Recruitment of synaptic vesicles to developing synapses

by

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Synapse formation begins with the recognition of appropriate targets and formation of incipient contacts, and culminates with the recruitment of pre- and postsynaptic proteins to points of cell-cell contact. It is still unclear how cell-cell contact translates into the recruitment of synaptic proteins. Previous studies have shown that the cadherin/β-catenin cell adhesion complex plays an important role in localizing synaptic vesicles to developing synapses. This dissertation discusses work elucidating the transduction pathway that is activated following cell-cell contact, leading to the recruitment and retention of synaptic vesicles to presynaptic compartments. Using rat and mouse primary hippocampal cultures as a model system, we have demonstrated that β-catenin mediates the localization of synaptic vesicles to synapses through its recruitment of the PDZ scaffold protein, scribble, and its subsequent recruitment of the Rac/Cdc42 guanine nucleotide exchange factor, β-pix. We further demonstrate that β-pix enhances actin polymerization at these discrete sites, which is important for the “trapping” of synaptic vesicles as they translocate along the axon. We have demonstrated that cadherin, β-catenin, scribble, and β-pix form a complex at developing synapses using immunohistochemistry coupled with immunoprecipitation assays using synaptosomal fractions. Knockdown of β-catenin, scribble or β-pix using RNA interference (RNAi) disrupts the appropriate localization of synaptic vesicles at synapses. We have ordered this pathway and have shown that β-catenin is important for the recruitment and clustering of scribble to synapses, but that scribble knockdown does not affect β-catenin localization. We have also demonstrated that scribble knockdown disrupts the clustering of β-pix at synaptic sites. This complex has shown to control vesicle localization through β-pix-mediated enhancement of actin polymerization at these discrete sites. Indeed, β-pix knockdown results in decreased actin polymerization at synapses. Importantly, restoring actin polymerization at synapses through cortactin overexpression rescues the mislocalization of synaptic vesicles. This work provides novel insights into the molecular and cellular mechanisms underlying the development presynaptic compartments.
The work in chapter 2, entitled “Scribble interacts with β-catenin to localize synaptic vesicles to synapses” has been published as:


All experiments and data analysis for this manuscript was done by myself with following exceptions: Immunocytochemical experiments in Figure 2.2E-M was done by Dr. Shernaz X. Bamji, the senior author in this manuscript. Immunocytochemical experiments and data analysis in Figure 2.4C-F and Figure 2.6 were done with the assistance of the second author, Mytyl Aiga. Validation of scribble RNA interference constructs in Figure 2.5 was done by the third author, Eileen Yoshida. A subset of the antibodies and DNA constructs for scribble were generously provided by our collaborator, Dr. Patrick O. Humbert. The manuscript was written and figures prepared by me under the supervision of the senior author, Dr. Shernaz X. Bamji.

The work in chapter 3, entitled “β-pix modulates actin-mediated recruitment of synaptic vesicles to synapses” is currently under revision as:


All the experiments and data analysis were done by myself. The manuscript was written and figures prepared by me under the supervision of the senior author, Dr. Shernaz X. Bamji.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>PREFACE</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xi</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 PRIMARY CULTURED HIPPOCAMPAL NEURONS</td>
<td>1</td>
</tr>
<tr>
<td>1.2 THE CNS SYNAPSE</td>
<td>3</td>
</tr>
<tr>
<td>1.2.1 The presynaptic terminal</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2 The postsynaptic terminal</td>
<td>9</td>
</tr>
<tr>
<td>1.2.3 Synaptogenesis</td>
<td>11</td>
</tr>
<tr>
<td>1.3 CADHERIN/B-CATENIN ADHESION COMPLEXES</td>
<td>17</td>
</tr>
<tr>
<td>1.3.1 The cadherin and catenin family</td>
<td>17</td>
</tr>
<tr>
<td>1.3.2 Localization of cadherin and β-catenin at synapses</td>
<td>18</td>
</tr>
<tr>
<td>1.3.3 Function of cadherin at synapses</td>
<td>19</td>
</tr>
<tr>
<td>1.3.4 Function of β-catenin at synapses</td>
<td>21</td>
</tr>
<tr>
<td>1.4 SCRIBBLE</td>
<td>25</td>
</tr>
<tr>
<td>1.4.1 Structure of scribble</td>
<td>25</td>
</tr>
<tr>
<td>1.4.2 Localization of scribble at synapses</td>
<td>26</td>
</tr>
<tr>
<td>1.4.3 Function of scribble at synapses</td>
<td>26</td>
</tr>
<tr>
<td>1.5 β-PIX</td>
<td>27</td>
</tr>
<tr>
<td>1.5.1 Structure of β-pix</td>
<td>27</td>
</tr>
<tr>
<td>1.5.2 Localization of β-pix at synapses</td>
<td>28</td>
</tr>
<tr>
<td>1.5.3 Function of β-pix at synapses</td>
<td>29</td>
</tr>
<tr>
<td>1.6 THE ACTIN CYTOSKELETON</td>
<td>31</td>
</tr>
<tr>
<td>1.6.1 Regulation of actin organization</td>
<td>31</td>
</tr>
<tr>
<td>1.6.2 Localization of actin at synapses</td>
<td>34</td>
</tr>
<tr>
<td>1.6.3 Function of actin at presynaptic compartments</td>
<td>34</td>
</tr>
<tr>
<td>1.6.4 Function of actin at postsynaptic compartments</td>
<td>38</td>
</tr>
<tr>
<td>1.6.5 Actin and synaptic plasticity</td>
<td>40</td>
</tr>
</tbody>
</table>
2. SCRIBBLE INTERACTS WITH β-CATENIN TO LOCALIZE SYNAPTIC VESICLES TO SYNAPSES

2.1 INTRODUCTION ................................................................. 43
2.2 MATERIALS AND METHODS ................................................ 46
   2.2.1 RNAi constructs and recombinant DNAs ................................. 46
   2.2.2 Neuron cultures ................................................................ 46
   2.2.3 Semi-quantitative RT-PCR .................................................. 47
   2.2.4 Immunohistochemistry ...................................................... 47
   2.2.5 FM 4-64 analyses ........................................................... 47
   2.2.6 Immunoblot analysis ......................................................... 48
   2.2.7 Generation of GST fusion proteins and GST pull down assays .... 49
   2.2.8 Confocal imaging ............................................................ 49
   2.2.9 Image analysis and quantification ........................................ 49
2.3 RESULTS .............................................................................. 52
   2.3.1 Scribble interacts with β-catenin and localizes at synapses ........ 52
   2.3.2 SVs are mislocalized in hippocampal neurons lacking scribble ...... 57
   2.3.3 SV recycling defects in cultured hippocampal neurons lacking scribble 66
   2.3.4 β-catenin localizes scribble to synapses ................................. 68
2.4 DISCUSSION ........................................................................ 73
   2.4.1 Scribble interacts with β-catenin at synapses ......................... 74
   2.4.2 Role of scribble in the synaptic localization of SVs ............... 74

3. β-PIX MODULATES ACTIN-MEDIATED RECRUITMENT OF SYNAPTIC VESICLES TO SYNAPSES

3.1 INTRODUCTION ................................................................. 77
3.2 MATERIALS AND METHODS ................................................ 79
   3.2.1 RNAi constructs and recombinant DNAs ................................. 79
   3.2.2 Neuron cultures ................................................................ 79
   3.2.3 Immunohistochemistry ...................................................... 79
   3.2.4 FM 4-64 analyses ........................................................... 80
   3.2.5 Immunoblot analysis ......................................................... 80
   3.2.6 Confocal imaging ............................................................ 81
   3.2.7 Image analysis and quantification ........................................ 81
3.3 RESULTS .............................................................................. 83
   3.3.1 Increase actin polymerization enhances SV clustering ............. 83
   3.3.2 β-pix interacts with cadherin/β-catenin/scrabble complexes at synapses 89
   3.3.3 Localization of β-pix at synapses is decreased in scribble knockdown cells 90
   3.3.4 Perturbation of β-pix activity disrupts synaptic actin .................. 91
   3.3.5 Perturbation of β-pix activity disrupts the localization of SVs at synapses .... 93
   3.3.6 Cortactin overexpression rescues the SV clustering phenotype observed in β-pix knockdown cells ................................................................. 96
3.4 DISCUSSION ....................................................................... 98
   3.4.1 Actin localizes synaptic vesicles to developing synapses ........ 98
   3.4.2 Scribble regulates β-pix localization at synapses .................... 99
   3.4.3 β-pix regulates actin polymerization at synapses .................... 100
3.4.4 Dynamic regulation of cadherin adhesion complexes and sv localization .... 100

4. CONCLUSION ............................................................................................................. 102
  4.1 OTHER SIGNALS THAT LOCALIZE SYNAPTIC VESICLES .......... 102
  4.2 LOCALIZATION OF PICCOLO-BASSOON TRANSPORT VESICLES .......................................................................................................................... 103
  4.3 TRANSSYNAPTIC EFFECTS ................................................................................. 104
  4.4 THE ROLE OF THIS COMPLEX AT MATURE SYNAPSES .......... 104
  4.5 SIGNIFICANCE ..................................................................................................... 105

BIBLIOGRAPHY ............................................................................................................. 106
LIST OF TABLES

Table 1 Analysis of scribble localization at synapses.......................................................... 53
LIST OF FIGURES

Figure 1.1: The trisynaptic circuit of the hippocampus ........................................ 1
Figure 1.2: A schematic of a CNS glutamatergic synapse. ........................................ 4
Figure 1.3: Ultrastructure of a synapse in 15DIV hippocampal culture ....................... 4
Figure 1.4: Molecular model of an average CNS synaptic vesicle .............................. 5
Figure 1.5: The vesicle cycle .................................................................................. 7
Figure 1.6: Schematic diagram of interactions of CAZ proteins and the resulting network at the active zone ................................................................. 8
Figure 1.7: Morphology of dendritic protrusions, filopodia and spines ....................... 9
Figure 1.8: Organization of proteins and protein-protein interactions in the PSD. 11
Figure 1.9: Multiple cellular mechanisms for CNS synaptogenesis ........................... 15
Figure 1.10: Schematic of the structure of classic cadherins .................................. 17
Figure 1.11: Schematic of the structure of β-catenin ............................................ 22
Figure 1.12: Schematic of the structure of human scribble .................................... 25
Figure 1.13: Schematic of different isoforms of PIX .............................................. 28
Figure 1.14: The turnover of an actin filament ...................................................... 31
Figure 1.15: The actin polymerization machinery triggers dendritic nucleation for protrusion at the leading edge ................................................................. 33
Figure 1.16: Signaling pathways from Rho, Rac, and Cdc42 to the regulation of actin polymerization and depolymerization ............................................... 39

Figure 2.1: Scribble immunoreactivity in cultured hippocampal neurons .................. 53
Figure 2.2: Scribble localizes to synapses ............................................................ 54
Figure 2.3: Scribble associates with β-catenin at synapses ..................................... 56
Figure 2.4: RNAi-mediated knockdown of scribble protein levels in primary neurons ............................................................................................................. 59
Figure 2.5: Scribble mRNA level is decreased in RNAi-1 expressing neurons .......... 60
Figure 2.6: SVs are more diffusely distributed along the axon in neurons expressing scribble RNAi constructs ................................................................. 64
Figure 2. 7: Density, area and intensity of bassoon immunoreactive puncta remain constant in RNAi expressing cells. ................................................................. 65
Figure 2. 8: Deficits in SV recycling following scribble knockdown. ..................... 67
Figure 2. 9: β-catenin localization is not affected in scribble RNAi-expressing cells. ................................................................................................................. 69
Figure 2. 10: Scribble is diffusely distributed along the axon in cells lacking β-catenin. ................................................................. 71

Figure 3. 1: GFP-actin and Syn-RFP are localized to synapses. ......................... 84
Figure 3. 2: The density and IntDen of GFP-actin and Syn-RFP clusters are increased following ALLN treatment. ................................................................. 86
Figure 3. 3: The density and IntDen of GFP-actin and Syn-RFP clusters are increased in neurons expressing cortactin.......................... 88
Figure 3. 4: β-pix interacts with cadherin, β-catenin and scribble at synapses. ...... 90
Figure 3. 5: The IntDen of synaptic GFP-actin clusters is decreased in neurons with reduced β-pix activity................................................................. 92
Figure 3. 6: The localization of synaptic vesicles at synapses is disrupted in neurons with reduced β-pix activity................................................................. 95
Figure 3. 7: Cortactin expression rescues the disruption in synaptic vesicle localization in β-pix knockdown cells. .................................................. 97

Figure 4. 1: Schematic of SV assembly at presynaptic compartments.................. 102
LIST OF ABBREVIATIONS

ADF/cofilin – actin depolymerizing factor/cofilin
AMPA – α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
Cad – cadherin
CAM – cell adhesion molecule
CNS – central nervous system
DIV – day in vitro
GABA - γ-aminobutyric acid
GFP – green fluorescent protein
GlyR – glycine receptor
FRET - fluorescence resonance energy transfer
NMDA – N-methyl-D-aspartic acid
GABA – gamma-aminobutyric acid
HA – haemagglutinin
HBSS – hank’s balanced salt solution
HEK – human embryonic kidney
LIMK – LIM kinase
LTP – long-term potentiation
LTD – long-term depression
mEPSC – miniature excitatory postsynaptic current
PBS – phosphate buffered saline
PDZ – PSD-95/Disc-large/ZO-1
PSD-95 – Postsynaptic density
PSD-95 – Postsynaptic density protein 95
RNAi – RNA interference
S-SCAM – Synaptic scaffolding molecule
SV – synaptic vesicle
VAMP – vesicle-associated membrane protein
VGLUT-1 – Vesicular glutamate transporter-1
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1. INTRODUCTION

1.1 PRIMARY CULTURED HIPPOCAMPAL NEURONS

The hippocampus is a part of the limbic system in the brain and consists of two main interlocking parts, the Ammon’s horn and the dentate gyrus (DG). The Ammon’s horn or Cornu Ammonis (CA), also known as the hippocampus proper, consists primarily of pyramidal neurons, while the dentate gyrus consists primarily of granule cells. The CA region and dentate gyrus are interconnected to each other and to neighboring entorhinal cortex through the trisynaptic circuit (Fig. 1.1). Simplified, the surface layers of entorhinal cortex project to DG granule cells through the perforant pathway. DG granule cells then project to pyramidal neurons in the CA3 region via the mossy fiber pathway. CA3 pyramidal neurons project and synapse onto CA1 pyramidal neurons via the Schaffer collateral pathway.

Figure 1. 1: The trisynaptic circuit of the hippocampus.

The major input is carried by axons of the perforant path, which convey polymodal sensory information from neurons in layer II of the entorhinal cortex to the dentate gyrus. Perforant path axons make excitatory synaptic contact with the dendrites of granule cells. Granule cells project, through their axons (the mossy fibers), to the proximal apical dendrites of CA3 pyramidal cells, which, in turn, project to CA1 pyramidal cells through Schaffer collaterals and to CA3 and CA1 pyramidal cells through commissural connections. Adapted with permission from (Neves et al., 2008).
Hippocampal neurons were first dissociated and successfully grown in culture by Dr. Banker in 1977 (Banker and Cowan, 1977). Since then, primary hippocampal cultures have become one of the most popular model systems to study synapse formation and remodeling. There are a number of reasons for this. First, these cultures are homogenous and make physiologically relevant neuronal connections. Hippocampal cultures are typically prepared from the CA1-3 region of the hippocampus at embryonic day 17-18 (E17-18). At this stage of development, approximately 90% of the neurons are pyramidal neurons, with few interneurons and nonneuronal cells (Kaech and Banker, 2006). Second, cultured hippocampal neurons exhibit key phenotypic features that resemble those observed in vivo. For instance, they form dendritic spines and make extensive, synaptically connected networks. Third, compared to in vivo models which are extremely complex, hippocampal cultures are simple, relatively easy to genetically and pharmacologically manipulate, and easy to image. Fourth, over the past three decades, hippocampal cultures have been well characterized and the results are consistent from laboratory to laboratory, which provide an extensive database for new studies in these cultures. Based on these reasons, in this dissertation, primary cultured hippocampal neurons were used as a model system to examine the molecular mechanism underlying synapse assembly.
1.2 THE CNS SYNAPSE

The human brain contains approximately 50 to 100 billion neurons, that are organized into an intricate network through specialized sites called synapses. A synapse is the point of contact between neurons that allows neurons to propagate electrical and chemical signals from one to another. Synapses are typically characterized by the neurotransmitters that they utilize to propagate signals. Commonly found neurotransmitters include glutamate, GABA, glycine, acetylcholine, dopamine and serotonin. Glutamatergic synapses, transmit the majority of excitatory signals in the brain, and are often referred to as “excitatory synapses”, while GABAergic synapses, transmit the majority of inhibitory signals, are often referred to as “inhibitory synapses”. Excitatory synapses are formed between axons and dendritic spines and generate excitatory postsynaptic potentials that increase the probability of the postsynaptic neuron to reach action potential threshold (Levinson and El-Husseini, 2005). In contrast, inhibitory synapses are formed on the shaft of dendrites and generate inhibitory postsynaptic potentials that decrease the probability of the postsynaptic neuron to reach action potential threshold (Levinson and El-Husseini, 2005). In addition to the neurotransmitters and neurotransmitter receptors utilized at these specialized synapses, excitatory and inhibitory synapses also differ in morphology and protein composition (Levinson and El-Husseini, 2005). This current dissertation will only focus on glutamatergic synapses.

Synapses are composed of several specialized domains, including the presynaptic compartment, the synaptic cleft and the postsynaptic terminal (Fig 1.2). Presynaptic compartments are typically formed within small varicosities that decorate the length of axons. On the ultrastructural level, the presynaptic terminal is characterized by the presence of hundreds of synaptic vesicles (SVs) filled with neurotransmitters (Fig 1.3). A few SVs are in physical contact with the plasma membrane at release sites, also called active zones. Action potentials depolarize presynaptic membranes and result in the fusion of SVs with the presynaptic membrane and the release of neurotransmitters into the synaptic cleft; a narrow gap approximately 20 nm in width between the pre- and postsynaptic neurons. Released neurotransmitters diffuse across the cleft and bind to their receptors anchored in the postsynaptic density (PSD), an electron dense meshwork of proteins at the postsynaptic membrane. The activation of these receptors then leads to depolarization of the postsynaptic membrane.
Figure 1. 2: A schematic of a CNS glutamatergic synapse.

Hundreds of synaptic vesicles are present in the presynaptic terminal. Some SVs are docked at the active zone. The presynaptic terminal is separated from the postsynaptic terminal by the synaptic cleft. A number of trans-synaptic adhesion molecules span this cleft and provide a molecular connection between the pre- and postsynaptic membranes. Glutamate receptors, including AMPA and NMDA receptors, are found in the postsynaptic membrane, where they are associated with a large number of scaffolding and signaling proteins that together comprise the postsynaptic density. Adapted with permission from (McAllister, 2007).

Figure 1. 3: Ultrastructure of a synapse in 15DIV hippocampal culture.

The synapse is formed onto a dendritic spine (SP), indicating that it is excitatory in nature. Docked synaptic vesicles can be seen along a prominent synaptic cleft (arrowheads). Adapted with permission from (Waites et al., 2005).

1.2.1 THE PRESYNAPTIC TERMINAL

Synaptic vesicles

Synaptic vesicles are small vesicles with a diameter range of 35-50 nm. Proteomic work has revealed the molecular composition of SVs (Fig. 1.4)(Takamori et al., 2006). SV proteins can be broadly categorized into four groups, that are not mutually exclusive, including: 1) integral SV membrane proteins, such as synaptophysin; 2) neurotransmitter transporters, such as VGLUT1-3 (vesicular glutamate transporter); 3) proteins involved in SV fusion, including the
calcium sensor, synaptotagmin and members of the SNARE (soluble NSF attachment protein receptors) complexes, synaptobrevin/VAMP (vesicle-associated membrane protein); and 4) proteins associated with SV surface, such as synapsins and Rab3 GTPases (Takamori et al., 2006).

During synaptogenesis, SV precursors appear in the axon (discussed in detail in section 1.2.3) and are recruited to points of cell-cell contacts. Although the signals that control SV accumulation at sites of contact are not fully understood, several lines of evidence have suggested that the cadherin/β-catenin cell adhesion complex plays a role in this process. This will be further discussed in detail in section 1.3.3 and 1.3.4.

Within the terminal, SVs can be functionally organized into three distinct pools: the readily releasable pool (RRP), the recycling pool and the reserve pool (RP) (Cingolani and

**Figure 1.4: Molecular model of an average CNS synaptic vesicle.**

An outside view of a SV based on space-filling models of all macromolecules at near atomic resolution. Adapted with permission from (Takamori et al., 2006).
The RRP constitutes approximately 1 to 2% of the total SVs in terminal. SVs in the RRP are thought to dock at the active zone and are immediately available for exocytosis. The RRP can be depleted upon high-frequency stimulation. The recycling pool constitutes approximately 5 to 50% of SVs and represents the total number of SVs that undergo exo- and endocytosis during stimulation. The RP constitutes approximately 50 to 90% of SVs in the terminal. The RP of SVs is accessible for neurotransmitter release during intense stimulation. It has been estimated that there are over 200 SVs per synapse in cultured hippocampal neurons, of which only 4 to 8 SVs are docked at active zone and 21 to 25 are involved in recycling, suggesting the majority of SVs in the terminal are at rest (Sudhof, 2004). The large number of resting vesicles may serve as a reservoir for the synapse to respond to repetitive stimulation.

Upon depolarization, docked SVs undergo fusion at the active zone. Following fusion, SVs may either be locally retrieved back into the terminal without loss of their identity (kiss-and-run), collapse into the presynaptic membrane (Fig. 1.5), or undergo one or several rounds of kiss-and-run fusion before complete fusion with the plasma membrane (Shupliakov and Brodin, 2010). The membrane of SVs undergoing complete fusion is retrieved by clathrin-mediated endocytosis and participates in the formation of new SVs (Fig. 1.5). The majority of these newly formed SVs are directly transported back to the SV cluster in the terminal, whereas a small population is transported to an endosomal compartment (Shupliakov and Brodin, 2010).
**Figure 1.5: The synaptic vesicle cycle.**

Synaptic vesicles partially or completely fuse with the presynaptic membrane at the active zone and release neurotransmitter into the synaptic cleft. The membrane of the fused vesicles then diffuses laterally to the areas outside the active zone where it is retrieved by clathrin-mediated endocytosis. Clathrin-coated vesicle formation involves several morphologically distinct steps, from clathrin coat binding, invagination of the coated bud, constriction and fission of the ‘neck’ of the bud and the subsequent stripping of the clathrin coat from the newly formed vesicle. The vesicle is then either directly transported back to the cluster of synaptic vesicles or translocated to a primary endosomal compartment. During endocytosis and migration to the release site vesicles are refilled with neurotransmitters. Adapted with permission from (Shupliakov and Brodin, 2010).

**Cytomatrix at the active zone**

The active zone is tightly associated with an electron-dense cytoskeletal matrix, called the cytomatrix at the active zone (CAZ). Electron micrographs of CNS synapses show that the CAZ forms a web-like structure extending approximately 50 nm into the cytoplasm (Schoch and Gundelfinger, 2006). A number of proteins have been shown to associate with the CAZ, including: 1) cell adhesion molecules, such as cadherins, neurexins, and integrins; 2) cytoskeletal proteins, such as actin; 3) scaffolding proteins, such as CASK, mint and veli; 4) proteins involved in SV fusion, such as Munc18 and members of the SNARE complex,
including SNAP25 (synaptosomal associated protein 25) and syntaxin; and 5) voltage-gated calcium channels (Schoch and Gundelfinger, 2006). So far, five protein families that are enriched in the CAZ have been functionally analyzed: the Munc13 family, RIMs (Rab3-interacting molecule), bassoon and piccolo, ELKS (ERC/CAST) and liprin-α (Schoch and Gundelfinger, 2006). These proteins interact with each other through multiple protein-protein binding domains, forming a large single complex (Fig. 1.6). This huge scaffold provides a platform for embedded SVs and are important regulators of synapse formation and plasticity (Schoch and Gundelfinger, 2006). For instance, mice expressing truncated bassoon exhibit a decrease in synaptic transmission and spontaneous epileptic seizures (Altrock et al., 2003).

Figure 1. 6: Schematic diagram of interactions of CAZ proteins and the resulting network at the active zone.
Reproduced with permission from (Schoch and Gundelfinger, 2006).
1.2.2 THE POSTSYNAPTIC TERMINAL

**Spines**

Glutamatergic synapses are typically formed onto dendritic spines - actin-rich protrusions that vary in length from 0.5-2 µm, and which have diverse morphologies (Svitkina et al., 2010). These include stubby spines, elongated thin spines, and mushroom spines, the most predominant form in the adult brain (Fig. 1.7). It has been proposed that spines emerge directly from dendritic filopodia-tapered membranous extensions, longer than 2 µm (Ziv and Smith, 1996). In support of this idea, the loss of filopodia during development is accompanied by an increase in the number of spines, and early dendritic filopodia become shorter and less motile, as animals mature (Portera-Cailliau et al., 2003; Zuo et al., 2005a; Zuo et al., 2005b). However, so far the link between dendritic filopodia and spines has not been directly shown. Several lines of evidence have shown that spines are critical in synapse development and function. Indeed, changes in the shape and size of spines are correlated with the strength of glutamatergic synaptic connections (Hotulainen and Hoogenraad, 2010). Moreover, alteration of spine morphology is found in patients with mental retardation and schizophrenia (Ramakers, 2002).

**Figure 1.7: Morphology of dendritic protrusions, filopodia and spines.**

Schematic representation of morphologies of a filopodium and three types of dendritic spine: thin type, stubby type and mushroom type. Gray disks represent the PSD structure and chains of red circles represent F-actin. Adapted with permission from (Sekino et al., 2007).
**Postsynaptic Density**

At the ultrastructural level, the postsynaptic terminal of a glutamatergic synapse is characterized by the presence of a postsynaptic density. The postsynaptic density is a specialized 30-40nm-thick microdomain precisely apposed to the presynaptic active zone. An estimate based on mass spectroscopy suggests that there are upwards of 600 proteins present in the postsynaptic density (Collins et al., 2006). These proteins can be categorized into several groups, including: 1) cell adhesion molecules, such as neuroligin and cadherins; 2) cytoskeletal proteins; 3) membrane-bound channels and neurotransmitter receptors, such as ionotropic glutamate receptors, the NMDARs (N-methyl-D-aspartic acid receptors) and AMPARs (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors); 4) scaffolding proteins and adaptor proteins, such as PSD-95 and ProSAP/Shank; as well as 5) signaling molecules, such as protein kinases and phosphatases (Fig. 1.8) (Boeckers, 2006). Neurotransmitter receptors and other proteins are tethered to the postsynaptic density through interactions with scaffolding proteins, which are critical regulators of synaptic function (Boeckers, 2006). For instance, PSD-95 is important for the synaptic localization of many postsynaptic components, including NMDARs, AMPARs, neuroligin, Shank and GKAP (guanylate kinase-associated protein), through which PSD-95 influences synaptic strength and plasticity (Keith and El-Husseini, 2008).
Figure 1.8: Organization of proteins and protein-protein interactions in the PSD.

Only major families and certain classes of PSD proteins are shown [in approximate stoichiometric ratio and scaled to molecular size, if known]. Domain structure is shown only for PSD-95 (PDZ domain, SH3 domain, GuK domain). Abbreviations: AKAP150, A-kinase anchoring protein 150 kDa; CAM, cell adhesion molecule; CaMKII, calcium/calmodulin-dependent protein kinase II; Fyn, a Src family tyrosine kinase; GKAP, guanylate kinase-associated protein; H, Homer; IRSp53, insulin receptor substrate 53 kDa; KCh, K+ channel; mGluR, metabotropic glutamate receptor; nNOS, neuronal nitric oxide synthase; RTK, receptor tyrosine kinases (e.g., ErbB4, TrkB); SPAR, spine-associated RapGAP. Adapted with permission from (Sheng and Hoogenraad, 2007).

1.2.3 SYNAPTOGENESIS

The formation of synapses in the vertebrate occurs over a protracted period of time, from embryonic development to postnatal life. Synapse formation can also occur in adults, and is thought to contribute to learning and memory. The establishment of synapses is believed to begin with cell-cell contact mediated by filopodia originating from axons and dendrites, followed by the recruitment of pre- and postsynaptic proteins to these points of cell-cell contact.
These nascent synapses then expand in size, with an increase in the volume of the pre- and postsynaptic terminals, the number of SVs, and the area of the active zone and PSD (Waites et al., 2005). Synapse maturation is marked morphologically by the appearance of spines and functionally by the appearance of AMPA-mediated postsynaptic currents (Waites et al., 2005). This dissertation specifically focuses on the initial assembly of synaptic components, which will be discussed in greater detail below.

Assembly of the presynaptic compartment

Time-lapse studies have shown that synapse formation can occur on a timescale of minutes (Friedman et al., 2000), suggesting the requirement of a rapid and efficient method of delivering synaptic proteins. Indeed, presynaptic proteins are transported in heterogeneous clusters of proteins, called “transport packets” (McAllister, 2007). These presynaptic precursors are present in axons before most synapses have formed (Ziv and Garner, 2004). In young neurons, two types of transport packets have been identified and characterized: PTVs (piccolo-bassoon transport vesicles) and STVs (SV protein transport vesicles).

PTVs are 80 nm dense-core vesicles that transport active zone proteins, including piccolo and bassoon, the synaptic adhesion molecule, N-cadherin, as well as proteins important for SV exocytosis, including Munc13, Munc18, syntaxin, and SNAP25 (Zhai et al., 2001). The movement of PTVs is saltatory, with an average velocity greater than 0.35 \( \mu \text{m/s} \) (Shapira et al., 2003). PTVs can move in both directions, occasionally split into smaller clusters, or coalesce into less mobile larger clusters (Shapira et al., 2003). Evidence suggests that active zones are assembled from proteins carried on 2-3 PTVs (Shapira et al., 2003).

STVs transport SV-associated proteins and other proteins important for exo- and endocytosis, such as VAMP-2, synapsin, synaptotagmin and calcium channels (Ahmari et al., 2000; Zhai et al., 2001). They are morphologically different than PTVs. The morphologies of STVs range from pleomorphic, tubulo-vesicular organelles (Ahmari et al., 2000) to clusters of SVs (Kraszewski et al., 1995). Rapid movement of STVs has been reported, ranging from 0.1-1 \( \mu \text{m/s} \) (Kraszewski et al., 1995; Dai and Peng, 1996; Nakata et al., 1998; Ahmari et al., 2000). STVs can split or coalesce and their movement is also saltatory (Kraszewski et al., 1995; Ahmari et al., 2000). Anterograde and retrograde transport have both been observed for STVs, suggesting that these packets are transported by multiple motor proteins (Kraszewski et al., 1995; Dai and Peng, 1996; Nakata et al., 1998; Ahmari et al., 2000). To date, three microtubule motors have been identified for STV anterograde transport, conventional kinesin-1, KIF1a and
KIF1Bβ2, whereas dynein has been shown to mediate the retrograde transport of STVs (Goldstein et al., 2008).

**Assembly of the postsynaptic compartment**

Similar to presynaptic proteins, many postsynaptic components, such as glutamate receptors and postsynaptic scaffolding proteins, are present in the dendrite before synapses have formed (Craig et al., 1993; Washbourne et al., 2002; Washbourne et al., 2004; Gerrow et al., 2006). However, inconsistent findings cloud our understanding on how these components assemble at points of cell-cell contact. In the case of NMDARs, several lines of evidence have led to two opposing theories. Some studies reveal that NMDARs are transported in discrete transport packets. Similar to presynaptic transport packets, these postsynaptic precursors move within dendrites bidirectionally with an average velocity of 6-8 um/s (Washbourne et al., 2002; Washbourne et al., 2004). Retrospective immunostaining suggests that many other proteins are also transported with these packets, including a scaffolding protein SAP-102, and an exocyst protein, Sec 8 (Sans et al., 2003; Washbourne et al., 2004). A subset of these NMDAR transport packets also carries AMPARs (Washbourne et al., 2002). In contrast, evidence using time-lapse imaging has suggested that the recruitment of NMDARs does not involve discernible discrete transport organelles (Bresler et al., 2004).

How the postsynaptic density assembles is also under debate. Several early studies have reported the presence of immobile clusters of PSD-95 in the dendrite prior to cell-cell contact (Rao et al., 1998; Friedman et al., 2000; Sans et al., 2000; Bresler et al., 2001; Marrs et al., 2001; Washbourne et al., 2002), whereas others have observed mobile PSD-95 clusters (Prange and Murphy, 2001; Gerrow et al., 2006). One study shows that these PSD-95 containing mobile organelles also carry other components of the postsynaptic density, such as GKAP and Shank (Gerrow et al., 2006). However, another study shows that the recruitment of Shank 2 and -3 does not involve discernible transport organelles (Bresler et al., 2004). It is unclear why different observations are seen in these time-lapse imaging studies. The difference could be attributable to different type of neurons and different age of cultures being used in these studies.

**Different models of synaptogenesis**

Recent application of fluorescently tagged proteins and time-lapse imaging has drawn our attention to the time course of synaptogenesis and allowed us to tease out the hierarchy of synaptic assembly. Several models have been proposed on how synapse formation may occur
based on four elegant studies (McAllister, 2007). In the study by Friedman et al., presynaptic differentiation occurs well before postsynaptic development (Friedman et al., 2000). In this model, STVs first accumulate to nascent synapses, followed by a sequential recruitment of the presynaptic active zone scaffold protein, bassoon, the postsynaptic scaffold protein, PSD-95, followed by AMPARs and NMDARs (Fig. 1.9a). In the study by Washbourne et al., pre- and postsynaptic proteins are recruited to sites of cell-cell contact simultaneously with a time course under 10 mins (Washbourne et al., 2002). In this model, NMDARs are recruited just prior to STVs. PSD-95 recruitment is less robust, sometimes coincident with NMDARs and sometimes not present even 1 hr following NMDAR recruitment (Fig. 1.9b). In the study by Gerrow et al., postsynaptic assembly precedes the recruitment of presynaptic proteins (Gerrow et al., 2006). In this model, PSD scaffolding proteins, PSD-95, GKAP and Shank and synaptic adhesion molecule neuroligin are transported via preassembled packets. These transport packets stabilize and then recruit STVs in 2 hrs (Fig. 1.9c). In the study by Sabo et al., synapses are formed at predefined sites along the axon (Sabo et al., 2006). In this model, STVs pause repeatedly at specific sites along the axon prior to postsynaptic contact and dendritic filopodia later selectively stabilize at these pause sites (Fig. 1.9d). Interestingly, these STVs undergo spontaneous fusion with the plasma membrane at the pause sites, suggesting that these STVs may release diffusible molecules, such as glutamate, which may stabilize rapidly extending and retracting postsynaptic filopodia. The disparity in the order of synaptic assembly could be attributable to different type of neurons and different age of cultures being used in these studies. Although a consensus has not yet been reached, these pioneer studies suggest that both axonal and dendritic filopodia possess the intrinsic ability to initiate synaptic assembly.

To complicate matters further, another study has challenged the necessity of axonal-dendritic contacts during synapse formation (Krueger et al., 2003). In this study, functional SV release sites that share the same exocytic and endocytic machinery as mature synapses are observed in isolated axons. Due to the lack of postsynaptic contracts, these SV release sites are termed “orphan synapses”.

Figure 1.9: Multiple cellular mechanisms for CNS synaptogenesis.
(a) Glutamatergic synapses can form in about an hour after the initial accumulation of STVs and PTVs. After ~30 min, PSD-95 accumulates at these sites followed by glutamate receptors. (b) Glutamatergic synapses can form even faster, on a timescale of several minutes. In young cortical neurons, STVs and NMDARs are both found in transport packets in the axons and dendrites, respectively, before synapse
formation. Contact between cells leads to the rapid and simultaneous recruitment of STVs and NMDARs at nascent synapses within ~7 min. PSD-95 is recruited to these sites with a variable time course, and AMPARs are recruited an hour following initial recruitment of NMDARs. (c) Glutamatergic synapses can also form at prespecified sites along dendritic shafts of hippocampal neurons, defined by stable preformed scaffold complexes associated with neuroligin. In this scenario, complexes of scaffolding proteins (including PSD-95, Shank, and GKAP) are mobile within dendrites before synapses are formed. When these complexes associate with neuroligin, they often become stabilized in the dendritic membrane. A significant proportion of these complexes then recruit STVs to form synapses within 2 h of their stabilization. (d) There are also predefined sites along the axon shaft where en passant synapses selectively form. These predefined sites are stable locations along the axon where STVs cycle with the plasma membrane and presumably release diffusible molecules before synapses are formed. Filopodia from dendritic growth cones are selectively attracted to, and stabilized at, these sites. Following stabilization of this contact at this predefined site, the presynaptic terminal is formed and additional pre- and postsynaptic proteins are recruited to form a nascent synapse. Adapted with permission from (McAllister, 2007).

**Synaptic cell adhesion molecules**

Pre- and postsynaptic membranes are held in apposition by transsynaptic cell adhesion molecules. This class of molecules includes the cadherin family, neurexin/neuroligin family, integrins, SynCAM, NCAM, SALMs (synaptic cell adhesion-like molecules) (Togashi et al., 2009) and the newly discovered LRRTMs (leucine rich-repeat transmembrane molecules) (Linhoff et al., 2009). CAMs have been considered to be attractive candidates to trigger synaptogenesis for several reasons. First, many of these molecules are present early in synapse development and are recruited to cell-cell contact sites before synapse have formed (Zhai et al., 2001; Gerrow et al., 2006). Secondly, CAMs are expressed trans-synaptically and can coordinate synapse differentiation bidirectionally. Thirdly, several CAMs have been shown to be capable of inducing presynaptic differentiation in co-culture assays and overexpression of these molecules in neurons increases the number of synapses (Waites et al., 2005). Among these molecules the cadherin family is one of the best studied and will be discussed in greater detail in section 1.3.
1.3 CADHERIN/B-CATENIN ADHESION COMPLEXES

1.3.1 THE CADHERIN AND CATENIN FAMILY

The cadherin superfamily includes cadherins, protocadherins, desmogleins, and desmocollins, Flaminogo/CELSRs and FAT (Takeichi, 2007). These members are characterized by the presence of extracellular cadherin repeats, containing the sequence important for calcium binding. However, the number of these repeats can vary greatly among members (Takeichi, 2007). This dissertation focuses on the classic cadherin subfamily. The classic cadherin subfamily can be further subdivided into types I and II based on the difference in their adhesive properties (Patel et al., 2006; Takeichi, 2007). With a few exceptions (Inuzuka et al., 1991), type I classic cadherins typically form homophilic interactions, whereas type II classic cadherins make both homo- and heterophilic interactions (Shimoyama et al., 2000). Classic cadherins are composed of five extracellular cadherin repeats, which mediate the interactions of cadherins, one transmembrane domain, and a short cytoplasmic tail, which interacts with catenin family (Fig.1.10). Classic cadherins have been shown to exert key roles in cell sorting, cell motility, neurite outgrowth, as well as synapse formation and maintenance (Shapiro et al., 2007).

![Figure 1.10: Schematic of the structure of classic cadherins.](image)

Catenins bind to the cytoplasmic tail of the classic cadherins. The catenins can be divided into three subfamilies, including the α-catenin subfamily, β-catenin subfamily and p120catenin subfamily (Arikkath, 2009). The α-catenin subfamily includes αN-catenin, αE-catenin, and αT-catenin. The β-catenin subfamily includes β-catenin and plakoglobin. The p120catenin subfamily includes δ-catenin, p120catenin, ARVCF (armadillo repeat gene deleted in velocardiofacial syndrome), and p0071. In addition to cadherins, catenins interact with variety of proteins including effectors of the actin cytoskeleton, PDZ (postsynaptic density-95/Discs large/zona occludens-1) proteins, protein kinases and phosphatases (Arikkath, 2009).
Catenins regulate cadherin-based adhesion, the trafficking of cadherins, the stability of cadherins at the plasma membrane, and act to link cadherins to variety of intracellular signaling pathways (Arikkath, 2009). This dissertation focuses on β-catenin, which will be discussed in greater detail in the following sections.

1.3.2 LOCALIZATION OF CADHERIN AND B-CATENIN AT SYNAPSES

Several classic cadherins have been identified at synapses, including, N-cadherin (neural-cadherin), E-cadherin (epithelial-cadherin), R-cadherin (retina-cadherin), cadherin-6 and cadherin-7 (Obst-Pernberg and Redies, 1999). It has been proposed that cadherins regulate synapse specificity by targeting different cadherins to different subclasses of synapses. For instance, although both N- and E-cadherins are found at hippocampal synapses, their distribution is mutually exclusive (Fannon and Colman, 1996).

N-cadherin and β-catenin are present in axons and dendrites before synapse formation and then cluster at developing synapses (Benson and Tanaka, 1998). N-cadherin is initially present at both excitatory and inhibitory synapses and becomes restricted to only excitatory synapses later in development (Benson and Tanaka, 1998). Interestingly, β-catenin remains present at both excitatory and inhibitory synapses, suggesting that other cadherin isoforms may replace N-cadherin at inhibitory synapses at a more mature stage (Benson and Tanaka, 1998). Although specific cadherins that localize to inhibitory synapses have yet to be identified, it has been shown that cadherin-11 and -13 are capable of promoting the formation of inhibitory synapses in hippocampal neurons (Paradis et al., 2007). Further work is required to examine the localization of these cadherins at inhibitory synapses.

The localization of cadherin within individual synapses is developmentally regulated (Elste and Benson, 2006). In young hippocampal cultures (5-6DIV), cadherins are evenly distributed throughout the active zone, whereas in more mature cultures (14DIV), cadherins are localized to regions immediately surrounding the active zone (Uchida et al., 1996) (Elste and Benson, 2006). The redistribution of cadherins at different developmental stages is also observed in vivo (Elste and Benson, 2006).

N-cadherin can be transported in preassembled transport packets. In hippocampal neurons, N-cadherin is transported in PTVs with active zone components to developing synapses prior to the accumulation of SVs (Zhai et al., 2001). In Rohon-Beard neurons of the
embryonic zebrafish spinal cord, N-cadherin is transported by two types of trafficking packets: tubulovesicular structures that move preferentially in the anterograde direction and punctate structures that move bidirectionally (Jontes et al., 2004).

### 1.3.3 FUNCTION OF CADHERIN AT SYNAPSES

**The role of cadherin in synaptic vesicle clustering**

N-cadherin plays an important role in localizing SVs to synapses. Perturbation of cadherin function through expression of a dominant-negative N-cadherin, which does not contain the extracellular domain, results in a mislocalization of SVs along the axon (Togashi et al., 2002). SV recycling is also perturbed in these neurons (Togashi et al., 2002). Fluorescence recovery after photobleaching (FRAP) experiments at individual synaptic boutons reveals a strong impairment of SV accumulation in N-cadherin knockdown cells (Stan et al., 2010). Moreover, in neurons derived from N-cadherin knockout embryonic stem cells, there is an impairment in the availability of SVs for exocytosis during high frequency stimulation (Jungling et al., 2006). Cadherin regulation of SV localization is developmentally regulated, and cadherins are more important in mediating vesicle localization in young cultures compared to old (Togashi et al., 2002; Bozdagi et al., 2004). Interestingly, in contrast to a decrease in the number of synaptic vesicles when cadherin is disrupted in mammalian culture systems, disruption of *Drosophila* DN-cadherin results in an aberrant over-accumulation of SVs at synapses formed between photoreceptor cells and their target interneurons (Iwai et al., 2002). It is currently unknown whether there is a disparity between vertebrate and invertebrate systems or whether differences in SV phenotypes arise from differences in synapse type.

Two recent studies have demonstrated a role for postsynaptic N-cadherin in the control of presynaptic SV localization (Stan et al., 2010; Aiga et al., 2011). This transsynaptic machinery involves the cooperation of N-cadherin, neuroligin-1 and S-SCAM (synaptic scaffolding molecule) (Stan et al., 2010; Aiga et al., 2011). N-cadherin recruits neuroligin-1 to postsynaptic compartments via S-SCAM, and neuroligin-1 in turn, enhances the clustering of SVs and the subsequent formation of synapses (Stan et al., 2010) (Aiga et al., 2011). Indeed, N-cadherin localizes to synapses prior to the clustering of neuroligin-1 (Aiga et al., 2011). Overexpression or knockdown of N-cadherin results in an increase or a decrease in neuroligin-1 density, respectively (Aiga et al., 2011). More importantly, knockdown of N-cadherin abolishes the clustering of SVs triggered by neuroglin-1 overexpression (Stan et al., 2010).
The role of cadherin in regulating spine morphology

N-cadherin is an essential regulator of spine morphology. Inhibition of cadherin activity leads to alterations of spine morphology, such as elongated spines and bifurcated spine heads, along with concomitant disruption of PSD-95 localization (Togashi et al., 2002; Xie et al., 2008). Several lines of study have revealed N-cadherin controls spine morphology via its interaction with the catenins and their subsequent regulation of the actin cytoskeleton. Indeed, ablation of α-catenin (Abe et al., 2004), β-catenin (Okuda et al., 2007), p120catenin (Elia et al., 2006), or δ-catenin (Arikkath et al., 2009), results in a similar spine phenotype to that observed following disruption of N-cadherin function. Enhancing cadherin activity by treatment with preclustered Fc-N-cadherin causes an enlargement of existing spines (Xie et al., 2008). N-cadherin dependent spine enlargement requires the function of a Rac guanine nucleotide exchange factor, kalirin-7, and a scaffolding protein AF-6/afadin (Xie et al., 2008). N-cadherin recruits kalirin-7 to spines via AF-6, and activates Rac (Xie et al., 2008). Knockdown of kalirin-7 prevents Fc-N-cadherin induced spine enlargement (Xie et al., 2008).

N-cadherin is required for activity induced spine remodeling. The activation of AMPARs induces the enlargement of spine heads, and N-cadherin activity is essential for this rearrangement (Okamura et al., 2004; Saglietti et al., 2007). Indeed, N-cadherin directly interacts with GluR2, and regulates the localization of GluR2 in the spines (Saglietti et al., 2007). Inhibition of N-cadherin activity results in the abrogation of AMPAR induced spine remodeling (Okamura et al., 2004; Saglietti et al., 2007). N-cadherin also mediates LTP (long term potentiation) induced long-term spine stabilization (Mendez et al., 2010).

The role of cadherin in regulating synaptic plasticity

N-cadherin is required for regulating short-term plasticity at synapses (Jungling et al., 2006). In neurons derived from N-cadherin knockout embryonic stem cells, short-term plasticity is strongly altered with an enhanced synaptic depression (Jungling et al., 2006). N-cadherin regulates the changes by a transsynaptic mechanism (Jungling et al., 2006). Studies from chimeric cultures consisting of wild-type neocortical neurons and embryonic stem cell-derived neurons reveal that, with N-cadherin absent postsynaptically, there is an increase in short-term synaptic depression, similar to that observed in N-cadherin knockout neurons (Jungling et al., 2006).
N-cadherin is important for regulating long-term plasticity, such as LTP (Tang et al., 1998; Bozdagi et al., 2000; Bozdagi et al., 2010). Inhibition of N-cadherin functions prevents the induction of late-phase LTP (Bozdagi et al., 2000). At mature synapses, N-cadherin controls the persistent expression of LTP (Bozdagi et al., 2010). Given the important role of N-cadherin in LTP, it is not surprising to note that N-cadherin plays a role in learning and memory (Schrick et al., 2007). Mice injected with peptide that blocks N-cadherin dimerization in the dorsal hippocampus exhibit impaired formation of long-term contextual fear memory (Schrick et al., 2007). The role of N-cadherin in memory consolidation is thought to be mediated by IQGAP (IQ motif containing GTPase activating protein) and Erk (extracellular signal-regulated kinase) signaling (Bozdagi et al., 2010). Interestingly, N-cadherin seems dispensable in LTD (long term depression) expression (Bozdagi et al., 2010). Other forms of cadherins are also involved in LTP regulation, however, they play a different role than N-cadherin. For instance, when cadherin-11 is ablated, LTP is unexpectedly enhanced and fear or anxiety-related response is decreased (Manabe et al., 2000).

Conversely, synaptic plasticity can also regulate N-cadherin’s function. Neuronal activity increases the level of N-cadherin in the spine by enhancing N-cadherin protein synthesis (Bozdagi et al., 2000; Mendez et al., 2010). This accumulation is accompanied by an increase in N-cadherin dimerization, which leads to the strengthening of cadherin-based adhesion (Bozdagi et al., 2000; Tanaka et al., 2000).

### 1.3.4 FUNCTION OF B-CATENIN AT SYNAPSES

β-catenin is a common cytoplasmic interacting partner for all classic cadherins. It is composed of a N-terminal domain, through which it associates with α-catenin; a central domain consisting of 12 armadillo repeats that bind to cadherin and the LEF/TCF transcription factor; and a PDZ-binding motif at the very C-terminus that enables β-catenin to interact with PDZ containing proteins (Fig. 1.11). In addition to its role in regulating cadherin-based adhesion, β-catenin is also involved in the Wnt signaling pathway. Briefly, Wnt activity inhibits the axin/GSK-3 (glycogen-synthasekinase)/APC (adenomatous polyposis colo) complex, which normally promotes the proteolytic degradation of β-catenin. This results in an accumulation of β-catenin and mediates the translocation of β-catenin into the nucleus, its interaction with TCF/LEF transcription factors, and the induction of Wnt target genes (Behrens et al., 1996).
**The role of β-catenin in synaptic vesicle clustering**

Presynaptic β-catenin plays a large role in regulation of SV localization (Bamji et al., 2003). In β-catenin conditional knockout mice, there is a significant decrease in the number of SVs recruited to individual synapses and an impaired response to prolonged repetitive stimulation (Bamji et al., 2003). The phenotype corresponds to a dispersion of SVs along the axon in cultured neurons lacking β-catenin (Bamji et al., 2003). The ability of β-catenin to localize SVs is mediated by its association with cadherin, and the recruitment of PDZ containing protein(s) to synapses (Bamji et al., 2003). Indeed, deletion of the cadherin-binding armadillo repeats and the PDZ-binding motif of β-catenin both results in a mislocalization of SVs along the axon (Bamji et al., 2003).

The role of β-catenin on SV localization is regulated by its phosphorylation state (Bamji et al., 2006; Lee et al., 2008). It has been shown that phosphorylation of β-catenin on tyrosine 654 greatly decreases the affinity of β-catenin for cadherins (Roura et al., 1999; Piedra et al., 2001). Dissolution of cadherin-β-catenin complexes by this phosphorylation mobilizes SVs at existing synapses, and results in small SV clusters “splitting” away from synaptic sites (Bamji et al., 2006). A pathway that regulates tyrosine phosphorylation of β-catenin has also been shown to be important for SV localization (Lee et al., 2008). This pathway includes p120catenin, the cytoplasmic tyrosine kinase Fer and the protein phosphatase SHP-2 (Lee et al., 2008). Disruption of any component in this pathway results in a mislocalization of SVs along the axon (Lee et al., 2008). Interestingly, presynaptic Fer depletion also prevents the localization of active zone constituents, such as bassoon (Lee et al., 2008).
The role of β-catenin in regulating spine morphology

β-catenin is localized to postsynaptic compartments and plays an important role in regulating spine morphology and postsynaptic strength (Okuda et al., 2007). Ablation of postsynaptic β-catenin leads to an increase in the density of thin elongated spines and a decrease in the density of mushroom spines (Okuda et al., 2007). These morphological changes are accompanied with a decrease in the amplitude of mEPSCs (miniature excitatory postsynaptic currents) (Okuda et al., 2007). The ability of β-catenin to modulate synaptic responses depends on its interaction with cadherins and PDZ proteins. Indeed, deletion of a central armadillo domain that binds to cadherins and a C-terminal domain that binds to PDZ proteins decreases the amplitude of mEPSCs (Okuda et al., 2007).

β-catenin is a primary regulator of N-cadherin endocytosis in the spines (Tai et al., 2007). Following depolarization by high concentration of KCl or NMDAR stimulation, β-catenin moves from dendritic shafts into spines and exhibits increased binding to N-cadherin (Murase et al., 2002; Tai et al., 2007). This enhanced association decreases the rate of N-cadherin endocytosis, resulting in an accumulation of N-cadherin in the plasma membrane (Tai et al., 2007). The distribution of β-catenin is dependent on the phosphorylation state of the tyrosine residue at position 654 (Murase et al., 2002). The Y654F-β-catenin point mutation (phosphorylation-prevented) results in the concentration of β-catenin within spines, whereas Y654E-β-catenin (phosphorylation-mimic) accumulates in dendritic shafts (Murase et al., 2002). Maintaining β-catenin localization in the spines by expressing Y654F-β-catenin-GFP, leads to the stabilization of surface N-cadherin (Tai et al., 2007), an increase in the size of PSD-95 and associated SV clusters, and an increase in the frequency of mEPSCs (Murase et al., 2002). These presynaptic changes caused by redistribution of postsynaptic β-catenin suggest that postsynaptic cadherin/β-catenin complexes provide retrograde signals to promote presynaptic development.

Transsynaptic control of β-catenin on presynaptic development

As discussed in the previous section, the transsynaptic control of N-cadherin on presynaptic development is mediated by S-SCAM and neuroligin (Aiga et al., 2010; Stan et al., 2010). Another study suggests that APC, a β-catenin binding protein, is also involved in this process at nicotinic synapses in avian ciliary ganglion neurons (Rosenberg et al., 2010). β-catenin and APC form a complex at synapses and this complex is important for the postsynaptic
localization of S-SCAM and neuroligin (Rosenberg et al., 2010). Blockade of APC and β-catenin interactions decreases postsynaptic accumulation of S-SCAM and neuroligin, retrogradely decreases the localization of neurexin and active zone proteins, and subsequently leads to defects of presynaptic terminals (Rosenberg et al., 2010).

The role of β-catenin in memory consolidation

Given the important role of β-catenin in controlling synapse development and synaptic strength, it has long been hypothesized that β-catenin is directly involved in crucial events that mediate learning and memory. However, examination of the role of β-catenin in these processes has been delayed due to the embryonic lethality of the β-catenin knockout animals. Until recently, an elegant study using an inducible genetic approach examines the effects of temporal and region-specific deletion of β-catenin within the amygdala and demonstrates that β-catenin is required for fear-memory consolidation (Maguschak and Ressler, 2008). During fear-memory consolidation, there are changes in β-catenin mRNA and phosphorylation, as well as its association with cadherin (Maguschak and Ressler, 2008). More importantly, the deletion of β-catenin prevents the transfer of newly formed fear learning into long-term memory (Maguschak and Ressler, 2008).

The role of β-catenin mediated Wnt signaling at neuromuscular junctions

As discussed above, the functions of β-catenin at synapses are largely dependent on its association with cadherin. However, β-catenin mediated Wnt signaling has been demonstrated to play an important role in clustering of acetylcholine receptors (AchRs) at neuromuscular junctions (NMJs) (Zhang et al., 2007; Li et al., 2008; Wang et al., 2008). β-catenin/Wnt signaling suppresses the expression of Rapsyn, and subsequently inhibits the clustering of AchRs at NMJs (Zhang et al., 2007; Wang et al., 2008). In absence of muscle β-catenin, AchR clusters are increased in size and distributed throughout a wider region (Li et al., 2008). Interestingly, muscle β-catenin provides a retrograde signal to promote the differentiation of motoneurons (Li et al., 2008). Indeed, muscle-specific ablation of β-catenin causes both morphologic and functional defects in motoneuron terminals (Li et al., 2008).
1.4 SCRIBBLE

Scribble was first discovered as a polarity protein that determines apical and basolateral compartments in *Drosophila* from a screen for maternal effect mutations that disrupt epithelial morphogenesis (Bilder and Perrimon, 2000). In *scribble* mutants, cells present with aberrant shapes, and the monolayer organization of embryonic epithelia is completely disrupted, hence the name scribble (Bilder and Perrimon, 2000). This drastic phenotype is accounted by the loss of cell polarity in the absence of scribble. In epithelial cells, *Drosophila* scribble is localized to septate junctions in the basolateral domain, which is essential for appropriate localization of apical proteins (Bilder and Perrimon, 2000). The ability of scribble to determine cell polarity is thought to function by excluding the apical determinant crumbs from the basolateral domain (Bilder and Perrimon, 2000).

1.4.1 STRUCTURE OF SCRIBBLE

Scribble is composed of sixteen leucine-rich repeats (LRRs) and four PDZ (PSD-95/Dlg/ZO-1) domains (Bilder and Perrimon, 2000) (Fig. 1.12). It belongs to a family of LAP (LRR and PDZ) proteins, including Erbin, Lano and Densin-180 (Bryant and Huwe, 2000). Studies have shown that the LRR domain is essential for the localization of scribble at the plasma membrane in epithelial cells and is required for the cell polarization in *Drosophila* (Albertson et al., 2004; Zeitler et al., 2004). Sequence analysis reveals that all four PDZ domain of scribble are Type 1A, which can interact with the consensus sequence S/TXV at the carboxy terminus of proteins (Bilder and Perrimon, 2000). Indeed, scribble has been shown to associate with variety of proteins through its PDZ domains, including tumor suppressor, APC, zyxin-related protein LPP (Lipoma Preferred Partner), junction-associated protein ZO-2, NOS1AP (neuronal nitric oxide synthase adaptor protein) and Rac/Cdc42 specific guanine nucleotide exchange factor, β-pix (Audebert et al., 2004; Metais et al., 2005; Petit et al., 2005; Takizawa et al., 2006; Richier et al., 2010).

Figure 1.12: Schematic of the structure of human scribble.
Adapted with permission from (Bilder and Perrimon, 2000).
1.4.2 LOCALIZATION OF SCRIBBLE AT SYNAPSES

Scribble distributes to both axons and dendrites in neurons and colocalizes with variety of synaptic markers, suggesting its localization at synapses (Audebert et al., 2004; Takizawa et al., 2006; Moreau et al., 2010; Richier et al., 2010). A detailed analysis using electron microscopy shows significant scribble localization at spine heads, closely associated with the postsynaptic density and within presynaptic boutons (Moreau et al., 2010). The postsynaptic localization of scribble at Drosophila neuromuscular junctions involves the function of fasciclin 2, a cell adhesion molecule and amphiphysin, a curvature inducing protein in endocytosis (Zelhof et al., 2001; Kohsaka et al., 2007). Indeed, in the mutants of fasciclin 2 or amphiphysin, synaptic localization of scribble is reduced and it mislocalizes throughout the muscle (Zelhof et al., 2001; Kohsaka et al., 2007). However, in the presynaptic compartment at NMJs, dlg (Discs large) controls the localization of scribble by the interaction with GUK-holder (Mathew et al., 2002). The machinery that regulates the synaptic localization of scribble in vertebrates has yet been discovered.

1.4.3 FUNCTION OF SCRIBBLE AT SYNAPSES

Scribble plays an important role in regulating SV localization at Drosophila NMJs (Roche et al., 2002). Indeed, loss-of-function mutations in scribble result in an accumulation of SVs at the NMJ, and a decreased number of presynaptic active zones (Roche et al., 2002). Moreover, several forms of plasticity are drastically altered in scribble mutants, accompanied by impaired vesicle dynamics (Roche et al., 2002). These results suggest that scribble is an important regulator of SV turnover.

Scribble is also essential for the development of dendritic spines in neurons (Moreau et al., 2010; Richier et al., 2010). Indeed, the disruption of scribble causes a decrease in spine number and an increase of filopodia-like protrusions. Moreover, these morphological changes result in a weakened synaptic strength and plasticity (Moreau et al., 2010). Interestingly, animals with one allele of mutant scribble exhibit an increased hippocampal-dependent learning ability and an impairment of social interaction - two features relevant to autistic spectrum disorders (Moreau et al., 2010). The function of scribble in controlling spine development is thought to be dependent on its regulation of the actin cytoskeleton through its association with β-pix (Moreau et al., 2010; Richier et al., 2010).
1.5 β-PIX

β-pix was discovered from a screen designed to identify the binding partners for PAK (p21-activated kinase) (Bagrodia et al., 1998; Manser et al., 1998). Two proteins isolated from this screen were named α- and β-pix (PAK-interacting exchange protein) (Manser et al., 1998), also known as p85SPR and p85COOL-1 (cloned out of library), respectively (Bagrodia et al., 1998). Sequence analysis revealed that β-pix is a guanine nucleotide exchange factor for small GTPases, Rac and Cdc42 (Manser et al., 1998). Indeed, β-pix can promote exchange of GTP for GDP for Rac1 in vitro (Manser et al., 1998).

1.5.1 STRUCTURE OF B-PIX

β-pix is composed of multiple functional domains (Fig.1.13). At the N-terminus, β-pix possesses a Src homology 3 domain that is required for its interaction with PAK (Manser et al., 1998). Two domains of β-pix are found to be important for PAK activity, including an 18-amino acid sequence designated as “T1 insert”, which inhibits the ability of β-pix to activate PAK (Feng et al., 2002), and a proline-rich region, which inactivates PAK though interactions with POPX-1 and -2 phosphatases (Koh et al., 2002). The GEF activity of β-pix for Rac and Cdc42 is defined by the presence of a Dbl-family exchange homology and a pleckstrin homology domain (Manser et al., 1998). β-pix interacts with G protein-coupled receptor kinase-interacting protein 1 (GIT 1) through its GIT-binding domain and (Koh et al., 2001), and to PDZ proteins, such as scribble (Audebert et al., 2004) and Shank (Park et al., 2003) through its C-terminal PDZ binding motif. Moreover, a coiled-coil region with a leucine zipper domain is found to be important for the formation of dimerization of β-pix (Kim et al., 2001; Koh et al., 2001).

Different isoforms of β-pix have been reported (Kim et al., 2000; Koh et al., 2001) (Fig.1.13). Unlike β-pix, which is ubiquitously expressed, β-pix-b, β-pix-c and β-2-pix are predominantly expressed in the brain (Kim et al., 2000; Koh et al., 2001). Compared to β-pix, β-pix-b and -c both possess an additional proline-rich region and these isoforms have been implicated in brain development (Kim et al., 2000). The third isoform, β-2-pix contains a serine-rich region at the C-terminus instead of the coiled-coil sequence that presents in other β-
pix variants (Koh et al., 2001). Nonetheless, most studies including those at the synapse, have focused on the ubiquitously expressed β-pix.

![Figure 1.13: Schematic of different isoforms of PIX.](image)

**Abbreviations:** CH, calponin homology domain; SH3, Src homology 3; DH, Dbl homology domain; PH, pleckstrin homology domain; inverted triangle, deletion in αPIX; I, insert sequence of ~30 amino acids in β1PIX and β2PIX not present in αPIX; II, insert sequence of ~60 amino acids present in β2PIX, β1PIX-b and β1PIX-c. The numbers represent the percentage similarity between the domains of αPIX and β1PIX. β1PIX-b, β1PIX-c and β2PIX are all splice variants of β1PIX. Adapted with permission from (Koh et al., 2001).

### 1.5.2 LOCALIZATION OF B-PIX AT SYNAPSES

In *Drosophila*, β-pix has been shown to localize to the postsynaptic sites at NMJs (Parnas et al., 2001). In mammals, β-pix is distributed within both axons and dendrites in neurons and colocalizes with variety of synaptic markers, indicating its localization at synapses (Park et al., 2003; Zhang et al., 2003). β-pix has been found to form a complex with the presynaptic scaffolding protein, piccolo, in the rat brain indicating its localization at presynaptic terminals (Kim et al., 2003). Similarly, β-pix can also be co-immunoprecipitated with the postsynaptic density protein, Shank, from the crude synaptosomal fractions indicating its localization at postsynaptic terminals (Park et al., 2003).

Several proteins have been implicated in regulating β-pix localization. In neuroendocrine PC12 cells, β-pix is recruited by scribble to the plasma membrane upon membrane depolarization and scribble/β-pix are both essential in controlling Ca$^{2+}$ dependent exocytosis (Audebert et al., 2004). In neurons, the postsynaptic localization of β-pix can be
regulated by Shank, with Shank overexpression enhancing β-pix localization at spines (Park et al., 2003). GIT1 is also indicated to be important for synaptic localization of β-pix (Zhang et al., 2003). Indeed, the expression of dominant negative β-pix, lacking the GIT1 binding domain, results in the mislocalization of β-pix along processes (Zhang et al., 2003). It is unclear whether GIT1 plays a role in localizing β-pix to pre or postsynaptic sites. However, given GIT1 is expressed on both sides, it is possible that this machinery is used by β-pix to target to pre- and postsynaptic compartments.

1.5.3 FUNCTION OF B-PIX AT SYNAPSES

β-pix has been shown to play a large role in postsynaptic development at Drosophila NMJs (Parnas et al., 2001). Mutations in dpix, a Drosophila homologue of mammalian β-pix, cause mislocalization of various postsynaptic proteins, including PAK, dlg, the cell adhesion molecule Fas II, and the glutamate receptor subunit GluRIIA, and an almost complete depletion of subsynaptic reticulum (Parnas et al., 2001). Unexpectedly, these mutants exhibit relatively mild electrophysiological deficits, with a small reduction in evoked excitatory junctional potentials (EJPs), and the amplitude of spontaneous miniature excitatory junctional potentials (mEJPs) (Parnas et al., 2001). Interestingly, a 40% decrease is found in the frequency of mEJPs, suggesting there is a presynaptic defect, although no dramatic change in the structure of presynaptic boutons is noted (Parnas et al., 2001).

In CNS synapses, β-pix is essential for regulating spine morphogenesis (Zhang et al., 2003; Zhang et al., 2005; Saneyoshi et al., 2008). Disruption of the synaptic localization of β-pix results in a significant decrease in spine and synapse density (Zhang et al., 2003). β-pix controls spine morphology through its ability to regulate actin cytoskeleton. Indeed, synaptically localized β-pix locally activates Rac in the spine and in turn, organizes the actin network (Zhang et al., 2005). β-pix’s GEF activity can be elevated by phosphorylation of Ser516 in β-pix by calmodulin-dependent kinase I (CaMKI) and inhibition of the phosphorylation decreases spine formation (Saneyoshi et al., 2008). Moreover, this spine phenotype can be recapitulated by using a dominant negative version of Rac (RacN-17), further suggesting that β-pix controls spine formation by activating Rac (Zhang et al., 2003). A study has further demonstrated that β-pix/Rac activates PAK, which further phosphorylates myosin II.
regulatory light chain (MLC) and β-pix/Rac/PAK/MLC complex is essential for controlling spine morphogenesis and synapse formation (Zhang et al., 2005).
1.6 THE ACTIN CYTOSKELETON

1.6.1 REGULATION OF ACTIN ORGANIZATION

Actin is the major constituent of the cytoskeleton present in almost all eukaryotic cells. Actin exists in either a monomeric form G-actin (globular actin), or in a polymerized form, F-actin (filamentous actin) (Chen et al., 2000). F-actin is formed by weak noncovalent interactions of G-actin, which allows for rapid turnover of filaments in response to cellular stimulation. F-actin is asymmetric, with a fast-growing barbed-end (or plus end) and a slow-growing pointed end (or minus end). The assembly of F-actin is coupled with hydrolysis of ATP. Under physiological conditions, ATP bound G-actin is incorporated at the barbed end. ATP-actin hydrolyzes and converts into ADP-actin as G-actin shifts along filaments to the pointed end. ADP bound G-actin then dissociates from the pointed end and enters a new round of turnover cycle (Fig.1.1). Whether ends of filaments grow or shrink is dependent on the cytosolic concentration of available G-actin in the surrounding area. The minimal concentration of G-actin required for assembly is called the critical concentration (Cc). Because Cc at the pointed end is higher than that at the barbed end, G-actin is added to the barbed end and lost from the pointed end. At steady states, the net assembly of G-actin at the barbed end equals the net disassembly at the pointed end, a process known as actin treadmilling.

Figure 1. 14: The turnover of an actin filament.
The filament turnover at steady state involves a sequence of actin assembly, ATP hydrolysis, Pi release, filament disassembly and nucleotide exchange. Adapted with permission from (Chen et al., 2000).

The rate of F-actin turnover in vivo is 100-200-fold faster than that with pure actin in vitro, suggesting the rapid turnover of F-actin in cells is achieved by the assistance from other
proteins (Zigmond, 1993). Indeed, a cohort of proteins has been identified as regulators of actin remodeling (Disanza et al., 2005). These proteins can be generally categorized into 4 groups according to the way they influence actin organization (Fig.1.15). The first group of proteins are the F-actin nucleators, including the Arp2/3 complex and formins. The Arp2/3 complex branches new actin filaments at 70 degrees angles from the mother filaments, whereas formin promote nucleation of the unbranched filaments. The second group is proteins that accelerate actin depolymerization. For instance, ADF (actin depolymerization factor)/cofilin preferentially bind to ADP bound G- and F-actin and specifically increases the rate of depolymerizaion at the pointed ends. The third group is proteins that interact with G-actin. For instance, profilin promotes nucleotide exchange of ADP-G-actin to ATP-G-actin and enhances the assembly at barbed ends. In contrast, β-thymosin sequesters G-actin and prevents spontaneous nucleation. The fourth group includes proteins that cap the ends of filaments. One example is gelsolin, which binds to the barbed end and terminates the elongation of that end. These actin regulatory proteins are targets of a number of signaling pathways, among which Rho GTPases have emerged as central players in actin remodeling. Indeed, Rac, Cdc42 and RhoA control the formation of actin-enriched structures, lamellipodia, filopodia and stress fibers, respectively (Disanza et al., 2005).
Figure 1.15: The actin polymerization machinery triggers dendritic nucleation for protrusion at the leading edge.

The activated Arp2/3 complex nucleates and branches actin filaments at the leading edge, pushing the membrane forward. Capping proteins control the half-life of filaments, and by blocking a large fraction of barbed ends, promote site-directed elongation of uncapped filaments. ADF/cofilin promotes dissociation of ADP-actin from filament pointed ends and severs preexisting filaments, generating new barbed ends. Profilin catalyzes the exchange of ADP for ATP on monomeric actin molecules, which become available for new polymerization at barbed ends. Reproduced with permission from (Disanza et al., 2005).
1.6.2 LOCALIZATION OF ACTIN AT SYNAPSES

Actin filaments are the major component of the active zone and are abundantly distributed throughout the terminal (Hirokawa et al., 1989). The presynaptic localization of actin has been examined using light microscopy and fluorescently labeled probes. In the reticulospinal giant synapse of lamprey, actin is localized to the active zone and in the core of vesicle clusters (Shupliakov et al., 2002; Bloom et al., 2003). F-actin is also found at the endocytic domain lateral to the active zone, and the amount of F-actin at these regions is significantly increased upon neuronal stimulation (Bloom et al., 2003). In mammalian hippocampal synapses, G-actin is homogeneously distributed throughout neuronal processes, whereas F-actin is concentrated synaptic terminals (Zhang and Benson, 2002). Studies using GFP-actin show that actin largely colocalizes with SVs especially enriched in the region surrounding vesicle clusters (Morales et al., 2000; Colicos et al., 2001; Sankaranarayanan et al., 2003). Actin filaments often associate with synapsin filaments, approximately 30nm in length (Landis et al., 1988; Hirokawa et al., 1989). Synapsin filaments are found exclusively in the regions occupied by SVs, linking SVs to actin filaments (Hirokawa et al., 1989). This actin-synapsin network is thought to be essential in maintaining vesicle localization at synaptic terminals. However, mice lacking three synapsin isoforms I, II and III show a relatively normal vesicle organization, raising the question about the role of synapsins in clustering SVs (Siksou et al., 2007).

The enrichment of actin filaments in dendritic spines has been shown at the ultrastructural level and at the light microscopic level (Drenckhahn et al., 1984).

1.6.3 FUNCTION OF ACTIN AT PRESYNAPTIC COMPARTMENTS

Actin and mobilization of the reserve pool of synaptic vesicles

As described above, actin filaments are localized to presynaptic terminals where they surround the RP vesicles (Hirokawa et al., 1989; Sankaranarayanan et al., 2003). This subcellular localization suggests that F-actin may function as a physical barrier to maintain synaptic vesicles at synaptic terminals (Dillon and Goda, 2005). Indeed, in Drosophila, cytochalasin D, an actin depolymerization drug, depletes the RP vesicles at NMJs (Kuromi and Kidokoro, 1998). Similarly, in mammalian hippocampal neurons, depolymerization of actin using latrunculin A, leads to a near complete loss of SV clusters along the axon (Zhang and
Interestingly, this process appears developmentally regulated. As the neuron matures, vesicle clustering becomes increasingly resistant to latrunculin A treatment, such that by 2-4 weeks, SV distribution is independent of F-actin (Zhang and Benson, 2001); (Sankaranarayanan et al., 2003). At frog NMJs, latrunculin A does not alter the localization of SVs, which may reflect the dispensable role of F-actin in later developmental stage (Richards et al., 2004). This change correlates well with the acquisition of presynaptic scaffolding proteins, such as bassoon, suggesting these large scaffolds may substitute for F-actin in regulating vesicle organization in mature synapses (Zhang and Benson, 2001).

F-actin not only prevents SVs from dispersing along the axon, but also restricts SV mobility within the terminal and suppresses the recruitment of vesicle to the active zone (Cingolani and Goda, 2008). Recent studies using fluorescence recovery after photobleaching (FRAP) discover that in hippocampal neurons, the mobility of vesicles at synaptic terminals is generally low (Shtrahman et al., 2005), and significantly increases following actin depolymerization (Jordan et al., 2005). Accordingly, studies from the frog NMJ (Wang et al., 1996) and chick sympathetic neurons (Bernstein et al., 1998) both suggest that F-actin impose a barrier for mobilization of SVs from the RP. However, opposite results are also documented. For instance, at Drosophila NMJs, F-actin is shown to be facilitatory for vesicle mobilization (Kuromi and Kidokoro, 1998; Nunes et al., 2006). Indeed, dominant negative and loss-of-function mutants of N-ethymaleimide sensitive factor (Nsf), exhibit a decrease in F-actin level and a concomitant reduction in vesicle mobility in presynaptic boutons, which results in a decrease in the size of readily releasable pool (Nunes et al., 2006). Moreover, cytochalasin D causes a defect in evoked synaptic transmission (Kuromi and Kidokoro, 1998). Since vesicle endocytosis and exocytosis appear normal upon cytochalasin D treatment, this defect is likely due to the impairment of vesicle recruitment from the RP caused by actin depolymerization (Kuromi and Kidokoro, 1998). A study of mammalian Calyx of Held also corroborates the facilitatory role of actin in vesicle mobilization (Sakaba and Neher, 2003). How may F-actin recruit RP vesicles to the active zone within the presynaptic terminal? Some studies support a model, such that F-actin serves as cytoskeleton tracks and facilitates SV movement via actin-based motors (Cingolani and Goda, 2008). Myosin V is an SV-associated protein and interacts with syntaxin, a t-SNARE in presence of Ca$^{2+}$ (Watanabe et al., 2005). It is tempting to speculate that within the terminal, SVs trafficked along F-actin “tracks” could be captured at the active zone by interaction of myosin V with syntaxin. Indeed, the interaction of myosin V and
syntaxin is important for vesicle exocytosis in chromaffin cells (Watanabe et al., 2005). Moreover, disruption of myosin binding to F-actin by inhibition of myosin light chain kinase, blocks mobilization of RP vesicles within the presynaptic terminal (Ryan, 1999).

**Actin and exocytosis of synaptic vesicles**

F-actin has been shown to exert a dual role in controlling of vesicle fusion and neurotransmitter release (Cingolani and Goda, 2008). First, in terminals that are large and release neurotransmitters at a fast rate, F-actin has a facilitatory role for SV exocytosis. In support of this idea, latrunculin A treatment causes impairment of vesicle exocytosis at NMJs of frog (Richards et al., 2004) and snake (Cole et al., 2000). However, at Drosophila NMJs, depolymerization of F-actin has little impact on vesicle release (Kuromi and Kidokoro, 1998). Secondly, terminals that are small and release neurotransmitters with low probability would use F-actin as a brake to limit vesicle fusion. Indeed, in hippocampal synapses, latrunculin A leads to an increase in the frequency of mEPSCs and the size of evoked EPSCs (Morales et al., 2000). Given that the size of the RRP and its rate of refilling are not affected, this increase is likely due to the elevated release probability following actin depolymerization (Morales et al., 2000). Consistent with the inhibitory role of F-actin on exocytosis, latrunculin A increases the destain rate of FM, a styryl dye in hippocampal neurons (Sankaranarayanan et al., 2003) and cytochalasin D increases the mEPSC frequency in hippocampal slices (Meng et al., 2002). Moreover, the inhibitory role of F-actin on exocytosis is further supported by the analysis of LIMK-1 knockout mice (Meng et al., 2002). LIM kinase-1 promotes actin polymerization by phosphorylating and thereby inhibiting ADF/cofilin (Kuhn et al., 2000). Indeed, an abnormal organization of the actin cytoskeleton is observed in these mice with a concomitant increase in the mEPSC rate (Meng et al., 2002). However, the results from hippocampal synapses are not always consistent. For instance, latrunculin B has been shown to decrease mEPSC frequency in rat hippocampal slices, suggesting a positive role of F-actin on exocytosis (Kim and Lisman, 1999). Moreover, several studies have shown that depolymerization of F-actin has no effect on basal transmission (Krucker et al., 2000; Sankaranarayanan et al., 2003). These confusing findings could attribute to the difference in the age of tested neurons and the protocol used to modulate actin polymerization. Interestingly, a recent study has raised the possibility that varied temperature can lead to different actin-dependent properties (Jensen et al., 2007).
**Actin and endocytosis of synaptic vesicles**

A role of actin in vesicle exocytosis has been established by a study of reticulospinal giant synapse of the lamprey (Shupliakov et al., 2002). At resting synapses, very thin actin filaments are found adjacent to the plasma membrane, around SV clusters. In response to an exhaustive stimulation, these filaments proliferate and extend toward the vesicle cluster in the centre of presynaptic boutons. Some vesicles are tethered along these filaments. These results suggest that F-actin guides the transport of endocytosed vesicles to RP. The authors propose that such mechanism may involve actin treadmilling that are used by infectious agents (Merrifield et al., 1999) and actin-based motor (Taunton, 2001). In support of the second hypothesis, perturbation of myosin shows an accumulation of SVs between the endocytic zone and the RP. Moreover, this paper also demonstrates a role of F-actin in formation of clathrin-coated pits (CCPs). Disrupting actin filaments using the C2 toxin leads to an increase in the number of unconstricted CCPs at the plasma membrane.

Surprisingly, at CNS synapses, the involvement of actin in vesicle retrieval is less dominant. Indeed, disruption of actin filaments using actin polymerization drugs shows little effect on SV endocytosis at hippocampal synapses (Morales et al., 2000; Sankaranarayanan et al., 2003; Gaffield et al., 2006). However, a recent study using FRAP analysis demonstrates a novel actin-dependent component in SV recycling (Darcy et al., 2006a). In this study, the authors show that endocytosed SVs are not restricted to their ‘host bouton’ as people have long assumed, but rather shared between neighboring boutons (Darcy et al., 2006a). Recycled SVs are incorporated into the mobile SV clusters along the axon and are available to synapses during stimulation (Darcy et al., 2006a; Staras et al., 2010). This constitutive sharing of recycled SVs requires dynamic actin turnover, because jasplakinolide, an inhibitor of actin turnover, greatly suppresses this process (Darcy et al., 2006a). However, how actin dynamics control the sharing remains to be established. Actin may be important for the transportation of recycled SVs or actin may play a role in regulating the release and entry of these vesicles into the bouton.

**Actin and presynaptic unsilencing**

In the developing brain, many synapses are incapable of neurotransmission under basal conditions (known as the “silent synapses”) and can be converted to functional synapses by repetitive stimulation (Kerchner and Nicoll, 2008). Two studies have demonstrated a crucial role of actin polymerization in presynaptic unsilencing (Shen et al., 2006; Yao et al., 2006). Both studies demonstrate that stimulation enhances actin polymerization resulting in a
concomitant increase in the presynaptic release (Shen et al., 2006; Yao et al., 2006). BDNF and Cdc42 act upstream of actin polymerization to regulate this process (Shen et al., 2006). Indeed, interference of BDNF signaling, inhibition of Cdc42 activation or disruption of actin polymerization abolishes the induced presynaptic maturation (Shen et al., 2006; Yao et al., 2006). Conversely, activation of Cdc42 by BDNF or enhancement of actin polymerization by jasplakinolide is sufficient to trigger this unsilencing (Yao et al., 2006). How actin polymerization converts silent bouton into functional ones is not clear. The fast speed of conversion (within minutes, (Yao et al., 2006)) suggests that all the presynaptic machinery is in place and actin polymerization acts as a switch to turn on synaptic transmission.

1.6.4 FUNCTION OF ACTIN AT POSTSYNAPTIC COMPARTMENTS

**Actin and dendritic spines**

The dendritic spine is an actin-enriched structure. Both G- and F-actin are present in the spines. The degree of actin polymerization (the ratio of G-/F-actin) affects the various aspects of spine morphology. A variety of proteins that regulate actin polymerization are concentrated in the spine, and alteration of these proteins results in abnormal spine morphology (Hotulainen and Hoogenraad, 2010). For instance, the Arp2/3 complex, the main nucleator of actin filaments, is abundant in the spine (Racz and Weinberg, 2008) and plays a large role in the expansion of the spine head (Wegner et al., 2008; Hotulainen et al., 2009). Knockdown of the p34 subunit of the Arp2/3 complex using RNAi causes a decrease in the number of spines and an increase number of filopodia (Hotulainen et al., 2009). Similar changes are also achieved by disrupting the localization of Arp2/3 complex (Hotulainen et al., 2009). Consistently, depletion of the Arp2/3 complex activator, cortactin reduces the number of spines (Hering and Sheng, 2003). Signals that promote actin depolymerization are also important in spine morphology. In absence of ADF/cofilin, neurons exhibit fewer spines but long dendritic protrusions (Hotulainen et al., 2009). ADF/cofilin can be phosphorylated and thus inactivated by LIMK (Foletta et al., 2003). LIMK-1 knockout mice exhibit “sessile spines’ with a very thick spine neck and a relatively small spine head (Meng et al., 2002). Some actin binding proteins modulate spine dynamics in response to neuronal stimulation. Profilin promotes actin polymerization by exchanging ADP to ATP on the barbed-end of actin filaments. Interestingly, profilin only localizes to a subset of dendritic spines in resting neurons whereas targets to many more spines when postsynaptic NMDARs are activated (Ackermann and Matus, 2003). This translocation subsequently causes an inhibition of actin-based spine dynamics (Ackermann and Matus, 2003).
In fibroblasts, the major signaling modules in the regulation of the actin cytoskeleton are the small Rho GTPases, including Rac, Rho and Cdc42. These small GTPases have been extensively studied in neurons and play a distinct role in controlling spine morphogenesis. For instance, activation of Rac1 decreases the number of spines and increases the number of dendritic protrusions (Nakayama et al., 2000; Zhang et al., 2003), whereas activation of RhoA reduces spine length and density (Nakayama et al., 2000; Govek et al., 2004). Inhibition of Cdc42 blocks spine formation and leads to an increased in filopodia number (Irie and Yamaguchi, 2002; Wegner et al., 2008). These different effects on spine morphology are due to the stimulation of different actin regulatory pathways. RhoA functions by inhibition of cofilin activity and Rac and Cdc42 function mainly by activating the Arp2/3 complex (Rodriguez et al., 2003)(Fig. 1.16).

These elegant studies point out the importance of regulation of the actin cytoskeleton in spine morphogenesis. However, as shown in the examples above, many actin regulatory proteins are present in the spine. How these proteins coordinate to organize dendritic actin networks remain to be established.

Figure 1.16: Signaling pathways from Rho, Rac, and Cdc42 to the regulation of actin polymerization and depolymerization.

Abbreviation: ROCK, Rho associated coiled-coil containing kinase, PAK, p21 activated kinase; LIM-K; LIM domain containing kinase; IRSp53, insulin receptor substrate p53. Adapted with permission from (Luo, 2002).
Actin and localization of postsynaptic components

The actin cytoskeleton is associated with many PSD scaffolding molecules and is essential to maintain the localization of these proteins (Kuriu et al., 2006). At excitatory synapses, depolymerization of F-actin using latrunculin A rapidly eliminates the dynamic fraction of major PSD scaffolding proteins, GKAP, Shank and PSD-Zip54 at synapses, whereas the synaptic localization of PSD-95 remains unaltered (Kuriu et al., 2006). The redistribution of these proteins is dependent on actin dynamics. In response to neuronal activity, more GKAP accumulates to spines, however, a fraction of Shank and PSD-Zip45 moves away from the spine (Kuriu et al., 2006). Inhibition of actin dynamics by jasplakinolide abolishes the activity-dependent redistribution of these scaffolds (Kuriu et al., 2006). At inhibitory synapses, pharmacological disruption of F-actin leads to a reduction in the number of gephyrin clusters, a glycine receptor scaffolding protein (Kirsch and Betz, 1995; Charrier et al., 2006).

Actin is enriched in the PSD and is important for anchoring receptors. At excitatory synapses, disruption of F-actin using latrunculin A leads to a decrease in both the number of NMDAR1 clusters and GluR1 clusters (Allison et al., 1998). At inhibitory synapses, latrunculin A treatment decreases the localization of GlyR (glycine receptor) (Charrier et al., 2006). Using a single particle tracking technique, this study suggests that actin regulates GlyR number at synapses by controlling the rate of GlyR moving in and out of synapses (Charrier et al., 2006).

Actin exerts a dual role in the regulation of AMPAR endocytosis. On one hand, F-actin prevents AMPARs being endocytosed. Indeed, disruption of F-actin by latrunculin A greatly accelerates AMPAR internalization, whereas stabilization of F-actin by jasplakinolide blocks this turnover (Zhou et al., 2001). On the other hand, F-actin is also required for AMPAR endocytosis. It has been shown that the internalization of AMPARs is dependent on myosin VI, an actin motor, which directs receptor traffic along stable F-actin (Osterweil et al., 2005).

1.6.5 ACTIN AND SYNAPTIC PLASTICITY

Actin and short-term plasticity

Synapses are consistently undergoing remodeling by neuronal activity, which causes structural changes of the synapse. As the major cytoskeletal component at synapses, the actin network is also shaped by these activities. Time-lapse imaging of GFP-actin at an individual synapse shows that a single tetanic stimulus transiently moves presynaptic actin towards the synaptic junction (Colicos et al., 2001). Accordingly, postsynaptic actin moves away from the
junction and laterally expands in the spine head (Colicos et al., 2001). These results imply a physiological role for the remodeling of actin in maintaining neurotransmission (Dillon and Goda, 2005). Changes in the presynaptic compartment could reflect a facilitation of actin in some aspects of the SV cycle, such as recruiting more SVs to the RRP and assisting SV fusion at the active zone, as discussed in the previous section. Similarly, the expansion of spine actin network could modify the PSD and increase the insertion of postsynaptic receptors.

**Actin and long-term plasticity**

Unlike the transient remodeling of actin network described above, some form of synaptic activity can stably reorganize actin cytoskeleton and these changes last for weeks (Fukazawa et al., 2003). For instance, repetitive spaced tetanic stimuli induce presynaptic actin to redistribute and form new puncta along the axon (Colicos et al., 2001). Interestingly, clusters of actively cycling SVs accumulate at these new actin puncta sites within 2 hours (Colicos et al., 2001). These newly formed actin clusters appear before functional synapses have formed, as if actin is a “spatial tag” for synaptogenesis (Colicos et al., 2001). Meanwhile, rearrangement of postsynaptic actin results in the sprouting of protrusions toward the presynaptic actin clusters (Colicos et al., 2001).

G- and F-actin are both present in the dendritic spine. Using an elegant FRET-based imaging technique, Okamoto et al. show that tetanic stimulation, known to induce LTP, causes a rapid but persistent shift of actin equilibrium toward F-actin in the spine (Okamoto et al., 2004). This increase of F-actin is dependent on NMDAR activation through the inhibition of ADF/cofilin activity (Fukazawa et al., 2003). Conversely, prolonged low-frequency stimulation, known to induce LTD shifts the actin equilibrium toward G-actin in the spine (Okamoto et al., 2004). However, another study showed that low-frequency stimulation stabilizes actin (Star et al., 2002).

Accumulating lines of evidence has suggested that F-actin is required in the expression of LTP. Inhibition of actin polymerization using a variety of drugs blocks both the formation and the maintenance of the LTP (Kim and Lisman, 1999; Krucker et al., 2000; Fukazawa et al., 2003; Ramachandran and Frey, 2009). However, polymerization of actin is not sufficient for the induction of LTP (Okamoto et al., 2004).
**Actin and learning and memory**

Given its important role in LTP expression, it is not surprising that the regulation of the actin cytoskeleton is essential in learning and memory. Intra-hippocampal injections of F-actin depolymerization drugs latrunculin A or cytochalasin D prevents the acquisition of context-dependent fear learning (Fischer et al., 2004). Genetic studies of various actin regulatory proteins, including LIMK1, which inhibits ADF/cofilin activity (Meng et al., 2002), WAVE1, which activates Arp2/3 complex (Soderling et al., 2007), and profilin (Ackermann and Matus, 2003) show impairments in learning and memory.

Studies from patients with neuronal disorders have demonstrated a profound role of Rho GTPase signals in regulating brain function. For instances, a number of genes mutated in patients carrying the heritable forms of mental retardation encode modulators of Rho GTPases. These genes include ARHGEF6 (or α-pix), PAK3, OPHN1 (Oligophrenin-1), LIMK1 and FMR1 (Ramakers, 2002). Recently, schizophrenia risk factor DISC1 has been linked to the regulation of Rac1 activity (Hayashi-Takagi et al., 2010). Interestingly, patients with mental retardation and schizophrenia show abnormal dendritic arborization, which is similar to the effects when actin network is disrupted (Ramakers, 2002). It is possible that mutation in these genes causes an impaired Rho GTPase signaling, which leads to an abnormal organization of actin cytoskeleton and thus translate into dysfuncitn of the brain. Moreover, inhibition of Rho GTPase signaling in animal model has led to deficits in learning and memory (Dash et al., 2004; Hayashi et al., 2004; Shuai et al., 2010).
2. SCRIBBLE INTERACTS WITH β-CATENIN TO LOCALIZE SYNAPTIC VESICLES TO SYNAPSES

An understanding of how synaptic vesicles are recruited to and maintained at presynaptic compartments is required to discern the molecular mechanisms underlying presynaptic assembly and plasticity. We have previously demonstrated that cadherin/β-catenin complexes cluster synaptic vesicles at presynaptic sites. Here we show that scribble interacts with the cadherin/β-catenin complex to coordinate vesicle localization. Scribble and β-catenin are colocalized at synapses and can be co-immunoprecipitated from neuronal lysates, indicating an interaction between scribble and β-catenin at the synapse. Using an RNA interference approach, we demonstrate that scribble is important for the clustering of synaptic vesicles at synapses. Indeed, in scribble knockdown cells, there is a diffuse distribution of synaptic vesicles along the axon, and a deficit in vesicle recycling. Despite this, synapse number and the distribution of the presynaptic active zone protein, bassoon, remain unchanged. These effects largely phenocopy those observed following ablation of β-catenin. In addition, we show that loss of β-catenin disrupts scribble localization in primary neurons but that the localization of β-catenin is not dependent on scribble. Our data supports a model by which scribble functions downstream of β-catenin to cluster synaptic vesicles at developing synapses.

2.1 INTRODUCTION

Synapse formation begins with the recognition of appropriate targets and formation of incipient contacts, and is followed by the recruitment of pre- and postsynaptic proteins to points of cell-cell contact (Ziv and Garner, 2004). The formation of presynaptic active zones, sites of neurotransmitter release, is believed to occur by the insertion of large dense-core vesicles containing multiple active zone components in a preassembled form (Ahmari et al., 2000; Zhai et al., 2001). Similarly, clusters of pleiomorphic vesicles associated with synaptic vesicle (SV) proteins are recruited and localized to regions underlying active zones (Ziv and Garner, 2004). These elements were previously thought to be relatively stable in mature synapses, however, emerging evidence from time-lapse imaging suggests that there is constant movement of SVs and other synaptic proteins into and out of individual synapses (Krueger et al., 2003; Bamji et
Indeed, a high degree of sharing of SVs between synapses has been reported (Darcy et al., 2006a, b), and may be important for regulating synapse maintenance, efficacy and plasticity (Staras, 2007). Elucidating the molecular mechanisms underlying SV recruitment and localization will therefore not only shed light on presynaptic assembly, but will also be informative with respect to mechanisms underlying presynaptic plasticity.

The cadherin/β-catenin adhesion complex has been shown to play a crucial role in the recruitment of SVs to synapses (Iwai et al., 2002; Togashi et al., 2002; Bamji et al., 2003; Jungling et al., 2006). We have previously demonstrated that some of the effects of cadherin on SV localization are attributed to the function of its intracellular binding partner, β-catenin (Bamji et al., 2003; Bamji et al., 2006). Indeed, in the absence of β-catenin, SVs do not cluster at synapses, resulting in an impaired response to prolonged repetitive stimulation. Domain analyses reveal that the armadillo domain of β-catenin (which binds cadherin), as well as its C-terminal PDZ binding motif, are essential for proper SV localization, indicating that β-catenin mediates this effect by acting as a scaffold to tether PDZ protein(s) to cadherin clusters (Bamji et al., 2003). Moreover, transient disruptions of cadherin/β-catenin interaction enhances the overall mobility of SVs, and more specifically, enhances the efflux of SVs from established synapses (Bamji et al., 2006). As regulation of SV localization is thought to be involved in presynaptic plasticity during development, these data suggest that the cadherin/β-catenin adhesion complex may play a role in this process. However, thus far, it is unclear how cadherin/β-catenin complexes regulate SV localization.

Scribble, a member of the LAP (leucine-rich repeats and PDZ domains) protein family (Yamanaka and Ohno, 2008), has been shown to localize to Drosophila neuromuscular junctions (NMJ) (Mathew et al., 2002), where it plays a role in regulating SV localization (Roche et al., 2002). Loss-of-function mutations in scribble result in an accumulation of SVs at the NMJ, and a decreased number of presynaptic active zones (Roche et al., 2002). Moreover, several forms of plasticity are drastically altered in scribble mutants, accompanied by impaired vesicle dynamics (Roche et al., 2002). These results suggest that scribble is an essential regulator of SV turnover. A relationship between scribble and the cadherin/β-catenin complex has been demonstrated in various cell types and tissues (Navarro et al., 2005; Nguyen et al., 2005; Kamei et al., 2007). For instance, scribble has recently been shown to exist in a complex with β-catenin and adenomatous polyposis coli (APC) in the mouse brain (Takizawa et al., 2006; Kalla et al., 2006; Tsuriel et al., 2006). Indeed, a high degree of sharing of SVs between synapses has been reported (Darcy et al., 2006a, b), and may be important for regulating synapse maintenance, efficacy and plasticity (Staras, 2007). Elucidating the molecular mechanisms underlying SV recruitment and localization will therefore not only shed light on presynaptic assembly, but will also be informative with respect to mechanisms underlying presynaptic plasticity.
Our results suggest that scribble is recruited by β-catenin to presynaptic compartments, where it recruits SVs to developing synapses.
2.2 MATERIALS AND METHODS

2.2.1 RNAI CONSTRUCTS AND RECOMBINANT DNAs

To suppress expression of endogenous scribble, three RNA interference (RNAi) constructs specifically against mouse scribble were transiently transfected into mouse hippocampal neurons. RNAi-1 and RNAi-2 are short hairpin RNA (shRNA) sequences corresponding to mouse scribble (GenBank accession no. NM_134089) nucleotides 3396 - 3416 and 1280 - 1300, respectively and were expressed using the pRETROSUPER vector (Ludford-Menting et al., 2005). RNAi-3 is a commercially available cocktail of 3 short interfering RNA (siRNA) oligonucleotides against mouse scribble (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). An shRNA specifically against human scribble (GenBank accession no. NM_015356) nucleotides 1842 - 1862 was used as a control (RNAi-C) (Dow et al., 2007).

The Syn-GFP construct was a kind gift from T. Nakata. GFP-hScrib has been reported previously (Dow et al., 2003). RFP-hScrib construct was generated by replacing GFP with RFP at NheI/BsrGI sites of GFP-hScrib. Mouse β-catenin was cloned by standard PCR and inserted into the BamHI site of pGEX vector to generate GST-β-cat, GST-β-cat ΔArm, and GST-β-cat ΔPDZb.

2.2.2 NEURON CULTURES

Hippocampi from E18 rat, E18 mouse, or P1 B6.129-Ctnnb1tm2Kem/KnwJ mouse (JAX® Mice and Services, Bar Harbor, ME) were prepared as previously described (Xie et al., 2000), and plated at a density of 130 cells/mm², 170 cells/mm², 260 cells/mm², respectively. For western analysis, hippocampal neurons were transfected with RNAis using the Amaxa Nucleofector System (Amaxa Inc., Gaithersburg, MD) according to manufacturer’s instructions prior to plating. For all other analyses, neurons were transfected using Lipofectamine 2000 (Invitrogen Canada Inc., Burlington, ON) at 7 DIV and imaged at 10 DIV unless otherwise noted.
2.2.3 SEMI-QUANTITATIVE RT-PCR

P1 mouse hippocampal cells were transfected with GFP plus scribble RNAi-C or RNAi-1 using Amaxa nucleofection. Total RNA was isolated using RNeasy Kit (Qiagen Canada, Mississauga, ON) and reverse transcribed using the SuperScript First Strand Synthesis system for RT-PCR kit (Invitrogen Canada Inc., Burlington, ON). The resulting cDNA fragments were amplified by PCR using the following primers: mouse scribble primers (5’-CAG CCA AAG CTG AGC GAC G-3’; 5’-GAC AAA GGC AAG CGT CCA C-3’), mouse GAPDH primers (5’-CTG AAC GGG AAG CTC ACT-3’; 5’-GTC ATA CCA GGA AAT GAG C-3’), and GFP primers (5’-GTG AGC GGC GAG GAG-3’; 5’-CTT GTA GTT GCC GTC GTC-3’). PCR reaction was run on a 1% agarose gel and visualized by SYBR Safe DNA gel stain (Invitrogen Canada Inc., Burlington, ON). The gel image was acquired using the Alphalmager Imaging system (Alpha Innotech Corp., San Leandro, CA). To quantify band intensity, images were imported into ImageJ and the mean gray value was analyzed.

2.2.4 IMMUNOHISTOCHEMISTRY

Neuron cultures were fixed in 4% paraformaldehyde/ 4% sucrose for 10 min, permeabilized in 0.1% Triton-X for 10 min, and blocked in 10% goat serum for 1 hr at room temperature. Primary antibodies were applied in 1% goat serum overnight at 4°C and secondary antibodies were applied in 1% goat serum for 1 hr at room temperature. Primary antibodies: mouse anti-tau (Sigma, Saint Louis, MO), mouse anti-synaptophysin (Sigma, Saint Louis, MO), rabbit anti-scribble (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), guinea pig anti-VGLUT-1 (Synaptic Systems, Goettingen, Germany), mouse anti-β-catenin (Zymed Laboratories, Inc. South San Francisco, CA), mouse anti-bassoon (Assay Designs, Inc., Ann Arbor, MI), mouse anti-PSD-95 (Affinity BioReagents, Golden, CO) and mouse anti-scribble (Dow et al., 2003). Secondary antibodies: Alexa 488, Alexa 633 and Texas Red-conjugated goat anti-mouse or anti-rabbit (Molecular Probes, Eugene, OR).

2.2.5 FM 4-64 ANALYSES

FM 4-64 experiments were done as previously described (Gerrow et al., 2006). Briefly, 15 µM FM 4-64 (Molecular Probes, Eugene, OR) was loaded for 30 s into presynaptic terminals using a hyperkalemic solution of 90 mM KCl in modified HBSS, where equimolar NaCl was
omitted for final osmolality of 310 mOsm. Neurons were rinsed three times and maintained in HBSS without Ca$^{2+}$ for imaging. 1 mM ADVESAP-7 (Sigma, Saint Louis, MO) was added to quench non-specific signal. Three images were captured every 30 s to confirm that the positive FM 4-64 sites were stationary presynaptic terminals. Unloading was done using the hyperkalemic solution described above and neurons were rinsed three times with NeuroBasal media for continued imaging.

### 2.2.6 IMMUNOBLOT ANALYSIS

**Preparation of protein lysates:** Brain tissues or cultured hippocampal neurons were homogenized in approximately 4 vols (w/v) of lysis buffer containing (50mM Tris pH 7.4, 150mM NaCl, 1.0% NP-40, 10% glycerol) and centrifuged at 14 000 g for 30 min at 4°C.

**Crude synaptosomal fraction (P2):** E18 rat were decapitated and brains were rapidly removed and placed in ice cold homogenization buffer (320mM sucrose, 4mM Hepes and 1mM EGTA). The tissue was homogenized in a homogenizer (Canadian Laboratory Supplies, Winnipeg, MB) by 6 gentle up and down strokes at 2300 rpm. The homogenate was centrifuged at 1312 g for 10 min to remove nuclei and cell debris. The resulting supernatant was centrifuged at 14 481 g for 15 min to remove small cell fragments and total soluble proteins. The resulting pellet was resuspended in homogenization buffer and centrifuged at 17 522 g for 15 min to obtain the crude synaptosomal fraction, P2. P2 was resuspended in lysis buffer described above and used for immunoprecipitation and GST pull down assays.

**Immunoprecipitation:** P2 synaptosomal preparations were incubated overnight at 4°C with an anti-β-catenin antibody, an anti-scribble antibody or pre-immune serum. The following day, 50 µl of protein A/G–Sepharose (GE healthcare, Chicago, IL) was added, and the bead-bound immunocomplexes were recovered after 2 hr, washed 4 times with lysis buffer, solubilized with loading buffer, separated by SDS–PAGE and analyzed by means of immunoblotting with antibodies against scribble, β-catenin, synaptophysin or cadherin (Zymed Laboratories, Inc. South San Francisco, CA).

**Western blot analysis:** Proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL) on a Bio-Rad Versadoc 4000 (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON). The brightness and contrast of entire images was moderately adjusted using Photoshop (Adobe Systems Canada, Toronto, ON) after recommended,
scientifically acceptable procedures, and no information was obscured or eliminated from the original (Rossner and Yamada, 2004).

### 2.2.7 GENERATION OF GST FUSION PROTEINS AND GST PULL DOWN ASSAYS

GST-β-catFL, GST-β-cat ΔPDZb and GST were expressed in *E. coli* DH5α cells after induction with 0.1mM isopropyl-β-D-1-thiogalactopyranoside for 3 h at 37 °C. Bacteria were pelleted and lysed in PBS, 5mM DTT, 1mM PMSF in the presence of protease inhibitors (Roche Diagnostics, Indianapolis, IN) by passing through French Press twice (Thermo Fisher Scientific, Inc., Waltham, MA). Fusion proteins were purified on Glutathione Sepharose 4 Fast Flow (GE healthcare, Chicago, IL) following the manufacturer’s protocol. The amount of GST fusion protein input was verified by Coomassie blue staining.

For protein binding assays, Sepharose-bound GST fusions or GST were incubated with E18 synaptosomal preparations for 2 hr at 4°C and followed by 4 washes with PBS. The proteins bound to the Sepharose beads were solubilized with loading buffer and loaded onto a SDS-PAGE. Proteins bound to the GST fusion protein were detected using antibodies against scribble and cadherin.

### 2.2.8 CONFOCAL IMAGING

Transfected hippocampal neurons were imaged using an Olympus Fluoview 1000 confocal microscope (10X/0.30 UPlan FL N; 20X/0.75 UPlan SApo; 60X/1.4 Oil Plan-Apochromat). All images in a given experiment were captured and analyzed with the same exposure time and conditions. To enhance visualization of the diffuse localization of Syn-GFP in scribble knockdown cells, the intensity levels of those representative pictures were enhanced using Adobe Photoshop.

### 2.2.9 IMAGE ANALYSIS AND QUANTIFICATION

**Colocalization analyses:** Images of were analyzed using ImageJ with a colocalization plugin downloaded from the program’s website (http://rsb.info.nih.gov/ij/plugins/colocalization.html). Points of colocalization were defined as regions greater than 4 pixels in size where the intensity ratio of the two channels was greater than 50. All the puncta were examined in a field.
Numbers of colocalized puncta were expressed as a percentage of the total number of puncta, which were analyzed at threshold 85 (for VGLUT-1), 80 (for PSD-95), 80 (for scribble), 60 (for Synaptophysin), 80 for β-catenin, 50 (for Syn-GFP) or 40 (for RFP-hScrib). To examine the percentage of synapses associates with scribble, a ‘mask’ was made of VGULT-1 and PSD-95 overlay images, and the percentage was then determined by the presence of scribble on this ‘mask’.

**Density analyses:** To determine the density of puncta along axons, puncta were manually counted and the lengths of Syn-GFP labeled axons were measured using Image J. The density was expressed as the number of puncta over 100 µm axon length.

**Fluorescence intensity histogram:** To examine the distribution of Syn-GFP fluorescence signal along the axon, a short line representing approximately 30 µm was drawn along the axon and through the major axis of Syn-GFP fluorescence signal. The distribution of fluorescence intensity along this line was determined by the Fluoview 1000 software, and the histogram generated using Excel (Microsoft Canada Co., Mississauga, ON).

**Coverage of Syn-GFP and RFP-hScrib fluorescence:** Variability in Syn-GFP and RFP-hScrib fluorescence can occur depending on the density of cells and the density of processes in a given region. To minimize variability, transfected neurons were identified, and the density of processes in the region observed using brightfield. Images were obtained from regions with similar density of processes. With few exceptions, our cultures exhibit similar densities throughout the coverslip. To quantify the length of Syn-GFP and RFP-hScrib fluorescence signal, images were imported into Image J and the major axis lengths of Syn-GFP and RFP-hScrib fluorescence signal (Feret’s diameter) were analyzed at a set threshold of 50 and minimum pixel size of 10. The distribution of Syn-GFP fluorescence signal was also expressed as the Syn-GFP fluorescence coverage, which represents the sum of the length of Syn-GFP fluorescence signal along a 10 µm axon length.

**Intensity analyses:** Quantification of the intensity of Syn-GFP fluorescence at colocalized bassoon and PSD-95 sites were done using Image J. Sites of colocalized bassoon and PSD-95 puncta were first determined by the colocalization plug-in described above and saved as selections (a “mask”). The intensity of Syn-GFP fluorescence was quantified on bassoon/PSD-95 selections.

**Puncta size analyses:** To quantify the size of FM 4-64, bassoon, or β-catenin puncta along a
single transfected neuron, a selection (“mask”) was initially made of the GFP signal. The area of the puncta within the selection was then quantified using Image J at threshold 100 (for FM 4-64), 80 (for β-catenin) and 100 (for bassoon).
2.3 RESULTS

2.3.1 SCRIBBLE INTERACTS WITH β-CATENIN AND LOCALIZES AT SYNAPSES

*Drosophila* scribble has been implicated in the development of synapse structure and function (Roche et al., 2002). The mammalian homolog of scribble has a primary structure similar to that of its fly homolog (Santoni et al., 2002), and localizes to distinct regions in the brain, including the hippocampus (http://www.brain-map.org/). However the function of scribble in neurons remains unknown. To determine the distribution of scribble in hippocampal neurons, 10 DIV primary hippocampal cultured neurons were immunostained for scribble. Scribble immunoreactivity was observed throughout the cell, being localized to both dendrites and axons (Fig. 2.1). Previous work has demonstrated that scribble is localized to synapses in cultured cerebellar neurons (Audebert et al., 2004; Takizawa et al., 2006). Analysis of hippocampal cultures demonstrates a similar synaptic distribution for scribble (Fig. 2.2A-D), whereby 84.8% of excitatory synapses, identified by colocalization of the presynaptic vesicular glutamate transporter, VGLUT-1, and the excitatory postsynaptic marker, PSD-95 were associated with scribble immunoreactive puncta (Table 2.1). Scribble puncta that are not associated with VGLUT-1 and PSD-95 may represent populations at inhibitory synapses or at non-synaptic sites (Fig. 2.2B, D; arrows). Moreover, the majority of synaptophysin, an integral synaptic vesicle (SV) marker, colocalizes with scribble (Fig. 2.2E-G, Table 2.1). Scribble positive sites that are not associated with synaptophysin may represent mobile scribble clusters or scribble clusters that are at nascent synapses prior to the recruitment of synaptic vesicles.
Figure 2.1: Scribble immunoreactivity in cultured hippocampal neurons.
Confocal image of a 10 DIV neuron immunolabelled with anti-scribble (A) and the axonal marker, tau (B). Scribble is observed in both tau-positive (arrows) and tau-negative (arrowheads) processes, indicating its localization in both axons and dendrites (A, C). Asterisks denote the cell body for reference. Scale bar = 10 µm.

Table 1 Analysis of scribble localization at synapses.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Ave. ± SE</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>% VGLUT-1/PSD-95 co-localized with scribble</td>
<td>84.8 ± 1.82</td>
<td>21 images, 2 cultures</td>
</tr>
<tr>
<td>% synaptophysin co-localized with scribble</td>
<td>68.3 ± 5.69</td>
<td>12 images, &gt;3 cultures</td>
</tr>
<tr>
<td>% Syn-GFP co-localized with scribble</td>
<td>74.6 ± 9.54</td>
<td>10 cells, 3 cultures</td>
</tr>
<tr>
<td>% Syn-GFP co-localized with RFP-hScrib</td>
<td>75.7 ± 6.53</td>
<td>9 cells, &gt;3 cultures</td>
</tr>
<tr>
<td>Density of scribble immunoreactive puncta/100um</td>
<td>8.14 ± 1.19</td>
<td>10 cells, 3 cultures</td>
</tr>
<tr>
<td>Density of RFP-hScrib puncta/100um</td>
<td>9.56 ± 1.23</td>
<td>11 cells, &gt;3 cultures</td>
</tr>
<tr>
<td>% β-catenin co-localized with scribble</td>
<td>72.0 ± 3.10</td>
<td>12 images, &gt;3 cultures</td>
</tr>
<tr>
<td>% β-catenin/scribble co-localized with bassoon</td>
<td>87.9 ± 1.31</td>
<td>10 images, 2 cultures</td>
</tr>
<tr>
<td>% bassoon co-localized with β-catenin/scribble</td>
<td>70.6 ± 1.65</td>
<td>10 images, 2 cultures</td>
</tr>
</tbody>
</table>
Figure 2.2: Scribble localizes to synapses.

(A-M) Confocal images of 12 DIV hippocampal cultures. (H-M) To determine the subcellular localization of fluorescently-tagged proteins in individual neurons, cells were transfected at <1% efficiency with Lipofectamine 2000 at 10 DIV. Endogenous scribble is localized in a punctate pattern and highly colocalizes with VGLUT-1 (A) and PSD-95 (B) puncta, indicating its localization at excitatory synapses (A-D). Scribble-positive puncta that do not colocalize with VGLUT-1/PSD-95 puncta are also observed (for examples see arrows). Endogenous scribble also colocalizes with synaptophysin (E-G), and Syn-GFP (H-J). RFP-hScrib is localized in a puncta pattern and colocalizes with Syn-GFP (K-M). Scribble-positive puncta that do not colocalize with synaptophysin (arrows), and synaptophysin puncta that do not colocalize with scribble (arrowheads) are observed (E-G). Similarly, the majority of Syn-GFP puncta colocalize with scribble (H-J). Scribble-positive puncta that do not colocalize with Syn-GFP represent immunostaining on cells not transfected with Syn-GFP (H-J; arrows). Arrows indicate RFP-hScrib puncta that do not colocalize with Syn-GFP (K-M). Scale bars = 5 µm.
GFP-tagged synaptic vesicle marker proteins have been widely used to mark presynaptic sites in vertebrates, and do not compromise the secretory physiology of the synapse (Sankaranarayanan and Ryan, 2000). Moreover, our work has previously demonstrated that the pattern of synaptophysin-GFP (Syn-GFP) expression is comparable to that of endogenous SV proteins (Bamji et al., 2003). Endogenous scribble puncta were found to colocalize with Syn-GFP to approximately the same degree, further demonstrating the faithful use of this tagged marker (Fig. 2.2H-J, Table 2.1). To determine whether RFP-conjugated human scribble (RFP-hScrib) is appropriately expressed, neurons were co-transfected with RFP-hScrib and Syn-GFP. The percent colocalization of RFP-hScrib and Syn-GFP was highly similar to that observed with endogenous scribble and synaptophysin (Fig. 2.2K-M, Table 2.1). Finally, the density of RFP-hScrib clusters was similar to that of endogenous scribble (Table 1, p>0.05), and RFP-hScrib was found associated with endogenous synaptophysin clusters (data not shown). Thus, our data indicate that Syn-GFP and RFP-hScrib are faithful markers for assaying appropriate localization patterns of the corresponding endogenous proteins.

We next examined the association of endogenous scribble and β-catenin in neurons. Immunohistochemical analysis demonstrated that a large proportion of β-catenin puncta colocalize with scribble puncta. These colocalized clusters were highly enriched at bassoon immunoreactive sites, suggesting that scribble colocalizes with β-catenin at synapses (Fig. 2.3A-E, Table 2.1). To further demonstrate that scribble associates with β-catenin at synapses, crude synaptosomal fractions from E18 brains were prepared and immunoprecipitated with antibodies against scribble and β-catenin. Co-immunoprecipitation assays demonstrate that scribble associates with β-catenin and cadherin at synapses (Fig. 2.3F). The interaction between β-catenin and scribble was further confirmed using GST pull down assays (Fig. 2.3G).
Figure 2.3: Scribble associates with β-catenin at synapses.

(A-E) Confocal images of 12 DIV hippocampal cultures demonstrating colocalization of scribble, β-catenin and the presynaptic marker, bassoon. Higher magnifications of the inset from panel (A) are shown in (B-E). The majority of scribble puncta colocalized with β-catenin and bassoon. β-catenin puncta that do not colocalize with scribble (arrows), scribble puncta that do not colocalize with β-catenin (open arrows), and bassoon puncta that do not associate with β-catenin and scribble (arrowheads) are observed. Colocalized scribble and β-catenin puncta that are not associated with bassoon positive sites are also observed (open arrowheads). (F, G) Scribble and β-catenin can be co-immunoprecipitated from synaptosomal fractions. Synaptosomal fractions from E18 brains were immunoprecipitated using β-catenin or scribble antibodies, separated by SDS-PAGE, and immunoblots probed with antibodies specific to scribble, β-catenin, synaptophysin or cadherin. The input lane corresponds to 40 µg of the crude synaptosomal fraction, P2. Rabbit IgG was used as a control. Scribble and β-catenin were co-immunoprecipitated with one another and with cadherin, but not with the synaptic protein, synaptophysin, indicating the specificity of the immunoprecipitation. N= 2 different preparations. (G) Synaptosomal fractions from E18 brains were affinity purified with GST-β-cat FL, GST-β-cat ΔPDZb or
GST alone bound to glutathione-Sepharose beads. Coomasie blue staining was used to verify the levels of GST and GST fusion proteins used for GST pull-down assay (arrows). Bound proteins were eluted and blots probed with anti-scribble or anti-cadherin. Scale bars = 5 µm.

β-catenin consists of three domains: an N-terminal domain that interacts with α-catenin, a central domain of twelve armadillo repeats that binds to cadherin and LEF/TCF transcription factors (Daniels et al., 2001; Ivanov et al., 2001), and a C-terminal domain that interacts with transcriptional regulators and contains a PDZ binding motif (Perego et al., 2000). Our previous work demonstrated that the PDZ binding motif is essential for the clustering of SVs at synaptic junctions (Bamji et al., 2003). To investigate whether this domain is important for the interaction between β-catenin and scribble, β-catenin deletion mutant lacking the PDZ binding motif (GST-β-catΔPDZb) was generated (Fig 2.3G). Full-length β-catenin (GST-β-cat FL) was able to pull down scribble and cadherin from synaptosomal fractions. In contrast, deletion of the C-terminal 10 amino acids containing the PDZ binding motif abolished the association between β-catenin and scribble. As expected, GST-β-catΔPDZb was able to pull down cadherin from synaptosomal fractions (Fig. 2.3G). Together, this demonstrates that scribble associates with cadherin and β-catenin at synapses via the PDZ binding motif of β-catenin.

2.3.2 SVS ARE MISLOCALIZED IN HIPPOCAMPAL NEURONS LACKING SCRIBBLE

To study the role of scribble at synapses, scribble protein levels were attenuated in hippocampal neurons using RNAi. To minimize possible “off-target” effects, two short hairpin RNAs (RNAi-1, 2) and one cocktail of 3 short interfering RNA oligonucleotides (RNAi-3), were used. The efficacy of the RNAis was examined using western blot analysis. Although the efficiency of transfection was on average only 35.9 ± 2%, a significant decrease in scribble protein levels was observed in cultures transiently transfected with RNAi-1, 2 or 3, as compared to cultures transfected with control RNAi (RNAi-C; Fig. 2.4A, B). In contrast, β-catenin levels remained similar in scribble RNAi-expressing cells (Fig. 2.4A). (It is important to note that Fig. 3B represents raw data and has not been normalized for transfection efficiency). Similarly, quantitative RT-PCR revealed significant decreases in scribble transcripts in cultures transfected with RNAi (Fig. 2.5). The efficiency of scribble depletion was similar between all three RNAi treatments, and used interchangeably in subsequent experiments. To further confirm the
knockdown of scribble protein in neurons expressing RNAi, cultured hippocampal neurons were transfected with either RNAi-C or each one of the 3 RNAis and immunolabeled for scribble. Cells were co-transfected with Syn-GFP to mark RNAi transfected neurons. At low magnifications, expression levels of scribble in cell bodies of control cells were abundant (Fig 2.4C, D asterisks), whereas in cells expressing RNAi-1, somatic scribble levels were dramatically decreased (Fig. 2.4E, F, asterisks). This was consistently observed in neurons expressing each of the 3 RNAis, indicating an effective knockdown of scribble in neuronal cultures.

Interestingly, the pattern of Syn-GFP expression in RNAi-expressing neurons was altered compared to control neurons. In wild-type cells, discrete Syn-GFP puncta were observed along the axon (Fig. 2.4D’; 2.6A, B). In contrast, in scribble RNAi-expressing cells, discrete Syn-GFP puncta were not observed. (Fig. 2.4F’; 2.6F, G). To confirm the distribution of SVs in RNAi-tranfected cells, cultures were immunostained with synaptotagmin, another integral SV marker. Synaptotagmin expression appeared similar to that of Syn-GFP, with fewer large immunopositive clusters along RNAi expressing axons (Fig. 2.4J-L).
Figure 2.4: RNAi-mediated knockdown of scribble protein levels in primary neurons.

(A, B) Neurons were transfected with control RNAi (RNAi-C), or three distinct scribble RNAis (RNAi-1 to 3) with approximately 35% transfection efficiency using the Amaxa nucleofector system. Immunoblot analysis revealed a decrease in scribble protein levels in all three RNAi expressing cultures, whereas β-catenin levels remained constant. (B) Quantification of immunoblots represents raw data and has not been normalized for transfection efficiency (35.9 ± 2%). N=3 immunoblots from 3 separate cultures. *p<0.05 using Student’s T-Test. (C-L) Confocal images of 10 DIV hippocampal neurons co-transfected using Lipofectamine 2000 (<1% transfection efficiency) with Syn-GFP plus either RNAi-C (D, G) or RNAi-1 (F, J), and immunostained for scribble (C, E) or synaptotagmin (H, K). The neuron expressing RNAi-1 shows a clear reduction in scribble immunostaining in the cell body (E) compared to...
RNAi-C expressing cell (C). Asterisks denote transfected neurons and arrows denote untransfected neurons. Syn-GFP is punctate in RNAi-C expressing cells (D’, G), but more diffusely localized in cells expressing RNAi-1 (F’, J). (G-L) Endogenous synaptotagmin is diffusely expressed in RNAi-1 expressing cells. In RNAi-expressing cells Syn-GFP and synaptotagmin are colocalized and display a punctate distribution (G-I). In contrast, in neurons expressing RNAi-1, both Syn-GFP and synaptotagmin are more diffusely distributed (J-L). Synaptotagmin-positive puncta that do not colocalize with Syn-GFP represent immunostaining on cells not transfected with Syn-GFP and RNAi-1. Scale bars = 20 µm.

Figure 2.5: Scribble mRNA level is decreased in RNAi-1 expressing neurons.

Neurons were transfected with control RNAi (RNAi-C), or RNAi-1 with approximately 35% transfection efficiency using the Amaxa nucleofector system. (A) RT-PCR demonstrating a reduction in scribble mRNA levels in cells transfected with RNAi-1 compared to cells transfected with RNAi-C. Genomic DNA contamination was determined with RNAi-1 -RT and RNAi-C -RT (no reverse transcriptase). PCR with primers specific to GAPDH were used to normalize samples. PCR with primers specific to GFP were used to verify similar transfection efficiencies. (B) Scribble mRNA expression level was presented by densitometry normalized to GAPDH ± SE. Scribble mRNA expression was decreased by 68 ± 6.3% in RNAi-1 expressing neurons compared to control. N=2 different preparations.

To further investigate the pattern of SV localization in neurons, and specifically its localization at synaptic sites, cells were transfected with Syn-GFP and RNAi-C or each of the 3 RNAis, and immunostained with bassoon and PSD-95 to label pre- and postsynaptic sites, respectively. In control neurons, the fluorescence intensity of the Syn-GFP positive puncta was high, as observed in the pseudo-coloured, low-magnification image (Fig. 2.6A), and in the intensity distribution histogram (Fig. 2.6K). Discrete fluorescence intensity peaks representing individual Syn-GFP puncta were observed, and the level of fluorescence intensity between peaks (inter-punctal intensity) was relatively low (Fig. 2.6A, K). These Syn-GFP positive puncta were localized to synapses as observed by its colocalization with PSD-95 and bassoon
(Fig. 2.6B-E). In contrast, in RNAi-expressing cells, the fluorescence intensity distribution of Syn-GFP was relatively uniform along the axon, with an overall increase in basal intensity compared to the inter-punctal intensity of wild-type cells (Fig. 2.6F, L). Although Syn-GFP fluorescence was more diffusely distributed along the axon, PSD-95 and bassoon colocalization (synapses) was still evident in these cells (Fig. 2.6G-J).

We next measured synaptophysin levels to ensure that changes in the pattern of Syn-GFP distribution in scribble knockdown cells were not due to overall changes in synaptophysin expression. Both synaptophysin and β-catenin levels remained similar in scribble RNAi-expressing cells compared to control (Fig. 2.6M), suggesting that attenuation of scribble perturbs the distribution of SV clusters, while leaving the overall expression of SV proteins undisturbed.

Variability in the “punctate-ness” of Syn-GFP was observed in RNAi-expressing cells. To determine the distribution of vesicles along the axon, the Feret’s diameter (defined as the greatest distance possible between any two points along the boundary of a region of interest, hereafter called the “length”) of the Syn-GFP fluorescence signal was determined as described previously (Bamji et al., 2003). To avoid bias, all transfected neurons on each coverslip were imaged and quantified. A 28.6-34.4% increase was observed in the average length of Syn-GFP fluorescence signal in knockdown cells compared to wild-type. Although the increase was significant, the difference between wild-type and knockdown cells was minimized due to an increased density of small Syn-GFP puncta in knockdown cells which we believe arise from small clusters of SVs that are not retained at synapses. To address this, the sum of the length of Syn-GFP fluorescence signal per 10 µm axon length (hereafter referred to as the “coverage”) was used to represent the distribution of SVs along the axon (Fig. 2.6N). No significant difference was seen in Syn-GFP coverage between neurons expressing Syn-GFP alone and Syn-GFP plus RNAi-C. In contrast, the coverage of Syn-GFP along the axon was over 2-fold greater in RNAi-expressing neurons compared to neurons expressing RNAi-C. The magnitude of the phenotype was similar for all three RNAis. Importantly, co-expression of RFP-hScrib that is insensitive to our RNAis rescued this phenotype, indicating that the observed effects were primarily due to specific interference with scribble function (Fig. 2.6N).

To further assess the distribution of SVs at synapses, the intensity of Syn-GFP fluorescence at synapses (defined here as points of colocalization between PSD-95 and bassoon) was quantified. A significant decrease in Syn-GFP fluorescence intensity at synapses was
observed in RNAi-transfected cells compared to control cells (Fig. 2.6O), suggesting that SV number is specifically diminished at synaptic junctions upon scribble knockdown. Synapse number was also quantified by counting the density of bassoon-positive puncta that colocalized with PSD-95 in control and RNAi-transfected cells, and no significant difference was observed (Fig. 2.6P).
Figure 2.6: SVs are more diffusely distributed along the axon in neurons expressing scribble RNAi constructs.

(A-J) Confocal images of 10 DIV hippocampal neurons co-transfected with Syn-GFP plus scribble RNAis using Lipofectamine 2000 (<1% transfection efficiency) and immunostained for bassoon and PSD-95. Synapses were defined as regions where bassoon and PSD-95 puncta colocalized. (A, F) Images of neurons transfected with RNAi-C (A) and RNAi-1 (F) and pseudo-colored for fluorescence intensity. Insets from panels (A) and (F) are shown in higher magnification (B-E, G-J) and selected for histogram analyses (K, L). In RNAi-C expressing neurons, Syn-GFP is punctate and colocalizes with PSD-95 and bassoon (B-E, arrows). In RNAi-1 expressing neurons, Syn-GFP fluorescence is more diffusely distributed along the axon, however discrete bassoon and PSD-95 colocalization is still observed (G-J, arrows). Immunopositive bassoon and PSD-95 puncta that do not colocalize with Syn-GFP may represent immunostaining on untransfected neurons. (K, L) Histograms of Syn-GFP fluorescence intensity along a selected axon length in neurons expressing RNAi-C (A, B) or RNAi-1 (F, G) demonstrate that the distribution of Syn-GFP fluorescence is more uniform in scribble knockdown cells, and lack distinct punctal and inter-punctal regions. (M) Using the Amaxa nucleofector system, neurons were transfected with control RNAi (RNAi-C), or scribble RNAis (RNAi-1 to 3), and the expression level of synaptophysin and β-catenin was analyzed using western blot. Despite the fact that synaptophysin protein levels are similar in cultures transfected with RNAi-C and RNAi-1 to 3 (M), the coverage of Syn-GFP along the axon (the sum of the length of Syn-GFP fluorescence signal per 10 µm axon length ± SE) is greater in RNAi-expressing cells (N). N= 30-60 cells, and >2000 puncta from >3 separate cultures. ***p<0.001 using Student’s T-Test. (O) A significant decrease in the average intensity of Syn-GFP fluorescence at sites of bassoon/PSD-95 colocalization was observed in RNAi-expressing cells. N= >23 cells, and 106-158 colocalized bassoon and PSD-95 puncta from at least 3 separate cultures. *p> 0.05 using Student’s T-Test. (P) The density of bassoon/PSD-95 clusters along transfected axons (the average number of colocalized immunopositive puncta per 100 µm of axon length ± SE), was similar in control and RNAi-expressing cells. (Q, R) Schematic of the effect of scribble knockdown on SV localization. (Q) In wild-type neurons, SV clusters are localized to synapses, and some clusters are also observed in perisynaptic regions. (R) In scribble knockdown cells, SV are more diffusely distributed along the axon and less SVs are accumulated at synapses. Green bars above each axon mark the length of SV clusters and thickness reflects the relative intensity of Syn-GFP clusters. Although the number of SVs in wild-type and knockdown cells are similar, the Syn-GFP fluorescence coverage is greater in knockdown cells, highlighting the diffuse distribution of SVs along the axon. Scale bars = 5 µm.

To determine the role of scribble on presynaptic development beyond its role in SV localization, the expression pattern of the presynaptic cytoskeletal matrix protein, bassoon, was
examined. Bassoon is recruited to synapses in large dense-core vesicles along with other components of the active zone, including piccolo, N-cadherin, syntaxin, SNAP-25 and chromogranin B, independently of the vesicles that transport SV proteins to synapses (Zhai et al., 2001). The density, size and intensity of endogenous bassoon was similar between control and scribble RNAi expressing neurons (Fig. 2.7). These data suggest that scribble is involved in some, but not all steps, of synapse assembly.

**Figure 2.7: Density, area and intensity of bassoon immunoreactive puncta remain constant in RNAi expressing cells.**

Quantification of 10 DIV hippocampal neurons transfected with Syn-GFP and the indicated RNAi. The density area and intensity of endogenous bassoon is similar in control and RNAi expressing neurons. N= >23 cells, and > 90 bassoon puncta from at least 3 separate cultures. p> 0.05 using student’s T-Test.

Taken together, our results demonstrate an important role for scribble in localizing SVs to synapses. In cells expressing scribble RNAi, discrete SV clusters are rarely observed, and the intensity of Syn-GFP fluorescence at synapses significantly decreases. Interestingly, scribble knockdown does not appear to affect the localization of other presynaptic proteins such as bassoon, or the density of synapses along the axon. This phenotype is very similar to the observation in β-catenin knockout neurons (Bamji et al., 2003).
2.3.3 SV RECYCLING DEFECTS IN CULTURED HIPPOCAMPAL NEURONS LACKING SCRIBBLE

Vesicle recycling is required to maintain SV pools and enable efficient neurotransmission at synapses (Sudhof, 2004; Schweizer and Ryan, 2006). To determine whether the defects in vesicle localization in scribble knockdown cells are associated with impaired presynaptic function, the efficiency of vesicle recycling was studied by stimulating neurons with a high-K+ solution in the presence of FM 4-64, a fluorescent dye that marks sites of endocytosis. In control cultures, uptake of FM 4-64 was observed at Syn-GFP sites (Fig. 2.8B, C). However, in cultures expressing scribble RNAi, this uptake was strongly suppressed, with greatly reduced density and size of FM 4-64-positive puncta compared to control cells (Fig. 2.8E, F, G). To confirm that this was a synaptic activity-dependent phenomenon, we assessed FM 4-64 unloading by re-stimulating labeled neurons. This treatment almost completely removed FM 4-64 dye in both control and experimental cultures (Fig. 2.8B’, E’), suggesting activity-dependent vesicle recycling.
Figure 2. 8: Deficits in SV recycling following scribble knockdown.

(A-F) Confocal images of 10 DIV neurons transfected with Syn-GFP and the indicated RNAi using Lipofectamine 2000 (<1% transfection efficiency). Neurons were loaded with FM 4-64 and three images were captured every 30 s to confirm that the positive FM 4-64 sites were stationary presynaptic terminals. Arrows indicate FM 4-64 positive sites on transfected axons. FM dyes were then unloaded to demonstrate specificity (A’-F’). (F’ arrowhead) The FM 4-64 positive site not observed in dye “load” image (E), but observed following dye “unload” (E’) most likely represents a mobile FM 4-64 positive puncta on an untransfected neuron. The FM 4-64 cluster in the transfected neurons (arrow) is not observed after de-staining. The density (G) and size (H) of FM 4-64 positive puncta ± SE were reduced in cells expressing RNAi-3 compared to control. N= 17 cells; >85 FM-4-64 puncta from >3 separate cultures. * p<0.05 using Student’s T-Test. Scale bar = 5µm.
2.3.4 β-CATENIN LOCALIZES SCRIBBLE TO SYNAPSES

Our previous work has demonstrated a role for β-catenin in the localization of SVs (Bamji et al., 2003). In light of our above results showing that scribble is also required for normal localization of SVs, and that scribble and β-catenin exist in complex with each other, we next determined whether scribble acts in concert with β-catenin to localize SVs. First, the localization of β-catenin in scribble knockdown neurons was examined. In wild-type cells, endogenous β-catenin displayed a punctate expression pattern and colocalized with Syn-GFP (Fig. 2.9A-C). In scribble knockdown cells, there was a diffuse pattern of Syn-GFP expression, however the β-catenin remained punctate (Fig. 2.9D-F). Moreover, the density of β-catenin along the axon, as well as the area of β-catenin, remained similar in control and scribble RNAi-transfected cells (Fig. 2.9G, H). This suggested that the localization of β-catenin at synapses is not dependent on scribble.
Figure 2.9: β-catenin localization is not affected in scribble RNAi-expressing cells.

(A-F) Confocal images of 10 DIV hippocampal neurons transfected with Syn-GFP and RNAi-C (A-C) or RNAi-3 (D-F) using Lipofectamine 2000 (<1% transfection efficiency) and immunostained for β-catenin. Syn-GFP puncta colocalize with β-catenin puncta in cells expressing RNAi-C (B, C; arrows). Neurons expressing RNAi-3 exhibit a diffuse pattern of Syn-GFP (D), however β-catenin expression remains punctate (E, F; arrows). The average density (G) and size (H) of β-catenin puncta ± SE is similar in control and RNAi-expressing cells. N>10 cells, and >250 puncta from 3 cultures. p>0.05 using Student’s T-Test. Scale bar =10 μm.
We next tested the role of β-catenin in the recruitment and synaptic localization of scribble. Hippocampal neurons prepared from B6.129-Ctnnb1^{tm2Kem}/KnwJ mice (homozygous β-catenin flox mice) were transfected with a vector expressing the Cre recombinase to ablate β-catenin. This methodology has previously been shown to efficiently ablate β-catenin in vitro (Bamji et al., 2003). In control cells, RFP-hScrib was expressed in a punctate pattern and colocalized with Syn-GFP and PSD-95 at synapses (Fig. 2.10A-D). In contrast, expression of the Cre recombinase in β-catenin flox neurons resulted in the expected diffuse pattern of Syn-GFP expression, as well as a diffuse pattern of RFP-hScrib expression (Fig. 2.10E-H). Indeed, the average length of RFP-hScrib fluorescence along the axon was significantly greater in cells lacking β-catenin (Fig. 2.10O). Interestingly, PSD-95 puncta at postsynaptic sites apposed to transfected axons remained punctate (Fig. 2.10G).

We have demonstrated that the PDZ binding motif of β-catenin is important for the interaction between β-catenin and scribble (Fig. 2.3G). To test whether the PDZ binding motif is important for the synaptic localization of scribble, neurons were transfected with either β-cat FL or β-catΔPDZb, plus Syn-GFP and RFP-hScrib. In cells expressing β-cat FL, Syn-GFP and RFP-hScrib had a punctate distribution, and were largely colocalized, similar to that observed with endogenous synaptophysin and scribble proteins (Fig. 2.10I-K; Fig. 2.1E-G). As previously demonstrated, cells expressing β-catΔPDZb exhibited a diffuse pattern of Syn-GFP expression (Fig. 2.10L) (Bamji et al., 2003). Interestingly, in these cells, scribble was also diffusely distributed along the axon (Fig. 2.10M, P). These data suggest that β-catenin plays an important role in localizing scribble to synaptic sites, and that scribble acts downstream of β-catenin to localize SVs.
Figure 2.10: Scribble is diffusely distributed along the axon in cells lacking β-catenin.

(A-H) Hippocampal neurons cultured from 10 DIV B6.129-Ctnnb1tm2Kem/J (homozygous β-catenin flox) mice were co-transfected using Lipofectamine 2000 (<1% transfection efficiency) with Syn-GFP and RFP-hScrib (A-D) or Syn-GFP, RFP-hScrib and a construct expressing the Cre recombinase to ablate β-catenin (E-H). Confocal images demonstrate that Syn-GFP and RFP-hScrib are colocalized and are apposed to postsynaptic PSD-95 at synapses in control cells (C, D; arrows). Neurons expressing the Cre recombinase exhibit a diffuse expression (E, F), whereas PSD-95 expression remains punctate (G, H;
arrows). Immunopositive PSD-95 puncta that do not colocalize with Syn-GFP may represent immunostaining on neurons that are not transfected. (I-N) Confocal images of 10 DIV hippocampal neurons transfected with Syn-GFP and RFP-hScrib plus either β-cat FL (I-K) or β-catΔPDZb (L-N). In β-cat FL expressing neurons, Syn-GFP puncta largely colocalize with RFP-hScrib puncta (I-K; arrows), whereas cells expressing β-catΔPDZb exhibit a diffuse pattern of Syn-GFP and RFP-hScrib expression (L-N). The normalized average coverage of RFP-hScrib along the axon ± SE is greater in cells lacking β-catenin (Cre+ cells) (O) and in cells expressing β-catΔPDZb (P) indicating a more diffuse distribution along the axon. N>7 cells and >500 puncta from 3 different cultures. *p<0.05, **p<0.001 using Student’s T-Test. Scale bars = 10 µm.
2.4 DISCUSSION

Increasing evidence suggests that there is a strong correlation between the number of SVs localized at presynaptic compartments and synaptic efficacy (Murthy et al., 2001; Cabin et al., 2002; Taschenberger et al., 2002; Bamji et al., 2003; Thiagarajan et al., 2005). As synapses mature, vesicle pool size and exocytotic efficiency (amount of exocytosis per Ca\(^{2+}\) influx) increases, resulting in more effective transmission following bursts of neuronal activity (Taschenberger et al., 2002). Evidence suggests that altering neuronal activity can impact the number of SVs localized to the presynaptic zone of mature synapses. For example, when hippocampal synapses in culture are pharmacologically silenced for several days, synaptic strength increases. Amongst changes in other synaptic components, this corresponds to an increase in the total number of docked vesicles and the total number of vesicles per synapse (Murthy et al., 2001), as well as an increase in the rate of vesicle turnover (Thiagarajan et al., 2005). Changes in the number of SVs per synapse can also alter synaptic efficacy. Indeed, in both α-synuclein (Cabin et al., 2002) and β-catenin (Bamji et al., 2003) knockout mice, there is a decrease number of total SVs per synapse, and a concomitant impairment in the synaptic response to prolonged repetitive stimulation. Understanding the mechanism of SV localization to synapses is therefore essential to our understanding of synapse development and function.

Recent studies demonstrate that the cadherin/β-catenin adhesion complex plays a central role in recruiting and retaining SVs to synapses (Iwai et al., 2002; Togashi et al., 2002; Bamji et al., 2003; Bamji et al., 2006; Jungling et al., 2006). β-catenin was shown to act downstream of cadherin to mediate SV localization (Bamji et al., 2003). As both the internal armadillo repeats that bind cadherin, and the PDZ binding motif of β-catenin are important for vesicle localization, β-catenin is believed to mediate SV clustering by acting as a scaffold (Bamji et al., 2003). In this study, we identify the PDZ protein downstream of β-catenin that localizes SVs to synapses. We demonstrate that β-catenin recruits scribble to synapses and forms a complex with scribble through its C-terminal PDZ binding motif. Scribble, in turn, regulates the recruitment and localization of SVs.
2.4.1 SCRIBBLE INTERACTS WITH β-CATENIN AT SYNAPSES

The present study indicates an interaction between scribble and β-catenin. Indeed, scribble can be co-immunoprecipitated with β-catenin from synaptosomal preparations, and largely colocalizes with β-catenin at synapses in cultured hippocampal neurons. GST pull down assays showed that the PDZ binding motif at the C-terminus of β-catenin is essential for this interaction. Our data demonstrating that β-catenin’s PDZ binding motif is important for synaptic localization for scribble and SV, suggests that scribble could be the downstream regulator to mediate β-catenin’s role in SV localization.

It is still unclear, however, whether this interaction is direct or indirect. Scribble has four PDZ domains and it is possible that one or more of these directly interact with β-catenin PDZ binding domain. Indirect interactions are also plausible. For example, it has been suggested that scribble and β-catenin may interact indirectly via their association with adenomatous polyposis coli (APC) (Takizawa et al., 2006). We do not believe that APC is acting as an intermediary for the association between scribble and β-catenin at synapses as β-catenin interacts with APC via its armadillo domain (Su et al., 1993), and our data suggest the PDZ binding motif of β-catenin is important for its association with scribble.

2.4.2 ROLE OF SCRIBBLE IN THE SYNAPTIC LOCALIZATION OF SVS

Interestingly, the role of β-catenin and scribble in regulating SV localization is independent of the initial steps of presynaptic development (Zhai et al. 2001), and bassoon remains punctate in β-catenin knockout cells (Bamji et al., 2003) and scribble knockdown cells. These data indicate that multiple signaling pathways are involved in presynaptic development and that these cues can act independently to regulate synapse assembly and function. In vitro studies demonstrate that synapse number remained similar in both β-catenin knockout cells and scribble knockdown cells. It would be interesting to see whether disrupting scribble in vivo affects synapse number. Indeed, in β-catenin conditional knockout mice, there was a significant increase in synapse number, which is potentially due to compensatory mechanisms (Bamji et al., 2003).

As disruption of intercellular cadherin interactions also results in SV mislocalization (Togashi et al., 2002), it is likely that cadherin, β-catenin and scribble act in concert to regulate
SV localization. How does scribble localize SVs? There are at least two broad possible answers: 1) scribble binds directly to SVs which in turn “traps” SVs at synapses, or 2) scribble acts as a scaffold to recruit other proteins to modulate SV localization.

The role of cadherin, β-catenin and scribble in regulating SV localization has been demonstrated in both invertebrate and vertebrate systems. Disruption of Drosophila N-cadherin (DN-cadherin) results in an aberrant over-accumulation of SVs at synapses formed between photoreceptor cells and their target interneurons (Iwai et al., 2002). Similarly, in Drosophila scribble mutants, there is an over-accumulation of SVs at the NMJ (Roche et al., 2002). In contrast, in vertebrate hippocampal cultures, disruption of intercellular N-cadherin contacts results in a mislocalization of SVs that are more diffusely distributed along the axon (Togashi et al., 2002). Moreover, ablation of β-catenin in conditional knockout mice results in a decreased number of vesicles associated with synapses, corresponding with a more diffuse pattern of SV localization in cultured hippocampal neurons (Bamji et al., 2003). In N-cadherin knockout embryonic stem cell-derived neurons, there is a clear presynaptic defect in the availability of vesicles for exocytosis, and a coincident alteration in short-term plasticity properties (Jungling et al., 2006). A detailed quantification of the number of SVs per synapse is lacking in this study, however the gross similarity between wild-type and mutant mice may be due to the expression of additional classic cadherins at the hippocampal synapse. It is currently unknown whether there is a disparity between vertebrate and invertebrate systems or whether differences in SV phenotypes arise from differences in synapse type.

It has previously been shown that the number of SVs at presynaptic compartments can contribute to the regulation of synaptic function (Cabin et al., 2002; Bamji et al., 2003), and that this can be modulated by homeostatic mechanisms in response to changes in synaptic activity (Murthy et al., 2001; Thiagarajan et al., 2005). Synaptic activity has also been shown to modulate synaptic adhesion. Indeed, alterations in activity can modulate both N-cadherin expression levels (Bozdagi et al., 2000) and levels of N-cadherin dimerization (Tanaka et al., 2000). These findings suggest that synaptic activity may regulate SV localization through modulation of cadherin/β-catenin adhesion complexes. Understanding the molecular mechanisms underlying SV recruitment and localization is therefore important not only for our understanding of the development and maintenance of synapses, but also for our understanding of how these signaling pathways could be used by neurons to modulate synaptic properties. Our
data demonstrate a critical role for scribble in localizing SVs and provide an important link between scribble and the cadherin/β-catenin complex.
3. β-PIX MODULATES ACTIN-MEDIATED RECRUITMENT OF SYNAPTIC VESICLES TO SYNAPSES

Presynaptic compartments are formed through the recruitment of preassembled clusters of proteins to points of cell-cell contact, however, the molecular mechanism(s) underlying this process remains unclear. We demonstrate that clusters of polymerized actin can recruit and maintain synaptic vesicles to discrete sites along the axon, and that cadherin/β-catenin/scrabble/β-pix complexes play an important role in this event. Previous work has demonstrated that β-catenin and scribble are important for the clustering of vesicles at synapses. We demonstrate that β-pix, a Rac/Cdc42 guanine nucleotide exchange factor (GEF), forms a complex with cadherin, β-catenin and scribble at synapses and enhances localized actin polymerization in rat hippocampal neurons. In cells expressing β-pix siRNA or dominant-negative β-pix that lacks its GEF activity, actin polymerization at synapses is dramatically reduced, and synaptic vesicle localization is disrupted. This β-pix phenotype can be rescued by cortactin overexpression suggesting that β-pix-mediated actin polymerization regulates vesicle localization.

3.1 INTRODUCTION

Synapse formation begins with the formation of incipient contacts, and culminates with the recruitment of synaptic proteins to points of contact. Presynaptic proteins are transported in preassembled clusters along axons in at least two types of transport packets; large dense-core vesicles containing active zone components, and synaptic vesicle transport packets, pleiomorphic vesicles containing synaptic vesicle-associated proteins as well as proteins critical for exo- and endocytosis (McAllister, 2007). It is still unclear what signals mediate the accumulation of these transport packets at developing synapses.

Recent work has resurrected the concept that artificial cell contact is sufficient to induce the clustering of synaptic vesicles (SVs) and that actin polymerization is required in this process. Indeed, vesicles accumulate at points of contact between axons and beads coated with poly-D-lysine, poly-L-lysine, or growth factors (Burry and Hayes, 1986; Burry et al., 1986; Lee
and Peng, 2006; Lucido et al., 2009). Actin polymerization at contact points precedes vesicle clustering, and treatment with latrunculin A abolishes the accumulation of vesicles at discrete sites along the axon (Kuromi and Kidokoro, 1998; Zhang and Benson, 2001; Lee and Peng, 2006; Lucido et al., 2009). This suggests, but does not directly demonstrate, a role for actin in SV clustering. Indeed, although gross actin depolymerization disrupts vesicle clustering, this can be attributed to a variety of factors including the weakening of strong cell-cell adhesion. It also remains unclear what signals enhance the preferential polymerization of actin at sites of cell-cell contact.

The cadherin/β-catenin adhesion complex has been shown to play an important role in clustering SVs at synapses (Iwai et al., 2002; Togashi et al., 2002; Bamji et al., 2003; Bamji et al., 2006; Lee et al., 2008). Perturbation of intercellular cadherin interactions (Togashi et al., 2002) or ablation of β-catenin (Bamji et al., 2003), dramatically impairs the accumulation of SVs at contact sites. We have recently demonstrated that cadherin/β-catenin complexes localize SVs to contact sites by recruiting the PDZ protein, scribble, to developing synapses, and that the mislocalization of SVs is phenocopied in scribble knockdown cells (Sun et al., 2009).

In this study, we identify a molecular pathway through which cell-cell contact can translate to the recruitment and localization of SVs to incipient synapses. We first demonstrate a positive role for actin in clustering SVs at synapses. We next demonstrate that β-pix, a Rac/Cdc42 specific guanine nucleotide exchange factor (GEF), can enhance actin polymerization at synapses and can recruit SVs to synapses. Knockdown of β-pix results in the mislocalization of SVs along the axon, which can be rescued by enhancing actin polymerization through cortactin overexpression. This directly implicates actin in mediating β-pix’s effects on SV clustering. Finally, we show that β-pix forms a complex with cadherin, β-catenin and scribble at synapses, and that scribble is important for the localization of β-pix at synapses. Together, our data suggest that cadherin/β-catenin/scribble complexes recruit β-pix to sites of cell-cell contact, and that this enhances the local polymerization of actin, which can “trap” SVs as they translocate along the axon.
3.2 MATERIALS AND METHODS

3.2.1 RNAI CONSTRUCTS AND RECOMBINANT DNAs

To suppress expression of endogenous scribble, a short hairpin RNA against mouse scribble (GenBank accession no. NM_134089) nucleotides 3396 – 3416 was transfected into mouse hippocampal neurons (Sun et al., 2009). An shRNA specifically against human scribble (GenBank accession no. NM_015356) nucleotides 1842 - 1862 was used as a control (shRNA control) (Dow et al., 2007; Sun et al., 2009). To suppress expression of endogenous β-pix, three interfering RNA oligonucleotides against rat β-pix (Invitrogen Canada Inc., Burlington, ON) were transiently transfected into rat hippocampal neurons. Sequences of siRNA-1 and siRNA-2 correspond to rat β-pix (GenBank accession no. NM_053740) nucleotides 21-45 and 1779-1803, respectively. A mixture of siRNA-1 and -2 (siRNA-M) was used to assess the additive effect of these two siRNAs. siRNA negative control duplexes were also purchased from Invitrogen (Invitrogen Canada Inc., Burlington, ON) and used as a control (siRNA-C).

GFP-actin, GFP-β-pix, HA-β-pix DN-β-pix, Cort-HA, Syn-RFP and Syn-GFP constructs were kind gifts from Michael Colicos, Eunjoon Kim, Rick Horwitz, Lorrine Santy, Tim O’Connor, Louis Reichardt and Tadashi Nakata, respectively.

3.2.2 NEURON CULTURES

Hippocampi from E18 rat and mouse were prepared as previously described (Xie et al., 2000) and plated at a density of 130 cells/mm² and 170 cells/mm², respectively. Neurons were transfected using Lipofectamine 2000 (Invitrogen Canada Inc., Burlington, ON) at 6-8 DIV according to manufacturer’s recommendations and imaged at 8-10 DIV. Neurons were treated with 20 µM ALLN (Calbiochem, San Diego, CA) for 24 hours at 7 DIV.

3.2.3 IMMUNOHISTOCHEMISTRY

Neuron cultures were fixed in 4% paraformaldehyde/ 4% sucrose for 10 min, permeabilized in 0.1% Triton-X for 10 min, and blocked in 10% goat serum for 1 hour at room temperature. Primary antibodies were applied in 1% goat serum overnight at 4°C and secondary antibodies were applied in 1% goat serum for 1 hour at room temperature. Primary antibodies:
rabbit anti-synaptophysin (Abcam Inc., Cambridge, MA), mouse anti-PSD-95 (Affinity BioReagents, Golden, CO), guinea pig anti-bassoon (Synaptic Systems, Goettingen, Germany), rabbit anti-bassoon (Synaptic Systems, Goettingen, Germany), mouse anti-HA (Cedarlane Laboratories Ltd., Burlington, ON) and rabbit anti-β-pix (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary antibodies: Alexa 488, Alexa 633, Cy5 and Texas Red-conjugated goat anti-mouse, anti-rabbit or anti-guinea pig (Molecular Probes, Eugene, OR).

### 3.2.4 FM 4-64 ANALYSES

FM 4-64 experiments were done as previously described (Gerrow et al., 2006). Briefly, 15 µM FM 4-64 (Molecular Probes, Eugene, OR) was loaded for 30 s into presynaptic terminals using a hyperkalemic solution of 90 mM KCl in modified HBSS, where equimolar NaCl was omitted for final osmolality of 310 mOsm. Neurons were rinsed three times and maintained in HBSS without Ca\(^{2+}\) for imaging. 1 mM ADVESAP-7 (Sigma, Saint Louis, MO) was added to quench non-specific signal. Three images were captured every 30 s to confirm that the positive FM 4-64 sites were stationary presynaptic terminals. Unloading was done using the hyperkalemic solution described above and neurons were rinsed three times with NeuroBasal media for continued imaging.

### 3.2.5 IMMUNOBLOT ANALYSIS

**Preparation of protein lysates:** Brain tissues or cultured cells were homogenized in approximately 4 vols (w/v) of lysis buffer containing (50mM Tris pH 7.4, 150mM NaCl, 1.0% NP-40, 10% glycerol) and centrifuged at 14 000 g for 30 min at 4°C.

**Crude synaptosomal fraction (P2):** E18 rat brains were homogenized in ice cold buffer (320mM sucrose, 4mM Heps and 1mM EGTA) using a homogenizer (Canadian Laboratory Supplies, Winnipeg, MB) by 6 strokes at 2300 rpm. The homogenate was centrifuged at 1312 g for 10 min to remove nuclei and cell debris. The resulting supernatant was centrifuged at 14 481 g for 15 min to remove small cell fragments and total soluble proteins. The resulting pellet was resuspended in homogenization buffer and centrifuged at 17 522 g for 15 min to obtain the crude synaptosomal fraction, P2. P2 was resuspended in lysis buffer described above and used for immunoprecipitation assays.
**Immunoprecipitation:** P2 synaptosomal preparations were incubated overnight at 4°C with either anti-β-catenin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-scribble (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-β-pix or pre-immune serum. 50 µl of protein A/G–Sepharose (GE healthcare, Chicago, IL) was added to the synaptosomal fractions, and the bead-bound immunocomplexes were recovered after 2 hours, washed 4 times with lysis buffer, solubilized with loading buffer, separated by SDS–PAGE and analyzed by means of immunoblotting with antibodies against cadherin, β-catenin, scribble, β-pix, or synaptophysin.

**Western blot analysis:** Proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL) on a Bio-Rad Versadoc 4000 (Bio-Rad Laboratories (Canada Ltd., Mississauga, ON). The brightness and contrast of entire images was moderately adjusted using Photoshop (Adobe Systems Canada, Toronto, ON) after recommended, scientifically acceptable procedures, and no information was obscured or eliminated from the original (Rossner and Yamada, 2004).

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**3.2.6 CONFOCAL IMAGING**

Neurons were imaged using an Olympus Fluoview 1000 confocal microscope (60X/1.4 Oil Plan-Apochromat). For time-lapse imaging, neurons were imaged every 12 sec for 10 min. All images in a given experiment were captured and analyzed with the same exposure time and conditions. To analyze the localization of GFP-β-pix, weakly-transfected cells exhibiting a comparable pattern of expression to endogenous β-pix were chosen blindly to the conditions being analyzed. Image acquisition was optimized based on this cell and every transfected cell in the dish was imaged using the same parameters. All cells that exhibited areas of saturation in the look up tables were discarded from the analysis.

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**3.2.7 IMAGE ANALYSIS AND QUANTIFICATION**

**Density analyses:** To determine the density of puncta along axons, axon length was measured using Image J and density expressed as the average number of puncta per 100 µm of axon length.

**Analysis of puncta area and Integrated Density (IntDen):** Images were thresholded using Image J based on a subjective evaluation of “real” clusters compared to background noise. Once the threshold was set for a given experiment, the same threshold was used throughout the
analyses. Puncta area and Integrated Density (IntDen; the product of an area and the average gray value within that area), were then determined using Image J. To examine the IntDen at synapses, puncta associated with synaptic markers were identified and the IntDen measured using Image J.

**Integrated Density of Syn-GFP fluorescence at synapses:** To quantify the IntDen of Syn-GFP at synapses, a “synaptic mask” was made of regions of colocalization between PSD-95 and bassoon (Fig. 3.6) using ImageJ with a colocalization plugin downloaded from the program’s website (http://rsb.info.nih.gov/ij/plugins/colocalization.html). Points of colocalization were defined as regions greater than 4 pixels in size where the intensity ratio of the two channels was greater than 50. The IntDen of Syn-GFP fluorescence within this mask was then determined. In Figure 3.7, masks were made of PSD-95 immunoreactive signals.

**Coverage of Syn-GFP fluorescence:** To quantify the length of Syn-GFP fluorescence signal, the length of the major axis of Syn-GFP fluorescence signal (Feret’s diameter) was analyzed using Image J. Syn-GFP fluorescence coverage represents the sum of the Feret’s diameter of Syn-GFP fluorescence signal per 10 µm axon length.

**Localization of β-pix at synapses:** To examine the localization of β-pix at synapses, a “mask” was made of regions of colocalization between bassoon and PSD-95 using the colocalization plugin, and synaptic β-pix defined as β-pix puncta that associated with bassoon/PSD-95 clusters. The number of β-pix puncta that localized to synapses and the number of synapses that associate with β-pix was expressed as a percentage of the number of total puncta. All puncta in a field were analyzed.

**Time-lapse imaging of GFP-actin and Syn-RFP:** To determine the distribution of Syn-RFP clusters, and its association with GFP-actin clusters, puncta were divided into stable and mobile categories. Puncta that remained stationary for the duration of the 10 min imaging period were identified as stable puncta, and were otherwise classified as mobile puncta.
3.3 RESULTS

3.3.1 INCREASE ACTIN POLYMERIZATION ENHANCES SV CLUSTERING

To examine the role of actin in clustering SVs at synapses, fluorescently-tagged actin and synaptophysin were used to determine the distribution of these proteins. Fluorescently-tagged actin has been widely used to determine the localization of actin at both pre- and postsynaptic terminals. Tagged actin constructs have proved to be particularly useful when focusing on presynaptic actin, and alleviate the eclipsing effect of postsynaptic actin observed using conventional immunostaining or phalloidin dyes (Fischer et al., 1998; Morales et al., 2000; Colicos et al., 2001; Sankaranarayanan et al., 2003; Saneyoshi et al., 2008; Lucido et al., 2009). In this study, GFP-actin driven by the PDGF promoter to minimize actin overexpression was utilized to examine the distribution of actin along the axon (Colicos et al., 2001). When expressed in neurons, GFP-actin was expressed in a punctate pattern along axons and largely colocalized with the postsynaptic marker, PSD-95, and the presynaptic marker, bassoon, suggesting an enrichment of GFP-actin at synapses as previously reported (Fig. 3.1A, A’, (Fischer et al., 1998; Morales et al., 2000; Colicos et al., 2001; Sankaranarayanan et al., 2003)). Thus, GFP-actin is a faithful marker for assaying the localization of endogenous actin.

Fluorescently-tagged SV marker proteins have been widely used to mark presynaptic sites in vertebrates, and do not compromise the secretory physiology of the synapse (Sankaranarayanan et al., 2003). Our work has previously demonstrated that the pattern of synaptophysin-GFP (Syn-GFP) expression is comparable to that of endogenous SV proteins (Bamji et al., 2003; Sun et al., 2009), and we now observed appropriate synaptophysin-RFP (Syn-RFP) colocalization with PSD-95 and bassoon at synapses (Fig. 3.1B, B’).
Figure 3.1: GFP-actin and Syn-RFP are localized to synapses.

Confocal images of 15 DIV hippocampal neurons transfected with GFP-actin (A) or Syn-RFP (B) and immunolabeled with synaptic markers, PSD-95 and bassoon. Bassoon and PSD-95 immunopositive puncta that are not colocalized with GFP-actin or Syn-RFP may represent puncta on untransfected cells. Higher magnifications of the insets from A and B are shown in A’ and B’, respectively. GFP-actin and Syn-RFP were highly colocalized with PSD-95/bassoon (open arrowheads). Scale bars=5 µm.
Recent work has demonstrated that repressing calpain protease activity using the calpain inhibitor, ALLN, enhances actin polymerization at discrete sites along neurites (Mingorance-Le Meur and O'Connor, 2009). To globally enhance actin polymerization in hippocampal cultures, cells were treated with 20 µM ALLN. To quantify actin clusters, we measured the integrated density (IntDen) of each puncta. This is the product of the area and mean grey value of each puncta, and most accurately represents the amount of protein per puncta. The density and IntDen of GFP-actin clusters were significantly increased 24 hours following ALLN treatment, suggesting an enhancement of actin polymerization in neurons (Fig. 3.2A-D). The density and IntDen of Syn-RFP clusters that were associated with GFP-actin clusters were specifically increased, whereas the density and IntDen of Syn-RFP clusters that were not associated with GFP-actin clusters, remained unchanged (Fig. 3.2A-D). The increase in SV clustering was not attributable to increased synaptophysin expression, as western blot analysis revealed no change in synaptophysin protein levels following ALLN treatment (Fig. 3.2E). Instead, the increase in SV density and IntDen was likely attributable to the accumulation of small SV clusters that were originally below the imaging threshold. Taken together, these results demonstrate that ALLN treatment enhances actin polymerization at discrete sites along the axon and mediates the recruitment of SVs to these sites. Despite the increase in SV cluster density, there was no significant change in the density of synapses, as determined by the density of colocalized Syn-RFP and PSD-95 puncta, 24 hours following ALLN treatment (Fig. 3.2A-C).
**Figure 3.2: The density and IntDen of GFP-actin and Syn-RFP clusters are increased following ALLN treatment.**

(A, B) Confocal images of 8 DIV hippocampal neurons transfected with GFP-actin and Syn-RFP and immunolabeled with PSD-95. Neurons were treated either with 20 µM ALLN or with DMSO vehicle 24 hours prior to fixation. Open arrowheads in A, B indicate synapses as defined by colocalized Syn-RFP/GFP-actin/PSD-95 clusters, whereas closed arrowheads indicate sites of colocalization between GFP-actin and Syn-RFP, but not PSD-95. ALLN treatment increased the density (A, B open plus closed arrowheads; C) and IntDen (D) of GFP-actin clusters. The density and IntDen of Syn-RFP clusters that were associated with GFP-actin were specifically increased (C, D). N= 23-33 cells per condition from 3 separate cultures. *p<0.05, **p<0.01; student’s t-test. Scale bar = 10 µm. (E) Synaptophysin protein levels were similar in control and ALLN treated cells (α-tubulin was used as a loading control). N= 3 different blots from 3 separate cultures.

Bath treatment of neurons with ALLN can enhance actin polymerization at both pre and postsynaptic compartments, and may enhance SV density by trans-synaptic mechanisms. To determine whether presynaptic actin recruits SVs in a cell autonomous manner, actin polymerization was enhanced in individual neurons through expression of HA-tagged cortactin (Cort-HA). Cortactin is an actin-binding protein that promotes actin polymerization by stabilizing Arp2/3 complexes (Cosen-Binker and Kapus, 2006). In neurons, cortactin is
proteolysed by calpain, resulting in the suppression of membrane protrusion (Mingorance-Le Meur and O'Connor, 2009). Cort-HA was distributed in a punctate pattern along the axon, and was highly colocalized with GFP-actin clusters (Fig. 3.3B). Overexpression of Cort-HA enhanced actin polymerization in transfected cells, as shown by a significant increase in the density and IntDen of GFP-actin clusters (Fig. 3.3A-D). Similar to that observed in ALLN treated cells, the density and IntDen of Syn-RFP clusters that were associated with GFP-actin clusters were specifically increased, whereas the density and IntDen of Syn-RFP clusters that were not associated with GFP-actin clusters remained unchanged (Fig. 3.3A-D). Overexpression of Cort-HA did not alter the density of synapses, defined as colocalized Syn-RFP/PSD-95 clusters (Fig. 3.3E-G).

The majority of synapses along transfected axons were associated with Cort-HA clusters (87.4±4.3% of synapses contained Cort-HA, N=21 cells from 3 separate cultures; Fig. 3.3F) in accord with previous work demonstrating the enrichment of endogenous cortactin at synapses (Boeckers et al., 1999; Naisbitt et al., 1999). To examine how increased cortactin at synapses impacts the localization of SVs specifically to synapses, the IntDen of Syn-GFP at synapses was measured. The IntDen of Syn-GFP was specifically increased at synapses that were associated with Cort-HA clusters, whereas there was no significant change in the IntDen of Syn-GFP at synapses not associated with Cort-HA clusters (Fig. 3.3E, F, H).

Two pools of SVs have previously been identified by time-lapse analyses; a relatively stable pool that remains stationary for hours and localizes primarily at presynaptic boutons, and a mobile pool that translocates along the axon in a saltatory manner (Kraszewski et al., 1995; Dai and Peng, 1996; Nakata et al., 1998; Ahmari et al., 2000; Bresler et al., 2004). To determine whether enhanced actin polymerization increases the density of stable or mobile SV clusters, neurons co-transfected with GFP-actin and Syn-RFP plus or minus Cort-HA were imaged using time-lapse microscopy. The density of total SV clusters was increased in cells expressing Cort-HA, which was attributable to a specific increase in the density of stable Syn-RFP clusters (Fig. 3.3I). Moreover, the density of stably colocalized GFP-actin/Syn-RFP clusters was 3-fold greater in cells expressing Cort-HA than in control cells (Fig. 3.3J). Together, these results demonstrate that actin clusters can recruit SVs and stably localize them at discrete sites. We next examined how cell-cell contact enhances the local polymerization of actin and enhances the localization of SVs to nascent synapses.
Figure 3. 3: The density and IntDen of GFP-actin and Syn-RFP clusters are increased in neurons expressing cortactin.

(A, B) Confocal images of 8 DIV hippocampal neurons co-transfected with GFP-actin and Syn-RFP plus either empty vector or Cort-HA and immunolabeled for HA. GFP-actin, Syn-RFP and Cort-HA were highly colocalized (B, open arrowheads). In neurons expressing Cort-HA, the density (A, B, open arrowheads, C) and IntDen (D) of GFP-actin clusters and Syn-RFP clusters associated with GFP-actin clusters were increased, whereas the density and IntDen of Syn-RFP clusters that were not associated with GFP-actin clusters were not significantly changed (A, B, closed arrowheads, C, D). N=23-31 cells per condition from 3 separate cultures. (E, F) Confocal images of 8 DIV hippocampal neurons co-transfected with Syn-GFP plus either empty vector or Cort-HA and immunolabeled for PSD-95 and HA. Arrowheads indicate synapses, which are defined as sites of colocalization between Syn-GFP and PSD-95. A subset of Cort-HA clusters localized to synapses (F, arrowheads). Synapse density remained unaltered in Cort-HA expressing cells (G); however, the IntDen of Syn-GFP puncta in these cells was significantly increased at synapses associated with Cort-HA clusters (+Cort-HA), but unaffected at synapses not associated with Cort-HA (-Cort-HA) (H). N=19-24 cells per condition from 3 separate cultures. Time-lapse analysis demonstrated...
an increase in the density of stable Syn-RFP clusters (I) and stably colocalized GFP-actin/Syn-RFP clusters (J) in cells expressing Cort-HA. N=16-17 cells per condition from 3 separate cultures. *p<0.05, **p<0.01, ***p<0.001; student’s t-test. Scale bars = 10 µm.

3.3.2 β-PIX INTERACTS WITH CADHERIN/β-CATENIN/SCRIBBLE COMPLEXES AT SYNAPSES

We have previously shown that β-catenin/scribble complexes are important for clustering SVs at developing synapses (Bamji et al., 2003; Sun et al., 2009). To further elucidate the mechanism through which this occurs, and to determine whether this complex is involved in the regulation of actin polymerization, we took a candidate approach to search for the protein(s) that associates with β-catenin/scribble complexes to localize SVs. In mouse brains, scribble forms a complex with β-pix, a Rac/Cdc42 GEF, which promotes actin polymerization via regulation of Rac/Cdc42 activity (Audebert et al., 2004). We hypothesized that β-catenin/scribble complexes act as scaffolds to localize β-pix to developing synapses and that β-pix, in turn, enhances the localized polymerization of actin and the subsequent accumulation of vesicles at these sites.

Endogenous β-pix was distributed in a punctate pattern in neurons and highly colocalized with the pre and postsynaptic markers, bassoon and PSD-95, respectively (Fig. 3.4A, 79.3 ± 2.0% of β-pix clusters colocalized with bassoon/PSD-95 clusters, N=7 images from 2 separate cultures). This is in accord with previous studies showing that β-pix immunoprecipitates with the presynaptic scaffold protein, piccolo, and localizes to spines (Kim et al., 2003; Park et al., 2003; Zhang et al., 2003). β-pix clusters that were not associated with PSD-95 and bassoon may represent populations at inhibitory synapses or at non-synaptic sites.

To examine whether β-pix interacts with β-cateinin/scribble complexes at synapses, crude synaptosomal fractions from E18 brains were prepared and immunoprecipitated with antibodies against cadherin, scribble, β-catenin and β-pix. Co-immunoprecipitation assays demonstrated that these 4 proteins could associate with one another, but not with synaptophysin (Fig. 3.4B). Together, these results demonstrate that cadherin, β-catenin, scribble and β-pix exist in a complex at synapses.
3.3.3 LOCALIZATION OF β-PIX AT SYNAPSES IS DECREASED IN SCRIBBLE KNOCKDOWN CELLS

To examine whether scribble is involved in localizing β-pix to presynaptic sites, cells were transfected with GFP-β-pix plus a short hairpin RNA against scribble (Scrib shRNA). We have previously utilized this shRNA to attenuate scribble protein levels in hippocampal neurons (Sun et al., 2009). GFP-β-pix is diffusely localized when expressed at high levels (Zhang et al., 2003), therefore only weakly transfected cells exhibiting a punctate pattern of GFP-β-pix, that was comparable to endogenous β-pix, were analyzed (see Methods). Weakly transfected cells were identified blindly to the conditions being analyzed. We assessed the localization of GFP-β-pix at synapses by analyzing the IntDen of those GFP-β-pix clusters specifically associated with synapses (defined as points of colocalization between PSD-95/bassoon). The IntDen of synaptic GFP-β-pix clusters was significantly decreased in cells expressing Scrib shRNA (Fig. 3.4C-E). These results suggest that scribble is involved in localizing β-pix to presynaptic compartments.

Figure 3.4: β-pix interacts with cadherin, β-catenin and scribble at synapses.
Confocal images of 15 DIV hippocampal cultures immunolabeled for β-pix, and synaptic markers, PSD-95 and bassoon. β-pix clusters are highly colocalized with PSD-95/bassoon in neurons. (B) β-pix, scribble and β-catenin can be coimmunoprecipitated from synaptosomal fractions. Synaptosomal fractions from brains were immunoprecipitated using β-pix, β-catenin or scribble antibodies and separated by SDS-PAGE. β-pix, scribble, β-catenin and cadherin coimmunoprecipitated with one another, but not with the synaptic protein, synaptophysin, indicating the specificity of the immunoprecipitation. The input lane corresponds to the crude synaptosomal fraction, P2. Rabbit IgG was used as a control. N= 3 different blots from 3 separate preparations. (C, D) Confocal images of 8 DIV hippocampal neurons transfected with GFP-β-pix plus either shRNA control or Scrib shRNA and immunolabeled for PSD-95 and bassoon. The IntDen of synaptic GFP-β-pix clusters, defined by GFP-β-pix at sites of colocalization between PSD-95 and bassoon, was smaller in cells expressing Scrib shRNA (C, D, arrowheads; E). N=26-27 cells per condition from 3 separate cultures. ***p<0.001; student’s t-test. Scale bars = 10 µm.

3.3.4 PERTURBATION OF β-PIX ACTIVITY DISRUPTS SYNAPTIC ACTIN

To study the function of β-pix at synapses, β-pix protein levels were attenuated in neurons using RNA interference. To minimize possible “off-target” effects, 2 short interfering RNA oligonucleotides (siRNA-1-2) against rat β-pix were used. siRNA negative control duplexes were used as a control (siRNA-C). Given the low transfection efficiency of primary cultured hippocampal neurons, the efficacy of β-pix siRNAs was examined in HEK 293 cell line using western blot analysis. β-pix siRNAs significantly attenuated the expression of GFP-β-pix (Fig. 3.5A). A mixture of siRNA-1 and -2 was also used to further control for off-target effects (siRNA-M).

To determine the effects of β-pix knockdown on actin, cells were co-transfected with GFP-actin plus β-pix siRNAs. The density of GFP-actin was unaltered in neurons expressing β-pix siRNAs (Fig. 3.5B-D); however the IntDen of synaptic GFP-actin clusters (GFP-actin clusters associated with PSD-95/bassoon co-clusters) was decreased in these cells (Fig. 3.5B, C, E).

To determine whether the GEF activity of β-pix regulates actin clusters at synapses, cells were co-transfected with GFP-actin plus either HA-tagged full-length β-pix (FL-β-pix) or a dominant-negative β-pix (DN-β-pix), that contains two mutations in the Dbl homology domain..
(L238R and L239S) and lacks GEF activity (Manser et al., 1998; Zhang et al., 2003). FL-β-pix did not alter GFP-actin at synapses, however, the IntDen of synaptic GFP-actin clusters in cells expressing DN-β-pix was significantly reduced (Fig. 3.5F). These results suggest that β-pix GEF activity is important for actin polymerization at synapses.

**Figure 3.5:** The IntDen of synaptic GFP-actin clusters is decreased in neurons with reduced β-pix activity.

(A) GFP-β-pix expression in HEK 293 cells is decreased in cells expressing β-pix siRNA. N= 2 different blots from 2 separate experiments. (B, C) Confocal images of 8 DIV hippocampal neurons transfected with GFP-actin plus scrambled or β-pix siRNAs and immunolabeled for PSD-95 and bassoon. The density of GFP-actin clusters was unaltered in β-pix siRNAs expressing neurons (D). However, the IntDen of synaptic GFP-actin clusters (GFP-actin at sites of PSD-95 and bassoon
colocalization) was decreased in cells expressing β-pix siRNAs (B, C, arrowheads, E). N= 16-24 cells per condition from 2 separate cultures. *p<0.05, ***p<0.001; one-way ANOVA with Dunnett’s post hoc. (F) The IntDen of synaptic GFP-actin clusters was significantly attenuated in neurons expressing dominant-negative β-pix (DN-β-pix). N= 11-15 cells per condition from 2 separate cultures. ***p<0.001; one-way ANOVA with Tukey post hoc. Scale bar = 10 µm.

3.3.5 PERTURBATION OF β-PIX ACTIVITY DISRUPTS THE LOCALIZATION OF SVS AT SYNAPSES

To test whether β-pix is involved in the clustering of SVs at synapses, Syn-GFP localization was examined in β-pix knockdown neurons. In control cells, Syn-GFP was distributed in a punctate pattern (Fig. 3.6A). In contrast, the distribution of Syn-GFP fluorescence in siRNA-1-expressing neurons was relatively uniform along the axon, with only few discrete Syn-GFP clusters observed (Fig. 3.6B).

Variability in the “punctate-ness” of Syn-GFP was observed in siRNA-expressing neurons. To determine the distribution of vesicles along the axon, the Feret’s diameter (defined as the greatest distance possible between any two points along the boundary of a region of interest, hereafter called the “length”) of the Syn-GFP fluorescence signal was first measured and Syn-GFP florescence coverage (the sum of the length of Syn-GFP fluorescence signal per 10 µm axon length) was then determined as previously described (Bamji et al., 2003; Lee et al., 2008; Sun et al., 2009). To avoid bias, all transfected neurons on each coverslip were imaged and quantified. There was no significant difference in the coverage of Syn-GFP fluorescence in neurons expressing Syn-GFP alone and Syn-GFP plus siRNA-C (Fig. 3.6E). In contrast, in siRNA-expressing neurons the coverage of Syn-GFP fluorescence along the axon was approximately 2-fold greater than controls (Fig. 3.6E). In addition, the IntDen of Syn-GFP fluorescence at synapses (within masks made of regions of overlap between PSD-95/bassoon) was significantly reduced in siRNA expressing cells compared to controls, further demonstrating that the clustering of SVs at synapses is dependent on β-pix (Fig. 3.6F). The decreased IntDen of Syn-GFP fluorescence at synapses could not be attributed to changes in the density or area of bassoon and PSD-95 clusters, which were similar in wildtype and β-pix knockdown cells (Fig. 3.6G, H).
To determine whether the GEF activity of β-pix is required for the appropriate clustering of vesicles at synapses, cells were co-transfected with Syn-GFP plus either FL-β-pix or DN-β-pix. Expression of FL-β-pix did not alter Syn-GFP fluorescence coverage or the IntDen of Syn-GFP at synapses compared to control cells (Fig. 3.6C, E, F). In contrast, neurons expressing DN-β-pix exhibited a diffuse pattern of Syn-GFP expression, which was reflected in an increased coverage of Syn-GFP fluorescence along the axon and an attenuated IntDen of Syn-GFP fluorescence at synapses (Fig. 3.6D, E, F). Taken together, these results suggest that β-pix regulates vesicle localization through its GEF activity.

To determine whether deficits in vesicle clustering in β-pix knockdown cells are associated with impaired presynaptic function, the efficiency of vesicle recycling was studied by stimulating neurons with a high-K⁺ solution in the presence of FM 4-64, a fluorescent dye that marks sites of endocytosis. In cells expressing β-pix siRNA-1, this uptake was strongly suppressed, with greatly reduced density of FM 4-64-positive puncta compared to control cells (Fig. 3.6I).
Figure 3.6: The localization of synaptic vesicles at synapses is disrupted in neurons with reduced β-pix activity.

(A-D) Confocal images of 9 DIV hippocampal neurons transfected with Syn-GFP plus the indicated β-pix construct and immunolabeled for PSD-95 and bassoon. In control neurons (A) and those expressing full length β-pix (FL-β-pix) (C), Syn-GFP exhibited a punctate distribution and colocalized with PSD-95 and bassoon (arrowheads). In contrast, neurons expressing β-pix siRNA-1 (B) or dominant-negative β-pix (DN-β-pix) (D), exhibited a more diffuse pattern of Syn-GFP distribution, and the coverage of Syn-GFP (the sum of the length of Syn-GFP fluorescence signal per 10 µm axon length ± SE) was increased (B, D, E). A significant decrease in the IntDen of Syn-GFP fluorescence at synapses was observed in β-
pix siRNA-expressing neurons and those expressing DN-β-pix (F). The area (G) and density (H) of PSD-95 and bassoon were similar in control and siRNA-expressing neurons. N = 18-29 cells per condition from 3 separate cultures. *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA with Tukey post hoc. (I) Neurons transfected with Syn-GFP plus either control or β-pix siRNA-1 were loaded with FM 4-64. The density of FM 4-64 puncta was reduced in cells expressing β-pix siRNA-1. N = 30-31 cells per condition from 3 separate cultures. ***p<0.001; student’s t-test. Scale bars = 10 µm.

3.3.6 CORTACTIN OVEREXPRESSION RESCUES THE SV CLUSTERING PHENOTYPE OBSERVED IN β-PIX KNOCKDOWN CELLS

To further test whether the mislocalization of SVs in β-pix knockdown cells is attributable to decreased polymerization of actin at synapses, we determined whether this phenotype can be rescued by overexpressing cortactin. The IntDen of GFP-actin at synapses (defined as those GFP-actin clusters that were associated with PSD-95) was restored to wildtype levels in β-pix knockdown cells overexpressing Cort-HA (Fig. 3.7A). Overexpression of cortactin in β-pix knockdown cells also rescued the mislocalization of SVs, resulting in an increased clustering of SVs along the axon. Indeed, in neurons co-expressing β-pix siRNA-1 plus Cort-HA, discrete Syn-GFP clusters that colocalized with PSD-95 were observed, similar to those observed in control cells (Fig. 3.7D, F). Both the Syn-GFP fluorescence coverage (Fig. 3.7B), and the IntDen of Syn-GFP at synapses (Fig. 3.7C) were significantly rescued by cortactin overexpression. Taken together, these observations strongly suggest a model by which cadherin/β-catenin/scríbble complexes localize β-pix to nascent synapses where β-pix promotes actin polymerization through its GEF activity, and enhances the recruitment of SV clusters to these areas of actin polymerization.
Figure 3. 7: Cortactin expression rescues the disruption in synaptic vesicle localization in β-pix knockdown cells.

The IntDen (A) of synaptic GFP-actin clusters was decreased in neurons expressing β-pix siRNA, but unchanged in neurons expressing β-pix siRNA plus Cort-HA compared to control. N= 13-20 cells per condition from 2 separate cultures. (D-F) Confocal images of 9 DIV hippocampal neurons transfected with Syn-GFP and β-pix siRNA plus or minus Cort-HA and immunolabeled for PSD-95. β-pix siRNA-1-expressing neurons exhibited a more diffuse localization of Syn-GFP (E) and Syn-GFP coverage was increased compared to control (B). In neurons expressing both β-pix siRNA-1 and Cort-HA, discrete Syn-GFP clusters were observed at PSD-95 sites (F, arrowheads). Syn-GFP coverage in these neurons was significantly decreased compared to cells expressing β-pix siRNA-1 alone, however, Syn-GFP coverage was not completely rescued to control levels (B). The IntDen of Syn-GFP fluorescence (within “masks” of PSD-95 clusters) was significantly decreased in β-pix siRNA-1-expressing neurons, and unchanged in neurons expressing siRNA-1 plus Cort-HA compared to control (C). N= 13-20 cells per condition from 2 separate cultures. *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA with Tukey post hoc. Scale bar = 10 µm.
3.4 DISCUSSION

The formation of contacts between axons and dendrites is thought to be mediated by transynaptic adhesion molecules. These adhesion molecules stabilize transient, dynamic axodendritic contacts and activate intracellular signals that recruit synaptic proteins (McAllister, 2007). The cadherin adhesion complex has previously been shown to play a large role in localizing SVs to developing synapses. In N-cadherin knockout cultures (Stan et al., 2010), or cultures treated with peptides that block intercellular cadherin interactions (Togashi et al., 2002), SVs do not accumulate at contact points. We have shown that presynaptically-localized cadherin can mediate this effect through its association with β-catenin (Bamji et al., 2003; Bamji et al., 2006; Lee et al., 2008), and scribble (Sun et al., 2009). Recently, it has also been shown that cadherins can mediate SV localization transynaptically through the recruitment of neuroligin-1 to postsynaptic compartments (Aiga et al., 2010; Stan et al., 2010). In this study, we further examine the effects of presynaptic cadherin adhesion complexes in recruiting SVs to sites of contact. We demonstrate that scribble is important for the recruitment of β-pix to developing synapses, and that scribble and β-pix form complexes with cadherin and β-catenin. Moreover, we show that β-pix-mediated enhancement of actin polymerization is important for the localization of SVs to discrete sites along the axon.

3.4.1 ACTIN LOCALIZES SYNAPTIC VESICLES TO DEVELOPING SYNAPSES

SVs do not cluster appropriately at developing synapses in the presence of actin depolymerizing agents (Kuromi and Kidokoro, 1998; Zhang and Benson, 2001). Although this suggests a role for actin in SV clustering, it is also plausible that this is a secondary effect to reduced cell-cell adhesion, and it was unclear whether actin actively localizes SVs to nascent synapses. Utilizing gain-of-function assays, we demonstrate that enhancing actin polymerization increases the clustering of SVs. Indeed, there was a direct correlation between the density and IntDen of actin and SV clusters in all conditions analyzed. Increasing the polymerization of actin by itself is not sufficient to induce the formation of new synapses. Synapse formation requires intercellular communication and coordination of developing pre and postsynaptic compartments. Enhanced actin polymerization along axons can therefore cluster SVs, but not induce clustering of postsynaptic proteins at these sites.
A conventional way to enhance actin polymerization is to stabilize polymerized actin using jasplakinolide (Sankaranarayanan et al., 2003; Darcy et al., 2006a; Lucido et al., 2009). However, this method compromises dynamic actin reorganization, which is essential for the mobilization of SVs. Indeed, inhibiting actin turnover using jasplakinolide greatly reduces the transport of SVs along the axon (Darcy et al., 2006a) and nearly abolishes the accumulation of SVs at poly-D-lysine bead-induced contact sites (Lucido et al., 2009). We therefore promoted actin polymerization by treating cells with the calpain protease inhibitor, ALLN. Calpain maintains neurite consolidation by suppressing actin polymerization along neurites, and inhibiting calpain activity enhances actin polymerization (Mingorance-Le Meur and O’Connor, 2009). There are a number of substrates for calpain including cortactin, cadherin and β-catenin (Perrin et al., 2006; Abe and Takeichi, 2007; Jang et al., 2009). As calpain inhibition and cortactin overexpression can enhance GFP-actin cluster density and IntDen, we concluded that inhibition of calpain enhances SV localization through its effects on actin. SV clustering is unlikely to be attributable to elevated levels of cadherin and β-catenin, because overexpression of these proteins does not increase SV density (Scheiffele et al., 2000; Bamji et al., 2003; Sara et al., 2005; Latefi et al., 2009; Linhoff et al., 2009).

How does polymerized actin “trap” SVs that are being transported along microtubules? It has been known for some time that the actin motor, myosin-V, can form a complex with the microtubule motor, kinesin, and that this heteromotor complex enables vesicles to be transported on both microtubules and actin filaments. The direct interaction of motors from both filament systems may represent the mechanism by which the transition of vesicles from microtubules to actin filaments is regulated (Langford, 2002).

### 3.4.2 SCRIBBLE REGULATES β-PIX LOCALIZATION AT SYNAPSES

β-pix has previously been shown to co-immunoprecipitate with the presynaptic protein, Piccolo, and to colocalize with synaptic markers at dendritic spines (Kim et al., 2003; Park et al., 2003; Zhang et al., 2003). Shank (a postsynaptic scaffolding protein) and GIT1 (G protein-coupled receptor kinase-interacting protein 1) have been shown to be important for the localization of β-pix to postsynaptic compartments (Park et al., 2003; Zhang et al., 2003). In the present study, we show that the scaffold protein, scribble, is important for the localization of β-pix at presynaptic compartments. Cadherin and scribble are also essential for the appropriate localization of β-pix in other cell types. For instance, in epithelial cells, β-pix is recruited to
points of cell adhesion in an E-cadherin-dependent manner (Liu et al., 2010). Moreover, scribble can localize β-pix to the cell membrane in PC12 cells (Audebert et al., 2004). As the presynaptic localization of β-pix was not completely abolished upon scribble knockdown, it is possible that other proteins such as GIT1, which is expressed both pre and postsynaptically (Kim et al., 2003; Zhang et al., 2003), may also play a role in this event.

### 3.4.3 β-PiX Regulates Actin Polymerization at Synapses

Polymerized actin is concentrated at pre- and postsynaptic terminals (Dillon and Goda, 2005). In the present study, we used GFP-actin to specifically examine the presynaptic localization of actin. Although GFP-actin labels both G- and F-actin, it has been previously shown that GFP-actin clusters are largely colocalized with phalloidin immunoreactive signals, suggesting that these clusters represent F-actin (Morales et al., 2000; Nunes et al., 2006).

β-pix activates Rac and Cdc42 (Bagrodia et al., 1998; Manser et al., 1998), which are known regulators of actin remodelling. Rac/Cdc42 can promote actin polymerization through the Scar/WASP/Arp2/3 and PAK/LIMK-1/ADF/cofilin pathways (Rodriguez et al., 2003). Previous work has demonstrated that β-pix can regulate spine formation through modulation of actin (Parnas et al., 2001; Park et al., 2003; Zhang et al., 2003; Zhang et al., 2005; Saneyoshi et al., 2008). Here, we suggest that β-pix exerts a similar role in regulating actin polymerization at the presynaptic compartment. Indeed, actin polymerization at presynaptic terminals was significantly decreased in cells lacking β-pix and in cells lacking the β-pix GEF activity.

### 3.4.4 Dynamic Regulation of Cadherin Adhesion Complexes and SV Localization

Previous work has shown that the cadherin adhesion complex can be dynamically regulated, and that this may be a mechanism to regulate presynaptic plasticity. Phosphorylation of β-catenin on tyrosine residue 654 can rapidly uncouple cadherin from β-catenin and result in the enhanced mobility of SVs (Bamji et al., 2006). The phosphorylation and dephosphorylation of β-catenin can be mediated by the tyrosine kinase, Fer, and the phosphatase, SHP2. These proteins have been shown to modulate SV localization through their regulation of β-catenin (Lee et al., 2008). β-catenin phosphorylation can also be regulated by leukocyte antigen related (LAR) tyrosine phosphatase (Kypta et al., 1996). Interestingly, the synaptic localization of LAR
depends on liprin-α1, (Hoogenraad et al., 2007), the vertebrate homolog of Sad-2. Sad-2 is important for the clustering of SVs and Sad-2 mutants display a diffuse localization of SVs along the axon (Zhen and Jin, 1999). It is possible that liprin-α1 modulates vesicle localization by recruiting LAR tyrosine phosphatase to nascent synapses where it maintains the integrity of the cadherin-β-catenin complex by dephosphorylating β-catenin.

Our results suggest that synaptically localized cadherin/β-catenin/scribble/β-pix complexes enhance actin polymerization at points of cell-cell contact, and that polymerized actin “traps” SVs as they translocate along the axon. These findings provide a mechanism by which cell-cell contract leads to the assembly of synaptic components.
4. CONCLUSION

In the current dissertation, the author demonstrates a pathway by which SVs localize to developing synapses. This pathway includes the cell adhesion complex cadherin/β-catenin, a scaffolding protein, scribble, a Rac/Cdc42 specific GEF, β-pix and actin (Fig. 4.1). Briefly, the stabilization of a cell-cell contact by cadherin/β-catenin causes a recruitment of scribble and β-pix, following by a deposition of actin filaments at the contact sites. These actin filaments “trap” SVs, as they translocate along the axon.

Figure 4.1: Schematic of SV assembly at presynaptic compartments.

4.1 OTHER SIGNALS THAT LOCALIZE SYNAPTIC VESICLES

In this study, knockdown of scribble and β-pix leads to a mislocalization of SVs along the axon, a phenotype seen when cadherin and β-catenin is perturbed (Togashi et al., 2002; Bamji et al., 2003). However, some SVs are still present at synapses in those genetically manipulated cells (Togashi et al., 2002; Bamji et al., 2003). This suggests that there may be other signals that control SV localization. One candidate is the neurexin/neuroligin adhesion complex. Neuroligins have previously been shown to transsynaptically cluster SVs through its binding to α-neurexin (Sudhof, 2008). However, how α-neurexin regulates SV localization remains unclear.
In this study, I demonstrate that β-pix regulates SV localization via its ability to promote actin polymerization. Even though the downstream signaling cascade by which β-pix mediates SV localization was not tested in this study, previous findings suggest that β-pix can enhance actin polymerization via Rac/Scar/WASP/Arp2/3 or Rac/PAK/LIMK-1/ADF/cofilin pathways (Rodriguez et al., 2003). A number of molecules have been shown to regulate actin polymerization in postsynaptic spines (Dillon and Goda, 2005). It would be of great interest to determine whether some of these signaling molecules are also present at presynaptic compartments. If so, it would be of interest to determine whether they contribute to vesicle localization. As an example, it is known that the actin-binding protein, profilin, is localized at the presynaptic compartment (Schoch and Gundelfinger, 2006). Profilin controls the turnover of actin filaments by promoting nucleotide exchange of ADP-actin to ATP-actin at barbed ends (Disanza et al., 2005). Is profilin acting in conjunction with Arp2/3 and ADF/cofilin to mediate SV localization? If so, what is the upstream signal? Pursuing answers to these questions will provide a better understanding of presynaptic assembly.

4.2 LOCALIZATION OF PICCOLO-BASSOON TRANSPORT VESICLES

In this study, knockdown of scribble and β-pix leads to a mislocalization of SVs along the axon, whereas the localization of bassoon is largely unaffected. Previous study has shown that perturbation of β-catenin does not affect the localization of bassoon (Bamji et al., 2003). These results suggest that cadherin/β-catenin/scribble/β-pix complex does not play an essential role in clustering bassoon at synapses. Bassoon is transported in piccolo-bassoon transport vesicles (PTVs), and these vesicles have been reported to localize to nascent synapses prior to the recruitment of SVs (Zhai et al., 2001). PTVs also transport N-cadherin (Zhai et al., 2001). It is possible that cadherin first localizes to nascent synapses, recruits β-catenin/scribble/β-pix, which in turn enhances actin polymerization and subsequently cluster SVs. In accordance with this hypothesis, several lines of evidence demonstrate that actin polymerization precedes the clustering of SVs (Colicos et al., 2001; Lucido et al., 2009).

The tyrosine kinase, Fer, has also been shown to localize SVs by regulating the association of cadherin/β-catenin complexes (Lee et al., 2008). Interestingly, knockdown of Fer, and disruption of Fer-p120ctn interactions also disrupt the proper localization of bassoon.
(Lee et al., 2008). These results suggest that Fer-p120ctn can regulate PTV localization and this ability is independent of cadherin/β-catenin complexes. It would be of interest to identify the pathway by which Fer-p120ctn mediates the recruitment of PTVs to synapses.

### 4.3 TRANSSYNAPTIC EFFECTS

The current study shows that SVs are not able to cluster at synapses in scribble and β-pix knockdown cells, whereas the localization of postsynaptic scaffold protein PSD-95 remains normal along the knockdown axon. Previous studies have shown that both N-cadherin and β-catenin are able to regulate synapse function transsynaptically (Murase et al., 2002; Stan et al., 2010). For instance, disruption of postsynaptic N-cadherin comprises SV clustering on the presynaptic side (Stan et al., 2010). Force maintaining association between N-cadherin and β-catenin in spines leads to an increase in the size of SV clusters at presynaptic compartments (Murase et al., 2002). It is interesting that a transsynaptic effect is not observed in scribble and β-pix knockdown cells. One possible reason is that the knockdown is transient and it requires longer time for a presynaptic change translates into a pronounced postsynaptic response. Nonetheless, some subtle changes in the postsynaptic cell may have been overlooked. The current study shows that there are defects in SV recycling in the scribble and β-pix knockdown cells. Therefore, the function of these synapses may have been weakened and the surface expression of receptors may have changed. It would be of interest to examine these postsynaptic responses thoroughly.

### 4.4 THE ROLE OF THIS COMPLEX AT MATURE SYNAPSES

Several lines of evidence suggest that cadherin/β-catenin/scribble/β-pix complexes may be less essential in SV localization at mature synapses (Zhang and Benson, 2001; Stan et al., 2010). For instance, disruption of postsynaptic N-cadherin causes a decrease in the clustering of SVs in young cultures (6-7 DIV), whereas, in older cultures (10-14 DIV), SV localization is not significantly affected (Stan et al., 2010). Moreover, in 5-6 DIV neurons, actin depolymerization drug causes a loss of SV clusters along the axon, and in 18-20 DIV neurons, SV clustering is largely unaffected by actin depolymerization (Zhang and Benson, 2001). The current study shows that knockdown of scribble and β-pix disrupts SV localization in 8-10 DIV neurons.
Despite the essential role of this complex in initial synapse assembly, these proteins remain at synapses at a mature stage as well. It would be of great interest to investigate the role of this complex in mature neurons. One possibility is that this complex may regulate SV localization and synapse function in response to neuronal activity.

Previous studies have shown that activity can regulate the expression or function of a number of components in this complex (Bozdagi et al., 2000; Tanaka et al., 2000; Colicos et al., 2001; Okamoto et al., 2004). Activity increases the level of N-cadherin in the spines by enhancing protein synthesis, and this accumulation is accompanied by an increase in N-cadherin dimmerization, which leads to the strengthening of cadherin-based adhesion (Bozdagi et al., 2000; Tanaka et al., 2000). Protocols that induce the formation of LTP can result in a persistent shift of actin equilibrium toward F-actin in the spines (Okamoto et al., 2004). Conversely, protocols that induce the formation of LTD shift the equilibrium toward G-actin (Okamoto et al., 2004). Repetitive spaced tetanic stimuli induce the formation of new actin clusters along the axon, followed by the accumulation of actively cycling SVs (Colicos et al., 2001). These findings have pointed out a potential role of this complex in regulating synapse function in response to neuronal activities.

4.5 SIGNIFICANCE

This research has answered many questions in the field of synaptic biology. First, this study provides a molecular mechanism through which presynaptic assembly occurs. Second, it shows the causation and purpose of preferential localization of actin polymerization in the presynaptic terminal. Third, it is the first study to examine the role of scribble and β-pix in the presynaptic terminal, and provides a better understanding of the function of these proteins. Fourth, it provides novel information about downstream regulators of cadherin function at synapses. Fifth, on a broader scale, it answers the question of how proteins are selectively localized to discrete subcellular domains, which is important for general cell biology. These findings open up exciting fields for new research in synaptic biology. Further investigation will be beneficial for a better understanding of brain development and function.


the transsynaptic control of vesicle accumulation. Proc Natl Acad Sci U S A 107:11116-11121.


