth. In the third stage (~DIV1.5), one of the neurites extends very rapidly and marks the first stage of axonal differentiation and the establishment of polarity. Stage 4 begins at approximately DIV4, and is characterized by the outgrowth of the remaining neurites and the appearance of proteins specific to dendrites. In stage 5 (from DIV7 on) elaborate maturation of axonal and dendritic arbors begins, including neurite branching, the formation of spines, and synaptogenesis. Throughout these stages (and beyond), a vast collection of proteins
and posttranslational factors are involved in the proper regulation of this development.

Neurite outgrowth, the process by which neurites extend/retract and find their paths, depends on a wide variety of [often interconnected] factors ranging from cytoskeletal/growth cone dynamics, neurotransmitter receptors, CAMs, extracellular signaling gradients, and localized intracellular calcium levels (for example, Pearce et al., 1987; Brewer and Cotman, 1989; Henley and Poo, 2004; Chen et al., 2006). Additionally, how these extending neurites ultimately reach their destination and begin the process of forming synaptic contacts is another developmental question of great interest. Like neurite outgrowth, synapse formation (or synaptogenesis), is a highly studied phenomenon that involves a wide variety of biological factors. At the later stages of neurite outgrowth, and the initial stages of synapse formation, axons and dendrites find their paths via molecular determinates/extracellular guidance cues (often traveling great distances, in the case of the axons), and ultimately establish trans cell-cell contacts and adhesion via CAMs. The synaptic apparatus, including classical CAMs and vesicle clusters (including 80 nm dense core vesicles, which may act as precursors of the active zone) initiate/facilitate the constitution of pre-synaptic protein complexes. Meanwhile, cytoskeletal and scaffolding proteins, and neurotransmitter (glutamate) receptor complexes facilitate the formation of the excitatory postsynaptic density (PSD). This cumulatively leads to the differentiation of pre- and post-synaptic specializations (for review, see Garner et al., 2002; Dityatev and El-Husseini, 2006). Neurotransmitter receptors and CAMs clearly have critical roles throughout the various stages neuronal development. As I examine in this
dissertation, two such classes of synaptic proteins include the NMDARs and the SALMs.

### 1.3 Synaptic adhesion-like molecules (SALMs)

SALMs are a newly discovered family of CAMs that were initially characterized by Dr. Robert Wenthold’s laboratory in 2006 (Wang et al., 2006). As I describe in Chapters 2 and 3 of this dissertation, SALMs have a variety of functions in neuronal development, including aspects of neurite outgrowth and synapse formation (Ko et al., 2006; Morimura et al., 2006; Wang et al., 2006; Seabold et al., 2008; Wang et al., 2008). Also known as Lrfn (leucine-rich and fibronectin III domain-containing), five family members have been identified thus far: SALM1/Lrfn2, SALM2/Lrfn1, SALM3/Lrfn4, SALM4/Lrfn3, SALM5/Lrfn5. In the mouse, they range from 626-788 amino acid residues in length. All SALMs contain a characteristic domain structure including six leucine-rich repeat regions (LRRs), an immunoglobulin C2-like domain (IgC2), a fibronectin type 3 domain (FN3), and a transmembrane region (TM) (Figure 1.1). Additionally, SALMs 1-3 contain a PDZ-binding domain (PDZ-BD) at their distal C-termini, while SALMs 4-5 do not. The SALMs share a considerable amount of sequence similarity, though there are regions of high variability in both the N-and C-termini that could lend distinct characteristics to their individual functions (Figure 1.2). Studies on SALMs and their functions thus far have focused on the rat and mouse representatives of this gene family. However, SALMs are present in all mammalian species, as well as in fish and amphibians (Morimura et al., 2006). Interestingly, additional sequence analysis using the
Figure 1.1: SALM protein domain structure. Schematic diagram illustrating the domain structure of the SALM family of proteins. The SALMs contain an N-terminal signal peptide, six extracellular Leucine-rich repeat (LRR) regions flanked by N- and C-terminal LRR regions, an Immunoglobulin C2-like (IgC2) domain, a Fibronectin type III (FN3) domain, a single transmembrane (TM) region, and a PDZ binding domain (PDZ-BD, present in SALMs 1-3).

(Wang and Wenthold, 2009)
Figure 1.2: SALM family sequence comparison. (A) Sequence analysis reveals that mouse SALMs contain six LRR regions (flanked by N- and C-terminal LRR sequences), an IgC2-like domain, a FN3 domain, a TM region, and a PDZ-BD at the distal C-termini (present in SALMs 1-3). Illustrated domain locations are based on the SALM1 sequence. Similar amino acid residues are highlighted by the yellow background, and the consensus sequence/strength is shown above the alignment. Consensus strength correlates with the length of the individual bars. Protein sequences for SALMs 1-5 (accession numbers NM_027452, NM_030562, NM_153388, NM_175478, and NM_178714, respectively) were aligned by the ClustalV method using MegAlign computer software. (B) Phylogenetic tree comparing the mouse SALMs was constructed with MegAlign based on the alignment produced in (A).
Figure 1.2: SALM family sequence comparison
Ensembl database (www.ensembl.org) indicates that the protein structure of the SALMs is quite conserved across these species. As depicted in Figure 1.3, the LRRs, IgC2, FN3, and PDZ-BD regions are all conserved across various SALM1 sequences, including those of human, mouse, platypus, chicken, and medaka (Japanese killifish). Analysis of the Drosophila genome indicates that SALMs share the closest sequence identity with a family of LRR and Ig-like domain containing transmembrane proteins called kekkon (kekkon 1-5), which function in the developmental regulation of EGF receptor activity during oogenesis (Ghiglione et al., 1999; Ghiglione et al., 2003). Phylogenetic analysis reveals that SALMs are closely related to a variety of leucine-rich molecules including the AMIGO, LINGO, NGL, PAL, and FLRT family of proteins (Chen et al., 2006). As I describe later in this dissertation (Chapter 2), SALMs and these highly related proteins have one major characteristic in common: their role in regulating neurite outgrowth.

Northern blot analysis indicates that all SALM transcripts are found in mouse and rat brain, and transcripts for SALMs 2, 3, and 4 are seen to some extent in testis (Ko et al., 2006; Morimura et al., 2006). Additionally, SALM4 transcripts are found in the gastrointestinal tract and kidney (Morimura et al., 2006). Temporal expression profile blots indicate that transcript levels for SALMs 2-4 show an incremental increase starting from E10.5, while SALM1 and SALM5 increase from around E11.5-E12.5 (Morimura et al., 2006). In situ hybridization reveals that SALM transcripts are distinctly expressed in a variety of brain regions, including the cerebral cortex, hippocampus, dentate gyrus, and olfactory bulb (Ko et al., 2006; Morimura et al., 2006; Homma et al., 2009). Recently, in situ hybridization in whole-mount
Figure 1.3: SALM1 species comparison. SALM1 protein sequences from a variety of species were acquired using the Ensembl database (www.ensembl.org). (A) The SALM1 sequence structure is highly conserved among a variety of mammalian, avian, and fish species including human (Homo sapien), mouse (Mus musculus), platypus (Ornithorhynchus anatinus), chicken (Gallus gallus), and Japanese killifish/medaka (Oryzias latipes). Similar amino acid residues are highlighted by the yellow background, and the consensus sequence/strength is shown above the alignment. (B) Phylogenetic tree comparing the SALM1 sequences of the various species. SALM1 proteins sequences were aligned by the ClustalV method using MegAlign. Ensembl gene IDs used for SALM1 human, mouse, platypus, chicken, and killifish were ENSG00000156564, ENSOANG00000014625, ENSGALG00000010050, and ENSORLG00000018222, respectively. Accession number NM_027452 was used for the SALM1 mouse sequence.
Figure 1.3: SALM1 species comparison

(Wang and Wenthold, 2009)
preparations of E10 mouse embryos indicate that SALM1 and SALM5 mRNA are specific to neuronal tissues, while SALMs 2-4 are not. The authors note that SALM3 mRNA was found in all tissues examined (Homma et al., 2009). Western blot and subcellular fractionation experiments showed that SALM proteins are highly expressed in the rat brain, and present in synaptosomal and postsynaptic density fractions (Ko et al., 2006; Wang et al., 2006). SALM protein expression levels exhibit some differentiation among family members. Protein levels for SALM1 are detectable from E18, display high expression from P1 to P21, and then decrease at P28 (Wang et al., 2006). Protein levels for SALM2 increase from P21 and remain high at 6 weeks (Ko et al., 2006). SALM2 protein is widely distributed in brain, and for example, has been detected in cortical pyramidal neurons, hippocampal CA3 and CA1 neurons, and cerebellar Purkinje cells (Ko et al., 2006). At the subcellular level, SALM2 proteins localize to cell bodies, neurites, and punctate structures that co-localize with the presynaptic protein, synapsin I (Ko et al., 2006). Ultrastructural analysis using immunogold electron microscopy (EM) shows that native SALM4 is present at a variety of presynaptic, postsynaptic and extrasynaptic sites in hippocampus, olfactory bulb, and cerebellar cortex (Seabold et al., 2008). Over-expressed SALMs are localized throughout the cell in the soma, axons, dendrites, and growth cones in young (<DIV7) neuronal cultures, both on the cell surface and intracellularly (Wang et al., 2006; Wang et al., 2008). In older (>DIV14) neuronal cultures, over-expressed SALMs are localized throughout the cell, on the cell surface, and at synapses (Ko et al., 2006; Wang et al., 2006, and unpublished observations). Additionally, Ko et al (2006) demonstrated that SALM2 is localized to excitatory, but
not inhibitory synapses, and that perturbation of SALM2 expression leads to aberrations in excitatory synaptic formation.

1.4 SALM-associated proteins and functional significance

The known binding partners for the SALMs include the PSD-95 family of membrane-associated guanylate kinase (MAGUK) proteins, the NR1 subunit of the NMDAR, and the SALMs themselves - through homomeric and heteromeric interactions (Ko et al., 2006; Morimura et al., 2006; Wang et al., 2006; Seabold et al., 2008). The SALMs were independently identified in two different laboratories through yeast two-hybrid screens using the PDZ domains of MAGUKs as bait (SAP97 in Wang et al., 2006, and PSD-95 in Ko et al., 2006). Classically described as scaffolding proteins that assist in the tethering of receptors and associated proteins at the PSD, MAGUKs have been linked to a variety of functions in the central nervous system (CNS), including the trafficking of NMDARs to the synapse and neurite outgrowth (Kim and Sheng, 2004; Charych et al., 2006). Overexpression of PSD-95 decreases dendritic branching in immature neurons, while knocking down PSD-95 increases it (Charych et al., 2006). Overexpression of SALMs in young neurons promotes neurite outgrowth, while overexpression of SALM 1-3 constructs lacking the PDZ-BD do not (Wang et al., 2008/Chapter 2 of this dissertation). This suggests that there may be a direct link in the process of neurite outgrowth and SALM-MAGUK associations, at least for SALMs 1-3. Interestingly, SALMs 4-5 lack a PDZ-BD, but still promote neurite outgrowth. This may indicate that they act via a different mechanism or through heteromeric associations with other SALMs to induce MAGUK-associated
neurite outgrowth. In mature neurons, deletion of the PDZ-BD has effects on synapse formation and morphology (Wang et al., 2006), further emphasizing the importance of associated PDZ proteins in SALM function. Additional evidence suggests that SALMs may interact, indirectly or directly, with various other postsynaptic proteins. For example, bead-induced aggregation experiments revealed co-clustering of AMPARs and GKAP with SALM2 (Ko et al., 2006). SALM1 has also been biochemically shown to directly interact with NMDAR subunits in brain tissue (Wang et al., 2006).

1.5 N-methyl D-aspartate receptors (NMDARs)

NMDARs are a class of calcium-permeable ionotropic glutamate receptors that have critical roles in the CNS throughout development and in the mature brain. NMDARs are ligand-gated channels that allow the conductance of both sodium and calcium ions. Activation of NMDARs requires the binding of two agonists, glutamate and glycine, and the release of a voltage-sensitive magnesium block through membrane depolarization. The structure, assembly, and trafficking of NMDARs to the synapse are of great importance to their function, and have been studied in detail. Briefly, functional NMDARs are heteromeric complexes comprised of NR1, NR2, and/or NR3 subunits. Each NMDAR subunit contains an extracellular N-terminal region, three transmembrane regions (M1, M3, M4), a membrane region that lines the pore of the channel (M2), and an intracellular C-terminal region that is highly variable among the subunits and contributes to differential protein interactions. NR1 subunits are obligatory in the functional NMDAR complex, and exist as eight different splice
variants (NR1-1a/b, NR1-2a/b, NR1-3a/b, NR1-4a/b), due to the alternative splicing of three exons (one near the NT and two near the CT) (for review, see Wenthold et al., 2008). Studies have identified seven of these of these splice variants, in vivo, which display distinctions in regional and developmental characteristics (Laurie and Seeburg, 1994; Laurie et al., 1995; Zukin and Bennett, 1995; Prybylowski and Wolfe, 2000). There are four NR2 subunits (NR2A, NR2B, NR2C, and NR2D) and two NR3 subunits (NR3A and NR3B), each of which is a separate gene product that contributes distinct functional characteristics onto the NMDAR (Dingledine et al., 1999; Matsuda et al., 2003 and for review, see Wenthold et al., 2008). The various NR2 and NR3 subunits also have distinct regional and developmental profiles (Ishii et al., 1993; Monyer et al., 1994; Laurie et al., 1997; Wong et al., 2002; Wee et al., 2008). Most NMDARs are heteromeric tetramers consisting of two NR1 subunits and two NR2 subunits, though NR1/NR2/NR3 complexes have also been reported (Perez-Otano et al., 2001; Al-Hallaq et al., 2002). Interestingly, NR1/NR3 complexes form an excitatory glycine receptor (Chatterton et al., 2002). In hippocampus, the majority of NMDARs are NR1/NR2A or NR1/NR2B diheteromers, though a population of NR1/NR2A/NR2B triheteromeric complexes is also present (Al-Hallaq et al., 2007).

1.6 Developmental changes in NMDAR properties

NMDAR function is dependent upon the subunit composition of the receptor. As mentioned earlier, the various subunits display differences in developmental, regional, and functional properties. NR2C subunits are highly expressed in adult
cerebellum, while NR2D is highly expressed throughout embryonic development and peaks at postnatal day 7 (P7) in brainstem and diencephalon (Monyer et al., 1994; Dunah et al., 1996; Takahashi et al., 1996). NR2A and NR2B subunits are the most common NR2 subunits in the forebrain. NR2B subunits are highly expressed early in development, while NR2A subunits are not. During the course of development, NR2A levels gradually increase while NR2B levels decline (for example, see Sans et al., 2000). Interestingly, through the use of visual deprivation paradigms, this developmental switch in subunit composition has been shown to be experience-dependent, and reversible (Quinlan et al., 1999a; Quinlan et al., 1999b). Recent studies from Dr. Quinlan’s laboratory have also demonstrated that this bi-directional synaptic plasticity occurs in mature, as well as juvenile brain (He et al., 2006; He et al., 2007), which could have important implications for the treatment of visual system disorders. The NR2 subunits also display unique physiological properties, as NR2B-containing receptors exhibit slower deactivation kinetics than NR2A-containing receptors (Carmignoto and Vicini, 1992; Vicini et al., 1998). Transgenic studies have demonstrated the critical importance of NMDAR subunits in development, and learning and memory. For example, knockout mice lacking NR1 or NR2B die shortly after birth (Forrest et al., 1994; Kutsuwada et al., 1996), while knocking out NR2A prevents the maturation of excitatory synapses in cerebellar granule cells (Fu et al., 2005), results in a loss of NMDAR-mediated currents (Townsend et al., 2003) and deficits in synaptic plasticity (Philpot et al., 2007). Interestingly, mice overexpressing NR2B show enhancements in leaning and memory during certain behavioral tasks (Tang et al., 1999).
1.7 NMDAR function throughout development

NMDARs are critical components of the nervous system throughout life. NMDARs are involved in a broad range of functions, including spine motility (Star et al., 2002), synapse formation, synaptic plasticity, and learning and memory (for review, see Wenthold et al., 2008; Yashiro and Philpot, 2008). In addition to postsynaptic function, NMDARs are found extrasynaptically (Tovar and Westbrook, 1999; Groc et al., 2006; Yi et al., 2007) and at presynaptic terminals (for review, see Corlew et al., 2008). NMDAR function also has implications in neuronal excitotoxicity and a variety of neurological disorders, including Huntington’s disease, Parkinson’s disease, autism spectrum disorders, schizophrenia, and epilepsy, and drug addiction (for review, see Lau and Zukin, 2007; Wenthold et al., 2008). While the roles of NMDARs synapses have been actively studied, their roles early in development are considerably less understood. In mammalian systems, NMDARs function in neuronal migration and neurite outgrowth (Behar et al., 1999; Manent et al., 2005; Manent et al., 2006). In Xenopus spinal neurons, NMDARs have been shown to influence filopodial dynamics and growth cone turning (Zheng et al., 1996). As I describe in Chapter 4, NMDARs have also been found at axonal growth cones (Ehlers et al., 1998; Herkert et al., 1998), though up until now, their function there has not been extensively investigated.

The protein interactions and posttranslational modifications that facilitate NMDAR trafficking are of critical importance to the function of NMDARs. Of particular interest are the interactions mediated by the PDZ-BDs of NMDAR subunits. NR1
subunits that contain the C2 exon (NR1-1a/b and NR1-2a/b) and NR2 subunits contain a PDZ-BD (-STVV) on their distal C-termini. Through these PDZ interactions, NMDARs directly associate with the MAGUKs. The MAGUK family of proteins comprises four members (PSD-95, SAP102, SAP97, and PSD-93), each of which contain three PDZ domains, an SH3 domain, and a guanylate kinase-like (GK) domain. In two projects that I contributed to in Dr. Wenthold’s laboratory, we described novel interactions between MAGUKs and their associated proteins that influence the trafficking of NMDARs to the synapse (Sans et al., 2003; Sans et al., 2005). Interactions between SAP102 and Sec8, a component of the mammalian exocyst complex, mediate the surface delivery of NMDARs (Sans et al., 2005). Next, in a project that I was highly involved in during my postbaccalaureate research and early graduate studies, we found that SAP102 and PSD-95 interact with the mammalian homolog of Drosophila Pins (mPins), also known as the LGN protein for multiple Leu-Gly-Asn repeats found in its primary structure. Our results indicate that this MAGUK-mPins interaction mediates the trafficking of NMDARs to the synapse, and has effects on synapse size and number (Sans et al., 2005). Interestingly, in Drosophila, Pins has a well characterized role in the asymmetric division of neuroblasts (Schweiguth, 2000; Bellaiche et al., 2001). Specifically, Pins interacts with the Drosophila MAGUK, Dlg, and participates in the Frizzled (Fz) signaling pathway that leads to the establishment of cell polarity (a pathway that is also present in vertebrates) (Schweiguth, 2000; Bellaiche et al., 2001). The role of mPins in mammalian neuronal development has not been characterized. Clearly, the roles of PDZ interactions with NMDARs, through direct (i.e. NMDAR-MAGUK) or indirect
interactions (i.e. NMDAR-MAGUK-mPins/Sec8) are of critical importance to their trafficking and function. NMDARs also interact directly and indirectly with a variety of CAMs. For example, neuroligin-1 has been shown to indirectly interact with NMDARs via PSD-95, and influence the trafficking of NMDARs, and NMDAR-mediated excitatory postsynaptic currents (EPSCs) (Irie et al., 1997; Chih et al., 2005; Chubykin et al., 2007). As mentioned earlier in this introduction, NMDARs also interact directly with SALM1, and could potentially interact indirectly with SALMs via MAGUK-SALM interactions. While the implications of these NMDAR-SALM interactions are not yet known, the previous literature and the studies presented in this dissertation raise several interesting questions about their potential functions together throughout development.
Chapter 2: SALMs promote neurite outgrowth

2.1 Introduction

Neurite outgrowth is a fundamental event in the development and maintenance of synaptic connections in the nervous system. As mentioned earlier, through highly regulated mechanisms, young neurons undergo axonal/dendritic polarization, and subsequent outgrowth of these neurites is essential to the establishment of synaptic connections that lead to brain function (da Silva and Dotti, 2002). CAMs are a diverse class of proteins that function in neurite outgrowth, synaptic development and maintenance, and cell adhesion at synaptic and non-synaptic sites (Craig and Banker, 1994; Dalva et al., 2007). Several CAMs are enriched at growth cones and are required for normal neurite outgrowth. For example, neural cell adhesion molecule (NCAM), N-cadherin, and L1-CAMs have been shown to regulate neurite outgrowth through various mechanisms, including changes in intracellular calcium levels, associations with cytoskeletal proteins at growth cones, and the activation of FGFR and MAPK signaling cascades (Meiri et al., 1998; Doherty et al., 2000; Utton et al., 2001; Francavilla et al., 2007). In humans, mutations in L1-CAMs lead to various neurological disorders, including hydrocephalus and MASA (mental retardation, aphasia, shuffling gait, and adducted thumbs) syndrome, and expression of constructs encoding L1 with these known mutations leads to deficits in neurite outgrowth (Moulding et al., 2000). While a wealth of information implicates CAMs in neurite outgrowth, the mechanism is highly complex and not completely understood. Previous studies showed that over-expression of SALM1 in young (DIV4) primary
hippocampal cultures promotes an increase in neurite outgrowth (Wang et al., 2006), while alterations in SALM2 expression affects synapse formation and may play a role in regulating the balance of excitatory and inhibitory synapses (Ko et al., 2006). Therefore, individual SALMs may have a range of different functions. Alternatively, all SALMs may have multiple roles and function in neurite outgrowth and synapse formation in developing animals, as well as maintenance of synapses in adults. In this chapter, we investigate these possibilities by studying the role of all SALMs in neurite outgrowth through a combination of protein over-expression, RNAi-mediated knock-down of expression, and blocking of function with antibodies to extracellular domains. Our results show that all SALMs promote neurite outgrowth, but with various phenotypes.
2.2 Methods

Antibodies

Anti-myc monoclonal (hybridoma purchased from ATCC, Manassas, VA, clone #9E10) and anti-hemagglutinin (HA) monoclonal (Covance, Denver, PA, clone #16B12) primary antibodies were used at a 1:1000 dilution. Anti-GFP polyclonal (Chemicon, Billerica, MA) was used at 1:2000. SALM3 antisera were used for immunostaining of transfected SALM3 due to the lack of an epitope-tagged cDNA construct. Antisera were produced in rabbits (Covance) as described in Seabold et al. (2008). Briefly, for detection of transfected full-length SALM3, polyclonal antibodies were generated using a peptide directed to the C-terminus of SALM3, (amino acids 621-636: [NH2]-CRGVGGSAERLEESVV-[COOH]). For detection of transfected SALM3ΔPDZ, polyclonal antibodies were generated using a peptide directed to the N-terminus of SALM3 (amino acids 377-389: [NH2]-TSAEGGRPGPSDI-[COOH]). For function-blocking antibody experiments, an antibody that recognizes the LRR regions of the SALMs was generated by expressing the SALM2 LRR region (residues 38-297) as a glutathione S-transferase (GST) fusion protein, and produced in rabbits (Covance).

cDNA constructs

Cloning and epitope-tagging of SALM cDNA constructs was performed by Dr. Kai Chang, as previously described (Wang et al., 2006; Seabold et al., 2008). The myc tag was inserted into the sequence of SALM1 before residue 21, the SALM2 myc tag was inserted before residue 33, the SALM4 myc tag was inserted before residue 24,
and the SALM5 HA-tag was inserted before residue 18. Non-tagged SALM3 cDNA was utilized in our experiments due to difficulties in detecting epitope-tagged SALM3 through immunocytochemistry. To generate SALM2 and SALM3 constructs lacking the PDZ-BD, we introduced stop codons into the constructs by site-directed mutagenesis (Stratagene, La Jolla, CA) at W759 for SALM2(Δ7) and E633 for SALM3(Δ4). The GFP construct (pEGFP-N2) was purchased from Clontech (Mountain View, CA). Dr. Chang generated the chimeras of SALM2 and SALM4 by creating a BamHI site after the transmembrane domain of SALM2 and SALM4. A single amino acid was then changed in the HA-SALM2 (D574S) and the myc-SALM4 (G566S) constructs for subcloning purposes. The C-terminal region of each construct was then excised and exchanged by utilizing the BamHI and EcoRI sites from the multiple cloning site in the pcDNA3.1+ vector.

**Primary hippocampal cultures and transfections**

Primary hippocampal neuronal cultures were prepared by Dr. Ya-Xian Wang, as previously described (Sans et al., 2005; Wang et al., 2006). Briefly, E18 hippocampi from Sprague-Dawley rats (Harlan, Indianapolis, IN) were dissected and dissociated with trypsin EDTA (Invitrogen, Carlsbad, California). Neurons were plated onto poly-ornithine/fibronectin-coated coverslips in 2% fetal bovine serum/Neurobasal medium (Invitrogen) in six-well culture plates. Neurons were plated at a density of 50,000 cells per ml of culture medium (50K) for transfections. Fifty percent of the culture media was changed to Neurobasal media plus B27 (Invitrogen) 72 hours after plating. All animal procedures were done in accordance with the National Institutes
of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23) under National Institute on Deafness and Other Communication Disorders protocol 1167-07. Transfections of DIV4 hippocampal cultures were performed using a calcium phosphate method (Clontech) with modifications. Briefly, Neurobasal/B27 culture medium was replaced with 5 ml DMEM one hour prior to transfection. One to five micrograms of cDNA was mixed with 2 M calcium solution, and added to the same volume of 2X HEPES buffered saline. The plasmid cDNA/calcium solution was incubated for 20 min at room temperature, and then applied to the neurons for 20 min. Neurons were washed twice with DMEM and cultured in the original Neurobasal/B27 medium at 37 °C, 5% CO₂. Control cells were co-transfected with empty vector (pcDNA 3.1+) and GFP (Green Fluorescent Protein construct) in each experimental group. Forty-eight hours after transfection (DIV6), neurons were fixed and processed for immunostaining. For neurite outgrowth experiments using RNAi plasmids, neurons were transfected at DIV2 and fixed for immunocytochemistry at DIV6 to allow sufficient time for knockdown of endogenous proteins.

**Immunocytochemistry**

Immunocytochemistry was performed as previously described (Sans et al., 2005). Neurons were fixed with 4% paraformaldehyde (PFA), washed with phosphate buffered saline (PBS) and permeabilized with 0.25% Triton-X/PBS. Neurons were then blocked with 10% normal goat serum (NGS)/PBS/0.1% Triton X-100 for one hour, and then incubated with primary antibodies at room temperature in 3%
NGS/PBS/0.1% Triton X-100 for one hour. Neurons were washed and incubated with Alexa Fluor 488 or 555 secondary antibodies (Molecular Probes, Carlsbad, California) for 30 minutes, washed and mounted on slides using Prolong Antifade Gold (Invitrogen). For surface staining experiments, live neurons were incubated with primary antibodies for 30 minutes at 4 °C, fixed with 4% PFA for 20 minutes, blocked with 10% NGS/PBS, and incubated with secondary antibodies for 30 minutes.

**RNAi constructs**

RNAi constructs were designed and synthesized using the Invitrogen miR RNAi custom synthesis service. Double-stranded oligos were synthesized and inserted into pcDNA6.2GW/EmGFP-miR vector, which utilizes an shRNA designed to have an RNAi effect in the context of micro RNA (miRNA) expression. The sequence region targeted for RNAi in SALM1 was 5’-TGC TGT ACT GAA GTC CAT CAA CTC ATG TTT TGG CCA CTG ACT GAC ATG AGT TGG GAC TTC AGTA. The sequence region targeted for SALM2 was 5’-TGC TGA GAA ATA GCG GTC AGT GAG ATG TTT TGG CCA CTG ACT GAC ATC TCA CTC CGC TAT TTCT. The sequence region targeted for SALM3 was 5’-TGC TGA AGA GTT GCC AAC CAG CCT GTG TTT TGG CCA CTG ACT GAC ACA GGC TGT GGC AAC TCTT. The sequence region targeted for SALM4 was 5’-TGC TGA AGA GTT GCC AAC GGC CTG TCA TGG TTT TGG CCA CTG ACT GAC CAT GAC AGC TGC TGC ATTT, and the region targeted for SALM5 was 5’-TGC TGA AGA GTT ACA GAC ACA ACG CTG TTT TGG CCA CTG ACT GAC AGC GTT GTC TGT CAG
ATTT. Control cells were transfected with RNAi plasmids containing an insert predicted to not target any known human, mouse, or rat gene (pcDNA 6.2-GW/EmGFP–miR-neg2): TGC TGT ATT GCG TCT GTA CAC TCA CCG TTT TGG CCA CTG ACT GAC GGT GAG TGC AGA CGC AATA.

**Image acquisition and neurite outgrowth analysis**

Images were taken using a Nikon E1000M microscope equipped with a CCD camera using a Plan Fluor 20x (0.5 NA) dry or Plan Apo 60x (1.4 NA) oil-immersion objective. For neurite outgrowth experiments, only transfected neurons without overlapping neurites from adjacent GFP-transfected cells were selected for analysis. All experiments were performed in triplicate. Partial images were systematically acquired for sections of each cell using the 20x objective until the entire cell was imaged. Partial images were digitally overlaid into a single composite image using Adobe Photoshop 7.0 (Adobe, San Jose, CA). Reconstructed images exhibiting the cell in its entirety were analyzed using Metamorph Neurite Outgrowth Module v7.0r3 (Molecular Devices, Sunnyvale, CA). If visual inspection of the resulting trace was incorrect, settings for each cell were individually adjusted until the resulting trace accurately represented that of the neuron. For SALM2 axonal measurements, dendrites were identified by MAP2 immunostaining and through morphological criteria. As described in Kaech and Banker (Kaech and Banker, 2006): dendrites were identified as processes that emerged gradually from the cell body, tapered with distance, had a radial orientation, and had a length of 200-300 μm. Axons were identified as processes that were thinner at their origin, exhibited less taper, often
turned at 90° angles, and extended over millimeters. Dendrites from reconstructed images were digitally severed from the cell body using Adobe Photoshop 7.0, and the resulting images were analyzed using Metamorph. Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA). Statistical significance between two groups were determined with a two-tailed, paired Student's \( t \)-test, while significance among multiple groups was determined with an analysis of variance (ANOVA), followed by the post-hoc Newman-Keuls multiple-comparison test. Differences were considered significant at \( p<0.05 \). All values are reported as mean ± SEM. For signal intensity studies, images were taken at consistent exposure times and contrast values, and imported to Metamorph for analysis of pixel intensity of transfected and non-transfected cells.
2.3 Results

2.3.1 Distribution of SALMs in neurons

SALM1 and SALM2 localize to both axons and dendrites (Ko et al., 2006; Wang et al., 2006). Additionally, SALM1 co-localizes with NMDA receptors (Wang et al., 2006), while SALM2 co-localizes with both pre- and post-synaptic proteins at excitatory synapses in mature neurons (Ko et al., 2006). To understand the roles of SALMs in neurite outgrowth, we began by characterizing the cellular localization and morphological effects of overexpressed SALMs early in neuronal development. Young primary hippocampal neurons (DIV4) were co-transfected with GFP and myc-SALM1, myc-SALM2, non-tagged SALM3, myc-SALM4, or HA-SALM5 cDNA constructs. Neurons transfected with GFP and pcDNA 3.1+ empty vector were used as a control, and immunocytochemistry was performed 48 hours after transfection. Transfected SALM constructs over-expressed their respective proteins by about 300%, as compared to endogenous SALM levels (data not shown). Over-expressed SALMs are localized throughout the cell in the soma, axons, dendrites, and growth cones (Figure 2.1) with a largely diffuse pattern. However, punctate staining is present and is particularly apparent when staining is restricted to SALMs present on the cell surface (Figure 2.2). Therefore, SALMs are present in intracellular pools represented by the diffuse staining as well as on the surface where they appear more clustered. The various SALMs qualitatively appear to have distinct effects on cell morphology. For example, SALM4-transfected neurons often had a dramatic increase in the number of shorter primary neurites protruding from the cell body (Figure 2.1D, arrows). These shorter neurites appeared to be dendritic, as MAP2
Figure 2.1: Localization and morphological characteristics of transfected SALM proteins.

DIV4 primary hippocampal cultures were co-transfected with SALM and GFP cDNAs. Neurons co-transfected with pcDNA3.1⁺ (empty vector) and GFP were used for control. Immunostaining was performed 48 hours later and the SALM localization and cell morphology were examined. Transfected SALMs localize throughout the cell body, axons, and dendrites. Representative examples of neurons transfected with SALMs 1-5 are shown in (A-E), respectively. Scale bars, 20 μm. Transfected SALMs are enriched at growth cones (A-E, fourth column). Scale bars, 10 μm. Transfected SALM4 and SALM5, which do not contain PDZ-BDs, often exhibit distinct phenotypes as compared to SALMs 1-3. (D) Myc-SALM4-transfected cells show a large increase in the number of short primary processes (arrows). HA-SALM5-transfected cells often display an increase in the number of primary processes crossing/overlapping with each other. (E) Transfected SALM5 accumulates at these crossing points (arrows). Representative examples of GFP and pcDNA3.1⁺ co-transfected (control) cell body and growth cone morphology are shown in (F).
Figure 2.1: Localization and morphological characteristics of transfected SALM proteins

(Wang et al., 2008)
Figure 2.2: SALMs are expressed on the surface of neurons. DIV4 primary hippocampal neurons were co-transfected with GFP and myc-SALM1, myc-SALM2, myc-SALM4, or HA-SALM5. Surface labeling with myc or HA antibodies was performed 48 hours after transfection. (A) Representative examples of myc-SALM2 surface labeling are shown. Scale bar, 20 μm. (B) SALM surface expression is punctate (arrows/inset), and accumulated at growth cones. Scale bar, 10 μm.
immunostaining localized at these neurites (data not shown). Many SALM5-transfected neurons showed another unique phenotype. The primary neurites often overlapped and appeared to adhere to one another at regions proximal to the cell body. SALM5 accumulated at these points of neurite adhesion (Figure 2.1E, arrows). These various changes in cell morphology of SALM-transfected neurons imply that individual SALMs may have different functions in the CNS.

2.3.2 SALMs promote neurite outgrowth

To further extend our initial qualitative observations indicating that SALMs may affect neurite outgrowth, we performed a detailed computer-assisted analysis of neuronal morphology using Metamorph Neurite Outgrowth Module (v7.0r3). The parameters tested included total neurite outgrowth (defined as the total skeletonized pixel area in μm), mean process length, number of primary neurites extending from the cell body, and number of total neurite branches. Primary hippocampal cultures were transfected at DIV4, a time when significant neurite outgrowth takes place (Dotti et al., 1988). Cultures were co-transfected with GFP (to visualize the entirety of each cell) and myc-SALM1, myc-SALM2, SALM3, myc-SALM4, or HA-SALM5 cDNA. Neurons were fixed and immunostained for GFP and SALM proteins 48 hours after transfection (DIV6); GFP staining was used to quantify neurite outgrowth and SALM staining served to verify SALM expression. Our results showed that SALMs promote neurite outgrowth with various phenotypes. SALMs 1-5 all promoted significant increases in total outgrowth and number of branches, as compared to the control (Figure 2.3B and D, respectively). Additionally, SALM4
Figure 2.3: SALMs promote neurite outgrowth. DIV4 primary hippocampal neurons were co-transfected with GFP and myc-SALM1, myc-SALM2, SALM3, myc-SALM4, HA-SALM5, or pcDNA 3.1+ vector (control). Immunostaining was performed 48 hours later, and neurite outgrowth was analyzed using Metamorph Neurite Outgrowth software (v7.0r3). Analysis was based on the transfected GFP signal. (A) Representative examples of transfected neurons. (B) Quantifications of neurite outgrowth: all five SALMs promote increases in total outgrowth, as compared to control, but do not promote increases in mean process length (C). The mean process length of myc-SALM4-transfected neurons is less than that of neurons transfected with myc-SALM1, SALM3, and HA-SALM5. (D) All five SALMs promote increases in the number of branches, as compared to control. Additionally, myc-SALM4 promotes increases number of branches as compared to the other SALMs. (E) Myc-SALM2 promotes increases in the number of processes, as compared to control, myc-SALM1, and HA-SALM5. Myc-SALM4 promotes increases in processes as compared to myc-SALM1, myc-SALM2, SALM3, HA-SALM5 (dotted lines) and control. (n=15-17, values shown are mean ± SEM, and analyzed by one-way ANOVA, * represents significance with respect to control, # represents significance with respect to other conditions, p<0.05. Scale bar, 100 μm.)
Figure 2.3: SALMs promote neurite outgrowth

(Wang et al., 2008)
promoted more branching than other SALMs (Figure 2.3D). SALM2 promoted an increase in the number of primary processes, as compared to the control, SALM1, and SALM5 (Figure 2.3E). Consistent with the dramatic increase in short primary neurites described earlier, the number of processes in SALM4-transfected cells more than doubled, as compared to control and other SALM-transfected neurons, including those transfected with SALM2 (Figure 2.3E). The mean process length of SALM4-transfected neurons was significantly less than that of SALM1, SALM3, and SALM5-transfected neurons (Figure 2.3C). Together, this increase in short primary neurites in SALM4 is a visually distinctive phenotype as compared to the other SALMs. Thus, SALMs promote neurite outgrowth with various distinct phenotypes, and the major outgrowth parameters that SALMs 1-5 modify are total outgrowth and neurite branching.

2.3.3 SALMs promote axonal and dendritic outgrowth

The increase in the number of MAP2-positive processes extending from the cell body, particularly evident with SALM4, indicates a change in dendrite growth. In order to determine if the SALM-mediated increases in number of processes were axonal as well, we co-transfected DIV4 neurons with SALM2 and GFP, immunostained for MAP2, and imaged the cells as described earlier. Since more than 90% of all GFP-transfected cells also expressed SALM2 (data not shown), we used GFP expression as a positive indicator of SALM2 transfection in these experiments. Along with MAP2 staining, the transfected cells were analyzed through the morphological criteria described in the methods to distinguish axons from dendrites.
(Figure 2.4A and B, top panels). Once axons and dendrites were identified, the dendrites were digitally removed from the cell and the resulting axon-only image was analyzed for neurite outgrowth (Figure 2.4A and B, bottom panels). Our results showed a 75% increase in total axonal outgrowth (3543 ± 371.42 μm for SALM2, 2029 ± 419.03 for control) (Figure 2.4C), 108% increase in axon number (2.43 ± 0.37 axons for SALM2, 1.17 ± 0.17 for control) (Figure 2.4F), and an 82% increase of axonal branches for SALM2 (119 ± 17.57 branches for SALM2, 65 ± 18.37 for control) (Figure 2.4E), as compared to control axons, showing that axon growth is also affected by SALM expression. Analysis of the non-axonal processes also showed increases in outgrowth with SALM2 expression. These results showed that the SALMs affect both axon and dendrite outgrowth, although the effects may vary with individual SALMs.

2.3.4 Application of antibodies directed to the extracellular LRR region of SALMs inhibits neurite outgrowth

While over-expression experiments suggest a role for SALMs in neurite outgrowth, they do not address a role for endogenous SALMs. Previous studies have demonstrated the efficacy of applying antibodies directed to extracellular domains of endogenous transmembrane proteins, including cell adhesion molecules like cadherin, L1-CAM, and NCAM, to block their activity (Lindner et al., 1983; Muller et al., 1996; Tang et al., 1998; Garcia-Castro et al., 2000). The extracellular domains of SALMs contain several functional regions of potential protein-protein interaction, including six highly conserved LRR regions, a FN3 domain, and an IgC2-like
**Figure 2.4: SALM2 promotes axonal outgrowth.** DIV4 primary hippocampal neurons were co-transfected with GFP and myc-SALM2 or pcDNA 3.1+ empty vector (control). Representative examples of control and myc-SALM2 transfected cells used for analysis are shown in (A) and (B), respectively. Axons and dendrites were identified by morphological criteria and the dendritic marker MAP2 (A and B, left). Dendrites were digitally separated from the cell body, and the resulting axon-only images were analyzed for neurite outgrowth (A and B, right). Scale bars for composite images and insets 100 μm and 50 μm, respectively. Myc-SALM2 promotes an increase in total axonal outgrowth (C), number of axonal branches (E), and number of axons (F), as compared to controls. (D) Myc-SALM2 does not promote increases in mean axon length. (n=7, values shown are mean ± SEM, and analyzed by unpaired students t-test, *p<0.05)
domain. Recently, Seabold et al. (2008) showed that application of polyclonal antibodies that bind to the LRR of SALMs (anti-LRR) inhibited SALM4 trans interactions in transfected heterologous cells. Directed to amino acids 38-297 of SALM2 (a region of high similarity among the SALMs), anti-LRR has been shown to interact with the extracellular domains of all SALMs expressed in heterologous cells (Seabold et al., 2008). To test the ability of anti-LRR application to inhibit SALM-mediated outgrowth, DIV4 primary hippocampal neurons were transfected with GFP and treated by bath application with anti-LRR. To control for variability due to the antibody application technique, parallel cultures were treated with antibodies directed to the intracellular C-terminus of SALM4 (anti-S4CT). Neurons were fixed and analyzed for neurite outgrowth 48 hours after antibody application. Representative examples of treated neurons are shown in Figure 2.5A. Anti-LRR-treated neurons showed a significant reduction in total outgrowth, mean process length, and neurite branches (Figure 2.5B, C, and D). There was no significant change in the number of processes (Figure 2.5E). While these results are in accordance with increases in neurite outgrowth and branches promoted by the over-expression of SALMs, we cannot rule out the possibility that anti-LRR antibodies interact with a variety of other endogenous proteins present on the surface of the neurons which have LRR domains, and that these interactions contribute to the changes in neurite outgrowth.

2.3.5 RNAi knock-down of SALM expression reduces neurite outgrowth

To further examine the function of SALMs in mediating neurite outgrowth, individual RNAi plasmid constructs were generated for SALMs 1-5 using pcDNA™6.2-
Figure 2.5: Application of antibodies directed to the extracellular LRR region of SALMs inhibits neurite outgrowth. DIV4 primary hippocampal neurons were transfected with GFP and treated by bath application with antibodies directed to the LRR region of SALM2 (anti-LRR) or to the C-terminus of SALM4 (anti-S4CT), as control. Immunostaining was performed 48 hours later, and neurite outgrowth was analyzed using Metamorph. (A) Representative examples of transfected/treated neurons. Neurons treated with anti-LRR showed a significant decrease in total outgrowth (B), mean process length (C) and number of branches (D), as compared to the control condition. Anti-LRR treatment did not have an effect on the number of processes (E). (n=15, values shown are mean ± SEM, and analyzed by unpaired students t-test, *p<0.05. Scale bar, 100 μm.)
GW/EmGFP-miR micro RNAi expression vectors, which co-express GFP. To test the efficacy of these RNAi constructs, each one was co-transfected with its respective SALM cDNA into neurons at DIV2. Over-expressed SALM levels were analyzed at DIV6 by fluorescence immunocytochemistry of transfected neurons and compared to those transfected with control RNAi. Co-transfection of each RNAi construct with the respective SALM cDNA resulted in significant reduction of SALM over-expression (Figure 2.6), with decreases in SALM over-expression of 28%, 75%, 60%, 61%, and 43% for SALMs 1-5, respectively. We initially transfected neurons with individual SALM RNAi constructs and measured neurite outgrowth. While there was a trend indicating a decrease in outgrowth and process length with the individual RNAi constructs (data not shown), the changes were not statistically significant. Since all SALMs promote neurite outgrowth and multiple SALMs are likely co-expressed in neurons (Morimura et al., 2006), loss of one SALM protein may have only a slight effect on outgrowth. Therefore, we examined the effects of knocking down all five SALMs on neurite outgrowth. DIV2 neurons were quintuple transfected with SALMs 1-5 RNAi constructs, and analyzed DIV6. GFP-positive RNAi-transfected cells showed a decrease in total outgrowth and mean process length (Figure 2.7B and C, respectively), with no change in the number of branches or processes (Figure 2.7D and E, respectively). These results indicate that cumulative knockdown of endogenous SALMs 1-5 expression effectively inhibits neurite outgrowth.
Figure 2.6: RNAi constructs reduce SALM over-expression. Individual RNAi plasmid constructs were designed and generated for SALMs 1-5 using pcDNA™6.2-GW/EmGFP-miR micro RNAi expression vectors, which co-expresses GFP. DIV4 primary hippocampal neurons were co-transfected individually with myc-SALM1, myc-SALM2, SALM3, myc-SALM4, or HA-SALM5 cDNA and their respective SALM RNAi plasmid (e.g. myc-SALM1 cDNA and SALM1 RNAi), or scramble RNAi negative control (e.g. myc-SALM1 cDNA and scramble RNAi). Expression levels of transfected myc-SALM1 with scramble RNAi or SALM1 RNAi are shown in rows A₁ and A₂, respectively. Expression levels of SALM2-SALM5 with scramble RNAi (GFP) or SALM RNAi (GFP) are shown in rows B-E, respectively. Scale bar, 20 μm. For the characterization of each SALM RNAi construct, images were taken and processed with identical exposure times and contrast levels. Expression of transfected SALMs was reduced by 28%, 75%, 60%, 61%, and 43% for SALMs 1-5, respectively (n=5).
Figure 2.6: RNAi constructs reduce SALM over-expression

(Wang et al., 2008)
Figure 2.7: RNAi knock-down of SALM expression reduces neurite outgrowth. Individual RNAi plasmid constructs were designed and generated for SALMs 1-5 (Invitrogen custom services). To knockdown the expression of endogenous SALM proteins, DIV2 neurons were quintuple transfected with SALMs 1-5 RNAi constructs (SALM12345 RNAi), and analyzed four days later (DIV6). (A) Representative examples of transfected cells. GFP-positive RNAi-transfected cells showed a significant decrease in total outgrowth and mean process length (B and C, respectively). The number of branches and processes were not significantly different, compared to control (D and E, respectively). (*n=10, values shown are mean ± SEM, and analyzed by unpaired students t-test, *p<0.05. Scale bar, 100 μm.)
2.3.6 Neurite outgrowth is mediated by the PDZ binding domains of SALMs

PDZ interactions between CAMs and PDZ domain-containing proteins are critical for a variety of mechanisms, including synapse formation and regulation, as well as neurite outgrowth (for review, see Dalva et al., 2007). For example, neurexin and neuroligin complexes regulate synapse formation through direct interactions with the PDZ proteins PSD-95 and CASK (Irie et al., 1997) and have been implicated in mediating neurite outgrowth (Grifman et al., 1998). SALMs 4 and 5 differ from SALMs 1-3 in that they do not have a PDZ-BD, and they have visually distinctive phenotypes that may be related to the lack of PDZ interactions (SALM4 promotes a dramatic increase in the number of short primary neurites, and SALM5 often induces primary neurites to overlap and apparently adhere to each other). To examine the roles of PDZ interactions in Salm-mediated neurite outgrowth, we generated mutant cDNA constructs of SALMs 1, 2, and 3 in which the distal C-terminal 4 (for SALMs 1 and 3) or 7 (for SALM2) amino acids corresponding to the PDZ-BD were deleted (SALM1ΔPDZ, SALM2ΔPDZ, and SALM3ΔPDZ, respectively). The SALMΔPDZ constructs were individually co-transfected with GFP into primary neurons at DIV4 and analyzed for neurite outgrowth. Deleting the PDZ-BD greatly attenuated the neurite outgrowth facilitated by the exogenous full-length SALMs 1-3. As seen in Figure 2.8B-E, the amounts of total outgrowth, mean process length, neurite branches, and number of processes were all similar to that of control. These results indicate that the PDZ-BD functions in neurite branching, which was the primary...
Figure 2.8: Neurite outgrowth is mediated by the PDZ domains of SALMs. Primary hippocampal neurons (DIV4) were co-transfected with GFP and myc-SALM1ΔPDZ, myc-SALM2ΔPDZ, SALM3ΔPDZ, or pcDNA 3.1+ vector (control). Immunostaining was performed 48 hours later, and neurite outgrowth was analyzed using Metamorph. (A) Representative examples of transfected neurons. Transfection of myc-SALM1ΔPDZ, myc-SALM2ΔPDZ, or SALM3ΔPDZ do not promote increases in total outgrowth (B), mean process length (C), number of branches (D), or the number of processes (E). \( n=10 \), values shown are mean ± SEM, and analyzed by one-way ANOVA, *\( p<0.05 \). Scale bar, 100 μm.)
Figure 2.8: Neurite outgrowth is mediated by the PDZ domains of SALMs

(Wang et al., 2008)
The individual SALMs display distinct phenotypes in promoting neurite outgrowth. The most visually apparent and unique phenotype is that of SALM4, which promotes dramatic increases in the number of short, primary processes extending from the cell body. By examining and replacing the domains of SALM4, we can investigate which domains are involved in regulating various facets of neurite outgrowth. The N-termini of the SALMs contain various protein-protein interaction domains that are highly homologous among the family members, while the C-termini show considerable sequence variability among the family members. Additionally, SALMs 1-3 have C-terminal PDZ-BDs that contribute to neurite outgrowth, while SALMs 4-5 do not. Therefore, it is conceivable that either the N- or C-terminal regions, or both, may contribute various functions in the mechanism of SALM-mediated neurite outgrowth, and that these functions may be unique to regions of the individual SALMs. To examine the domains involved in neurite outgrowth, Dr. Kai Chang generated chimera constructs with the N-termini and C-termini of SALM2 and SALM4. Myc-SALM2/4 contains the N-terminus of SALM2 and the C-terminus of SALM4, while HA-SALM4/2 contains the N-terminus of SALM4 and the C-terminus of SALM2.
When we transfected them into DIV4 cultures, both chimera constructs promoted a significant increase in total outgrowth (Figure 2.9B), which is consistent with both SALM2 and SALM4 enhancing outgrowth (Figure 2.3). However, the most apparent phenotype was that of HA-SALM4/2-transfected cells, as they additionally promoted an increase in total outgrowth compared to myc-SALM2/4 (Figure 2.9B). HA-SALM4/2 also promoted an increase in process number, as compared to both control and myc-SALM2/4, similar to the phenotype seen with transfected SALM4 (Figure 2.3). HA-SALM4/2 promoted a statistically significant increase in branching, as compared to control and myc-SALM2/4 (Figure 2.9D). Increases in mean process length were not statistically significant (Figure 2.9C), as was also seen for both SALM2 and SALM4 (Figure 2.3).

These results suggest that both the N and C termini play a role in neurite outgrowth. The most prominent result was that SALM4/2 chimera showed a significant increase in the number of processes, similar to that of SALM4, suggesting that the N-terminus plays a role in outgrowth. Total outgrowth was increased for both chimeras, which is similar to results seen with the over-expression of SALM2 and SALM4, as both increase outgrowth. However, SALM2ΔPDZ over-expression did not increase outgrowth (Figure 2.8), and the SALM2/4 chimera also lacks the PDZ-BD found in SALM2. This result may suggest that the SALM4 C-terminus contains another domain with a function similar to that of the PDZ-BD. On the other hand, while both SALM2 and SALM4 increased branching, only the SALM4/2 chimera increased branching. This supports our finding that the PDZ-BD of SALM2 is required for
Figure 2.9: Neurite outgrowth phenotype is determined by both the N- and C-termini of SALMs. We generated SALM2/SALM4 chimera constructs in which the N-termini and C-termini of SALM2 and SALM4 were switched. Myc-SALM2/4 contains the N-terminus of SALM2 and the C-terminus of SALM4, while HA-SALM4/2 contains the N-terminus of SALM4 and the C-terminus of SALM2. Chimera constructs were individually co-transfected with GFP at DIV4 and analyzed 48 hours later. (A) Representative examples of pcDNA3.1+ (control) and chimera-transfected cells. Both myc-SALM2/4 and HA-SALM4/2 exhibited increases in total outgrowth (B), but not mean process length (C), as compared to control. HA-SALM4/2 promoted increases in number of branches (D) and process number (E), as compared to both control and myc-SALM2/4. The HA-SALM4/2-mediated increase in process number is similar in phenotype to the increase seen with SALM4, indicating that the N-terminus of SALM4 confers this property. (n=10, values shown are mean ± SEM, and analyzed by one-way ANOVA, * represents significance with respect to control, # represents significance with respect to other conditions, p<0.05. Scale bar, 100 μm)
Figure 2.9: Neurite outgrowth phenotype is determined by both the N-and C-termini of SALMs

(Wang et al., 2008)
branching. Therefore, the mechanism of SALM-mediated outgrowth may involve an intricate interplay between multiple functional regions on each individual molecule, including the N-terminal domains, the C-terminus (including the PDZ-binding domains for SALMs 1-3), and conserved regions common to the various SALMs.
2.4 Discussion

2.4.1 Overview

In this chapter, we show that all five SALMs promote neurite outgrowth when over-expressed in young primary hippocampal cultures, with various phenotypes. Application of antibodies directed to the extracellular domain, or knockdown of endogenous SALMs using RNAi in hippocampal cultures, inhibits neurite outgrowth. The nature of the phenotype from over-expression is determined largely by the extracellular domain, although the PDZ-BD is required for most aspects of neurite outgrowth of SALMs 1-3. SALMs 4 and 5, which lack the PDZ-BD, also effectively promote neurite outgrowth, suggesting that their intracellular C-termini either function somewhat differently from SALMs 1-3 or that they have alternative interacting mechanisms to substitute for the PDZ interaction. In Table 1, we list a summary of the SALM-mediated neurite outgrowth characteristics known thus far.

The extracellular domain structure of the SALMs is homologous with that of a variety of related proteins that regulate neurite outgrowth, including AMIGO, NGL, LINGO, FLRT, NLRR, and PAL protein families (Chen et al., 2006). Similar to the SALMs, these proteins contain extracellular LRRs and variations in the presence and number of Ig-like and FN3 domains. Like SALMs, the AMIGO family members contain six LRRs and promote neurite outgrowth in hippocampal neurons via their extracellular region (Kuja-Panula et al., 2003). In contrast, LINGO proteins contain twelve LRRs and negatively regulate neurite outgrowth and regeneration in brain and spinal cord by enhancing myelin-mediated inhibition and participating in the Nogo/OMgp/MAG-NgR signaling pathway (Mi et al., 2004). Therefore, the SALMs join a list of
Table 1: Summary of SALM-mediated neurite outgrowth characteristics. (A-E) The individual SALMs promote neurite outgrowth and various differentiation when transfected into young hippocampal cultures, as described in Wang et al. (2006), and Wang et al. (2008). (F) When axons of SALM2-transfected neurons were digitally isolated and analyzed for neurite outgrowth characteristics, the resulting “axon-only” images show increases in total axonal outgrowth, axonal branches, and number of axons. (G, H) Application of function-blocking antibodies generated to the extracellular LRR region of SALMs, or knocking down SALM expression by RNA interference (quintuple transfection of SALMs 1-5 RNAi constructs) inhibits neurite outgrowth. (I-K) Transfection of SALM 1-3 constructs lacking the PDZ-BD has no significant effect on neurite outgrowth, indicating a role of PDZ interactions in SALM-mediated outgrowth. (L, M) Transfection of chimera constructs in which the N- and C-termini of SALM2 and SALM4 were switched indicates roles for both the N- and C-termini in SALM mediated outgrowth. (M) Transfection of SALM4/2 (containing the N-terminus of SALM4 and the C-terminus of SALM2) resembles the SALM4 results (D), indicating that the SALM4 N-terminus contributes to the outgrowth effects. (L) While the SALM2/4 chimera promotes a statistically significant increase in total outgrowth, the magnitude of the increase is less than that of either full-length SALM2 (B) or SALM4 (D). In addition, there is no increase in neurite branching. This construct (containing the N-terminus of SALM2 and C-terminus of SALM4, which lacks a PDZ-BD), resembles SALM2ΔPDZ in both structure and outgrowth effects (J), further indicating the role of the C-terminus in SALM-mediated neurite outgrowth.
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<th>Total outgrowth</th>
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<td>F) SALM2 axons</td>
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<td>J) SALM2ΔPDZ</td>
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(Wang and Wenthold, 2009)

Table 1: Summary of SALM-mediated neurite outgrowth characteristics
proteins with a general domain structure (X number of LRR ± IgC2 ± FN3) that regulate neurite outgrowth. However, the nature of the outgrowth is variable, likely reflecting specific combinations of the protein-interaction domains, interactions with extracellular binding partners, and intracellular signaling mechanisms.

2.4.2 The extracellular N-termini of SALMs are involved in neurite outgrowth

Several factors could play a role in the differential effects of SALM over-expression on neurite outgrowth. For example, a different distribution of the SALMs within the neuron may lead to selective outgrowth of axons or dendrites. To address this question, we expressed epitope-tagged constructs, and found that all five SALMs are distributed throughout the neuron, including in the growth cone. These results indicate that distribution alone is unlikely to be responsible for the different effects of the individual SALMs. Alternatively, the differential outgrowth effects of the SALMs could be due to the different properties of interactions with other proteins through their extracellular domains. Our results with the SALM2 and SALM4 chimeras show that the N-terminal regions of SALMs play a major role in regulating neurite outgrowth. This is not unexpected since the extracellular regions of related proteins, such as AMIGO (Kuja-Panula et al., 2003), have been shown to mediate neurite outgrowth. While the N-termini are highly conserved among the SALMs, there are regions of high variability. Additionally, sequence analysis reveals that there is a potential N-linked glycosylation site in the first LRR region of SALMs 1-3, but not SALMs 4 and 5 (data not shown). Glycosylation is a highly regulated post-
translational modification which influences a wide variety of functional consequences including cell adhesion, signal transduction, and the trafficking of proteins to the cell surface (for review, see Scheiffele and Fullekrug, 2000).

Our findings showing that the N-termini play a role in neurite outgrowth suggest that the N-termini of individual SALMs may interact with different binding partners or with the same binding partners, but with different affinities. The mechanisms by which the SALMs may interact or signal through extracellular domains has not been extensively studied. However, Seabold et al. (2008) recently reported that certain SALMs form trans associations in heterologous cells. SALMs 4 and 5 form homomeric, but not heteromeric, trans associations. Therefore, these interactions may play a role in determining SALM 4 and 5 neurite outgrowth phenotypes. Multiple mechanisms remain to be investigated for the SALMs. In addition to direct interactions through extracellular domains of molecules on the cell surface, secreted and cleaved fragments of adhesion molecules can function in neurite outgrowth. For example, NCAM is proteolytically cleaved by the metalloprotease TACE, and the inhibition of this cleavage inhibits NCAM-mediated neurite outgrowth (Kalus et al., 2006). A similar mechanism may be in play for the SALMs.

2.4.3 SALM-mediated neurite outgrowth involves PDZ domain interactions

A distinguishing feature of SALMs 1-3 is the presence of a PDZ-BD at the C-terminus. PDZ interactions mediate a variety of essential processes in the CNS,
including the clustering and trafficking of ion channels and facilitation of signaling at
the synapse (El-Husseini et al., 2000; Kim and Sheng, 2004). PDZ proteins are also
involved in neurite outgrowth (Hoogenraad et al., 2005; Charych et al., 2006).

GRIP1, an AMPA receptor-associated multi-PDZ domain protein, mediates dendritic
formation and outgrowth by regulating EphB receptor trafficking (Hoogenraad et al.,
2005). Firestein and colleagues have shown that PSD-95 regulates dendritic
branching in an activity-independent manner (Charych et al., 2006). Overexpression
of PSD-95 decreases dendritic branching in immature neurons, while knocking down
PSD-95 increases it (Charych et al., 2006). Previous studies (Ko et al., 2006; Wang
et al., 2006) showed that SALMs 1 and 2 interact with PSD-95 and other MAGUKs,
and that this interaction is required to recruit PSD-95 to presumed synaptic locations.
Therefore, it is possible that interactions between SALMs and MAGUKs may be
important for the proper regulation of early neuronal development. Our results show
that the PDZ-BDs (in SALMs 1-3) and the N-termini of the SALMs are involved in
neurite outgrowth. Other adhesion molecules mediate outgrowth-related mechanisms
through PDZ interactions. NrCAM (an L1-CAM) binds to MAGUKs and this
association regulates trafficking to the membrane (Davey et al., 2005; Dirks et al.,
2006). Neuroligin-1 contains LRR and Ig-like regions and also has been implicated
in regulating neurite outgrowth (Grifman et al., 1998). The synaptic localization of
neuroligin-1 is mediated by PDZ interactions (Irie et al., 1997), and neuroligin-1
regulates the balance of excitatory and inhibitory synaptic contacts through
interactions with the third PDZ domain of PSD-95 (Song et al., 1999). Previous
studies (Ko et al., 2006; Wang et al., 2006) showed that SALMs 1 and 2 interact with
members of the MAGUKs and that this interaction is required to recruit PSD-95 to presumed synaptic locations.

The PDZ-BD has significantly different roles in trafficking of the individual SALMs; in heterologous cells and mature neurons, SALM1ΔPDZ is excluded from the cell surface (Wang et al., 2006). SALM2ΔPDZ, however, is expressed on the cell surface throughout the neuron (data not shown). The requirement of the PDZ-BD for normal function of SALMs 1-3 suggests that either the PDZ interaction is required for the clustering of the SALM molecule, or that the PDZ protein is required to organize the SALM C-termini with other molecules associated with signaling. The slight, but not statistically significant, increase in total outgrowth of SALMs 1-3-ΔPDZ, would be consistent with the interpretation that the PDZ interaction is required for efficient coupling to intracellular signaling cascades; without it, only a minor effect remained, likely due to random associations. Therefore, SALMs may mediate neurite outgrowth through PDZ-mediated second messenger signaling cascades, which are disrupted in these mutant constructs. Among these, MAPK cascades and FGFR cascades at growth cones have been characterized in mediating neurite outgrowth in response to stimuli from CAMs such as L1, N-cadherin, and NCAM (Doherty et al., 2000). SALMs 4 and 5 do not contain PDZ-BDs, but over-expression still effectively promotes neurite outgrowth. A likely explanation is that they contain another motif(s) in their C-termini that substitute for the PDZ-BD, but these remain to be identified.
2.4.4 Dual functions for SALMs

The results of this chapter, which indicate that all SALMs can enhance neurite outgrowth, agrees with previous studies showing that SALM1 helps recruit proteins to the synapse (Wang et al., 2006) and that SALM2 plays a role in determining the number of excitatory synapses (Ko et al., 2006). Together, these studies suggest that SALMs participate in at least two functions, neurite outgrowth and synapse formation. Although not yet demonstrated functionally, their presence in mature synapses suggests a role in synapse maintenance in adults. This dual role is not without precedent among adhesion molecules, as the cadherins are critical to the development of the CNS and have roles in neurite outgrowth and synapse formation (for review, see Redies, 2000). N-cadherin mediates calcium-dependent cell adhesion and has been demonstrated to promote neurite outgrowth through various methods including over-expression studies (Matsunaga et al., 1988), function-blocking antibodies (Bixby et al., 1987), and as substrates for neuronal culture (Bixby and Zhang, 1990). N-cadherins also localize to growth cones, and mediate growth cone migration (Letourneau et al., 1990). In mature neurons, N-cadherins are components of NMDA receptor complexes (Husi et al., 2000), and function in synaptic plasticity and long term potentiation (LTP) (Tang et al., 1998), and in the formation and maintenance of synapses (Fannon and Colman, 1996). L1-CAMs also are involved in various mechanisms during development including neurite outgrowth, growth cone motility, and axonal fasciculation (Kamiguchi and Lemmon, 1997, 1998; Hortsch, 2000). L1-CAMs mediate neurite outgrowth in developing neurons through direct interactions with ankrynB (Nishimura et al., 2003), and via MAP kinase signaling...
(Whittard et al., 2006). L1-CAMs are also important in LTP, synapse alignment, and synapse formation (Godenschwege et al., 2006; Triana-Baltzer et al., 2006). This suggests that some of the processes involved in synapse formation and neurite outgrowth are shared, but raises questions about how these two processes are differentially regulated. For example, what determines the proportions of SALMs dedicated to synapse formation compared to neurite outgrowth, or what developmental change signals the switch in functions. We initially hypothesized that the PDZ interaction would not be relevant to neurite outgrowth, but our results showed that the PDZ-BD is required for neurite outgrowth. Therefore, the PDZ-BD cannot be used to differentiate between the two processes, unless different PDZ interactions regulate outgrowth earlier in development, while others regulate synapse formation.
Chapter 3: Adhesive properties and trafficking of SALMs

3.1 Introduction

Trafficking of synaptic proteins and the formation of cell-cell adhesions are critical components in the initial stages of synapse formation. The adhesive interactions mediated by CAMs are critical factors in several steps of this process. For example, cadherins are critical to proper synapse formation. Loss of cadherin junctions leads to delays in synapse formation, and those that are formed are reduced in size (Bozdagi et al., 2004). Neuroligins bind presynaptic neurexins to form functional synaptic terminals, and induce synaptic differentiation at both excitatory and inhibitory contacts (for review, see Dean and Dresbach, 2006; Lise and El-Husseini, 2006; Craig and Kang, 2007). Presynaptic α–neurexins also participate in the clustering of calcium channels (Missler et al., 2003; Zhang et al., 2005). SynCAM/nectins forms homomeric and heteromeric trans interactions to form functional synaptic terminals (Biederer et al., 2002; Sara et al., 2005; Fogel et al., 2007). NCAM promotes synapse stability through the binding of heparin sulfate proteoglycans (Dityatev et al., 2004).

CAMs are usually transmembrane proteins, and participate in the formation of cellular junctions through interactions between their extracellular protein domains. These CAM interactions can be cis, within the same membrane, or trans, across the cell-cell junctions of adjacent cells. These cis or trans interactions can be heteromeric (forming interactions with other distinct proteins), or homomeric
(forming dimers or higher-order multimers with a single protein type). For example, nectins form homomeric cis interactions (Takai and Nakanishi, 2003), while L1-type CAMs and axonin-1 form heteromeric cis interactions (Buchstaller et al., 1996; Stoeckli et al., 1996). Cadherins form homomeric trans interactions (Tepass et al., 2000), while neuroligin and neurexin form heteromeric trans interactions (Ichtchenko et al., 1995; Scheiffele et al., 2000). In Seabold et al. (2008), I participated in a study where we demonstrated that SALMs are able to form heteromeric and homomeric interactions with each other, though with some distinctions among the individual SALM family members.

Thus far, there are a few known roles of SALMs at synapses, but our understanding is far from complete. Transfected SALM2 increases the number of excitatory synapses and dendritic spines, while mislocalization of SALM2 decreases them (Ko et al., 2005). SALM1 has been shown to interact directly with PSD-95, via PDZ interactions, and influence the recruitment of PSD-95 and NR2A to dendritic spines (Wang et al., 2006). Overexpression of a SALM1 construct lacking the C-terminus (truncated after the TM region) leads to changes in spine morphology, and an increase in dendritic filopodia (Wang et al., 2006). SALMs are present in synaptic membranes and PSD subcellular fractions (Ko et al., 2006; Wang et al., 2006). Immunogold EM reveals that native SALM4 is present at both pre- and postsynaptic membranes of processes in the brain (Seabold et al., 2008). Additionally, the data indicate that SALM4 may be present at both excitatory and inhibitory synapses (Seabold et al., 2008). In contrast, SALM2 is reported to distribute exclusively to excitatory
synapses (Ko et al., 2006). In this chapter, we investigated the roles of SALMs in synaptic development. First, we directly examined the cell-adhesive properties of SALMs in neurons via co-culture experiments with transfected HeLa cells. Next, we investigated the localization of SALMs at interneurite junctions and branching points of axons and dendrites. Finally, we performed a series of preliminary experiments to investigate the roles of PDZ interactions in the trafficking of SALMs and their roles in synapse morphology.
3.2 Methods

Antibodies

Anti-myc monoclonal (hybridoma purchased from ATCC, Manassas, VA, clone #9E10) and anti-hemagglutinin (HA) monoclonal (Covance, Denver, PA, clone #16B12) primary antibodies were used at a 1:1000 dilution. Anti-GFP polyclonal antibodies (Chemicon, Billerica, MA) were used at 1:2000. SALM1 antisera were used for immunostaining of transfected SALM1 and SALM1ΔPDZ. Antisera were produced in rabbits (Covance) as described in Seabold et al. (2008). SALM1 polyclonal antibodies were generated using a peptide directed to the N-terminus of SALM1, amino acids 384–397: [NH2]-NSTSRMAPPKSRLS-[COOH] (designed by Dr. Wenthold).

cDNA constructs

Cloning of the SALM cDNA constructs was previously described in Chapter 2. GFP-SAP102 cDNA was generated by Dr. Nathalie Sans, as previously described (Sans et al., 2005). Briefly, SAP102 cDNA was subcloned into the pGFP-C3 mammalian expression vector (Clontech, Mountain View, CA).

Primary hippocampal cultures and transfections

Primary hippocampal cultures were prepared by Dr. Ya Xian Wang, as described in Chapter 2. Neurons were plated at a density of 50,000 cells per ml of culture medium (50K). Transfections were performed using the calcium phosphate method, as described in Chapter 2.
**Immunocytochemistry and co-culture experiments:**

Surface and total/permeabilized immunocytochemistry was performed as described in Chapter 2. For co-culture experiments, DIV7 hippocampal neurons were transfected with SALM cDNA at DIV7. Twenty-four hours after transfection, HeLa cells transfected with the same SALM family member were plated on top of the neurons. Cells were incubated overnight at 37 °C and then processed for total staining. Images were acquired using the 60x or 40x oil objective of an E-1000 Nikon microscope or 63x objective of an LSM 510 confocal microscope.
3.3 Results

3.3.1 SALM4 forms homomeric trans complexes in neurons

In Seabold et al. (2008), Dr. Gail Seabold showed that SALMs 1-3 co-immunoprecipitate with each other using brain tissue, indicating the formation of heteromeric interactions, while SALMs 4 and 5 do not. When individually expressed and co-plated in heterologous cells, only SALMs 4 and 5 are able to form homomeric trans interactions. In my contribution to Seabold et al. (2008), we directly investigated the ability of SALMs to form homomeric trans cell-cell adhesions in neurons though the use of neuron-HeLa cell co-cultures. HeLa cells and hippocampal neurons (DIV7) were individually transfected with SALM4 (one cell type with non-tagged SALM4 cDNA, and the other with myc-tagged SALM4 cDNA). The HeLa cells were subsequently trypsinized, collected, and plated onto the neurons 24 hours later (at DIV8), and allowed to incubate overnight. Immunocytochemistry was performed on the following morning (DIV9). Enrichment of SALM4 immunoreactivity in neurons was clearly present at points of contact between transfected HeLa cells and neuronal processes (Figure 3.1A-C). Based on morphological characteristics, as described and established in Chapter 2, these processes appear to include both dendrites and axons. The accumulation of staining appeared to occur when SALM4-transfected neuronal processes (Figure 3.1A-C) associated with SALM4-transfected HeLa cells. Neurons and HeLa cells were also transfected with SALM1 or SALM2 and co-cultured. No indication of an accumulation of either SALM1 or SALM2 was seen at points of contact between
**Figure 3.1: Recruitment of SALM4 in SALM-transfected heterologous cells and hippocampal neurons.** DIV7 hippocampal neurons were transfected with SALM4 cDNA. Twenty-four hours later, HeLa cells were transfected with SALM4 cDNA, plated (co-cultured) onto the neurons, and allowed to incubate overnight. At DIV9, the co-cultured cells were then fixed and permeabilized for total staining. SALM4 expressing cells were transfected with myc-tagged or non-tagged SALM4 and stained with anti-myc (red) or anti-SALM4 (green). (A) Near the soma of neurons, SALM4-transfected in HeLa cells induces an accumulation of SALM4 in dendrites of myc-SALM4-transfected neurons. (B) SALM4 is enriched at the point of contact between axons of SALM4-transfected neurons and myc-SALM4-transfected HeLa cells (arrows). (C) A similar enrichment occurs between the point of contact between myc-SALM4-transfected neurons and SALM4-transfected HeLa cells (arrows). (D–E) Clustering is not present in co-cultured cells transfected with SALM1 (D) or SALM2 (E).
Figure 3.1: Recruitment of SALM4 in SALM-transfected heterologous cells and hippocampal neurons

(Seabold et al., 2008)
neurons and HeLa cells (Figure 3.1D and E), consistent with Dr. Seabold’s findings in heterologous cells that these SALMs did not form trans-cellular associations. Interestingly, we did not see an accumulation of SALM4 at points of contact between transfected neurons and untransfected HeLa cells, or between untransfected neurons and transfected HeLa cells (data not shown). While endogenous SALM4 is present in untransfected hippocampal neurons, it is likely that the expression level may be insufficient to detect accumulations at points of contact with heterologous cells. Therefore, our data indicate that SALM4, but not SALM1 or SALM2, can form homomeric trans interactions in neurons.

3.3.2 SALMs accumulate at interneurite crossing and branching points

Interneurite contacts are points of adhesion between two neurites, either from the same cell, or from two adhering cells. These contacts can be axo-axonal, axo-dendritic, or dendo-dendritic. Establishment of interneurite junctions is critical for synaptogenesis and structural stability, and often involves CAMs (Togashi et al., 2006). NCAM, which shares a similar domain structure to SALMs, localizes to interneurite accumulations in hippocampal cultures. NCAM-containing junctions are found to recruit TGN organelles within minutes of interneurite contact initiation, and subsequently form functional synapses through accumulation of spectrin (a membrane-cytoskeletal linker protein), and the recruitment of NMDARs and CaMKIIα (Sytnyk et al., 2002; Sytnyk et al., 2006). In the course of our investigations of SALM-mediated neurite outgrowth (Chapter 2), we noticed that transfected SALMs are enriched at crossing points of neurites in young hippocampal
neurons, possibly interneurite junctions. To further explore this phenomenon, DIV4 primary hippocampal neurons were co-transfected with GFP and myc-SALM1, myc-SALM2, non-tagged SALM3, myc-SALM4, or HA-SALM5 cDNA. Immunocytochemistry was performed 48 hours later. We found that each transfected SALM localizes and accumulates at interneurite crossing and branching points of axons and dendrites. Myc-SALM4 transfections were used as representative examples in Figure 3.2, though all SALMs displayed similar accumulations. Interestingly, these points of accumulation were often at crossing points made by neurites from the same cell. Additionally, we saw examples of transfected SALM accumulations that cross with neighboring cells transfected with GFP-only, indicating that the transfected SALMs may interact with endogenous proteins (SALMs or other CAMs, etc.) (Figure 3.2B, white arrow). There were also examples of SALM accumulations along the shafts of axons or dendrites with no apparent interneurite contacts (Figure 3.2B, blue arrow). Due to the high density of these cultures, it is possible that these accumulations represent transfected SALMs that are interacting with neighboring non-transfected cells that could not be visualized. Another possibility is that SALMs are cleaved and secreted into the extracellular space. Perhaps these diffusible SALMs are interacting with membrane-bound SALMs to form these accumulations. To examine if synaptic proteins co-localize with SALMs at these interneurite contacts, we transfected DIV4 neurons with myc-SALM4, and immunostained for myc and the presynaptic marker, bassoon, 48 hours later. Our preliminary data indicate that bassoon co-localizes with these SALM accumulations (Figure 3.3). The immunofluorescence data clearly indicate that SALMs accumulate
**Figure 1.2: SALMs accumulate at interneurite junctions and branching points.**

DIV4 hippocampal neurons were co-transfected with SALM and GFP cDNA, and immunostained 48 hours later. (A) Transfected SALMs accumulate at branching points, and apparent points of contact between neurites of transfected neurons (arrows). Myc-SALM4 is shown here as a representative example, though all SALMs display similar accumulations. (B) Transfected SALMs may also form junctions with non-transfected neurites. The white arrows in (B) indicate a point of contact between a myc-SALM4 transfected neurite and a neurite only transfected with GFP. The blue arrows indicate a potential SALM accumulation with a neighboring non-transfected cell, or possibly a soluble extracellular protein.
Figure 3.3: (Preliminary data) SALM2 accumulations localize with the synaptic marker, bassoon. DIV4 hippocampal neurons were transfected with myc-SALM2 and immunostained for myc and native bassoon 48 hours later. Myc-SALM2 accumulations at interneurite junctions and branching points co-localized with the bassoon.
at these crossing points between neurites, though additional experiments should be performed to verify that the neurites are actually forming physical contacts or junctions at these points (for example, through 3-dimensional reconstruction using confocal microscopy).

### 3.3.3 SALM1 trafficking is mediated by PDZ interactions

PDZ interactions are important for the trafficking and function of many synaptic proteins. For example, the surface expression of the β1-adrenergic receptor (a catecholamine G protein-coupled receptor) is mediated by PDZ interactions with cystic fibrosis transmembrane conductance regulator-associated ligand (CAL; also known as PIST, GOPC, and FIG) (He et al., 2004). AMPA receptor trafficking and stabilization at the synapse involves interactions with the PDZ proteins, PICK1 and GRIP (Gardner et al., 2005; Liu and Cull-Candy, 2005 and for review, Greger and Esteban, 2007; Hanley, 2008). As mentioned in Chapter 1, NMDAR trafficking is facilitated by PDZ interactions involving the MAGUKs. TGF receptor surface expression is ablated by deletion of its PDZ-BD (Blobe et al., 2001). In Wang et al. (2006), Dr. Chang-Yu Wang demonstrated that deletion of the PDZ-BD of SALM1 significantly decreases its surface expression in DIV14 neurons, indicating that PDZ interactions mediate the surface expression of SALMs. To investigate the roles of PDZ interactions in SALM1 trafficking earlier in development, we transfected myc-SALM1 or myc-SALM1ΔPDZ into DIV4 neurons, and performed surface or total immunocytochemistry 48 hours later. Transfected myc-SALM1 localizes throughout the cell (Figure 3.4A2), and is expressed on the cell surface, including axons and
dendrites (Figure 3.4A1). Interestingly, while transfected myc-SALM1ΔPDZ is expressed throughout the cell (Figure 3.4B2), its surface expression was highly diminished at the soma and proximal neurites (Figure 3.4B1, white arrows). However, SALM1ΔPDZ was still expressed on the surface of distal neurites. While a comprehensive examination on this phenomena has yet to be accomplished, it appears that the neurites that express SALM1ΔPDZ at their distal portions may be axonal. Changes in synaptic protein polarization (differential expression in axons and dendrites) are common phenomena throughout development. For example, GAP43 is present in axons and dendrites early in development, but are primarily axonal as development proceeds (Goslin et al., 1990). Proteins may undergo polarization through various mechanisms, including selective targeting, selective removal/retention, or dendrite to axon translocation (Sampo et al., 2003; Wisco et al., 2003; Yap et al., 2008). As we describe in Chapter 4 of this dissertation, NMDARs are expressed in axons and growth cones early in development, but not later. How the PDZ-BD influences SALM1 trafficking and whether this trafficking is developmentally regulated could be important to proper synapse formation and function. These preliminary experiments examined surface and intracellular localizations of SALM1 and SALM1ΔPDZ constructs in parallel cultures. Future investigations may include a combination of surface plus permeabilized immunocytochemistry within the same cultures to examine and quantify changes in SALM1 and SALM1ΔPDZ surface and intracellular expression in individual neurons.
Figure 3.4: (Preliminary data) PDZ interactions influence the trafficking of SALM1.

DIV4 hippocampal neurons were transfected with myc-SALM1 or myc-SALM1 cDNA lacking the PDZ-BD (myc-SALM1ΔPDZ). Surface (A1, B1) or total (A2, B2) immunocytochemistry was performed 48 hours later. An antibody recognizing the N-terminus of SALM1 was used for surface labeling, while anti-myc was used for total labeling. (A1, A2) Myc-SALM1 (full length) localized throughout the cell, and on the cell surface. While myc-SALM1ΔPDZ was expressed throughout the cell (B2), its surface expression was highly diminished at the soma and proximal neurites (white arrow), with high levels of surface expression remaining at the distal, presumably axonal, processes (blue arrow). Arrows in B1 indicate the soma and distal neurite of a single transfected neuron.
3.3.4 Investigating interactions between SALM2 and SAP102

PDZ interactions are clearly important to SALM function. How SALMs traffic to the synapse and the proteins that associate with them along the pathway is of great importance to their function. As mentioned earlier, SALMs were initially identified via yeast two-hybrid screens using the PDZ domains of MAGUKs (SAP97 and PSD-95) (Wang et al., 2006; Ko et al., 2006). SALM2 has been shown to localize to excitatory synapses, and overexpression of SALM2 increases spine number and size (Ko et al., 2006). SAP102 is a MAGUK that is highly expressed at synapses and in the cytoplasm, and has been shown to influence NMDAR trafficking, as well as spine morphology, through associated proteins (Sans et al., 2003; Sans et al., 2005). Expression of SAP102 transcripts and protein levels increases within the first two postnatal weeks and declines later in development (Mueller et al., 1996; Sans et al., 2000). This developmental profile directly correlates with the formation of synapses in the rat cortex (Blue and Parnavelas, 1983). Therefore, to examine the effects of SALM2 and MAGUK interactions on synapse formation, we co-transfected myc-SALM2 and GFP-SAP102 into DIV14 primary hippocampal neurons. Interestingly, our preliminary observations show that neurons co-transfected with both constructs displayed very few spines, and those that were present were irregular in shape, or filopodial-like in morphology (Figure 3.5B). Co-transfecting neurons with GFP-SAP102 and myc-SALM2ΔPDZ (Figure 3.5C), results in a recovery of the spine number in size, similar to what is seen with transfected GFP-SAP102 alone (Figure 3.5A). These results raise a number of interesting questions about the potential roles of SAP102 and SALM2 in synapse formation and morphology. The observation that
overexpressing two proteins that positively influence spine number/size leads to a reduction of synapses was unexpected. However, there are possible explanations for this phenomenon. In Sans et al. (2005), we showed that mPins directly interacts with SAP102, which in turn facilitates the trafficking of NMDARs. While the mPins/SAP102/NMDAR complex traffics to the surface, co-expressing mPins and SAP102 without NMDARs leads to the mislocalization of the mPins/SAP102 complex into intracellular accumulations/inclusion bodies, possibly targeted for degradation (Sans et al., 2005). This indicates that when components of a multimeric complex are missing (or in this case, components are expressed in excess), the complex may mislocalize or undergo malfunction. In our experiment, it is possible that there is insufficient endogenous cargo (possibly NMDAR or other synaptic proteins) to compensate for the overexpressed SAP102 and SALM2, thereby resulting in the possible dysfunction of synapse formation and morphology.
Figure 3.5: (Preliminary data) SAP102 and SALM2 interactions potentially mediate spine morphology. DIV14 primary hippocampal neurons were transfected with GFP-SAP102 (A), GFP-SAP102 and myc-SALM2 (B), or GFP-SAP102 and myc-SALM2ΔPDZ (C). Total immunocytochemistry for GFP and myc was performed 48 hours later (myc channel not shown). While GFP-SAP102 transfected neurons showed numerous spines (A), neurons co-transfected with GFP-SAP102 and myc-SALM2 often showed few spines. The structures present were often thin or filopodia-like. (C) Neurons transfected with GFP-SAP102 and myc-SALM2ΔPDZ (lacking the PDZ-BD) had numerous spines, much like that of GFP-SAP102 alone (A), indicating that the PDZ interaction between SAP102 and SALM2 may potentially influence spine morphology.
Figure 3.5: (Preliminary data) SAP102 and SALM2 interactions potentially mediate spine morphology
3.4 Discussion

3.4.1 Overview

In this chapter, we investigated the localization of SALMs at cell-cell contacts, and provide insights into their trafficking and potential function at the synapse. While several of the studies presented here are preliminary, and require further repetition and refinements, our results demonstrate the potential importance of SALM function throughout development. First, we showed that members of the SALM family vary in their adhesive properties, as SALM4 forms homomeric trans interactions in neurons, while SALMs 1-3 do not. Next, we showed that all SALMs accumulate at interneurite junctions, and that synaptic proteins may also co-localize at these points. Finally, we showed that PDZ interactions may mediate the differential polarization of SALM trafficking, as well as their roles at synapses. Together, the experiments in this chapter provide valuable insights into SALM function at a developmental timepoint beyond that of its function in neurite outgrowth.

3.4.2 Adhesive properties and SALM trafficking

The mechanism of SALM trafficking and the dynamics of their early interactions in the secretory pathway are not yet known. A hypothetical model of SALM interactions is depicted in Figure 3.6. Early in the ER, SALMs may form combinations of dimers or higher-order multimers with each other, or other proteins. The constitution of such SALM multimers could contribute to the distinctiveness of
Figure 3.6: Hypothetical model of SALM multimerization and cis/trans synaptic interactions.

(A) SALMs may assemble as dimers or higher-order multimers within the ER, and traffic through the secretory pathway to the synapse. At the synapse, SALMs (specifically SALMs 4 and 5) may form trans complexes (B), or heteromeric cis and/or trans interactions with SALMs or other CAMs/unknown proteins (C). Another possible mechanism of regulating SALM function may involve proteolytic cleavage and release of the extracellular N-terminus into the synaptic cleft (D).
Figure 3.6: Hypothetical model of SALM multimerization and *cis/trans* synaptic interactions

(Wang and Wenthold, 2009)
SALM function, including their *cis/trans* interactions. The potential interplay between *cis* and *trans* interactions is highly complex, and may contribute to the variability among the SALM function. Formation of *cis* complexes may regulate the formation of *trans* complexes. For example, heteromeric *cis* interactions between axonin-1 and NgCAM (chicken L1-CAM) prevent the ability of axonin-1 to form homomeric *trans* interactions (Kunz et al., 1998; Sonderegger et al., 1998). Additionally, the formation of postsynaptic neuroligin-1/neurexin *cis* complexes inhibits the ability of postsynaptic neuroligin-1 to form *trans* interactions with presynaptic neurexin (Taniguchi et al., 2007). The *cis/trans* interactions of the various SALMs may work in concert to regulate various functions including intracellular signaling cascades and neurite outgrowth phenotypes of the SALMs. Further exploration of the trafficking, multimerization and establishment of SALM interactions on the cell surface could be essential to understanding SALM function.

### 3.4.3 SALMs localize at interneurite contacts

The accumulation of SALMs at interneurite contacts is compelling evidence of their adhesive properties in neurons. Young cultured neurons (<DIV7) exhibit highly motile dendritic filopodia that are believed to be critical in synaptogenesis (Jontes and Smith, 2000). The initial points of interneurite contacts are often between dendritic filopodia and axons, which may subsequently develop into dendritic spines or shaft synapses. Most synaptic contacts are on dendritic shafts before the first postnatal week, while the proportion of spines rises dramatically with age (Fiala et al., 1998). Additionally, interneurite contacts include non-synaptic cell adhesions that facilitate
and coordinate neuronal structural frameworks. Such contacts have been shown to be regulated by CAMs, including nectins, cadherins, and NCAM (Sytnyk et al., 2002; Togashi et al., 2006). Our preliminary evidence indicating the co-localization of bassoon and SALM at interneurite crossing points suggests that these may be sites of early synaptic contacts, though it is also possible they may also serve roles in general (non-synaptic or extrasynaptic) neuronal mechanical stability.

3.4.4 SALMs at the synapse

While we still have much to learn about the roles of SALMs at the synapse (and extrasynaptically), the data thus far indicate that they could be important for various facets of synaptic function and development. Overexpression of SALM2 increases the number of excitatory synapses and dendritic spines, while mislocalization of SALM2 decreases them (Ko et al., 2006). Knockdown of SALM2 reduces the frequency, but not amplitude of miniature EPSCs (mEPSCs), indicating a reduction of synapse number. This variability in localization among the various SALM family members could have important implications on synaptic development, and brings forth potentially interesting parallels between SALM and neuroligin function. Neuroligin-1 is present at excitatory synapses (Song et al., 1999), while neuroligin-2 is largely localized at inhibitory synapses (Varoqueaux et al., 2004). Neuroligins directly interact with PSD-95 via PDZ interactions (Irie et al., 1997), and overexpression of PSD-95 results in a change in the distribution of neuroligin-2 from inhibitory to excitatory synapses (Levinson et al., 2005). As mentioned earlier, neuroligins promote excitatory and inhibitory synapse formation, thereby mediating
the delicate balance of inputs in the nervous system. The balance of neuronal 
excitation to inhibition is critical to normal brain function, and disruption of this 
balance has been implicated in underlying a variety of neurological disorders 
including autism (Rubenstein and Merzenich, 2003). Additionally, several studies 
showed that various abnormalities in neuroligin genes are found in autistic 
individuals (for review, see Dean and Dresbach, 2006; Lise and El-Husseini, 2006; 
Craig and Kang, 2007). Localization of SALM proteins at inhibitory synapses has 
not yet been thoroughly investigated. The possibility that SALMs may function to 
regulate the balance of excitation to inhibition is highly intriguing, and deserves 
further study.

3.4.5 SALM function is mediated N- and C-terminal interactions

In the previous chapter, we showed that aspects of neurite outgrowth are dependent 
upon both the N- and C-termini of SALMs. Similarly, the studies in this chapter 
highlight the importance of both regions later in development. Here, we show that 
the N-termini of the SALMs mediate cellular adhesions, while the C-termini 
influence synapse morphology through PDZ interactions. Much of the recent SALM 
literature details the importance of both the N- and C-terminal domains in SALM 
biology. When individually expressed and co-plated in heterologous cells, only 
SALMs 4 and 5 are able to form homomeric trans interactions (Seabold et al., 2008). 
These interactions are due to the extracellular N-terminus, as demonstrated through 
the use of the SALM2/4 and SALM4/2 chimera constructs mentioned in Chapter 2. 
Additionally, application of antibodies directed to the N-terminal, extracellular LRRs
of SALMs blocks this interaction, indicating a function of the LRR region in these trans associations. Ko et al. (2006) demonstrated that bead aggregation of SALM2 induces the co-clustering of PSD-95 and other postsynaptic proteins, including GKAP and AMPARs. In Wang et al. (2006), the authors showed that overexpression of SALM1 in DIV14 neurons recruits NMDARs and PSD-95 to dendritic puncta, and that this recruitment is dependent upon the PDZ binding domain of SALM1. SALM1 also enhances surface expression of transfected NR2A, and co-immunoprecipitates with NR1 and NR2 subunits in brain, possibly via direct interactions at the N-termini. The C-terminus of SALM1 also modulates synapse morphology, as transfection of a SALM1 construct lacking the region following the TM (SALM1ΔCT) into DIV14 primary hippocampal cultures induces a dramatic increase in the appearance of thin filopodia-like structures (Wang et al., 2006). This observation is similar to our results in Figure 3.5, involving the co-overexpression of SALM2 and SAP102. These results may further illustrate the importance of the regulation between N- and C-terminal SALM interactions, as perhaps there is insufficient endogenous NMDARs (or other synaptic proteins) to compensate for the additional SAP102 and SALM2, thereby resulting in the dysfunction of synapse formation and morphology. The coordinated activity of both the N- and C-termini of SALMs throughout development could be instrumental to SALM function. Furthermore, it is possible that the various SALMs have unique interacting partners, and that these interactions may change during development. Together, the data indicate that SALMs have varied roles in the early stages of synapse formation, and are prime candidates for future study in the pursuit to understand mechanisms of neuronal development.
Chapter 4: Transient expression of NMDARs at 
axononal growth cones of young hippocampal neurons

4.1 Introduction

NMDARs are a class of ionotropic glutamate receptors that serves key functions in the nervous system, including synaptic plasticity, learning and memory, and neuronal development. While the roles of NMDARs at synapses continue to be well studied, their roles earlier in development are less understood. A number of studies have implicated NMDARs in neuronal migration and neurite outgrowth (for review, see Wenthold et al., 2008). In embryonic mouse cortex, NMDARs activity has been shown to promote neuronal migration (Behar et al., 1999). Studies in cerebellar granule cells demonstrated that NMDAR-mediated migration is calcium-dependent, (Komuro and Rakic, 1993; Kumada and Komuro, 2004), and that subunit composition may directly influence this process, as overexpression of NR2B-containing receptors increases migratory speed (Tarnok et al., 2008). NMDARs have also been shown to stimulate neuritogenesis in young neurons in a calcium-dependent manner, and promote various aspects of neurite outgrowth (Pearce et al., 1987; Brewer and Cotman, 1989; Rashid and Cambray-Deakin, 1992). In young hippocampal neurons, NMDAR subunits have been shown to localize to axonal growth cones and interact with neuronal intermediate filaments, implying direct functional connections to the cytoskeletal apparatus (Ehlers et al., 1998). However, reports of NMDAR localization at growth cones have produced somewhat differing results (Ehlers et al., 1998; Herkert et al., 1998). Using young hippocampal neurons,
Ehlers et al. (1998) showed that NR1 is present at axonal growth cones (but not in the axon shaft), while Herkert et al. (1998), described the enrichment of NR2B, but not NR1, at axonal growth cones. This discrepancy is not without precedent, as NMDAR localization by immunocytochemistry can be a challenging endeavor due to difficulties in producing highly specific primary antibodies to NMDAR subunits, especially those that can be used for surface labeling. The presence of NMDARs at axonal growth cones could have clear implications on early neuronal development, and in the formation and activity of presynaptic terminals. While classically described as postynaptic glutamate receptors, numerous studies over recent years have implicated the importance of presynaptic NMDARs in modulating synaptic function and plasticity (for review, see Corlew et al., 2008). Importantly, NMDAR activity allows the influx of calcium ions, a critical second messenger with numerous biological implications throughout development, with particularly vital roles in neurite outgrowth/retraction, and axonal growth cone motility (Henley and Poo, 2004). In *Xenopus* spinal neuron cultures, NMDARs have been shown to influence growth cone turning and filopodial asymmetry (Zheng et al., 1996). However, the roles of NMDARs at growth cones of mammalian neurons are unknown.

Discovered by Dr. Santiago Ramón y Cajal in 1890, growth cones are highly motile structures that are localized at the ends of growing axons and dendrites (for review of his observations, see Ramón y Cajal, 1988). Morphologically, growth cones are hand-like structures, conical in shape, that contain a microtubule-rich central (C) region at the distal end of the neurite shaft, surrounded by a peripheral (P) region that
contains a palm-like lamellipodial structure with finger-like filopodial protrusions extending in various directions that sense the extracellular environment to facilitate the pathfinding and outgrowth of the neurites (for review, see Gordon-Weeks, 2000). The lamellipodia and filopodia are actin-rich structures that undergo dynamic extension and retraction to generate the mechanical force to move the growth cone (Forscher and Smith, 1988). Growth cone turning and axon pathfinding are intricate processes that involve a highly diverse variety of factors; some examples include electric fields, membrane polarization, extracellular matrix molecules, CAMs, intracellular signaling pathways, and diffusible chemotropic factors (Gordon-Weeks, 2000; Farrar and Spencer, 2008). In 2003, Dr. Leslie Pick’s laboratory demonstrated that the Drosophila Insulin Receptor (DInR) functions in axon guidance via an association with the adapter protein Dock/Nck, indicating that a similar function of insulin receptors may exist in vertebrate development (Song et al., 2003). Additionally, a large collection of studies have demonstrated that calcium signals are key regulators of axonal growth cone turning and motility (for review, see Henley and Poo, 2004; Zheng and Poo, 2007). How calcium regulates growth cone motility is a topic of great interest and complexity. In general, global changes in calcium levels regulate changes in neurite outgrowth/retraction and growth cone motility rate, whereas localized changes in calcium levels mediate attractive/repulsive cues for growth cone turning. The collection of literature together indicates that specific/optimal levels of calcium activity are necessary for various changes in motility (i.e. small and large local calcium signals promote repulsion, whereas
moderate levels promote attraction) (Robles et al., 2003; Henley et al., 2004; Wen et al., 2004, and for review see, Kater et al., 1988; Gomez and Zheng, 2006).

Calcium signaling is clearly a fundamental component in axonal growth cone function. Therefore, NMDAR activity at growth cones could have dramatic implications ranging from regulating growth cone motility to synapse formation. In this chapter, we determined that NMDARs are present and functional at axonal growth cones of young hippocampal neurons. Through the use of fluorescence immunocytochemistry, we showed that NMDAR subunits are present at axonal growth cones and at the axon shaft early in development. Electrophysiological recordings indicated that local activation of NMDARs at growth cones mediates whole-cell currents. Furthermore, calcium imaging experiments showed that local activation of NMDARs at growth cones elicits calcium influx. Finally, we showed that the expression of NMDARs at axons and growth cones of hippocampal neurons is transient, as older neurons do not express NMDARs at distal axons. Our results indicate that NMDARs are functional components of axonal growth cones, and give further insights into aspects of NMDAR trafficking and activity throughout development.
4.2 Methods

Primary hippocampal cultures

E18 primary hippocampal cultures were prepared by Dr. Ya-Xian Wang as previously described in Chapter 2. Briefly, hippocampi from E18 rats were dissected, cells were dissociated, and plated onto coverslips at high density (50,000 cells per ml, or 50K) for transfection, or low density (0.5-5K) for electrophysiology and calcium imaging experiments. To minimize variability and contribution of somatic responses for electrophysiology and calcium imaging experiments, strict morphological and geometric criteria were utilized in the selection of neurons to analyze. Only isolated neurons (with no visible contacts with adjacent cells) that had morphologically distinct axonal growth cones positioned downstream of the perfusion flow, relative to the cell body, were chosen for examination.

Transfections and Immunocytochemistry

Transfections and immunocytochemistry were performed as described in Chapter 2. Neurons were transfected with cDNA constructs at various ages in culture (DIV4-14) using the calcium phosphate method. GFP-NR2A, GFP-NR2B, YFP-NR1-1 constructs were generously provided by Dr. Stefano Vicini. Immunocytochemistry was performed 48 hours after transfection, and visualized using a Nikon E1000M microscope equipped with a CCD camera using a Plan Fluor 20x (0.5 NA) dry or Plan Apo 60x (1.4 NA) oil-immersion objective. GFP rabbit-polyclonal antibodies were purchased from Chemicon (Billerica, MA) and used at 1:2000 dilution for surface and permeabilized immunocytochemistry. The NR1 rabbit monoclonal
antibody was from Chemicon, based on a 30 amino acid sequence in the NR1 C-terminal tail originally designed by Dr. Wenthold (amino acids 909-938: LQNQKDTVLPRRAIEREEGQLQLCSRHRES).

**Electrophysiology**

HEPES-based (pH 7.3, magnesium-free) extracellular recording solution contained 1.25 mM NaH$_2$PO$_4$, 150 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 10 mM glucose, 10 μM D-serine, and 0.2 mM CaCl$_2$. Somatic whole-cell voltage-clamp recordings were performed with borosilicate glass electrodes (6-8 MΩ) filled with a cesium-based internal solution containing 130 mM CsMeSO$_4$, 10 mM HEPES, 5 mM EGTA, 1 mM MgCl$_2$, 10 mM TEA-Cl, 2 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM phosphocreatine (tris), 2 mM QX-314. The solution was brought to pH 7.3 with CsOH. For whole-cell recordings at the growth cone, 10-20 MΩ electrodes were used. Whole-cell voltage clamp recordings were obtained from primary hippocampal neurons using a MultiClamp 700B (Molecular Devices, Sunnyvale, CA) patch clamp amplifier. Application of NMDA (100 μM) was performed using a Picospritzer III (Parker Hannifin, Cleveland, OH) pressure application system. Compounds were applied at 4-6 psi for 100 ms (500 ms for the whole-cell recordings at the growth cone shown in Figure 4.4). Cells were maintained near -70 mV for all conditions, and recordings were conducted and analyzed using Igor Pro (Wavemetrics, Portland, OR) software.
**Pharmacology**

The following pharmacological compounds were utilized, as described in the text. In control electrophysiology and calcium imaging experiments, NMDARs were blocked by adding 100 μM DL-APV (Tocris bioscience, Ellisville, MO) to the extracellular solution, and introduced into the recording chamber by perfusion. To block NR2B-containing NMDARs, 5 μM ifenprodil (Sigma-Aldrich, St. Louis, MO) was added to the extracellular solution. VSCCs were blocked with 0.1 μM ω-conotoxin MVIIC (Peptides International, Louisville, KY), 0.03 μM SNX-482 (Peptides International), 20 μM nimodipine (Sigma-Aldrich, St. Louis, MO), and 10 μM mibefredil (Sigma-Aldrich, St. Louis, MO). These compounds block VSCCs CaV2.1/2.2 (P/Q- and N-type), CaV2.3 (R-type), CaV1.2/1.3 (L-type), and CaV3 (T-type) classes of VSCCs, respectively. Replenishment of intracellular calcium stores was blocked using 10 μM cyclopiazonic acid (CPA). All reagents were dissolved in distilled water except for nimodipine and CPA, which were dissolved in DMSO.

**Calcium imaging**

Our standard extracellular solution was prepared with 2 mM CaCl₂ for these experiments. For AM-loading experiments, DIV4-7 primary hippocampal cultures were treated with fluo-4AM calcium indicator, prepared using methodologies reported in Dr. Wade Regehr’s laboratory (see Carter et al., 2002; and others). Briefly, fluo-4AM (50 μg) was solubilized in DMSO/0.8% pluronic F-127 to create a 1 mM stock solution. Fluo-4AM was applied to neurons in B27-supplemented neurobasal medium at a dilution of 1:100 and incubated at 37 °C for 3 minutes.
Neurons were then washed with fresh culture media and incubated for 30 minutes at
37 °C. For fluo-5F experiments, EGTA-free cesium-based internal solutions were
supplemented with 300 μM fluo-5F, and loaded into the cell through somatic whole-
cell voltage clamp techniques. NMDA (200 μM) was applied to axonal growth
cones, and calcium transients were imaged using a QImaging Rolera Mgi EM CCD
camera in conjunction with QCapture Pro 6 (QImaging, Surrey, BC) and IgorPro
software (Wavemetrics, Portland, OR). Images (100 ms exposures) were taken at 138
ms frame intervals, verified by output signals from the CCD camera. Calcium
transients were quantified using Metmorph v7.0r3 (Molecular Devices). Regions of
interest (ROIs) were selected to encompass the entire growth cone structure (P and C
areas), and background ROIs were selected within close proximity to the growth
cone. ΔF/F measurements were performed using the following formula \( \frac{F-F_0}{F_0} \),
where F is the background subtracted fluorescent value, and F_0 is the average of the
first seven background subtracted frames (baseline levels).
4.3 Results

4.3.1 NMDAR localization at growth cones of young hippocampal neurons

To investigate the localization of NMDARs at growth cones, we performed immunocytochemistry for endogenous NR1 in young (DIV3-6) hippocampal neurons using a rabbit monoclonal antibody that recognizes NR1 splice variants containing the C2 cassette (NR1-1a/b and NR1-2a/b). Recently produced by Chemicon, this antibody was based on a 30 amino acid segment in the C-terminus of NR1 (amino acids 909-938: LQNQKDTVLRRAIEREEGQLQLCSRHRES), originally designed by Dr. Wenthold (Petralia et al., 1994). Studies in Dr. Wenthold’s laboratory have shown that this antibody produces very clean labeling of NR1 proteins in immunoblot and immunohistochemical applications (unpublished observations). In our first experiment, we transfected DIV4 neurons with GFP and immunostained for total endogenous NR1 and GFP, 48 hours later (DIV6). The NR1 labeling displayed a punctate distribution throughout the soma, dendrites, axons, and growth cones. High magnification (100x) images show NR1 puncta at both the lamellipodia and filopodia of growth cones (Figure 4.1A). Similar to the experiments in Chapter 2, GFP was transfected to visualize the entirety of the cell so that axonal growth cone morphology could be clearly examined. As the transfected GFP signal is extremely low in neurites, the cells were immunostained with a GFP primary antibody and Alexa Fluor 488 secondary antibody to enhance the fluorescent signal. Control experiments were previously performed by immunostaining with the dendritic, axonal, and growth cone proteins (MAP2, tau, and GAP43, respectively), to establish the criteria for
Figure 4.1: Endogenous NR1 is expressed in axons and growth cones. (A) DIV4 primary hippocampal cultures were transfected with GFP cDNA to visualize the entirety the cell, and immunostained for endogenous NMDAR subunits 48 hours after transfection at DIV6. Endogenous NR1 displays a punctate distribution throughout the cell, and is present at axonal growth cones. Montage of 20x images displaying the entire cell (GFP signal). Inset images were taken at 100x magnification. Scale bars for (A) are 50 μm and 10 μm, for montage and insets, respectively. (B) DIV3 neurons were immunostained for NR1 and treated with phalloidin-488 to stain for actin. Scale bar, 50 μm.
distinguishing axons from dendrites of neurons at this age (Wang et al., 2008, Chapter 2, and unpublished observations). To investigate the presence of NR1 at an earlier time point, low density DIV3 hippocampal cultures were immunostained for total NR1 and labeled with phalloidin-488 (a marker for actin, which is present throughout the cell, including growth cones and filopodia). Differentiation of presumptive axon from dendrites was clearly evident at this early age, and NR1 puncta could be visualized throughout the cell, and in growth cones (Figure 4.1B). As this antibody was generated to the intracellular C-terminus of NR1, it could not be used for live surface labeling. In a companion study, Dr. Ronald Petralia utilized this same NR1 antibody to perform immunogold EM of hippocampus from P2 rats. He observed that NR1 localizes to membranes and filopodia of axonal growth cones, and at early presynaptic contacts (unpublished observations). To investigate the presence of NR2 subunits at axonal growth cones, GFP-NR2A or GFP-NR2B cDNA constructs were co-transfected with DsRed cDNA at DIV4, and immunostained for GFP two days later (DIV6). Similar to the soluble GFP, the transfected GFP-NR2A and NR2B signals are extremely low in neurites, and are only readily detectable in the soma with enhancement. Due to this technical issue, we were also able to reliably perform live-surface labeling with these transfected GFP-NR2A and GFP-NR2B proteins. The neurons were either fixed for permeabilized immunocytochemistry or labeled live to examine surface expression of transfected GFP-NR2A or GFP-NR2B (Figure 4.2). Our results show that exogenous GFP-NR2A and GFP-NR2B are present in discrete, punctate, locations along the entire cell, including the axon shaft, the lamellipodial structure and the filopodial tips of the growth cone. Both NR2B and NR2A show
Figure 4.2: Transfected NR2B is expressed on the surface of axons and growth cones. Primary hippocampal cultures were co-transfected with GFP-NR2B and DsRed cDNA constructs at DIV4. Surface or permeabilized immunocytochemistry was performed 48 hours later. (A) GFP-NR2B displays punctate expression on the surface of axons and growth cones. (B) Total/permeabilized staining of GFP-NR2B shows a substantial amount of GFP-NR2B at the axons and growth cones. Images are taken at 100x magnification.
similar results, indicating that they may utilize similar mechanisms to traffic to the
growth cones. These results indicate that NMDARs are present on, and may be
expressed on the surface of, axon shafts and axonal growth cones of young primary
hippocampal cultures.

4.3.2 NMDARs are functional at axonal growth cones

Our experiments thus far have established that NMDARs are present at axons and
growth cones of young primary hippocampal neurons. Next, we wanted to
investigate if these NMDARs are functional. To do this, we forged a collaboration
with Dr. Stephan Brenowitz of the NIDCD Section on Synaptic Transmission. With
his guidance, I was able to gain invaluable experience in electrophysiological
techniques. Low density primary hippocampal neurons were cultured by Dr. Ya-Xian
Wang, such that individual cells were isolated, and not visibly in contact with other
neurons or glia. At DIV3-6, the neurons were examined by whole-cell voltage clamp
(-70 mV holding potential). NMDA (200 μM) was then locally applied to the axonal
growth cone by pressure application (puffing, 4-6 psi) for 100 ms. Recordings were
performed in a HEPES-based, magnesium-free extracellular solution containing 0.2
mM CaCl₂, and the cesium-based internal recording solution contained 5 mM EGTA
to avoid calcium-mediated excitotoxicity. To localize the NMDA puff to the growth
cone, only cells that were in a precise geometry with the cell body positioned
upstream of the perfusion flow, and at least 100 μm from the growth cone, were
chosen for analysis (Figure 4.3A). The puffing pipets had resistances ranging from 4-
6 MΩ, and were positioned 30 μm from the growth cone. This application system
allows us to specifically target the NMDA application to the growth cones while avoiding the dendrites and cell body, though it is likely that regions of the distal axon were also within the area of the puffs (Figure 4.3E). For each cell examined, NMDA was puffed onto the cell body as a control. The pipet was then repositioned, and NMDA was puffed onto the growth cone for three trials (once per minute). Representative traces from a single cell (puffing at the cell body, growth cone, and growth cone in the presence of APV) are shown Figure 4.3B. Perfusion of extracellular solution containing the NMDAR antagonist DL-APV (100 μM) eliminated the currents mediated by NMDA puffs to the growth cone, indicating that this response is specific to NMDARs, and not due to a mechanical artifact.

Application of NMDA to the growth cone elicited a highly reproducible current with an average peak amplitude of 31 ± 3.4 pA compared to 265 ± 40.9 pA at the cell body (n=10) (Figure 4.3D). To further assess the characteristics of these currents, we mathematically integrated the area of the current traces to determine average changes in charge mediated by NMDA application to the growth cone before and after APV application (14.4 ± 5.1 fC and 0.038 ± 0.5 fC, respectively) (Figure 4.3C). All experiments were performed in the presence of 1 μM tetrodotoxin (TTX) to block voltage-sensitive sodium channels and prevent spontaneous action potentials.

Another strategy to directly measure NMDAR-mediated currents at growth cones is to patch the growth cone, and/or perform single channel recordings from outside-out patches made from the growth cone. To accomplish this, we developed a technique to patch the growth cone using high resistance pipets (10-20 MΩ) in conjunction with
**Figure 4.3: Functional NMDARs are present at axonal growth cones.** DIV3-6 primary hippocampal cultures were whole-cell voltage-clamped at the cell body and NMDA (200 μM) was puffed onto the growth cone or cell body by pressure application (100 ms, at 4-6 psi). (A) A composite DIC image of a representative patched cell and the application system. Recordings were performed in the presence of TTX (1 μM). To test the specificity of the NMDA-mediated response at growth cones, 100 M DL-APV was applied to the recording solution and NMDA was puffed onto the growth cone (B, bottom trace). The effects of NMDA were eliminated after application of DL-APV. (C) Average peak amplitudes for NMDA currents at the cell body and growth cones were 265 ± 40.9 pA and 31 ± 3.4 pA, respectively. (D) Average charge (fC) of currents elicited by applying NMDA to growth cones, before and after APV application (14.4 ± 5.1 fC and 0.038 ± 0.5 fC, respectively) (n=10, values shown are mean ± SEM, and analyzed by unpaired students t-test, *p<0.05). (E) To examine the area of the pressure application cloud, an electrode was loaded with Alexa Fluor 488 and puffed into the solution. Time-lapse frames are in 200 ms intervals. Fluorescent signals of the puff were pseudocolored based on pixel intensity. The fluorescent images were digitally overlaid onto a DIC image of the experimental system for visualization purposes.
Figure 4.3: Functional NMDARs are present at axonal growth cones
highly delicate micromanipulator operations. While this has been accomplished before in *Xenopus* spinal neurons (Nishiyama et al., 2003; Nishiyama et al., 2008), the technique has yet to be established in hippocampal cultures. Our preliminary efforts to patch the growth cones were successful, and NMDA application (200 μM) to the growth cone produced an inward current, as seen in Figure 4.4 (n=1). In this example (Figure 4.4A), a 500 ms puff of NMDA was performed, resulting in an increased decay time of the current compared to those of Figure 4.3. However, the success rate of patching the growth cone is still quite low (approximately 1 out of 10 trials were successful). Therefore, development of the technique requires further refinements and practice. Together, these electrophysiological data indicate that NMDARs at axonal growth cones are functional, as local activation of the receptors mediates an influx of positively charged ions.

### 4.3.3 NMDAR activation at growth cones promotes localized calcium influx

NMDARs are calcium-permeable ion channels. To further examine the functional significance of NMDARs at growth cones, we performed calcium imaging in young (DIV3-6) primary hippocampal neurons. In our preliminary experiments, we performed a qualitative analysis of NMDA-mediated calcium influx at growth cones using the high-affinity cell-permeable fluorescent calcium indicator, fluo-4AM. DIV3-6 neurons were loaded with fluo-4AM, and NMDA was applied to the growth cone by pressure application. Calcium transients were visualized using fluorescent microscopy, and occurred at the growth cones in a highly dynamic manner, with
Figure 4.4: (Preliminary data) Patching the growth cone. To further investigate the properties of NMDARs at growth cones, and to address the possibility of filtering, neurons were patched at the growth cone using 10-20 MΩ pipets. (A) Representative trace following a 200 μM NMDA application (500 ms puff). (B) DIC image of the patched growth cone. The internal solution was supplemented 150 μM Alexa Fluor 488 which diffused throughout the cell, indicating a successful patch (C).
signals appearing at various regions of the growth cone and propagating down the axon shaft (Figure 4.5B). For each cell examined, the addition of 100 μM DL-APV to the extracellular solution eliminated these calcium transients (Figure 4.5C). This response is NMDAR-specific, as the subsequent removal of the APV-containing ACSF led to a recovery of the calcium transients (Figure 4.5D). To examine the subunit-specific contributions of these NMDAR-mediated calcium transients, we repeated this experiment with the NR2B-containing receptor antagonist, ifenprodil (5 μM), instead of APV. Ifenprodil blocked the majority of the transients, indicating that NR2B-containing receptors are involved in this calcium influx (Figure 4.6). This is not unexpected, as NR2B-containing receptors are highly expressed at this young age (for example, see Sans et al., 2000).

While these results provide qualitative evidence that NMDARs at axonal growth cones are functional, and promote calcium influx, these experiments do not allow us to dissect the proportion of these calcium transients that are directly due to calcium influx through the NMDARs. It is possible that NMDAR-mediated depolarization and activation of voltage-sensitive calcium channels (VSCCs) and calcium induced calcium release (CICR) from intracellular stores may contribute to these transients. The neurons in these experiments were not voltage-clamped, thereby potentially allowing robust electrical activity. While diffusion of calcium ions is quite slow in the cytoplasm (coefficient of around 10 μm²s⁻¹) (al-Baldawi and Abercrombie, 1995; Murthy et al., 2000), calcium release mediated by IP₃ and ryanodine-sensitive
Figure 4.5: Activation of NMDARs at axonal growth cones induces calcium influx.

DIV3-7 cultures were loaded with fluo-4AM calcium indicator, and NMDA was puffed onto the growth cone while imaging. (A) DIC image of the experimental system. (B) Local application of NMDA (100 μM) to the growth cone induces calcium influx. (C) Introduction of APV (100 μM) into the extracellular solution eliminated the NMDA-mediated calcium influx. (D) Removal of APV from the bath led to the recovery of the NMDA-mediated response. Scale bar represents changes in raw fluorescence (pixel intensity).
Figure 4.5: Activation of NMDARs at axonal growth cones induces calcium influx
Figure 4.6: Functional NR2B-containing receptors are present at axonal growth cones.
DIV3-6 neurons were loaded with fluo-4AM calcium indicator, and NMDA was puffed onto the
growth cone while imaging. (A) DIC image of the experimental system. (B) Local application of
NMDA (100 μM) to the growth cone induces calcium influx. (C) Introduction of ifenprodil (5 μM)
into the extracellular solution eliminated the majority of the NMDA-mediated calcium influx. (D)
Removal of ifenprodil led to the recovery of the NMDA-mediated response. Scale bar represents
changes in raw fluorescence (pixel intensity).
Figure 4.6: Functional NR2B-containing receptors are present at axonal growth cones
channels can create localized bursts of highly concentrated calcium levels from intracellular stores (for review, see Zheng and Poo, 2007).

To address these factors, we utilized whole-cell voltage clamp techniques in conjunction with the addition of pharmacological agents to better isolate and examine the NMDAR-mediated component of the calcium influx at growth cones. Neurons were patched/loaded at the soma with a cesium-based internal solution supplemented with fluo-5F calcium indicator, and voltage-clamped at -70 mV membrane potential. To examine if VSCCs contributed to the calcium transients, a combination of blockers including 0.1 μM ω-conotoxin MVIIC, 0.03 μM SNX-482, 20 μM nimodipine, and 10 μM mibefredil, was added to the extracellular solution. These compounds block VSCCs CaV2.1/2.2 (P/Q- and N-type), CaV2.3 (R-type), CaV1.2/1.3 (L-type), and CaV3 (T-type) classes of VSCCs, respectively. TTX (1 μM) was also added to the solution to block voltage-sensitive sodium channels and spontaneous action potentials. In the presence of these blockers (VSCC+TTX), NMDA application to growth cones caused brief calcium transients at growth cones with an average of 36 ± 8.1% increase in fluorescence intensity (ΔF/F, n=7) (Figure 4.7E). The localization of the transients was often variable, sometimes occurring at discrete locations of the growth cone or distal axon (Figure 4.7A-D). In a parallel experiment, neurons were treated with 10 μM cyclopiazonic acid (CPA), which prevents the replenishment of intracellular calcium stores, and 1 μM TTX (CPA+TTX, n=4). In a third condition, cells were treated with only 1 μM TTX (n=5), and, as expected, application of APV to the system blocks the transients (data not shown). All three
**Figure 4.7: NMDARs mediate localized calcium influx at axonal growth cones.** DIV3-6 neurons were patched/loaded at the soma with a cesium-based internal solution supplemented with fluo-5F calcium indicator, and voltage-clamped at -70 mV membrane potential. Application of 200 μM NMDA to the growth cone elicits localized calcium transients. Panel (A) shows a representative example of a growth cone region before local application NMDA. (B) ΔF image analysis indicates the spatial distribution of calcium transients within the growth cone. ΔF was measured by subtracting baseline frames (before NMDA application) from frames showing the fluorescence levels at the peak of the response. In part C, we selected various regions of interest (ROIs) to analyze changes in fluorescence (ΔF/F) over time. ROIs were chosen at the growth cone (red) and axonal shaft (green). Background ROIs (blue) were chosen near the growth cone structures. (D) ΔF/F analysis reveals an increase in fluorescence at the growth cone, but not axon shaft, at the time of NMDA application. To isolate the NMDA-mediated component, we applied compounds to block VSCC or replenish intracellular calcium stores (E). These compounds show no significant effect on the calcium transients, indicating that the influx is mediated largely by NMDAR activity. VSCCs were blocked with the following compounds: 0.1 μM ω-conotoxin MVIIC, 0.03 μM SNX-482, 20 μM nimodipine, and 10 μM mibefredil (n=7). Replenishment of intracellular calcium stores was blocked using 10 μM CPA (n=4). TTX (1 μM) was included in all conditions (n=5 for TTX-only trials). ΔF/F measurements were performed using the following formula (F-F₀)/F₀, where F is the background subtracted fluorescent value, and F₀ is the average of the first seven background subtracted frames (baseline levels).
Figure 4.7: NMDARs mediate localized calcium influx at axonal growth cones
conditions produced similar changes in fluorescence at growth cones in both spatial and temporal parameters, indicating that the major component of this calcium influx was through the NMDARs (Figure 4.7E). Unfortunately, due to unknown technical difficulties related to patching/loading the neurons, we were unable to successfully perform these experiments while simultaneously blocking all VSCCs and intracellular stores. It is possible that the AM-loading technique may be utilized to address this technical limitation. Together, these results indicate that NMDARs at axonal growth cones are functional, and mediate localized influx of calcium into the cell. Furthermore, application of VSCC blockers or depletion of intracellular calcium stores had no significant effect on the calcium transients in growth cones, indicating that the observed calcium influx is primarily due to activation of NMDARs.

4.3.4 NMDAR localization later in development

Our studies thus far have demonstrated that NMDARs are present and functional at axons and axonal growth cones of young (DIV4-6) hippocampal neurons. As mentioned earlier, a growing body of literature has indicated roles of presynaptic NMDARs in modulating synaptic function and plasticity (Corlew et al., 2008). However, the function of the NMDARs throughout development, particularly during the period between neurite outgrowth and synapse formation, is still unclear. To examine the presence of NMDARs at axons later in development, two week-old hippocampal neurons were co-transfected with DsRed and YFP-NR1-1, GFP-NR2B, or GFP-NR2A and immunostained for GFP two days later (DIV16). Our results indicate that exogenous NMDAR subunits are highly expressed in the cell body and
in the dendrites, but are absent from distal axonal shafts and arborizations (Figure 4.8).

Previously, Herkert et al., 1998, reported that native NR2B is present at axons and growth cones in young neurons (DIV6), but is absent from axons later in development (DIV20) (Herkert et al., 1998). A recently published report indicates that NR2B is present throughout the cell at DIV3, but is restricted to somatodendritic sites by DIV5, and exhibits clustering with postsynaptic proteins by DIV10 (Song et al., 2009). Ehlers et al. (1998) found native NR1 at axons and growth cone of DIV10 hippocampal neurons. While there are some discrepancies in the timepoints between these studies, the trend is similar. Interestingly, the transfected YFP-NR1-1, GFP-NR2A, and GFP-NR2B often show several discrete puncta extending from the soma into the axon hillock and the proximal axon, with decreasing expression along the axon as its distance from the soma increases. The selective localization of polarized synaptic proteins depends on various mechanisms including selective targeting via protein interaction domains, selective removal or retention due to endocytic motifs, or dendrite to axonal translocation (Sampo et al., 2003; Wisco et al., 2003; Yap et al., 2008). This developmental change in NMDAR polarization is highly interesting and could lead to an understanding of fundamental aspects of NMDAR trafficking. Additionally, elucidating the developmental cues that initiate this polarization, the mechanism underlying this selective polarization, and the proteins and intracellular signaling pathways that are involved, may give insights into the roles of NMDARs in neurite outgrowth to synapse formation.
Figure 4.8: Transfected NMDARs do not localize at distal axons later in development. Primary hippocampal cultures were co-transfected with GFP-NR2B and DsRed cDNA constructs at DIV14. Immunocytochemistry was performed 48 hours later. Transfected YFP-NR1-1 (A), GFP-NRB (B), and GFP-NR2A (C) are expressed at the cell body and in dendrites, but are absent from the distal axonal processes (arrows). Images were taken at 40x magnification. Scale bars, 50 μm.
4.4 Discussion

4.4.1 Overview

We determined that NMDARs are present and functional at axonal growth cones of primary hippocampal neurons. The localization of NMDARs early in neuronal development was elucidated through our native and transfected immunocytochemistry, as well as immunogold EM studies performed by Dr. Ron Petralia (a companion study to this chapter). Our electrophysiological recordings and calcium imaging experiments demonstrate the first functional evidence of NMDAR activity at growth cones of hippocampal neurons. This suggests that NMDARs may mediate facets of calcium signaling that have long been demonstrated to be critical components of growth cone activity, including growth cone motility/turning and signaling pathways. Furthermore, we observed that the expression of NMDARs at axons is transient, suggesting that NMDARs may undergo mechanisms of targeted trafficking or endocytosis to achieve developmental polarization. NMDAR research in recent years has yielded a collection of established binding partners and synaptic trafficking and endocytic mechanisms. Applying this knowledge to investigating NMDAR trafficking earlier in development may help us establish the mechanism by which they achieve this selective polarization. The function or dysregulation of this transient expression could hold great potential relevance to proper neuronal development and synaptic activity.
4.4.2 NMDAR function in growth cone motility and intracellular signaling pathways

NMDARs have been implicated in neurite outgrowth and neuron migration early in development, though the mechanisms underlying these functions are unclear (Behar et al., 1999; Manent et al., 2005; Manent et al., 2006). In *Xenopus* spinal neurons, NMDARs influence filopodial dynamics and growth cone turning (Zheng et al., 1996). While this has yet to be demonstrated in mammalian systems, a similar effect would be conceivable due to the significant roles of localized calcium signaling on growth cone motility. Numerous signaling molecules implicated in growth cone functions have associations with NMDARs. Calcium/calmodulin-dependent protein kinase II (CaMKII) has critical roles in development and in synaptic plasticity (for review see Fink and Meyer, 2002; Lee, 2006). CaMKII isoforms are enriched at growth cones and function in various aspects of neurite outgrowth and growth cone motility (for review, see Zheng and Poo, 2007). Interestingly, CaMKII, calcineurin (CaN) and phosphatase-1 (PP1) have been shown to mediate calcium-dependent growth cone guidance (Wen et al., 2004). Generally, larger local calcium elevations activate CaMKII to promote growth cone attraction, while relatively lower levels promote repulsion via CaN-PP1 (Fink et al., 2003). αCaMKII, the most abundant CaMKII isoform, has been implicated in mechanisms of NMDAR-mediated postsynaptic plasticity (Zhou et al., 2007), while βCaMKII regulates neurite outgrowth and synapse formation (Fink et al., 2003). It is possible that calcium influx at growth cones via NMDARs activity mediates βCaMKII activity.
The various Rho GTPases (including Cdc42, Rac, and Rho) have also been implicated in essential developmental mechanisms including neurite outgrowth, neuronal migration, axon guidance, and growth cone motility (Luo, 2000; Fukata et al., 2003). For example, RhoA is involved in growth cone collapse and repulsion, while Rac and Cdc4d are involved in growth cone attraction (Dickson, 2001).

NMDARs have been implicated in the activity of Rho GTPases. Using in vivo time-lapse imaging of Xenopus laevis tadpoles, Dr. Hollis Cline’s laboratory has shown that NMDAR activity is required for Rho GTPase-mediated dendritic growth (Sin et al., 2002). MAGUKs directly associate with various effectors of Rho GTPases (such as SynGAP, kalirin-7, and citron) potentially providing direct links to NMDAR activity (Chen et al., 1998; Kim et al., 1998; Furuyashiki et al., 1999; Zhang et al., 1999; Penzes et al., 2001). Additionally, activation of VSCCs, intracellular calcium stores, and changes in membrane potential shifts have been implicated in various growth cone-mediated dynamics (Nishiyama et al., 2008, and for review, Zheng and Poo, 2007). While our data did not show a significant change in calcium transients before and after blocking VGCCs or replenishment of intracellular stores, it is possible that these elements provide some contribution to NMDAR function at growth cones. Clearly, local NMDAR activity could potentially be a critical factor in mediating many signaling mechanisms at growth cones.

4.4.3 Transient expression of NMDARs at axons and growth cones

Our data indicate that NMDARs are present at axons earlier, but not later, in development. The selective localization of proteins to dendritic or axonal regions is a
highly regulated process. Three primary mechanisms have been proposed to achieve molecular polarization: selective targeting of proteins to axonal or dendritic surfaces, non-specific targeting followed by selective retention/removal from axons or dendrites, and trancytosis from dendritic to axonal compartments. For example, VAMP2 traffics to the surface of both axons and dendrites early in development, but is preferentially endocytosed from the dendritic membrane due to an intracellular motif (Sampo et al., 2003). Wisco et al. (2003) and Yap et al. (2008), indicate that NgCAM traffics to dendrites and subsequently undergoes translocation to axons via endosomal recycling vesicles, while Sampo et al. (2003) indicate that axonal targeting of NgCAM is directly related to the presence of its extracellular FN3 domains. KIF5 (a member of the kinesin superfamily) is selectively targeted to axons (Nakata and Hirokawa, 2003), whereas KIF17 has been implicated in the dendritic targeting of NR2B via PDZ-associated interactions (Setou et al., 2000; Guillaud et al., 2003). The mechanism underlying this developmental shift in NMDAR polarization is not well understood, though a recent study gives some new insights into this process (Song et al., 2009). The authors show that the establishment of an ankyrin G- and F-actin-dependent cytoskeletal filter in the axon initial segment (AIS) selectively excludes KIF17-mediated axonal NR2B trafficking. Passage through this filter is dependent upon characteristics of motors, as KIF5-mediated VAMP2 transport and a KIF5/KIF17 chimera that binds NR2B successfully traffic to the axon. Disruption of F-actin removes such barriers (previously described in Winckler et al., 1999), thereby allowing the molecules to pass through the AIS. Knockdown of KIF5 decreases total NR2B levels by 30% while increasing NR2A expression by 24% (Guillaud et al.,
2003), indicating that KIF5-mediated transport contributes to only a portion of NMDAR trafficking.

NMDAR trafficking involves numerous reported mechanisms and protein associations that are likely working in concert to mediate this highly regulated and intricate process (for review, see Wenthold et al., 2008). In Chapters 2 and 3, we described the critical importance of CAMs in various mechanisms of neuronal development. NMDARs interact (either directly or indirectly) with numerous families of CAMs to mediate synaptic activity and plasticity, including cadherins and neuroligins (for review, see Huntley et al., 2002; Craig and Kang, 2007; Wenthold et al., 2008). While the implications of these NMDAR-CAM interactions at synapses have been well studied, their roles early in development are largely unknown. The direct interaction between SALMs and NMDARs is of particular interest, and could potentially function in several aspects of neuronal development, including the selective polarization of the molecules.

### 4.4.4 NMDAR function during development and disease

NMDARs have a wide variety of implications on neurological disorders. Unregulated NMDAR activity leads to excitotoxicity and cell death via excessive calcium influx. The mechanism of this excitotoxicity may involve characteristics of NMDAR subunit composition and subcellular localization (Hardingham and Bading, 2002; Lynch and Guttmann, 2002). Numerous studies have also linked NMDAR activity with Huntington’s disease, Alzheimer’s disease, Parkinson’s disease, autism
spectrum disorders, and schizophrenia (for review, see Lau and Zukin, 2007; Wenthold et al., 2008). While several of the neurological disorders listed above are characterized by progressive neurological decline that may not be developmentally regulated, an encompassing implication of this chapter is that NMDAR trafficking and function in development are highly regulated phenomena with ties to various associated proteins and signaling pathways that are critical for proper synaptic function. A dysregulation of this NMDAR trafficking and function at growth cones and/or axons could have severe effects on proper synaptic development. The continued study of NMDAR developmental biology may provide useful insights into the cause of neurological disorders in the future.
Chapter 5: General discussion

Many proteins have roles in both neurite outgrowth and synapse formation (two distinct, but highly interconnected mechanisms that provide a framework for brain function in the CNS). While the intricacies of the molecular cues guiding these processes are continually being deciphered, the transition from neurite outgrowth to synapse formation is still a topic of great speculation. One strategy to elucidate this connection is to investigate the factors that are involved in both processes.

The studies in this dissertation show that SALMs and NMDARs have multiple roles in development. SALMs promote aspects of neurite outgrowth (Chapter 2), and accumulate at interneurite crossing points (Chapter 3) in young neurons. SALM1 and SALM2 have previously been shown to influence synapse formation/morphology (Ko et al., 2006; Wang et al., 2006). Figure 5.1 illustrates the known functional characteristics of SALMs. Much of the evidence thus far regarding SALM interactions and function has involved in vitro molecular biological or biochemical techniques. The potentially unique functions among the individual family members indicate that the use of transgenic technology may be beneficial to further examine the roles of SALMs, in vivo. With the numerous implications of SALMs in developmental and synaptic phenomena, it would be interesting to examine the performance of SALM-transgenic animals on learning and memory tasks. The generation of transgenic animals could also be very useful to examine the roles of SALMs in mechanisms of synaptic plasticity, such as LTP or LTD through electrophysiology.
Figure 5.1: Functional characteristics of SALMs. A schematic diagram illustrating the known functions of SALMs. The various SALMs have distinct roles throughout development. Early in development, SALMs promote neurite outgrowth and various kinds of neurite differentiation (1A-F). Later in development, SALMs are involved in synapse formation/stabilization (2A-E).
Figure 5.1: Functional characteristics of SALMs

(Wang and Wenthold, 2009)
The roles of SALMs in synaptic transmission are largely unknown. In Ko et al. (2008), the authors show that overexpression of SALM2 had no effect on mEPSCs, but knocking down SALM2 decreased the frequency, but not amplitude of the mEPSCs, indicating a reduction in the number of excitatory synapses. Future studies could also include examining the effects of overexpressing or knocking down the other SALM proteins (or knocking down all of them) on spontaneous or evoked currents. Collectively, the work in this dissertation raises many interesting questions that could be the foundation for numerous future studies involving SALMs.

In Chapter 4, we showed that NMDARs function at axonal growth cones of young hippocampal neurons. Early in development, cytoplasmic calcium levels are critical regulators of neurite outgrowth and growth cone turning and motility. Our calcium imaging experiments showed that NMDARs mediate localized calcium influx at growth cones, indicating that they may contribute to such motility-based phenomena. NMDARs have been implicated in growth cone turning in *Xenopus* spinal neurons (Zheng et al., 1996), but their roles in mammalian neurons are unknown. We have begun preliminary work to repeat these experiments with rat primary hippocampal cultures, using the methods presented in Zheng et al. (1996). Under the guidance of Dr. Brenowitz, we are currently in the process of refining the experimental apparatus in the laboratory to systematically perform these investigations in growth cone turning and motility. In the process of this preliminary work, we made an unexpected observation. When we continually applied NMDA to the cell body of young neurons, there was a retraction of the axonal growth cone and axon (Figure 5.2A, see figure
legend for further experimental details). Specifically, we applied 200 μM NMDA to the cell body by pressure application, for 50 ms at a rate of 2 Hz. This retraction was reversible, as the growth cones proceeded to grow and elongate following termination of the NMDA application (Figure 5.2B). This ability to recover also indicates that the effects are not due to NMDAR-mediated excitotoxicity. Furthermore, application of 100 μM DL-APV to the chamber inhibits this retraction, indicating that this is an NMDAR-specific phenomenon. Interestingly, a recent paper showed that glutamate application to the cell body of young dentate granule cells induced axonal retraction (Yamada et al., 2008). The authors indicated that this retraction is due to somatic AMPA receptors, as application of NMDA or the type II metabotropic glutamate receptor agonist, ACPD, had no effect. Upon examination of their protocols, they apparently performed their experiments using Neurobasal medium, which contains 800 μM MgCl₂, in the extracellular solution. It is possible that they did not see an effect of NMDA application due to this factor, as the majority of the NMDAR activity would be inhibited due to the voltage-sensitive magnesium block. While these data do not directly indicate the role of the NMDARs at growth cones, it does give us insights into another role of NMDARs in development.
Figure 5.2: Somatic NMDAR activity promotes axonal retraction in young neurons. NMDA (200 μM) was continually applied to the soma of DIV4 hippocampal cultures at 50 ms intervals at a rate of 2 Hz. Time-lapse sequences of DIC images were collected using a Retiga 2000R CCD camera (QImaging) with the aid of QCapture Pro software at a rate of 1 frame every 10 seconds, for 30 minute trials. (A) Chronic activation of somatic NMDARs induces growth cone/axonal retraction, initially characterized by a rounding of the growth cone (arrow). (B) Growth cone morphology was restored (arrow), and axonal outgrowth resumed following cessation of NMDA application. The blue dashed line is provided for spatial reference. (C) DIC image of the application system.
It is possible that the NMDARs at growth cones and at the soma work together in a highly coordinated manner to regulate growth cone turning and axonal outgrowth/retraction through region-specific, localized, calcium influx and/or activation of intracellular signaling pathways. Therefore, NMDARs are functional at growth cones (Chapter 4) and at the soma (Figure 5.2) early in development, and are critical mediators of excitatory synaptic transmission later in development (for review, see Corlew et al., 2008; Wenthold et al., 2008). Future studies could entail elucidating the roles of growth cone-resident and somatic NMDARs on neurite motility, and investigating the potential relation between NMDARs at growth cones and presynaptic NMDARs.

Previous studies have shown that many proteins have multiple important roles throughout development. For example, DASM1 (dendrite arborization and synapse maturation 1) is an Ig, FN3, and PDZ-BD containing transmembrane protein and is involved in promoting both dendritic arborization early in development, and synapse maturation later in development (Shi et al., 2004a; Shi et al., 2004b). DASM1 functions in silent synapse formation by regulating AMPA receptor-mediated synaptic transmission through PDZ interactions with Shank and S-CAM, two PDZ domain proteins that are involved in spine maturation (Shi et al., 2004a). GRIP1 (glutamate receptor interacting protein 1) is an AMPA receptor-associated multi-PDZ domain protein that mediates the formation and outgrowth of dendrites in young neurons by regulating EphB receptor trafficking (Hoogenraad et al., 2005). In mature neurons, GRIP1 is involved in synaptic trafficking and stabilization of AMPARs (for
review, see Song and Huganir, 2002; Bredt and Nicoll, 2003). In young neurons, PSD-95 regulates dendritic branching (Charych et al., 2006). Among its many diverse synaptic roles, PSD-95 regulates synaptic localization of membrane proteins (Cline, 2005; Han and Kim, 2008), contributes to the formation and remodeling of the PSD (Marrs et al., 2001), and PSD-95 overexpression increases excitatory synapse formation (Ehrlich and Malinow, 2004).

This discussion suggests that some of the key players involved in synapse formation/stabilization and neurite outgrowth are shared, but also raises questions about how these two processes are differentially regulated. For example, what factors determine the proportions of SALMs and NMDARs dedicated to synapse formation compared to neurite outgrowth? What developmental change signals their switch in functions? Do different protein interactions regulate outgrowth earlier in development, while others regulate synapse formation? Specifically, do SALMs and NMDARs have different PDZ interactions for neurite outgrowth and synapse formation? SALMs are enriched on the surface of axonal and dendritic growth cones, though their polarization may be potentially regulated by PDZ interactions. NMDARs are localized to axonal growth cones early in development, but are absent from distal axons later in development. Does the contact of a axonal growth cone to an impending postsynaptic surface signal a change in SALM and/or NMDAR function, thereby switching modalities to initiate synapse formation? Alternatively, perhaps the mechanistic switch from neurite outgrowth to synapse formation is not so distinct, but rather a fluid transition in the process of development that utilizes the
same underlying machinery. Wang et al. (2006) indicated that SALM1 directly interacts with NR1. Perhaps SALMs and NMDARs directly interact and function at growth cones. Thus, there is an abundance of questions regarding both SALM and NMDAR function and the connection between neurite outgrowth and synapse formation. Striving to find the answers will lead to a greater understanding of synaptic proteins, such as CAMs and neurotransmitter receptors, and the development of the nervous system.
Chapter 6: Conclusions

Neuronal development depends on highly regulated coordination of events mediated by diverse classes of proteins. One of the central themes of this dissertation involves the dual (or multiple) roles of synaptic proteins during development and that the study of proteins with roles in both mechanisms may help unravel the mysteries connecting them.

First, I demonstrated that SALMs can promote neurite outgrowth when overexpressed in young hippocampal neurons. The various SALMs have the ability to mediate distinct changes in outgrowth characteristics, including increases in neurite branching, process length, and neuritogenesis.

Next, I performed a collection of experiments to examine the roles of SALMs at a developmental time point subsequent to neurite outgrowth. I found that SALMs accumulate at neuronal cell-cell adhesions, and may potentially participate in the initial stages of synapse formation.

Finally, I performed an examination of NMDARs at axonal growth cones of young hippocampal neurons in culture. The data indicate that NMDARs are present and functional at axonal growth cones, and that activation of NMDARs at growth cones elicits localized calcium influx. Interestingly, the expression of NMDARs at axons and growth cones early in development appears to be transient, as NMDAR subunits are not detected by immunocytochemistry at distal axons of older neurons.
These studies help elucidate functions and fundamental aspects of SALM biology, while providing new insights into the roles NMDARs early in development. These studies additionally raise a wide variety of interesting questions that may hopefully be utilized as the foundation of future studies, including examining direct interactions between SALMs and NMDAR subunits at growth cones, and at synapses later in development.
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