Constrained Diffusion in the Dendritic Endoplasmic Reticulum and Consequences for Early Secretory Receptor Trafficking and Postsynaptic Function

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Neurobiology in the Graduate School of Duke University

2009
ABSTRACT

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Abstract

The proper modification and trafficking of plasma membrane proteins are essential for normal neuronal function, such as dendrite morphogenesis, spine formation and synaptic plasticity. The secretory organelles including endoplasmic reticulum and Golgi apparatus are critical for the trafficking of these molecules as shown in fibroblasts. Although these secretory organelles have been observed in neurons including dendritic branches, their spatial organization and function in protein trafficking, neuronal development and plasticity are not clear yet. Here, I used photobleaching and photoactivation approaches combined with electron microscopy to show that although rapidly diffusing within the continuous network of the somato-dendritic ER, membrane proteins such as nascent AMPA receptors are confined by ER spatial complexity. The spatial range of ER membrane protein mobility becomes progressively confined over neuronal development and is rapidly restricted by synaptic activity. Thus, constrained lateral mobility within the ER provides a novel mechanism for compartmentalized trafficking of nascent receptors throughout dendrites. I also identified an ER protein as a novel microtubule-associated protein regulating dendritic ER spatial complexity, neuronal dendrite elongation and spine formation. Together, these results describe the spatial organization of dendritic ER and its role in regulating membrane protein trafficking, neuronal morphogenesis and postsynaptic functions.
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1. Introduction

Neurons are highly specialized cells with on average 250,000 μm² surface membrane areas, up to 10,000 times greater than epithelial or fibroblasts, and 1000 to 10,000 synapses for communication and signal processing. The capacity and complexity for neuronal signaling is not only determined by the enormous size of the neuronal surface area but also by the diverse morphology of multiple highly-branched dendritic trees and refined distribution of proteins and lipids. Normal neuronal development and function require appropriate protein expression and localization to correct regions at the cell surface at proper time. The creation and maintenance of neuronal morphology and function are determined by the delivery of newly synthesized proteins and lipids. These newly synthesized construction materials reach the neuronal surface via cellular organelles of the secretory pathway. Organization, signaling and function of the secretory pathway can regulate the absolute number and targeting of specific protein and lipid at neuronal surface and in turn regulate neuronal function.

In contrast to the relatively simple organization of secretory organelles in fibroblasts, neurons face more challenging questions. The morphology and function of neurons are not only polarized into axons and dendrites but also compartmentalized into micrometer-scale domains. A single spine or synapse can control the storage of memory independently. How to deliver a big population of lipid and protein molecules into dendrites and axons which are on a hundreds-of-micrometer scale, and at the same
time to target them to specific destinations on the cell surface which are on a nano-to-micrometer scale is an extremely interesting and important question. Despite the critical role of secretory trafficking in determining the morphology, cell surface molecule composition and function of neurons, the global and local organization of secretory machinery remains poorly understood.

1.1 Cellular Organelles of the Secretory Pathway

The secretory pathway is comprised of a series of cellular organelles including endoplasmic reticulum (ER), ER-Golgi-Intermediate Complex (ERGIC), Golgi apparatus and trans-Golgi network (TGN) which are involved in lipid and protein synthesis, modification and newly synthesized cargo transport, sorting and targeting to the plasma membrane (Jamieson and Palade 1967; Jamieson and Palade 1967; Jamieson and Palade 1968; Jamieson and Palade 1968; Novick, Ferro et al. 1981). Each compartment along the secretory pathway has special morphology, location and function which are maintained by specific resident proteins inside the organelle and by cytoskeleton proteins (Fig. 1-1) (Lippincott-Schwartz, Roberts et al. 2000). Protein and lipid cargoes move through these organelles in the secretory pathway by vesicular trafficking and there is also molecule exchange between different secretory organelles to keep these organelles dynamically stable (Rothman and Wieland 1996). The localization and efficiency of secretory organelles determine the trafficking pattern of vesicular carriers and finally characterize
Figure 1-1 Schematic representation of the secretory pathway.

Secretory cargo destined to be secreted or to arrive at the plasma membrane (PM) leaves the ER via distinct ER exit sites (ERES) that bud and translocate as tubular-vesicular structures (pre-Golgi or ERGIC) toward the (-) end of microtubules. Here they merge with Golgi membranes (Golgi), which in many mammalian cells are located near the Microtubule Organizing Center (MTOC). After passing through the Golgi complex and Trans-Golgi Network (TGN) secretory cargoes are packaged into post-Golgi transport intermediates (post-Golgi), which translocate plus-end directed along microtubules to the plasma membrane. (Adapted from Lippincott-Schwartz, Roberts et al. 2000)
Figure 1-1 Schematic representation of the secretory pathway.
the destination and distribution of newly synthesized lipids and proteins on the cell surface.

1.2 Endoplasmic Reticulum

The ER membrane was first observed by Keith R. Poter, Albert Claude, and Ernest F. Fullam in 1945 (Porter Keith R. 1945). The ER is the largest cellular organelle and is even called the “cell inside the cell”. It is a extended continuous network with typical reticular tubular structures from the nuclear envelope to the very periphery of the cell (Lee, Miller et al. 2004). It is one of the most important material factories in the cell for protein synthesis, folding, glycosylation, quanlity control, cargo concentration, forward trafficking to Golgi apparatus and even protein degradation (Sitia and Meldolesi 1992; Trombetta and Parodi 2003; Vandenberghe and Bredt 2004). The ER is also the biggest Ca\(^{2+}\) buffering pool in the cell (Petersen and Verkhratsky 2007). The uptake and release of Ca\(^{2+}\) from this intracellular store can tightly regulate intracellular Ca\(^{2+}\) concentration which is one of the most critical signaling molecules especially for neurons (Augustine, Santamaria et al. 2003). The ER can be charaterized by three dynamically segregated compartments, the nuclear envelope, ribosome-bound rough ER and ribosome-free smooth ER according to their morphology and function (Baumann and Walz 2001). Rough ER is responsible for all the secretory protein, integral membrane protein and partial cytoplasmic protein translation (Lippincott-Schwartz, Roberts et al. 2000; Stephens, Dodd et al. 2005). mRNAs for secretory proteins and integral membrane
proteins are targeted to ER by signal peptides and are translated by ER resident ribosomes (Rapoport 2007). The newly synthesized nascent peptides are inserted into the rough ER lumen (secretory protein) or membrane (integral membrane protein) via the interaction between the signal peptide and the Sec61α translocon complex spanning in the ER membrane (White and von Heijne 2004).

Rough ER is also the primary site for protein folding and modification. Different resident chaperones and enzymes in the ER help the proper folding, glycosylation and disulfide-bond formation of newly synthesized proteins (Trombetta and Parodi 2003). Specific mechanisms assure only the correctly folded, modified proteins or protein complexes exit the ER and progress to the next step of maturation in the Golgi apparatus (Ellgaard and Helenius 2001). This quality control mission is accomplished by mainly chaperones in the ER and is critical to prevent the secretion of improperly-folded or problematic protein products (Kim and Arvan 1998). Proteins which do not pass the quality control system are retained in the ER by chaperone binding and these proteins can be exported out of the ER through the AAA-ATPase Cdc48/p97 protein complex to the cytosol (Rothman 1989; Raasi and Wolf 2007). These exported proteins are ubiquitinated and destined to be degraded by proteosomes with the amino acids reused for protein synthesis (Trombetta and Parodi 2003). This pathway is called ER Associated Degradation (ERAD) which is important for normal ER maintenance and regulation of signaling protein (Vandenberghe and Bredt 2004). In some occasions, the
correctly folded and modified proteins or subunits of protein complexes can be retained in the ER by chaperone binding and these proteins provide a storage pool for integral membrane protein renewing and could also be important for up or down-regulation of signaling proteins on the cell surface (Greger, Khatri et al. 2002).

Smooth ER has the primary role of Ca²⁺ storage and release (Berridge 2002). Intracellular Ca²⁺ is pumped into the smooth ER through sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (SERCAs) and buffered by interactions with ER resident chaperones many of which are Ca²⁺ binding proteins (Corbett and Michalak 2000; Camello, Lomax et al. 2002). The ER lumen Ca²⁺ can be released into cytosol through ryanodine and inositol-trisphosphate (IP₃) receptors (Chen, Li et al. 1997; Meldolesi 2001). The function of ER Ca²⁺ release was originally identified for its critical role in muscle contraction (Franzini-Armstrong and Jorgensen 1994). It is also discovered to be important in intracellular signaling in neurons. There are brain specific isoforms of SERCAs and ryanodine receptors (Murayama and Ogawa 1996; Baba-Aissa, Van den Bosch et al. 1998). Ryanodine and IP₃ receptors are highly expressed in different brain regions. Ryanodine receptors are concentrated in the hippocampal neuronal axons and their postnatal expression is limited to hippocampus (Sharp, Dawson et al. 1993; Mori, Fukaya et al. 2000). On the contrary, IP₃ receptors are highly expressed in the cerebellum (Furuichi, Yoshikawa et al. 1989). Ca²⁺ release from different receptors influences the intracellular Ca²⁺ concentration dynamically and in turn regulates neuronal function.
1.3 ER Exit Sites

After proper folding, newly synthesized proteins export from the ER exit sites (ERES) which are defined by the COPII coats (Palade 1975; Kuehn and Schekman 1997). The formation of COPII vesicles is accompanied by the membrane curvature change and the membrane budding is facilitated by the COPII coats (Matsuoka, Orci et al. 1998; Pagano, Letourneur et al. 1999). COPII coats marked ERES are dynamically stable regions which are spatially segregated and COPII vesicles continuously bud from these relatively well defined regions (Hammond and Glick 2000; Stephens, Lin-Marq et al. 2000). At ERES, protein and lipid cargoes are selected and concentrated in the COPII vesicles by specific mechanisms and these products are destined for ER export and trafficking to the next modification station (Antonny and Schekman 2001).

COPII vesicles are composed of different proteins, which are originally identified from screen of lethal mutants of yeast *Saccharomyces cerevisiae* and Sec13 and Sec23 are required for ER to Golgi transport (Novick, Field et al. 1980). It is found in later studies that mammalian homolog of Sec13 and Sec23 are components of ER export vesicles (Tang, Peter et al. 1997; Hobman, Zhao et al. 1998). Other protein components of COPII coats are also identified in yeast screen including Sec24, Sec31 and Sar1 GTPase (Nakano and Muramatsu 1989; Wuestehube, Duden et al. 1996). It is shown that Sec23, Sec24, Sec13, Sec31 and Sar1 are the necessary components of COPII vesicles *in vitro*, and these
proteins are responsible for sorting and export of qualified lipid and protein cargoes out of ER (Matsuoka, Orci et al. 1998; Barlowe 2002).

Different COPII protein components can be shedded from the vesicle and be recycled for the second use in the later vesicle formation (Orci, Ravazzola et al. 1991). The formation of COPII vesicles include a sequencial recruitment of different protein components (Aridor, Fish et al. 2001). Sec16 is reported to determine the sites of ERES independent of COPII coat proteins (Connerly, Esaki et al. 2005; Watson, Townley et al. 2006). Sar1 is first recruited to the ER membrane and activated by Sec12, a GTP exchange factor (GEF) for Sar1 (Barlowe and Schekman 1993). Sar1-GTP recruits Sec23 and Sec24 the inner layer COPII coat proteins. Sar1, Sec23 and Sec24 are the main determinators for protein cargo selection and concentration into the vesicles (Aridor, Weissman et al. 1998). Later on, Sec13 and Sec31 the outer layer COPII coat proteins are recruited to the Sar1/Sec23/Sec24 protein complex and are in charge of COPII vesicle budding from the ER membrane. Sec13 and Sec31 also stimulate Sar1’s GTPase activity and upon the hydrolysis of Sar1-GTP to Sar1-GDP, two layers of COPII coat proteins are dissembled and recycled (Antonny and Schekman 2001). Surprisingly, although COPII vesicle formation and budding is a dynamic process, ERES are very stable (Hammond and Glick 2000; Stephens, Lin-Marq et al. 2000). In fabroblasts ERES are evenly distributed in the whole cell from nucleus neighboring regions to the very periphery. The detailed mechanisms for ERES location determination is not completely clear yet, but
illuminating the distribution mechanisms of ERES would be extremely useful to understand the active usage pattern of secretory organelles especially in polarized cells such as neurons.

1.4 ER to Golgi Transport: ERGIC

The ERGIC is the intermediate station between ER and Golgi apparatus composed of vesicles and tubular structures which are important for separating ER retained proteins and proteins for forward trafficking (Morin-Ganet, Rambourg et al. 1998; Martinez-Menarguez, Geuze et al. 1999). The coats of trafficking vesicles are changed from COPII proteins to COPI proteins and these COPI vesicles are also called vesiculotubular carriers (VTC) (Aridor, Bannykh et al. 1995; Stephens, Lin-Marq et al. 2000). Specific mechanisms guarantee the ER enzymes recycled back to ER lumen or ER membrane and protein products transported to Golgi apparatus (Appenzeller-Herzog and Hauri 2006). The morphology and trafficking in ERGIC is microtubule dependent and the vesicle movement is driven by microtubule minus end motor dynein (Malaisse and Orci 1979; Presley, Cole et al. 1997). Sec23 interaction with microtubule plus end associated dynactin protein complex attaches the vesicles to microtubules and ensures the forward trafficking (Vaughan, Tynan et al. 1999; Watson, Forster et al. 2005). Given its highly dynamic morphology and composition, ERGIC is rather an intermediate protein sorting and trafficking center than a stable cellular organelle.
1.5 The Golgi Apparatus

Golgi apparatus is first discovered by Camillo Golgi (Golgi 1898). It is a ribbon like continuous organelle composed of a series of membrane cisternae along the cis-to-trans axis at the perinuclear region (Rambourg and Clermont 1990; Clermont, Rambourg et al. 1994; Shorter and Warren 2002). The cis-face of Golgi opposed to the ERGIC accepts incoming vesicles from the ER and its trans-face is in charge of the protein export to the cell surface (Farquhar and Palade 1981; Clermont, Rambourg et al. 1995). Different enzymes are partitioned and reside in different cisternae of the Golgi stack (Worley 1946). Proteins are modified sequentially in the series of cisternae in a cis-to-trans manner and proceed the Golgi stack by vesicular trafficking (Palade 1975; Rothman 1994). Interestingly, on the contrary to the classic perinuclear ribbon structure in mammalian cells, Golgi morphology varies considerably among lower species. Drosophila oocytes have more than one thousand ERES-Golgi subunits distributed in the cytoplasm without a central Golgi apparatus (Herpers and Rabouille 2004). In yeast Saccharomyces cerevisiae the Golgi apparatus is composed of discrete tubular material spreaded in the cytoplasm (Preuss, Mulholland et al. 1992; Rambourg, Clermont et al. 1993; Rambourg, Clermont et al. 1995).

Interestingly, during mitosis in mammalian cells Golgi apparatus breaks down into vesicular fragments which is regulated by the phosphorylation of Golgi matrix and vesicle tethering proteins (Lucocq and Warren 1987; Lucocq, Berger et al. 1989; Shima,
Cabrera-Poch et al. 1998; Seemann, Pypaert et al. 2002). Golgi proteins are then separated into two daughter cells which is regulated by microtubules and the membranes are rebuilt into stacks after the cytokinesis (Lucocq, Berger et al. 1989; Souter, Pypaert et al. 1993; Shima, Cabrera-Poch et al. 1998; Seemann, Pypaert et al. 2002). The rebuilding of membranes is regulated by the reversal of Golgi matrix and vesicle tethering protein phosphorylation (Lowe, Gonatas et al. 2000). ER and Golgi membranes are in a dynamic equilibrium (Lippincott-Schwartz, Yuan et al. 1989). ER proteins transported to Golgi can be recycled back and the ER and Golgi membranes are interdependent (Cole, Ellenberg et al. 1998). There is evidence showing that the Golgi apparatus is derived from ER (Lippincott-Schwartz, Roberts et al. 2000). When the microtubule transport is disrupted, Golgi fragments can be formed near ERES (Hammond and Glick 2000).

The structure and function of Golgi apparatus are maintained by hundreds of integral and periphery Golgi resident proteins (Wu, Yates et al. 2000). The major function of Golgi apparatus is carbohydrate modification of newly synthesized proteins and lipids (Whur, Herscovics et al. 1969; Schachter, Jabbal et al. 1970; Farquhar and Palade 1998). The glycosylation enzymes including N-acetylglucosaminyltransferase, galactosyltransferase, mannosidase II and glycosyltransferase are concentrated in different Golgi cisternae and modify the newly synthesized protein sequentially (Whur,
Herscovics et al. 1969; Schachter, Jabal et al. 1970; Balch, Dunphy et al. 1984). Golgi is also the primary site for sphingolipid biosynthesis (Ichikawa and Hirabayashi 1998).

1.6 Golgi to Plasma Membrane Transport: TGN

In addition to protein and lipid modification, Golgi is also important for post-Golgi vesicle sorting and trafficking to the plasma membrane. In mammalian cells, these vesicles are transported from Golgi to the Trans-Golgi Network (TGN) and then to the plasma membrane, endosomes, lysosomes or back to the ER (Girod, Storrie et al. 1999; White, Johannes et al. 1999; Liljedahl, Maeda et al. 2001; Mullins and Bonifacino 2001).

During the sorting process, Golgi resident proteins need to be retained in the Golgi from other trafficking cargoes and constant membrane flux. At the trans-Golgi the proteins intended for regulated secretion are segregated away from constitutive membrane cargoes (Orci, Ravazzola et al. 1987). In polarized epithelial cells apical and basolateral cargoes are sorted at TGN and be transported to different plasma membrane domains (Griffiths and Simons 1986; Keller, Toomre et al. 2001).

Another important function of Golgi sorting is to orient the direction of secretory pathway and targe the protein cargoes to specific destinations on the cell surface. The role of Golgi in protein sorting and the orientation of trafficking suggest its important function in cell polarity and local domain formation. Golgi is the source for growing buds in yeast and the newly synthesized materials are transported to the buds exclusively (Tkacz and Lampen 1972; Tkacz and Lampen 1973; Novick and Schekman 2001).
1983; Ferro-Novick, Novick et al. 1984; Tschopp, Esmon et al. 1984). Golgi can also orient the secretory trafficking in the direction of migration in fibroblasts (Kupfer, Louvard et al. 1982; Etienne-Manneville and Hall 2001; Prigozhina and Waterman-Storer 2004).

### 1.7 Cellular Spatial Organization of Endoplasmic Reticulum

The morphology and localization of the ER and Golgi can determine the orientation of secretory cargoes and in turn regulate their final distribution on the cell surface. This local domain formation on the plasma membrane is critical for normal cell function especially for polarized cells such as neurons. The molecular mechanisms that form and maintain the spatial organization of the ER and Golgi is one of the most important questions to answer in the cell biology field.

The ER membranes form flattened sheets, cisternae and tubules which are observed using light microscopy to form an irregular polygonal network with characteristic three-way junctions in well-spread areas of the cell periphery (Lee and Chen 1988; Terasaki and Reese 1992; Voeltz, Rolls et al. 2002). Despite its complex organization, the ER is a continuous membrane compartment in fibroblasts and cerebellar purkinje cells (Fig. 1-2) (Terasaki, Slater et al. 1994). The function of cytoskeleton in ER architecture maintenance has been investigated in numerous studies and it is
**Figure 1-2** Spatial organization of the ER network and ERES in different cell types.

A, Representative ER network in a CV-1 cell stained with DioC6(3). Over 90% of the junctions in the ER network are triple junctions, linking three tubules (curved arrow), with double (arrow) and quadruple (arrowhead) junctions. Tubule branching and fusion were hypothesized as a possible mechanism for constructing the ER network. Bar represents 5.5 μm. (Adapted from Lee and Chen 1988)

B, The ER network and ERES in HeLa cells. ER tubules (green) were visualized by Myt1 immunofluorescence, which extend to the periphery of the cell. ERES COPII protein Sec13 (red) shows the localization of ERES. All the ERES are associated with ER and mark discrete ER subdomains. Bar represents 10 μm. (Adapted from Hammond and Glick 2000)

C, A single stage-9 *Drosophila* oocyte is surrounded by follicle cells. The ER network (green) is continuous in the oocyte. Within the oocyte, ERES-Golgi units represented by Gurken protein (red) are restricted to the dorsal/anterior corner near the nucleus (N) in dots. (Adapted from Levine and Rabouille 2005)

D, The dendritic ER in purkinje cell viewed with confocal microscopy labeled for IP$_3$ receptor. The ER network was found in dendrites and spines (arrow). (Adapted from Martone, Zhang et al. 1993)
Figure 1-2 Spatial organization of the ER network and ERES in different cell types.
found that microtubules and the ER are highly interdependent. When microtubules or the function of microtubule associated proteins are disrupted, the ER network retracts to the cell center in a number of experimental conditions, which include the depolymerization of the microtubule cytoskeleton by drugs or low temperature (Terasaki, Chen et al. 1986), the suppression of the heavy chain of the microtubule plus-end-directed motor kinesin (Feiguin, Ferreira et al. 1994) and the overexpression of the microtubule associated protein tau that impedes kinesin-mediated transport (Ebneth, Godemann et al. 1998). The dynamics of the ER membrane extension along microtubules has also been imaged by light microscopy both \textit{in vivo} (Waterman-Storer and Salmon 1998) and in cell-free systems, in which the elongation of the ER membrane can be inhibited by antibodies against kinesin heavy chain (Dabora and Sheetz 1988; Lane and Allan 1999). Microtubule motors can either drag membranes along underlying microtubules or drive the sliding of membrane-associated microtubules, whereas microtubule polymerization can promote the movement of an ER tubule bound to the dynamic microtubule tips (Waterman-Storer and Salmon 1998). Both plus-end-directed motors of the kinesin family and the minus-end-directed dynein motor have been implicated in microtubule-based membrane movements (Lane and Allan 1999). Considering the interdependent relationship between the ER membranes and microtubules, various nonmoter microtubule associated proteins have also been found to regulate the ER morphology.
It is known that microtubules and its associated motor proteins are critical for ER morphology regulation. Interestingly microtubule plus-end motor kinesin binding protein kinectin has a function in regulating the shape of ER membranes. Kinectin, an integral membrane protein is the major kinesin binding partner in membrane organelles including the ER and it is required for kinesin-driven motility (Toyoshima, Yu et al. 1992; Kumar, Yu et al. 1995; Blocker, Severin et al. 1997; Ong, Lim et al. 2000). Silencing of kinectin leads to a collapse of the ER to the center of the cell, which reveals its role in regulating the ER morphology (Santama, Er et al. 2004). Therefore, kinectin regulates the ER morphology potentially via attaching the ER membrane to the microtubule motor kinesin.

In previous studies it has been shown that stable attachment of ER membranes to microtubules is essential for maintaining the ER network morphology. Cytoskeleton Linking Membrane Protein of 63 kDa (CLIMP63) has revealed a stable interaction between the ER membrane and microtubules. CLIMP63 is a type II ER membrane protein which was identified in a search for organelle specific markers of the early secretory pathway (Schweizer, Ericsson et al. 1993; Schweizer, Rohrer et al. 1995). CLIMP63 is exclusively located in the ER shown by immuno-gold electron microscopy and CLIMP63 is excluded from the outer nuclear membrane by forming large immobile oligomers in the reticular ER (Schweizer, Ericsson et al. 1993; Klopfenstein, Klumperman et al. 2001). Purified CLIMP63 binds to microtubules and when overexpressed, CLIMP63
rearranges the ER along bundled microtubules, suggesting CLIMP63 functions in anchoring ER membranes to microtubules (Klopfenstein, Kappeler et al. 1998). Fibroblasts that overexpress CLIMP63 dominant negative microtubule binding deficient mutant exhibit a poorly extended ER without changing microtubule cytoskeleton (Vedrenne, Klopfenstein et al. 2005). These discoveries suggest that the CLIMP63 binding properties to microtubules rearrange the ER membranes. Overexpression of wild-type CLIMP63 increases the number of interacting points between the ER membrane and microtubules and therefore induces their coalignment, whereas overexpression of mutated CLIMP63 that does not bind to microtubules disrupts the anchoring of the ER to microtubules. CLIMP63 is required to stabilize the extended ER network in a microtubule dependent manner. Interestingly, CLIMP63 binding to microtubule is cell cycle dependent. During mitosis CLIMP63 binding to microtubule is inhibited and ER network is less developed switching from microtubule to actin dependent organization (McCullough and Lucocq 2005). Depolymerizing microtubule or microtubule dependent motor activity disruption caused ER membrane collapse is strikingly similar to the effect of CLIMP63 microtubule binding deficient mutants. Therefore, during the dynamic ER membrane extension on microtubules, CLIMP63 may act in synergy with kinesin motors to stabilize the ER tubules along the microtubules which would otherwise retract to the cell center because of membrane tension generated by the extensive tubule formation (Upadhyaya and Sheetz 2004; Koster, Cacciuto et al.
2005). Intriguingly, besides microtubule motor proteins, CLIMP63 is another essential structual protein in the ER which stably anchors the ER to microtubules and maintains its reticular tubular organization. The function of CLIMP63 in the dendritic ER organization and the postsynaptic function in neurons will be discussed in Chapter 4.

In addition to microtubule motor proteins and anchoring proteins, other microtubule associated proteins are also found to be important for ER-microtubule interaction and ER membrane structure maintenance, including ubiquitous Huntington’s disease protein huntingtin, of which a poly-glutamine expansion can cause a dominant progressive neurodegenerative disease. Interestingly, previous studies have suggested its role in intracellular trafficking and axon transport along microtubules (Li and Li 2004; Gunawardena and Goldstein 2005; Smith, Brundin et al. 2005). Huntingtin is a cytoplasmic protein mainly associated with microtubules (Gutekunst, Levey et al. 1995; Hoffner, Kahlem et al. 2002) and it also binds to various membranes, including clathrin-coated vesicles, recycling endosomes, ER, Golgi and plasma membranes. Knocking down of Huntingtin is found to induce an aberrant poorly extended ER network in mouse neuroblastoma and human glioblastoma cells, with leaving microtubules intact (Omi, Hachiya et al. 2005). Huntingtin binds to β-tubulin and dynactin via its neuronal partner huntingtin-associated protein (HAP1), which provides opportunities for direct and motor-based interactions with microtubules (Li, Gutekunst et al. 1998). Huntingtin may associate with ER membranes by different
mechanisms. It may bind to phospholipids of the ER bilayer directly through electrostatic interactions as by this mechanism huntingtin associates with plasma membranes (Kegel, Sapp et al. 2005). It may also bind to ER membrane by palmitoylation. It is shown that huntingtin is palmitoylated by palmitoytransferase HIP14 (Huang, Yanai et al. 2004). In addition, it may associate with the ER membrane through HAP1 by binding to IP₃R1 which is retained in the ER (Tang, Tu et al. 2003). Intriguingly, the involvement of huntingtin in ER structure maintenance may point to a role of ER organization in the development of Huntington’s disease.

Another interesting molecule which can regulate ER structure is p22 an EF-hand Ca²⁺-binding protein (Andrade, Zhao et al. 2004). It is a cytosolic microtubule binding protein and associates with microtubule in an N-myristoylation-dependent manner (Timm, Titus et al. 1999; Andrade, Pearce et al. 2004). P22 mediates the interaction between microsomes and microtubules. Ca²⁺ induces its conformation change and enhances its binding to microsomes. Injection of p22 antibody disrupts microtubules and vesiculates the ER membrane (Andrade, Pearce et al. 2004). This vesiculation is significantly different from typical ER membrane retraction after microtubule depolymerization. Therefore, it is possible that p22 regulates microtubule and ER structure independently. P22 may regulate ER membrane structure by promoting membrane fusion in a Ca²⁺ dependent manner (Barroso, Bernd et al. 1996). In other words, ER membranes would interact only transiently with microtubule-associated p22
in regions where Ca²⁺ signals allow for membrane binding. This intriguingly hypothesized p22 and Ca²⁺ dependent ER structure regulation is especially attractive in neurons considering the critical function of Ca²⁺ signaling in regulating neuronal function.

Vesicle-Associated Membrane Protein VAP-B is another characteristic ER morphology regulator (Amarilio, Ramachandran et al. 2005; Kaiser, Brickner et al. 2005). VAP proteins are type II integral membrane proteins proposed to function in secretory trafficking (Soussan, Burakov et al. 1999; Foster, Weir et al. 2000; Weir, Xie et al. 2001). In mammalian cells, VAP-B binds to a specific peptidic signal FFAT bearing Nir proteins, which is a family of cytosolic proteins involved in membrane trafficking and their interaction remolds ER structure (Loewen, Roy et al. 2003; Amarilio, Ramachandran et al. 2005). Overexpression of VAP-B together with Nir2 causes the formation of stacked membrane arrays, and on the contrary, overexpression of VAP-B with Nir3 makes ER membrane bundles along microtubules. This observation suggests that the same ER protein binding with different cytosolic partner can modulate ER morphology independently. Overexpression of dominant negative form of VAP-A protein which cannot bind to Nir proteins disrupts ER network (Kaiser, Brickner et al. 2005). Therefore the VAPs are a family of proteins which can regulate ER membrane morphology.

In fibroblasts the ER membrane is a dynamic structure with frequent morphing. Homotypic membrane fusion is usually considered to be involved in ER tubule polygon
formation and ER biogenesis. The fusion of intersecting tubules contributes to the formation of new polygons, whereas the fusion of two junctional tubules results in the net loss of a polygon within the peripheral ER network. Membrane fusion is also essential for the ER membrane reformation after mitosis. It is shown in previous studies that the reformation of ER network after microtubule depolymerization can be controlled by two sequential membrane fusion reactions. In the first step, NSF/α and γ-SNAP lead to vesicle aggregation and formation of thin membrane tubules connecting the disrupted ER elements. In the second fusion step, p97/p47/VCIP135 protein complex leads to complete ER network formation (Hetzer, Meyer et al. 2001; Uchiyama, Jokitalo et al. 2002; Kano, Kondo et al. 2005; Kano, Kondo et al. 2005). These two steps require the t-SNARE syntaxin 18, and knocking down of syntaxin 18 associated protein BNIP1 leads to a disintegration of the reticular ER (Nakajima, Hirose et al. 2004). P47 and VCIP135 are also required for ER tubule reformation (Uchiyama, Jokitalo et al. 2002) and p97 protein complex is involved in nuclear envelope reformation after mitosis (Hetzer, Meyer et al. 2001).

Although microtubule and its associated proteins play critical roles in regulating the ER membrane morphology, actin filaments are also essential for ER structure morphogenesis and maintenance. Plant and budding yeast cells use actin as tracks for cortical ER. Ultrastructural studies of plant cells have revealed the close association of ER tubules and actin bundles with the cortical cytoplasm (Du, Ferro-Novick et al. 2004).
and ER network can slide along stationary actin cables in lower-plant algae cells (Kachar and Reese 1988). Although most cortical ER tubules do not align with cortical actin filaments in yeast, the disruption of actin assembly results in a rapid and dramatic decrease in cortical ER dynamics (Prinz, Grzyb et al. 2000). Most interestingly, the absence of smooth ER in the dendritic spine of purkinje cells from dilute-lethal mice lacking the actin-based motor myosin Va indicates that actin is required for transporting ER from the dendritic shaft to the dendritic spine (Takagishi, Oda et al. 1996; Bridgman 1999). Actin-associated protein synaptopodin is strongly expressed by spine-bearing neurons in the olfactory bulb, striatum, cerebral cortex, and hippocampus, and in hippocampus it is preferentially located in the spine neck and is closely associated with spine apparatus which suggests that it could link the actin cytoskeleton of spines to intracellular calcium stores (Deller, Mundel et al. 2000). In animal cells microtubules are known to control outward ER extension, the transport of ER tubules towards the cell center is perturbed when actin assembly or myosin motor function is disrupted (Terasaki and Reese 1994; Waterman-Storer and Salmon 1998). Therefore, microtubule and actin filament may control different modes of ER tubule morphing and structure dynamics.

There are many other microtubule or actin filament associated proteins which can regulate ER morphology and ER membrane dynamics. Interestingly the myelin-associated membrane protein reticulon-4 (RTN4)/Nogo has been extensively studied
with regards to its neurite outgrowth inhibitory function, both in limiting plasticity in the healthy adult brain and regeneration during central nervous system injury. Surprisingly, Nogo and other reticulon paralogues are mainly localized to the ER, and are likely to have a role in modulating the morphology and functions of the ER (Voeltz, Prinz et al. 2006; Teng and Tang 2008). The molecular mechanisms for ER morphology regulation and its consequences in postsynaptic function will be further discussed in Chapter 4.

1.8 Cellular Spatial Organization of Golgi Apparatus

On the contrary to typical reticular tubular structures of ER, Golgi apparatus has stacked cisternae in mammalian cells. The morphology of Golgi apparatus is generated by cytoskeletal organization especially microtubules (Shorter and Warren 2002). Microtubules are oriented radially with the plus end extending out from the microtubule organizing center (MTOC) to the cell periphery in most of the interphase fibroblasts (Piehl and Cassimeris 2003). ER derived cargoes move inward along microtubules via the minus-end directed microtubule motor protein dynein to the perinuclear Golgi cisternae and these cargoes are critical for Golgi morphology maintenance. Disruption of microtubules or the function of dynein results in Golgi fragmentation (Rogalski, Bergmann et al. 1984; Ho, Allan et al. 1989; Vaisberg, Grissom et al. 1996). Cargoes from Golgi are transported to the cell surface along microtubules by microtubule plus-end directed motors such as kinesins (Kreitzer, Marmorstein et al.
Post-Golgi cargoes with sorting signals are transported to different cell domains by specific microtubule motor protein complexes (Nakagawa, Setou et al. 2000; Setou, Nakagawa et al. 2000).

Microtubules not only determine the morphology of Golgi apparatus at steady state, but also regulate Golgi localization and the secretory pathway orientation during cell polarity formation. During epithelial cell polarization Golgi apparatus is located to the apical region in the cytoplasm and proteins are only delivered to the apical two-third of the lateral plasma membrane (Bacallao, Antony et al. 1989; Kreitzer, Schmoranzer et al. 2003). Disruption of microtubules causes the mixture of the apical and basal proteins at the lateral plasma membrane (De Almeida and Stow 1991; Gilbert, Le Bivic et al. 1991; Kreitzer, Schmoranzer et al. 2003). Microtubule binding protein Adenomatous Polyposis Coli (APC) and the Par6-aPKC polarity complex regulate the microtubule organization and in turn regulate Golgi localization and directional membrane trafficking (Etienne-Manneville and Hall 2003).

In addition to microtubule, actin filaments can also regulate Golgi morphology and trafficking. Motor proteins for actin filament such myosin I (Fath, Trimbur et al. 1994), myosin II (Fath, Trimbur et al. 1994; Musch, Cohen et al. 1997) and myosin IV (Buss, Kendrick-Jones et al. 1998) are found in the Golgi membrane in mammalian cells. These motor proteins attach the vesicles to actin filaments and are important for membrane budding from the Golgi and cargo transport.
Another class of proteins, which are required for Golgi morphology maintenance are considered Golgi structural proteins and these proteins do not cycle between ER and the Golgi (Seemann, Pypaert et al. 2002). Peripheral membrane protein Golgi ReAssembly Stacking Protein (GRASPs), including GRASP55 and GRASP65 are required for forming the Golgi cisternae (Barr, Puype et al. 1997; Shorter, Watson et al. 1999). These proteins interact with Golgi-associated tethering proteins such as GM130 and Giantin (Nakamura, Rabouille et al. 1995; Puthenveedu and Linstedt 2001). Disruption of incoming COPI vesicles tethering to the cis-Golgi disrupts Golgi architecture (Levine, Rabouille et al. 1996). Both stacking and tethering proteins are critical for Golgi structure maintenance.

1.9 Neuronal Secretory Organelles

Neurons have all the same secretory organelles as fibroblasts, and the neuronal secretory pathway is critical for neuronal development, synaptic transmission and neuronal plasticity. However, considering the highly branched morphology of neurons and the enormous distance for secretory trafficking, the organization of secretory pathway must be different from that of the fibroblasts. Neurons not only have ER and Golgi apparatus in the cell body but there are also secretory organelles appearing in the neuronal dendrites and these satellite secretory organelles play important roles in dendrite formation and plasticity (Fig. 1-3) (Takagishi, Oda et al. 1996; Miyata, Finch et al. 2000; Horton, Racz et al. 2005; Ye, Zhang et al. 2007).
The ER is a continuous structure extending from the soma to the distal part of the dendrites and axons and it appears in spines shown by electron microscopy and immunofluorescence of ER marker proteins (Broadwell and Cataldo 1984; Martone, Zhang et al. 1993; Terasaki, Slater et al. 1994; Spacek and Harris 1997; Gardiol, Racca et al. 1999; Pierce, van Leyen et al. 2000). Smooth ER (SER) is a critical Ca²⁺ store in dendrites and spines in neuronal plasticity (Finch and Augustine 1998; Miyata, Finch et al. 2000; Nishiyama, Hong et al. 2000; Verkhratsky 2005). The volume of SER in the spine heads correlates with synaptic efficacies (Harris and Stevens 1988). In addition the ER is also a store for recycling membranes in dendrites (Cooney, Hurlburt et al. 2002). Dendritic ER is required for normal neuronal function. In purkinje cells of the ataxic mutant rat, ER is missing in dendritic spines (Dekker-Ohno, Hayasaka et al. 1996).

Rough ER (RER) is the first station in integral membrane protein and secretory protein synthesis and processing. Polyribosomes appear in neuronal dendrites and synapses (Steward and Levy 1982; Tiedge and Brosius 1996) associated with cellular organelles (Steward and Reeves 1988) and their dendritic localization is regulated by activity (Ostroff, Fiala et al. 2002; Steward and Schuman 2003). Chaperones for protein folding have also been observed in dendrites suggesting protein modification and
**Figure 1-3** Secretory organelles in neuronal dendrites by electron microscopy study.

A, Smooth ER forms a network in the dendrite of hippocampal neurons. SER in a single EM section appears as thin cisternae with wavy membranes (arrows). A mushroom spine (m) and a thin spine (t) originate from the dendrite on this section. Bar represents 1 μm. (Adapted from Cooney, Hurlburt et al. 2002)

B, Three-dimensional reconstruction of the SER in hippocampal neuronal dendrite demonstrates the cisternae form a network with larger flat compartments (arrowheads) connected by thin extensions (arrows). SER is found in three (*) out of seventeen spines originating from this segment of dendrite and two of them are mushroom spines with spine apparatuses. (Adapted from Cooney, Hurlburt et al. 2002)

C, Immunogold labeling for GM130 in adult rat hippocampus shows GM130-labeled Golgi outposts in the apical dendrite of a CA1 pyramidal neuron *in vivo*. Outposts are composed of stacks of cisternae with gold particles decorating the cis-face (arrows) of Golgi. Bar represents 1 μm. (Adapted from Horton, Racz et al. 2005)

D, Immunogold labeling for GM130 in the apical dendrite of a pyramidal neuron in adult rat somatosensory cortex shows a dendritic branch (*) with a nearby GM130-labeled Golgi stack (arrows). Bar represents 0.5 μm. (Adapted from Horton, Racz et al. 2005)
Figure 1-3 Secretory organelles in neuronal dendrites by electron microscopy study.
maturation happen in dendrites (Krijnse-Locker, Parton et al. 1995; Gardiol, Racca et al. 1999).

ERES also appear in dendrites and newly synthesized proteins can be exported from dendritic ERES. Protein export from ERES is not only critical for protein transport in the secretory pathway, but also important for regulating cell surface expression of signaling molecules including neuronal receptors. Surface expression of a number of receptors is controlled by the ER retention/retrieval or export signals (Bichet, Cornet et al. 2000; Margeta-Mitrovic, Jan et al. 2000; Standley, Roche et al. 2000; Ma, Zerangue et al. 2001; Scott, Blanpied et al. 2001; Mu, Otsuka et al. 2003). Some of these signals function at the ERES and are regulated by COPII protein binding. It is shown that a specific splicing isoform of NMDA receptor subunit NR1 can facilitate NMDA receptor export from ER which is regulated by neuronal activity (Mu, Otsuka et al. 2003). This specific splicing isoform of NR1 contains a C terminus sequence which can regulate its binding to COPII protein Sec23 and control its export from the ER. For AMPA receptor subunit GluR1 and GluR2, the ligand binding affinity, ligand binding domain conformation, Ca\(^{2+}\) permeability and tetramer formation can regulate their export from the ER (Greger, Khatri et al. 2002; Greger, Khatri et al. 2003; Penn, Williams et al. 2008; Coleman, Moykkynen et al. 2009), which will be further discussed in later sections in this introduction.
As the major check point for protein sorting and forward trafficking between the ER and Golgi apparatus, dendritic ERES could be one of the primary mechanism that respond to local neuronal activity change and control newly synthesized signaling molecule transport to the neuronal surface. Intriguingly, the number and distribution of ERES are related to neuronal stage (Aridor, Guzik et al. 2004), so it is very possible that the exit site formation is regulated by neuronal activity. It would be interesting to study how the localization of dendritic ERES and protein export efficiency from these ERES control neuronal development and postsynaptic function.

Neuronal Golgi was first discovered by Camillo Golgi in neurons (Golgi 1898). As another important station in the secretory trafficking, Golgi apparatus is primarily distributed in the somatic regions (Krijnse-Locker, Parton et al. 1995). It is found later that Golgi is also distributed in dendrites by immuno-gold labeling for Golgi protein TGN38, α-mannosidase II, giantin, and rab6 (Torre and Steward 1996; Pierce, van Leyen et al. 2000; Pierce, Mayer et al. 2001; Horton and Ehlers 2003). In these studies, Golgi markers are distributed in membrane-bound cisternae in apical dendrites and spines (Pierce, Mayer et al. 2001). Several other Golgi-associated neuron-specific proteins are found to distribute in distal dendrites and spines (Baillat, Moqrich et al. 2001; Camera, da Silva et al. 2003). The distribution of Golgi markers in dendrites suggests the neuronal Golgi organization is different from the centralized Golgi structure in fibroblasts. Intriguingly, recent studies have shown that Golgi is not only distributed in
dendrites but also concentrated at dendritic branch points of apical dendrites in hippocampal neurons (Horton, Racz et al. 2005; Ye, Zhang et al. 2007). Dendritic Golgi functions as a sorting center for newly synthesized lipids and proteins at dendritic branch points and it is critical for neuronal development of hippocampal neurons and DA neurons in *Drosophila* (Horton, Racz et al. 2005; Ye, Zhang et al. 2007).

The microtubule organization in neurons is quite distinct from non-neuronal cells. Rather than having microtubules with plus-ends projecting outwards in a radial array from a central microtubule organization center, microtubules distributed throughout the dendrites have a mixed polarity (Baas, Deitch et al. 1988; Sharp, Yu et al. 1995). On the contrary, in axons, plus ends of microtubules are oriented to the distal part (Baas, Deitch et al. 1988) and this polarity is dynein dependent (Zheng, Wildonger et al. 2008). The mixture of microtubule polarity in dendrites suggests that ER-to-Golgi transport could be bidirectional in dendrites, which is shown in light microscopy study in neurons (Horton and Ehlers 2003).

Existence of dendritic ER and Golgi apparatus is consistent with the observation of activity-regulated local protein synthesis and indeed, local translation is observed at special “hotspots” in dendrites (Kacharmina, Job et al. 2000; Aakalu, Smith et al. 2001; Job and Eberwine 2001). The local synthesis will be discussed in the following subsection in the introduction.
1.10 Synaptic Plasticity and Local Protein Synthesis

Neurons are the basic components of brain circuits and function. Neuronal activity and property directly control the memory storage in the brain and the cellular properties of neurons are regulated by important signaling molecules such as proteins and lipids. The synthesis of lipids and proteins in neurons play a critical role in normal brain function. The organization of the secretory pathway in neurons is different from that in fibroblasts. The ER and Golgi apparatus are also distributed in dendrites which make the local protein synthesis, modification and transport in dendrites possible.

Long-term synaptic plasticity discovered in different brain regions is thought to be the basis for learning and memory. A series of brief trains of high frequency stimulation to excitatory synapses in the central nervous system causes a sustained increase in the efficacy of synaptic transmission. This long lasting enhancement in synaptic strength is called Long Term Potentiation (LTP). First described by Bliss and Lomo in 1973 (Bliss and Gardner-Medwin 1973; Bliss and Lomo 1973), LTP is an excellent model and represents a broad range of types of synaptic modification which have fostered innumerable insights to molecular mechanisms of learning and memory (Malenka and Bear 2004). Recent studies revealed that there are different forms of synaptic plasticity which include NMDA receptor-dependent LTP, NMDA receptor-independent LTP, NMDA receptor-dependent Long Term Depression (LTD), mGluR-dependent LTD and endocannabinoid-dependent LTP (Kauer and Malenka 2007).
Because of the limited scope of this introduction and big volume of literatures on different forms of plasticity, I will only discuss a few forms of synaptic plasticity as following which require protein synthesis and neuronal secretory organelles.

LTP at CA3-CA1 synapses in mammalian hippocampus is the most thoroughly studied model of memory storage. NMDA receptor knockout mice and CaMKIIα mutant mice indicate hippocampal CA3-CA1 LTP would be critical for spatial memories (Tsien, Huerta et al. 1996; Giese, Fedorov et al. 1998; Miller, Yasuda et al. 2002). NMDA and AMPA type glutamate receptors play a central role in hippocampal LTP (Kauer, Malenka et al. 1988; Bliss and Collingridge 1993; Malenka and Nicoll 1999; Lu, Man et al. 2001; Bredt and Nicoll 2003; Malenka and Bear 2004). NMDA receptors behave as coincidence detectors for LTP induction in the hippocampus. Under basal conditions, the NMDA receptors are blocked by Mg²⁺ in a voltage dependent manner (Mayer, Westbrook et al. 1984; Ascher and Nowak 1988), such that the opening of the NMDA receptor channels require coincident postsynaptic depolarization and presynaptic release of glutamate (Mayer, Westbrook et al. 1984; Gustafsson, Wigstrom et al. 1987; Ascher and Nowak 1988). This permits the movement of extracellular Ca²⁺ into the postsynaptic terminus through activated NMDA receptor channels, which initiates postsynaptic signaling cascade for LTP expression (Jahr and Stevens 1987; Regehr and Tank 1990). Increasing postsynaptic Ca²⁺ induces the trafficking and insertion of AMPA receptors into synapses and increases postsynaptic transmission efficacies (Liao, Hessler
et al. 1995; Isaac, Nicoll et al. 1999; Shi, Hayashi et al. 1999; Liao, Scannevin et al. 2001; Shi, Hayashi et al. 2001; Bredt and Nicoll 2003; Takahashi, Svoboda et al. 2003). Increasing intracellular Ca\(^{2+}\) is necessary and sufficient for hippocampal LTP (Malenka, Kauer et al. 1988). Different signaling pathways and binding proteins are responsible for LTP induction and expression. CaMKII, PKA, PKC, PKM\(\zeta\), phosphatases such as PP1, PP2a, PP2b (Hu, Hvalby et al. 1987; Lisman 1989; Silva, Stevens et al. 1992; Frey, Huang et al. 1993; Pettit, Perlman et al. 1994; Lledo, Hjelmstad et al. 1995; Hrabetova and Sacktor 1996; Ling, Benardo et al. 2002; Lisman, Schulman et al. 2002; Esteban, Shi et al. 2003; Man, Wang et al. 2003), actin and AMPA receptor binding proteins are involved in this process (Kim and Lisman 1999; Krucker, Siggins et al. 2000; Bredt and Nicoll 2003). Although there is evidence showing that recycling endosomes are the direct source for AMPA receptor insertion during the early phase of LTP (Park, Penick et al. 2004), the source for AMPA receptors in LTP maintenance is not clear yet. The secretory pathway in dendrites may supply AMPA receptors for the hippocampal LTP (Broutman and Baudry 2001). Interestingly, SAP97 and stagazin bind to the AMPA receptor subunit GluR1 in the early secretory pathway and regulate AMPA receptor trafficking and function (Leonard, Davare et al. 1998; Chen, Chetkovich et al. 2000; Sans, Racca et al. 2001; Rumbaugh, Sia et al. 2003; Tomita, Adesnik et al. 2005; Vandenberghhe, Nicoll et al. 2005). Therefore, it is important to understand the early secretory pathways and
trafficking of AMPA receptors in the dendrites and examine how the receptor targeting is regulated.

This form of LTP has two temporal phases: the early phase and the late phase. Early-phase LTP (E-LTP) is induced by a single train of high frequency stimulation which can last for 1-2 hours. Late-phase LTP (L-LTP) is induced by three to four spaced trains of stimulation and has greater magnitude and longer duration (more than 3 hours) (Sweatt 1999; Kelleher, Govindarajan et al. 2004). It is proposed that both E-LTP and L-LTP induction are NMDA receptor dependent. AMPA receptor trafficking and postsynaptic efficacy change is required for the expression of both phases (Sweatt 1999; Kelleher, Govindarajan et al. 2004). Transcription and protein translation are necessary for the maintenance of L-LTP, but E-LTP does not require macromolecule synthesis (Frey, Krug et al. 1988; Nguyen, Abel et al. 1994; Huang and Kandel 1995; Crino, Khodakhah et al. 1998; Kelleher, Govindarajan et al. 2004). Besides trains of high frequency stimulation, neurotrophins such as BDNF, NT-3 and dopamine D1/D5 type receptor agonist can also induce long-lasting translation dependent LTP (Huang and Kandel 1995; Kang and Schuman 1995; Kang and Schuman 1996). Several cellular signaling cascades are activated in L-LTP. Activation of NMDA receptors, AMPA receptors and metabotropic glutamate receptors (mGluR) lead to activation of the small GTPase Ras, kinases such as PI3K, PKA, PKC, CaMKII, and other signaling molecules (Kelleher, Govindarajan et al. 2004; Lynch 2004). Among the downstream signaling
pathways, mitogen-activated protein kinase (MAPK) is regulated by activity. MAPK have been shown to regulate the activation of several translation factors, such as translation initiation factor eIF4E and p70S6K (Kelleher, Govindarajan et al. 2004; Kelleher, Govindarajan et al. 2004; Klann, Antion et al. 2004; Lynch 2004). This activation of translation machinery enhances target mRNA translation.

mGluR-dependent LTD is another form of plasticity which requires protein synthesis. mGluR-dependent LTD was first described at parallel fiber synapses on purkinje cells in the cerebellum and requires conjunct inputs from climbing fibers and parallel fibers (Ito, Sakurai et al. 1982; Watanabe, Fujiwara et al. 1989). Climbing fiber inputs lead to postsynaptic Ca^{2+} rise through voltage-gated calcium channels which is permissive for LTD and parallel fibers activate group I mGlu (mGluR1 and mGluR5) and downstream signal cascades (Linden, Dickinson et al. 1991; Aridor, Guzik et al. 2004). mGluR-dependent LTD can also be induced at hippocampal CA3-CA1 synapses and the activation of postsynaptic mGluR1 alone appears to be sufficient (Bolshakov and Siegelbaum 1994; Oliet, Malenka et al. 1997; Snyder, Philpot et al. 2001; Xiao, Zhou et al. 2001). Paired pulses at 1Hz can induce mGluR-dependent LTD at hippocampal CA3-CA1 synapses (Huber, Roder et al. 2001). In most cases, this type of LTD is mediated by rapid protein synthesis and the following clathrin-dependent endocytosis of synaptic AMPA receptors (Huber, Roder et al. 2001; Hou and Klann 2004; Pfeiffer and Huber 2006). Another presynaptic form of mGluR5-dependent LTD is expressed in the
hippocampus (Bolshakov and Siegelbaum 1994; Palmer, Irving et al. 1997; Doherty, Palmer et al. 2000; Fitzjohn, Palmer et al. 2001; Faas, Adwanikar et al. 2002; Zakharenko, Zablow et al. 2002) without affecting postsynaptic glutamate receptor response (Rammes, Palmer et al. 2003; Tan, Hori et al. 2003). Since mGluR5 is predominantly postsynaptic at these synapses, a retrograde mechanism may be involved in this form of LTD (Watabe, Carlisle et al. 2002).

“Input specificity” is a distinct feature of LTP. Isolated groups of synapses on the same postsynaptic cell can be regulated independently. Synaptic strength enhancement at one set of synapses would not influence the strength of other sets of synapses if the two groups of synapses are activated by different afferents (Andersen, Sundberg et al. 1977; Bliss and Collingridge 1993; Steward and Schuman 2001; Lynch 2004). If two sets of afferents are close (<50 μm), there would be heterosynaptic enhancement in the two different groups of synapses (Bonhoeffer, Staiger et al. 1989; Schuman and Madison 1994; Steward and Schuman 2001). As described above, transcription and protein translation are necessary for the maintenance of L-LTP and for memory storage, but how the newly synthesized proteins are transported and targeted to appropriate synapses and modify synaptic efficacy without influencing the strength of other inactivated synapses is not clear. There are several hypotheses to elucidate the mechanism.

One possible model for synaptic input specificity is “synaptic tagging,” which was first proposed by Frey and Morris in 1997 (Frey and Morris 1997). In a CA1 neuron,
if a CA3–CA1 pathway S1 is strongly tetanized to induce L-LTP, in the same postsynaptic cell a different CA3-CA1 pathway S2 can induce L-LTP by strong tetanus in a protein synthesis independent manner (30 minutes after S1 stimulus, during treatment with protein synthesis inhibitor anisomycin). In addition, giving a weak tetanic stimulation that ordinarily leads only to E-LTP through S2 can induce L-LTP if a strong tetanic stimulation is given to the CA1 cell through S1. Therefore, as repeated trains of tetanus can induce the protein synthesis required for L-LTP, E-LTP may create a protein synthesis independent “synaptic tag” and sequester the newly synthesized relevant proteins for establishment and maintenance of L-LTP (Frey and Morris 1997; Frey and Morris 1998). This synaptic tag produced by E-LTP can last around 2 hours (Frey and Morris 1998). However, the nature of synaptic tag is still a mystery. Kinases, adhesion molecules, cytoskeleton proteins, other relevant molecules, cellular organelles or cellular events are promising candidates (Martin and Kosik 2002).

Another interesting model is “local protein synthesis,” which has been revealed more and more in recent studies. Proteins can be translated in isolated neuronal dendrites (Kang and Schuman 1996; Ouyang, Kantor et al. 1997; Aakalu, Smith et al. 2001; Steward and Schuman 2003), and this local protein synthesis is critical for L-LTP (Kang and Schuman 1996; Martin, Casadio et al. 1997; Casadio, Martin et al. 1999; Miller, Yasuda et al. 2002). Different mRNAs are present in isolated dendrites, including those for proteins involved in LTP and LTD such as NMDA receptor subunit NR1, AMPA
receptor subunits GluR1/2/3/4, IP$_3$ receptor, and CaMKII$\alpha$ (Miyashiro, Dichter et al. 1994; Martone, Pollock et al. 1996; Steward and Schuman 2003). Some mRNAs can be transported to synapses in an activity dependent manner (Steward 1997; Krichevsky and Kosik 2001; Steward and Schuman 2001; Kanai, Dohmae et al. 2004). Therefore, it is proposed that mRNAs are translated in close proximity to stimulated synapses, allowing for the use of newly synthesized proteins by appropriate synapses for input specificity. AMPA receptor subunits GluR1 and GluR2 are translated in the dendrites under activity regulation (Ju, Morishita et al. 2004; Smith, Starck et al. 2005). There is also evidence showing local protein synthesis not only can be induced by activity change of particular synapses, but it can also further modulate the synaptic properties (Martin, Casadio et al. 1997; Casadio, Martin et al. 1999). As described above, local protein synthesis and dendritic cellular organelles are important for the strengthening and growth of synapses (Martin, Casadio et al. 1997; Casadio, Martin et al. 1999; Si, Giustetto et al. 2003; Si, Lindquist et al. 2003).

Although these proposed models for synaptic input specificity are attractive, there are many unresolved issues. For example, based on the local protein synthesis hypothesis, the spatial distribution of the mRNAs and protein products would be important for the synaptic specificity in context. However, integral membrane proteins are simultaneously translated and inserted into the ER membrane at the onset of the secretory pathway. During the folding and modification the nascent integral membrane
proteins are retained in the ER for a period of time. Therefore, the protein movement in the dendritic ER membrane could regulate the final location of these proteins which in turn could influence synaptic specificity. Studies of cellular organelles and protein transport in the dendritic secretory pathway would be critical for further understanding of the cellular mechanisms of LTP. The following studies summarized in this dissertation are the first report showing that the dendritic ER spatial organization restricts nascent integral membrane protein diffusion and regulates the neuronal postsynaptic function.

1.11 AMPA Receptor Function, Biogenesis and Trafficking in Plasticity

AMPA receptors are critical for the expression of various forms of synaptic plasticity in the brain and their biogenesis, trafficking, internalization and degradation directly regulate the induction and maintenance of synaptic plasticity (Malenka and Bear 2004). AMPA receptors are glutamate-gated cation channels with fast activation and deactivation kinetics (Greger, Ziff et al. 2007; Hansen, Yuan et al. 2007). Four AMPA receptor subunits (GluR1-4, or GluRA-D) have been found. A functional AMPA receptor consists of four subunits from a single subtype (Laube, Kuhse et al. 1998; Mano and Teichberg 1998; Rosenmund, Stern-Bach et al. 1998). The majority of AMPA receptors in the adult central nervous system are heteromers of GluR1/GluR2 or GluR2/GluR3 (Wenthold, Petralia et al. 1996; Greger, Ziff et al. 2007), which are non-rectifying cation channels without calcium permeability (Verdoorn, Burnashev et al. 1991). Homomeric
AMPA receptors formed by GluR1, GluR3 and GluR4 are calcium permeable inward rectifiers, but homomeric GluR2 channels lack calcium permeability and inward rectification (Dawson, Nicholas et al. 1990; Fletcher, Nutt et al. 1995; Andersen, Tygesen et al. 1996; Hennegriff, Arai et al. 1997; Varney, Rao et al. 1998). GluR2 subunits experience posttranscriptional mRNA editing by replacing the original glutamine (Q) codon by an arginine (R) codon (Seeburg and Hartner 2003). Interestingly, these edited GluR2 subunits form a stable pool in the ER and favor the assembly of heteromeric receptors (Greger, Khatri et al. 2002; Greger, Khatri et al. 2003). Another development regulated editing site R/G at amino acid 743 in the ligand binding domain produces analogous effect by reducing the assembly of GluR2 homomers and slows GluR2 maturation in the ER (Greger, Akamine et al. 2006). AMPA receptor subunit mRNAs are also subjected to splicing. The “flip” and “flop” isofoms generated by splicing differ in their sequences preceding the fourth transmembrane domain and have distinct pharmacologic, kinetic and trafficking properties (Sommer, Keinanen et al. 1990; Partin, Patneau et al. 1994; Koike, Tsukada et al. 2000; Coleman, Moykkynen et al. 2006). Both of these two forms of modification namely editing and splicing can regulate AMPA receptor dwelling and export from the ER. Therefore, they control the receptor channel property and trafficking at the same time.

All the AMPA receptor subunits share a similar structure containing the N-Terminal Domain (NTD), the glutamate Ligand Binding Domain (LBD), four
transmembrane domains and a cytoplasmic tail (Gouaux 2004). The second transmembrane domain only dips into the membrane from the cytoplasmic face and contributes to the channel pore of the fully assembled AMPA receptors. The LBD is consisted of two separated regions S1 and S2. S1 is located after NTD and S2 is located between the third and fourth transmembrane domains (Fig. 1-4) (Bredt and Nicoll 2003). Although the majority of AMPA receptors are heteromers of GluR1/GluR2 and GluR2/GluR3, the number and composition of AMPA receptors are regulated during development and vary in different brain regions (Shepherd and Huganir 2007). AMPA receptors are assembled in the ER as dimmers and then further assembled into tetromers and this tetramerization is critical for its export from the ER (Ayalon and Stern-Bach 2001; Safferling, Tichelaar et al. 2001; Greger, Khatri et al. 2003; Tichelaar, Safferling et al. 2004). GluR1/2 heteromers export from the ER but GluR2/3 heteromers are retained in the ER (Greger, Khatri et al. 2002). Both the Q/R editing in the channel pore and the R/G editing in the ligand binding domain contribute to the ER retention of GluR2 and favors the formation of heteromers instead of GluR2 homomers (Greger, Ziff et al. 2007). Intriguingly, the retained GluR2 in the ER is a pool of AMPA receptors and potentially control the surface AMPA receptor level and composition. In addition, homomeric AMPA receptors of the flop form are retained in
**Figure 1-4** The composition and structure of AMPA receptors.

A, The schematic structure of the AMPA receptor subunits and the tetrameric channel. The individual subunits are composed of four transmembrane domains, and the channel consists of four subunits, which are usually two dimmers. The dimmers are two different subunits, such as GluR1 and GluR2 or GluR2 and GluR3. (Adapted from Shepherd and Huganir 2007)

B, The structure of a single AMPA receptor subunit using GluR2 as an example. The LBD is composed of the S1 (purple) and S2 (green). The R/G editing site at 743 is indicated by a red diamond, the flip/flop by a blue curve. The ion channel domain within the lipid bilayer (blue) consists of three transmembrane (TM) segments (grey) and the re-entrant pore loop. The Q/R editing site is indicated by a red diamond. (Adapted from Greger, Ziff et al. 2007)

C, The crystal structure of an AMPA receptor complex. A hypothetical subunit dimmer is shown in cyan; the yellow dimmer lacks the extracellular portion for clarity. The vertical arrow runs through the twofold symmetric axis, and the four-fold symmetric ion channel axis. (Adapted from Greger, Ziff et al. 2007)

D, Back view of the crystal structure of a LBD domain which is composed of S1 (purple) and S2 (green). This LBD domain is shown from the back, with a view onto the elements forming the dimmer interface (D, J, β). Mutations in the LBD domain can disrupt AMPA receptor early secretory transport. (Adapted from Greger, Ziff et al. 2007)
Figure 1-4 The composition and structure of AMPA receptors.
the ER, whereas those of the flip form are transport to the cell surface efficiently (Coleman, Moykkynen et al. 2006). It is shown that the stabilization of S1 and S2 ligand binding domain interaction is critical not only for regulating the desensitization status of AMPA receptors, but importantly it also controls the receptor export from the ER (Greger, Ziff et al. 2007). Most interestingly, ligand binding ability is another mechanism, which regulates AMPA receptor export from the ER. Single mutation in GluR1 or GluR2 ligand binding domain which disrupts their ligand binding can make both of them retained in the ER, potentially by a quality control mechanism (Coleman, Moykkynen et al. 2009). All of these molecular mechanisms, which regulate AMPA receptor ER export or retention, provide us useful tools to study the nascent AMPA receptor properties in the ER, such as their mobility, trafficking and degradation. In Chapter 3 I will discuss some of the findings about nascent AMPA receptor diffusion by using ER retained AMPA receptor subunits as probes.

Besides self-regulation, many AMPA receptor interacting proteins can also control their ER retention or export. The ER chaperone protein BIP and calnexin are associated with AMPA receptors in the ER (Rubio and Wenthold 1999). The interaction between the Protein Interacting with C Kinase 1 (PICK1) (Xia, Zhang et al. 1999) and the c-terminal PDZ motif of GluR2 may be necessary for GluR2 export from the ER (Greger, Khatri et al. 2002). Synapse-Associated Protein 97 (SAP97), a member of the Membrane Associated GUanylate Kinase family (MAGUK) interacts with GluR1 c-terminus in the
ER (Leonard, Davare et al. 1998; Rumbaugh, Sia et al. 2003). This interaction is important for synaptic targeting of GluR1, though the detailed mechanisms are not clear yet (Hayashi, Shi et al. 2000). The transmembrane AMPA Receptor Regulatory Proteins (TARP), including stargazin (γ-2), γ-3, γ-4, γ-7, γ-8, can control the AMPA receptor secretory trafficking in multiple steps (Nicoll, Tomita et al. 2006; Kato, Zhou et al. 2007). Stargazin can facilitate the folding and assembly of AMPA receptors in the ER (Vandenberghe, Nicoll et al. 2005) and AMPA receptor trafficking from the ER to the cis-Golgi (Tomita, Chen et al. 2003; Bedoukian, Weeks et al. 2006). Following the synthesis in the ER, AMPA receptors are transported to the Golgi and modified with mostly complex glycosylation at N-glycosylation sites (Rogers, Hughes et al. 1991). In addition, AMPA receptor subunits are reversibly palmitoylated at two cystein sites (Hayashi, Rumbaugh et al. 2005). The increase of palmitoylation in the second transmembrane domain causes the accumulation of AMPA receptors in the Golgi (Hayashi, Rumbaugh et al. 2005) and on the contrary palmitoylation of the c-terminus inhibits the interaction of AMPA receptors with the actin binding protein 4.1N and decreases its internalization from the cell surface (Shen, Liang et al. 2000; Hayashi, Rumbaugh et al. 2005). Therefore, AMPA receptor interacting proteins can regulate its trafficking and function in a broad time window, even during their modification in the early secretory pathway.

AMPA receptor targeting to the plasma membrane is critical for synaptic function, and the trafficking of AMPA receptor during LTP is intensively studied
(Malenka and Bear 2004). Neurons have highly branched and complex morphology, therefore it is challenging to transport AMPA receptors to precise locations at the cell surface, especially considering newly synthesized AMPA receptors’ essential function in “input specific” synaptic plasticity. Vesicular trafficking and diffusion on the cell surface are considered two major ways to transport newly synthesized AMPA receptors to their destinations in synapses. The long distance microtubule dependent trafficking of AMPA receptors involves motor proteins such as dynein and kinesin and their adaptor proteins (Hirokawa and Takemura 2004). For example, conventional KIF5 (Setou, Seog et al. 2002) is linked to the c-terminus of GluR2 and GluR3 by the Glutamate Receptor Interacting Protein 1 or AMPA receptor Binding Protein (GRIP1/ABP) (Dong, O’Brien et al. 1997). Liprin-α links GluR2/GRIP1 complex with KIF1A (Wyszynski, Kim et al. 2002; Shin, Wyszynski et al. 2003). Disrupting the interaction in these dendritic transport complex decreases synaptic targeting of AMPA receptors (Setou, Seog et al. 2002). AMPA receptors can also be transported by actin dependent mechanisms. It is shown that AMPA receptors are transported into spines by actin motor myosin Vb during LTP (Wang, Edwards et al. 2008). It is also shown that the most dynamic movement of AMPA receptors occurs by lateral movement across the surface of neurons. A large internal store of AMPA receptors exchange rapidly with extrasynaptic somatic AMPA receptors, and these newly inserted AMPA receptors travel laterally along dendrites to reside stably at synapses (Adesnik, Nicoll et al. 2005). Therefore, diffusion is another
important way to transport receptors on top of intracellular vesicular trafficking. Considering the apparent continuity of dendritic ER and its role in protein synthesis, it will be extremely interesting to look at protein diffusion in dendritic ER during their modification and dwelling in the ER.

1.12 Studies of Protein Mobility in Fibroblasts and Neurons

Recent studies have revealed more and more that diffusion is an important aspect for neuronal signaling molecule properties during their transport and targeting. A variety of critical techniques for measuring protein diffusion have been developed and improved. These experimental methods allow us to visualize and quantify protein mobility on the smallest scale of nanometers. It is known that proteins are naturally mobile within a lipid membrane and undergo random Brownian motion, but their mobility is strongly influenced by physical obstacles and chemical reactions (Kusumi, Nakada et al. 2005). Different methods have been developed to optically track glutamate receptor movement, yielding important insights into the physical interaction and local environment of a receptor (Triller and Choquet 2005; Groc, Lafourcade et al. 2007). Heterologously expressed proteins can be tagged by green fluorescent protein (GFP) and the Fluorescence Recovery After Photobleaching (FRAP) can be measured. By using this method, the proportion of receptors that are exchangeable in a given bleached area, based on the extent of fluorescence recovery, which provides important information on the bulk dynamics of a receptor population can be quantified. From the FRAP
experiments, apparent diffusion coefficients of the mobile and exchangeable pool of proteins can also be calculated (Reits and Neefjes 2001; Chen, Lagerholm et al. 2006), which describes how fast the proteins move in a given environment, even though this value is averaged and is highly sensitive to the availability of exchangeable pools of unbleached molecules, the specific geometry of the bleached area, and the properties of the interface with neighboring structures including the presence of different physical barriers, such as chemical interactions, temporary cytoskeletal corrals, or restricted membrane geometry (Choquet and Triller 2003). Single-particle tracking (SPT) is another technique that can be used to measure protein mobility with high temporal and spatial resolution. Different from FRAP, SPT measures the movement of single molecule. Fluorescent probe tagged antibodies against proteins allow the visualization and mapping of protein movement and diffusion coefficients can be derived by plotting the receptor mean square displacement (MSD) over time (Qian, Sheetz et al. 1991; Kusumi, Sako et al. 1993; Saxton 1993). Semiconductor quantum dots (QDs) allow the measurements of molecule movement on a nanometer scale (Ehlers, Heine et al. 2007).

In the scope of this study, I used FRAP as one of the major techniques in the following chapters. Here I discuss the mathematical analysis of FRAP in more details. In FRAP experiments, fluorescent molecules are irreversibly photobleached in a small area of the cell by a high-powered focused laser beam. Subsequent diffusion of surrounding non-bleached fluorescent molecules into the bleached area leads to a recovery of
fluorescence, which is recorded at low laser power. FRAP experiments started 30 years ago using lipophobic or hydrophilic fluorophores, like fluorescein, coupled to proteins and lipids (Axelrod, Ravdin et al. 1976; Edidin, Zagyansky et al. 1976). Later on GFP made it possible to perform FRAP on living cells without disruption by microinjection. GFP-tagged molecules can be targeted to various sites in the cell and can be observed for long periods because the GFP molecule is very photostable. FRAP experiments provide information about the mobility of a fluorescent molecule in a defined compartment. Two parameters can be derived directly from FRAP: the mobile fraction of fluorescent molecules and the rate of mobility, which is related to the characteristic diffusion time, $\tau_D$ (half time $t_{1/2}$ for fluorescence recovery is also used in some occasions to describe the diffusion rate). It is shown in (Fig. 1-5) a typical fluorescence recovery curve, allowing the determination of the two parameters. The mobile fraction can be determined by comparing the fluorescence in the bleached region after full recovery ($F_\infty$) with the fluorescence before bleaching ($F_i$) and just after bleaching ($F_0$). The mobile fraction $R$ is defined as:

$$R = \frac{(F_\infty - F_0)}{(F_i - F_0)}$$

The mobile fraction can change in different circumstances, for example when the fluorescent protein interacts with other molecules or membranes. The mobile fraction
Figure 1-5 Fluorescence recovery after photobleaching (FRAP) analysis.

When a region in the fluorescent area (the ER in this figure) is bleached at time $t_0$ the fluorescence decreases from the initial fluorescence $F_i$ to $F_0$. The fluorescence recovers over time by diffusion until it has fully recovered $F_\infty$. The characteristic diffusion time or half time $t_{1/2}$ indicates the time at which the fluorescence has recovered. The mobile fraction can be calculated by comparing the fluorescence in the bleached region after full recovery $F_\infty$ with that before bleaching ($F_i$) and just after bleaching ($F_0$).

(Adapted from Reits and Neefjes 2001)
Figure 1-5 Fluorescence recovery after photobleaching (FRAP) analysis.
can also be affected by membrane barriers and microdomains in the membrane. These discontinuities can prevent, or temporarily restrict, the free diffusion of membrane molecules.

When motion due to active transport or unidirectional flow can be discounted, protein mobility in a cell is due to Brownian motion. The mobility is expressed as the diffusion coefficient $D$, which is related to the diffusion time $\tau_D$. Most formulas describing this relationship are based on the two-dimensional diffusion equation described by:

$$\tau_D = \omega^2 \gamma / 4D$$

where $\omega$ is defined as the radius of the focused circular laser beam at the $e^2$ intensity and $\gamma$ is a correction factor for the amount of bleaching (Axelrod, Ravdin et al. 1976). This equation assumes unrestricted two-dimensional diffusion in a circular bleached area, with no recovery from above and below the focal plane. In other circumstances, one-dimensional or three-dimensional diffusion models can be used, but usually in living fibroblasts, two-dimensional diffusion model is the most frequently used. In other more complex diffusion models, $D$ is not a constant but is changing with time, and in other words, $D$ is a function with spatial and temporal resolutions. This more complex diffusion model is called anomalous diffusion. Heterogeneities in the structure of the
diffusion environment, interaction with other molecules or other factors may count for the constrained anomalous diffusion, though the factors responsible and their relative contributions to hindering molecule motion have not been satisfactorily identified. The mean square displacement of a particle undergoing a random walk is linear in time. In the presence of potential energy traps with binding energies that vary over wide ranges in both space and time, the particle’s motion is constrained to anomalous subdiffusion, and the mean square displacement obeys a power law in time (Bouchard 1990):

\[ <r^2> = \Gamma t^\alpha = 4D(t)^*t \quad \text{with} \quad D(t) = 1/4\Gamma t^{\alpha-1} \]

where \( \Gamma \) is the transport coefficient, \( t \) is time of random walk, and \( \alpha \) the time exponent, which gives a measure of the degree to which the motion is restricted. This parameter \( \alpha \) is also called anomalous coefficient. For \( \alpha=1 \), this equation reduces to Brownian diffusion with a constant diffusion coefficient; for \( 0<\alpha<1 \) the motion is time dependent; on shorter time scale barriers have little effect, and the particle diffuses freely, but on longer time and length scales, interactions with the barriers become significant, and diffusion is restricted (Feder, Brust-Mascher et al. 1996).

The FRAP technique originally found success as a method to measure diffusion in cellular membranes (Liebman and Entine 1974; Poo and Cone 1974). However, the recent studies have a marked increase in the use of FRAP for studying protein mobility
in the cell interior. FRAP has not only been used to address diffusion rates, but also to assess protein dynamics and interactions with other cellular components (Houtsmuller, Rademakers et al. 1999; White and Stelzer 1999; Houtsmuller and Vermeulen 2001; Reits and Neefjes 2001; Carrero, Crawford et al. 2004; Kimura, Hieda et al. 2004). It is also used to study many other aspects of cell biology, including chromatin structure (Festenstein, Pagakis et al. 2003), transcription (Giese, Au-Yeung et al. 2003), cytoskeletal dynamics (Shaw, Kamyar et al. 2003), vesicle transport (Smith, Pfeiffer et al. 2003), cell adhesion (von Wichert, Haimovich et al. 2003) and mitosis (Howell, Moree et al. 2004).

FRAP is also used to measure protein mobility in different cellular organelles in the secretory pathway, including protein diffusion in the ER (Nehls, Snapp et al. 2000) and Golgi (Cole, Smith et al. 1996). Interestingly, FRAP has been widely used to examine soluble or membrane signaling molecule mobility in neurons and their regulation by the different factors. It is found that spines and dendritic shaft is tightly coupled diffusonally and electrotonically which is crucial for understanding the neuronal signal integration and synaptic plasticity (Svoboda, Tank et al. 1996). Surprisingly scaffolding proteins such PSD-95 and actin are mobile and exchangeable in spines (Star, Kwiatkowski et al. 2002; Blanpied, Kerr et al. 2008) and the morphology of spines can regulate Ca^{2+} diffusion in purkinje cells (Santamaria, Wils et al. 2006).

In addition to intracellular scaffolding and signaling molecules, the mobility of neuronal surface receptors is intensively studied by single particle tracking. These
Discoveries provide us enormous insights into the molecular composition and dynamics of synaptic proteins and illuminate valuable information about the molecular mechanisms regulating synaptic plasticity. Both the exchange of mobile glutamate receptors and stabilization within synapses have been demonstrated. It is shown that GluR2 revealed a wide range of diffusion coefficients for extrasynaptic AMPA receptors in hippocampal neurons, with a tendency for slower diffusion rates as neurons mature (Borgdorff and Choquet 2002). Organic-dye-labeled antibodies subsequently allowed for the tracking of GluR2 in both synaptic and extrasynaptic compartments and for visualizing receptor exchange at synapses (Tardin, Cognet et al. 2003). Interestingly GluR2 has confined movements in synaptic and extrasynaptic regions (Ashby, Maier et al. 2006; Sharma, Fong et al. 2006), and extrasynaptic GluR2 can enter into synaptic regions providing evidence that extrasynaptic receptors can act as a readily available pool to supply synapses. Synaptic activity including LTP, LTD and chronic modifications can regulate surface AMPA receptor diffusion (Borgdorff and Choquet 2002; Tardin, Cognet et al. 2003; Groc, Heine et al. 2004; Ashby, Maier et al. 2006; Sharma, Fong et al. 2006). The mobility of GluR1 was reduced at active synapses as compared to inactive synapses, and the dwelling time of GluR1 was significantly longer at active synapses (Ehlers, Heine et al. 2007). GluR1 also explores a greater area at the postsynaptic membrane in inactive synapses (Ehlers, Heine et al. 2007). Although the mobility of surface AMPA receptor and its potential function have been intensively studied in recent years, AMPA receptor
mobility in early secretory pathway inside the neuron is not yet clear, partially because of technical challenges. Most interestingly, a reverse proximal-to-distal increasing gradient is observed for AMPA receptors in CA1 pyramidal neurons (Magee and Cook 2000; Andrasfalvy and Magee 2001; Smith, Ellis-Davies et al. 2003). Such considerations highlight the important role of intracellular membrane trafficking coupled with receptor lateral diffusion for delivery of glutamate receptors to distal dendritic locations. It is known that secretory organelles are located in the whole dendrite from soma to distal part, therefore it will be extremely interesting to study the consequences of receptor movements in the secretory organelles and its contribution to this surface receptor gradient formation.

1.13 Experiment Rationale and Specific Aims

The goal of my thesis work is to study the trafficking of early secretory integral membrane protein cargoes in the dendritic ER; how the spatial organization of dendritic ER regulates the early secretory protein movement and their distribution in dendrites; whether dendritic ER spatial organization and early secretion protein trafficking in the ER is neuronal activity dependent and what are the molecular mechanisms and underlying signaling pathways. As I discussed in the previous sections, cellular organelles in the secretory pathway are important for neuronal development such as polarity, dendrite formation and normal synaptic functions. The local synthesis of integral membrane proteins such AMPA receptor subunits makes it even more
interesting to examine the biophysical properties and functions of dendritic secretory organelles. The dendritic ER is a critical station for protein and lipid synthesis and modification. All the integral membrane proteins dwell in the ER during folding and modification and explore the membrane surface of dendritic ER. I hypothesize that the dendritic ER is a platform for newly synthesized protein transport mainly mediated by Brownian diffusion. The spatial organization of dendritic ER, whose formation is neuronal activity dependent, constrains the protein diffusion in microdomains in the dendrite. This spatial organization of dendritic ER is also critical for surface targeting of newly synthesized receptors and regulates the postsynaptic functions.

Although it is known that integral membrane proteins are mobile in the ER membrane in fibroblasts and their mobility is regulated by chaperone protein binding and glycosylation (Nehls, Snapp et al. 2000), the mobility of early secretory protein in dendritic ER is not clear so far. The mobility of ER retained Ca²⁺ channels start to catch people’s attention (Fukatsu, Bannai et al. 2004) as the distribution of these channels regulate intracellular Ca²⁺ signaling directly. Three dimensional electron microscopy reconstruction study suggest a continuous ER membrane structure in CA1 hippocampal paramydal neurons (Spacek and Harris 1997; Cooney, Hurlburt et al. 2002). This discovery provides us insights into the stable spatial organization of dendritic ER. However, there is no systematic study to reveal the spatial organization of dendritic ER dynamically in live cells, in other words, whether the somato-dendritic ER is continuous
for protein diffusion, whether there are any diffusion barriers for protein movement in the ER, whether the dendritic ER structure is homogenous or heterogeneous in different dendritic regions, whether the ER spatial organization is regulated by synaptic input, whether the ER spatial organization constrains the protein trafficking in the dendrite and targeting to the cell surface, and finally what is its consequences for postsynaptic function are not characterized. Elucidating these mechanisms was the goal of the following Specific Aims.

AIM#1: To characterize the local constrains of nascent integral membrane protein diffusion and global organization of the dendritic ER by using fluorescent probes.

The ER membranes extend into dendritic shaft and some spines in hippocampal neurons. It is critical for the intracellular Ca\(^{2+}\) signaling, local translation and protein modification. To characterize the spatial organization of dendritic ER, I developed fluorescent probes which are retained in the ER to examine the dendritic ER structure. These fluorescent probes include ER-VSVG-GFP, SERCA2a-GFP, ER-GluR1-GFP and ER-GluR2-GFP. ER export signal in VSVGts and ligand binding sites in GluR1/2 are mutated to make them retained in the ER. Using live cell imaging approaches, combined with immunofluorescent labeling of secretory organelles, I found that dendritic ER membrane has tubular structures on the light microscoy level, in contrast to the typical tubular reticular structures usually observed in fibroblasts.
By using the ER fluorescent probes that have been developed, FRAP experiments were performed in different dendritic regions to examine the ER protein mobility. I found on local small scales the diffusion is an important aspect for ER protein dynamics and the protein mobility in dendritic ER is comparable to protein mobility on the plasma membrane, which indicates that intracellular ER membrane is potentially an important platform for protein trafficking. I also used these fluorescent probes to examine whether somato-dendritic ER is dynamically continuous, meaning whether ER membrane proteins can freely exchange in different regions in the soma and dendrites. It is found that there are no obvious diffusion barriers between different regions in the dendrites and proteins diffuse freely in the dendritic ER.

AIM#2: To examine the heterogeneity of dendritic ER spatial organization by using quantitative FRAP and 3D serial electron microscopy reconstruction.

The experiment results in AIM#1 suggests on both local small scale and global whole cell scale ER membrane proteins are mobile and exchangeable in different dendritic regions. Previous EM studies have shown that ER membrane volume in the dendritic shaft is correlated to the mushroom spine density in hippocampal pyramidal neurons (Spacek and Harris 1997). In order to describe the spatial organization of dendritic ER quantitatively I developed a FRAP based method. From the FRAP fluorescent recovery curve, half time for the recovery is calculated and a parameter “ER complexity index” was
defined to describe the ER spatial organization in terms of its influence on protein diffusion in the ER with higher ER complexity index meaning more restricted membrane protein diffusion in the dendritic ER. By using this quantitative FRAP and ER complexity analysis algorithm, membrane protein diffusion in the dendritic ER was examined with spatial and temporal resolutions.

It is found that dendritic ER membrane is more complex and protein movement is more restricted in the ER during neuronal development and apparent ER complexity is correlated with spine density. Golgi apparatus is important for neuronal polarity and dendritic branch formation during neuronal development (Horton and Ehlers 2003; Ye, Zhang et al. 2007), and it is enriched dendritic branch points working as a protein sorting center (Horton, Racz et al. 2005). As an upstream secretory organelle to Golgi apparatus, the ER could be more enriched and convoluted at Golgi possitive dendritic regions. In this way the newly synthesized proteins and lipids movement can be more restricted in the ER and they could have more chance to export to the downstream processing organelles. Therefore, I examined ER membrane complexity in different dendritic regions, and found that the ER complexity is not different between proximal and distal dendrites, but it is much more complex at dendritic branch points. In addition, by using photoactivable ER fluorescent probes I found protein diffusion in the ER within dendrites with dendritic branches is more restricted. It is also found that the ER is more complex in regions with possitive Golgi signals in dendritic shafts. The
correlation between ER complexity, restricted ER protein movement and the Golgi appearance gives us an interesting insight about the function of dendritic secretory pathway. The newly synthesized proteins and lipids can be processed by this “complex ER-Golgi” microdomains in the dendritic shaft and the modification and trafficking of these new synthesized materials could be more efficient, because of the integration of ER and Golgi secretory units.

I collaborate with Kristen Harris at UT Austin, and she kindly provided us serial sections of electron microscopy pictures in hippocampal CA1 neurons. Cam Robinson and Cyril Hanus did the three dimensional reconstruction on these serial images for the different dendritic regions and found that ER membrane is more enriched at dendritic branchpoints and very frequently associated with ribosomes. This finding provides us the biophysical bases for ER complexity. The ER membrane volume is higher at dendritic branch points. Because we did not quantify the ER membrane tubule branching and there could be other mechanisms that can regulate the ER membrane complexity, these other factors that influence the ER complexity cannot be excluded.

AIM#3: To study whether ER spatial organization is regulated by neuronal activity and its consequences in nascent AMPA receptor trafficking.

It is known that different forms of synaptic plasticity such as late phase LTP and mGluR dependent LTD require protein synthesis and secretory organelles. Based on the
previous findings in AIM#2 that the “complex ER-Golgi” microdomains which could make the local protein processing more efficient, I examined whether during the protein synthesis dependent synaptic plasticity the ER is more complex. I treat the hippocampal neurons with DHPG a type I mGluR agonist to activate mGluR1/5 and potentially start protein synthesis in the cultured neurons, and it is found that the dendritic ER is more complex and protein diffusion is more restricted after the type I mGluR activation and this regulation is PKC dependent. This discovery is consistent with our model that local ER-Golgi microdomains in the dendrite are important for the newly synthesized protein trafficking, processing and potentially the expression of different forms of synaptic plasticity. Interesting, NMDA receptor dependent LTD does not require protein synthesis and the dendritic ER complexity is not changed after the NMDA treatment. This finding adds another layer of understanding of our model that synaptic plasticity with different sources can modulate or control the function of neuronal secretory pathway independently.

As it is discussed in the previous sections, AMPA receptors are critical for the expression and maintenance of LTP, which has been intensively studied in the last decades. It is also found that AMPA receptor mRNA can be translocated into dendritic regions (Grooms, Noh et al. 2006) and be locally translated (Ju, Morishita et al. 2004). Therefore, secretory pathway in dendrites could be important for AMPA receptor synthesis and modification. Intriguingly, during mGluR activation the diffusion of
AMPA receptor subunit GluR1 is more restricted in the dendritic ER and this confined movement of AMPA receptors could make it export from the ER more efficiently and be targeted to the destination on the plasma membrane more specifically and precisely.

AIM#4: To study the molecular mechanisms which regulate ER spatial organization and its consequences in postsynaptic functions.

As discussed in the ER spatial organization and underlying mechanism section, microtubule and actin binding proteins can regulate ER morphology. It is shown that when ER membrane association with microtubules is enhanced by binding proteins, ER membranes make bundles along microtubules and when their association with microtubules is disrupted ER membranes shrink to the perinuclear region. CLIMP63 is an important microtubule binding protein which can regulate ER morphology dramatically (Vedrenne, Klopfenstein et al. 2005). Based on the finding in AIM#1, #2 and #3 that ER complexity is heterogenous in different dendritic regions, I examined whether this microtubule binding protein CLIMP63 can regulate ER complexity. It is discovered that the overexpression of CLIMP63-WT which enhanced the association between ER and microtubules makes the dendritic ER less complex and the overexpression of CLIMP63-3E a phospho-mimic mutant which dissociates ER from microtubules makes the ER more complex. Interestingly, CLIMP63 also regulates dendritic length and spine
density in neurons. CLIMP63 mediated ER complexity increase can produce shorter but more spiny dendrites.

The molecular mechanisms for CLIMP63 regulated ER complexity change is not clear yet, but the preliminary data show that it is a PKC dependent pathway. The mechanisms underlying CLIMP63’s effect on dendritic length and spine density is not clear either. One hypothesis is CLIMP63 dependent ER complexity change can regulate intracellular Ca\(^{2+}\) and the neuron morphology. The preliminary data, proposed model and future directions will be discussed in Chapter 4 and Chapter 5.
2. Materials and Methods

In this chapter, I will discuss in details all the materials and methods I used to generate the results in the following chapters. Serial electron micrograph data were provided by Dr. Kristen Harris (University of Texas at Austin, Austin, TX). Dr. Cyril Hanus and Dr. Cam Robinson (Duke University Medical Center, Durham, NC) finished the 3D reconstruction of the EM pictures for the CA1 pyramidal neurons. Tao Cui (Department of Electrical Engineering, Caltech, Pasadena, CA) finished the modeling and scripts for the diffusion analysis. All experiments involving animals followed all guidelines set by the National Institute of Health for the care and use of laboratory animals.

2.1 Cell-Culture and Transfection

Cos7 and HEK293T cells were grown in DMEM (Invitrogen) and HELA cells were grown in MEM (Invitrogen) supplemented with 10% fetal bovine serum, sodium pyruvate and glutamine, at 37°C in a 5% CO₂ atmosphere. Primary cultures of hippocampal neurons were obtained from E18 rat embryos. The entire hippocampus was isolated and dissociated with trypsin, and cells were plated at 75,000-100,000 per well onto poly-L-lysine-coated glass coverslips in 12-well plates in Neurobasal medium (Sigma, St. Louis, MO) supplemented with B27, glutamax, 5% bovine serum, and 1µg/ml gentamycin. FUDR (10µM) was added 6-7 days after plating, and cells were fed twice
weekly thereafter with Neurobasal medium prepared as above but without bovine serum. Neurons were grown at 37°C and in 5% CO2.

Cells were transfected with Lipofactamine 2000 (Invitrogen) according to manufacturer’s instructions. Most experiments in neurons, including FRAP and photoactivation, were performed 12-24 hours post-transfection in cells displaying moderate levels of exogenous protein expression.

### 2.2 DNA Constructs

GFP-tagged WT-GluR1 and GluR2 constructs (GluR1WT-PRK5-GFP and GluR2WT-PC3.1-GFP) were gifts from Dr. R. Huganir (Johns Hopkins University, Baltimore). HA-tagged WT-GluR2 (pGW1-GluR2WT-HA) and WT SAP97 constructs were gifts from Dr. M. Sheng (MIT, Boston). HA-tagged WT- GluR1 was generated from GluR1WT-PRK5-GFP by replacing GFP encoding sequence by PCR amplified HA after MluI digestion.

pCherry-N1 was derived from a mCherry encoding construct (Giepmans, Adams et al. 2006) given by Dr. R. Tsien (University of California at San Diego) by PCR and cloned as an Agel-BsrGI insert into pEGFP-N1 backbone (Clontech) where it replaced GFP.

The GFP-SERCA2a encoding plasmid (Fukatsu, Bannai et al. 2004) was a gift from Dr. K Mikoshiba (Institute of Medical Science, The University of Tokyo).
YFP-GT46 encoding plasmid (Pralle, Keller et al. 2000) was a gift from Dr. S. Grinstein (University of Toronto).

VSVGts045-GFP, GalT-GFP (Horton and Ehlers 2003) and βactin-mCherry (Helton, Otsuka et al. 2008) have been described previously. The ArfI-cherry encoding plasmid was derived from an ArfI-YFP construct (Majoul, Straub et al. 2001; Horton, Racz et al. 2005) and produced by PCR and EcoRI-BamHI cloning into pCherry-N1. The CMV promoter of this plasmid was attenuated as previously described (Hanus, Ehrensperger et al. 2006).

CLIMP63-WT, CLIMP63-ΔMBD, CLIMP63-3A, CLIMP63-3E (Vedrenne, Klopfenstein et al. 2005) constructs were gifts from Dr. H. Hauri (University of Basel, Switzerland).

PM-phluorin (PM-phluo) was generated by PCR amplification of the superecliptic phluorin (Miesenbock, De Angelis et al. 1998; Sankaranarayanan, De Angelis et al. 2000) from a plasmid given by Dr. G. Augustine (Duke University), and BglII-SalI cloning downstream to the signal peptide sequence of the cell-surface expression type I membrane protein backbone pDisplay (invitrogen). ER-VSVG (GFP or YFP-tagged) were generated from VSVGts045-GFP/YFP constructs (Horton and Ehlers 2003) by site-directed mutagenesis (Stratagene). Photoactivable ER-VSVG (ER-VSVG-paGFP) was produced by replacing GFP by paGFP (Patterson and Lippincott-Schwartz 2002) in our GFP-tagged ER-VSVG construct. ER-GluR1 and ER-GluR2 were generated
from GluR1WT-PRK5-GFP and GluR2WT-PC3.1-GFP by site-directed mutagenesis. All generated open reading frames were sequenced.

2.3 Antibodies

Rabbit polyclonal antibody against CLIMP63 cytoplasmic tail and ER-lumen tail amino acids were made by Open Biosystem. The cytoplasmic-terminus antigen: DKGAHPSGGADDVAKPP. The lumen-terminus antigen: KQDLTEKAVKEGESEINR. Phospho-peptides were generated to produce the phospho-specific antibody against CLIMP63 S3, S17 and S19. The peptide sequences are: P(pS3)AKQRGSKGGC, CGHGAA(pS17)PSDKGAH, CHGAASP(pS19)DKGAHPSG. These antibodies were used at 1:5,000 to 1:20,000 dilution in the immunoblot experiments.

2.4 Immunocytochemistry

The basic steps of our standard immunocytochemistry protocol were as follows: cells were fixed for 15 min at room temperature in 4% paraformaldehyde/PBS (Serva, Germany), and then incubated for 30min in 5% BSA/PBS (blocking solution) either directly after fixation (surface staining) or after a 15 min permeabilisation in 0.15% (w/v) TritonX100/PBS. Incubations with primary and secondary antibodies were performed in 1.5%BSA/PBS either overnight at 4°C (primary antibodies) or for 1 hour at room temperature (primary and/or secondary). Coverslips were mounted on glass slides in Gel-Mount medium (Electron Microscopy Sciences).
### Table 1 Antibodies used in experiments

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species</th>
<th>Application</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sec23</td>
<td>Rabbit (p)</td>
<td>IF</td>
<td>Abcam</td>
<td>1/300</td>
</tr>
<tr>
<td>BIP (GRP78)</td>
<td>Rabbit (p)</td>
<td>IF</td>
<td>Abcam</td>
<td>1/300</td>
</tr>
<tr>
<td>SAP97</td>
<td>Mouse (m)</td>
<td>IP</td>
<td>Stressgen</td>
<td>2μg</td>
</tr>
<tr>
<td>VSVG</td>
<td>Mouse (m)</td>
<td>IF</td>
<td>Horton and Ehlers 2003</td>
<td>1/300</td>
</tr>
<tr>
<td>GFP</td>
<td>Mouse (m)</td>
<td>WB</td>
<td>Clontech</td>
<td>1/500</td>
</tr>
<tr>
<td></td>
<td>Rabbit (p)</td>
<td>IP</td>
<td>BD Bioscience</td>
<td>2μg</td>
</tr>
<tr>
<td></td>
<td>Chicken (p)</td>
<td>IF</td>
<td>Chemicon</td>
<td>1/500</td>
</tr>
<tr>
<td>HA</td>
<td>Mouse (m)</td>
<td>WB</td>
<td>Covance</td>
<td>1/1000</td>
</tr>
<tr>
<td></td>
<td>Rabbit (p)</td>
<td>IP</td>
<td>Santa Cruz</td>
<td>2μg</td>
</tr>
<tr>
<td>CLIMP63</td>
<td>Mouse (m)</td>
<td>IF</td>
<td>Alexis</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mouse or rabbit IgGs, Rhodamine RX or FITC- conjugated</td>
<td>Goat (p)</td>
<td>IF</td>
<td>Jackson’s Lab</td>
<td>1/250</td>
</tr>
</tbody>
</table>

*m and p stand for mono- and polyclonal, respectively. All antibodies were IgGs.*
ER-exit sites were labelled using a different protocol adapted from the literature. Cells were fixed in ice-cold methanol for three minutes, rehydrated in PBS supplemented with 0.1% n-octyl-β-D-glucopyranoside (PBSO) and 100μM bis-sulfosuccinimidyl-sulphate (BS3, Pierce Chemical) for 30 minutes at room temperature. After extensive rinsing in PBSO, cells were quenched in 0.1% ethylenediamine-HCl (pH7.5) for 15 minutes at room temperature, rinsed in PBSO, incubated for one hour at room temperature in a blocking buffer of 3% w/v cold sea fish skin gelatine (Sigma) in PBSO and then incubated in primary and secondary antibody solutions as described above.

2.5 Co-immunoprecipitation

Transfected 293T cells were scrapped in PBS supplemented with 2mM EDTA and protease inhibitors (Roche) and disrupted by sonication. Membrane fractions were collected by ultracentrifugation (30 min at 50,000 rpm on a Beckman Ultracentrifuge) and lysed O/N at 4℃ in TNT lysis buffer (50mM Tris, 150mM NaCl, 1% w/v Triton X-100, pH 7.4) supplemented with protease inhibitors (Roche). Proteins of interest were then co-immunoprecipitated by sequential incubation with specific rabbit IgGs and protein G-coated sepharose beads (GE Healthcare) at 4℃ and were analysed by immuno-blotting after SDS-PAGE.
2.6 Live cell imaging, pharmacological treatments and intracellular calcium monitoring

Cells were monitored at 37°C in 150mM NaCl, 3mM KCl, 15mM Glucose (pH7.4) and either 2mM CaCl₂·MgCl₂ (standard E4) or 4mM CaCl₂ (low Mg²⁺ E4), for a maximal duration of 90min. DHPG, TTX, NMDA and AP5 (Tocris) were used at final concentrations of 100μM, 2μM, 20μM and 100μM, respectively.

DHPG effect was assayed in low Mg²⁺ E4 before and 30-45 min after addition of the drug. NMDA effect was assayed in low Mg²⁺ E4 supplemented with TTX before and 15-30min after a 3min exposure to the drug.

Intracellular calcium was monitored using Fluo4 imaging. Cells were loaded for 1 min at 37°C in standard E4 with 1μM Fluo4-AM (Molecular Probes) diluted from a 1mM solution made in DMSO and 10% pluronic acid, and were imaged as described for NMDA treatment.

2.7 Fluorescence microscopy

Confocal imaging was performed using a x60 objective (NA=1.4) on a Nikon inverted microscope equipped with a Hamamatsu spinning disk head and a diode-laser illumination device (Prairie), or a x63 objective (NA=1.32) on a Leica point laser scanning confocal SP2 AOBs inverted microscope. Most FRAP and photoactivation experiments were performed with the later microscope or using a x63 objective (NA=1.4) on a Zeiss LSM 5 (NA=1.4) fast-scanning laser confocal inverted microscope, with pinholes open at 5-6 airy units and a 2x2 pixel-binning. FRAP experiments shown for demonstration but
not quantification were performed at higher spatial resolution using a x60 objective (NA=1.4) on an Ultraview spinning disk inverted microscope (Perkin Elmer) without pixel-binning. All live-cell imaging experiments were performed at 37°C.

2.8 Fluorescence measurement

Average or integrated fluorescence intensities were measured in Metamorph (Universal Imaging Corporation) after background corrections, and in some instances after application of a 3x3 matrix low pass filter. To exclude ER fluorescence variations merely due to local heterogeneities of dendritic shapes, ER fluorescence variation coefficients throughout dendrites (standard deviation/mean) were normalized by the pixel-to-pixel fluorescence correlation index measured for GFP and a red cell-fill (mCherry). Fluorescence pictures shown in illustrations were pseudocolored in Metamorph or in Image J (NIH).

2.9 FRAP, photoactivation and FLIP data analysis

For FRAP analysis, fluorescence levels in the bleached area were normalized by the average fluorescence measured in unbleached cell areas and processed in Matlab (The Math-Works S.A.S). The time required for photobleaching was <0.5 sec and was neglectable compared to the duration of recovery monitoring. Only cells displaying a photobleaching above 70% of initial fluorescence in the bleached area, and a global photobleaching (due to picture acquisition) lower than 15% were analyzed. Experiments
in neuronal dendrites were performed in dendritic segments of comparable diameters at similar distances from the cell body.

**FRAP in fibroblasts.** The normalized fluorescence $F$ in the bleached area was analysed using the two-dimensional diffusion model described by Feder et al., (Feder, Brust-Mascher et al. 1996) and expressed in the form:

$$F(t) = \frac{F_1 + (R(F_0 - F_1) + F_1) \left( \frac{t}{t_{1/2}} \right)^\alpha}{1 + \left( \frac{t}{t_{1/2}} \right)^\alpha}$$

(1)

with $F_0$ the fluorescence intensity before bleaching, $F_1$ the fluorescence intensity immediately after bleaching, $R$ the recovered fluorescence fraction, and $t_{1/2}$ the recovery halftime.

**FRAP in neuronal dendrites.** Fluorescence recovery was analysed according to Fick’s second diffusion law (Crank 1980) assuming a three-dimensional anomalous diffusion in smooth cylindrical structures occurring only in the main dendritic axis, symmetrically at each side of bleached dendritic segments. Assuming the three-dimensional diffusion in cylindrical structures, with $C(r, t)$ the concentration of fluorophore at position $r$ within the photobleached segment, and time $t$, and the gradient concentration gradient $\nabla$ induced by photobleaching, Fick’s second diffusion law can be written as:
\[
\frac{\partial C(r,t)}{\partial t} = D(t)\nabla^2 C(r,t)
\]

(2)

The diffusion was considered to be potentially anomalous, implying a diffusion coefficient \(D\) varying with the transport coefficient \(\Gamma\) such as:

\[
D(t) = \frac{1}{4} \Gamma t^{a-1} = D_0 t^{a-1}
\]

(3)

with \(r\) included in the photobleached rectangular profile of width \(2\upsilon\) and length \(2w\), a symmetric recovery at each side of the bleached cylinder and an absence of diffusion in the direction \(n\) perpendicular to its axis, boundary conditions were:

\[
\begin{align*}
C(w, t) &= C(-w, t) = C_0, \ t \geq 0, \\
C(r, t) &= 0, \ t = 0, \\
\frac{\partial C(r, t)}{\partial n} \bigg|_{\Omega} &= 0,
\end{align*}
\]

(4)

Dendrites were considered to be smooth cylinders along which \(C(r, t)\) is equal in the entire planes orthogonal to the cylinder axis at position \(r\). The equation (2) could
then be approximated using a 1-dimensional model where the second Fick’s law becomes:

\[
\frac{\partial C(x,t)}{\partial t} = D(t) \frac{\partial^2 C(x,t)}{\partial x^2},
\]

(5)

with \(x\) the distance along dendrites from the center of the photobleached rectangle, and the symmetry allowed at \(x=0\), boundary conditions could thus be written in the form:

\[
C(w, t) = C(-w, t) = C_0, t \geq 0, \\
\frac{\partial C(x,t)}{\partial x} = 0, x = 0, t \geq 0 \\
C(x,t) = 0, t = 0, |x| < w
\]

(6)

The differential equation (5) was solved using the method of separation of variables and integration by parts, resulting in an equation of the form:

\[
C(x,t) = C_0 - \frac{4C_0}{\pi} \sum_{n=0}^{\infty} \frac{(-1)^n}{2n+1} \exp\left( - \frac{D_1(2n+1)^2 \pi^2 t^\alpha}{4\alpha w^2} \right) \cos\left( \frac{(2n+1)\pi x}{2w} \right)
\]

(7)

The normalized fluorescence intensity \(F(t)\) observed at time \(t \geq 0\) and calculated in the entire bleached rectangle could thus be expressed in the form:
\[
F(t) = R \left(1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \exp \left(-\frac{D_1 (2n+1)^2 \pi^2 t^\alpha}{4 \alpha w^2} \right)\right)
\]

which was used to calculate the recovered fraction R, the diffusion coefficient \(D_1\), the anomalous coefficient \(\alpha\) and the recovery halftime \(t_{1/2}\). Practically, the sum used for the integration was truncated with \(n\) varying from 0 to a nonnegative integer \(N\), taken from 1 to 10.

The diffusion of photoactivated molecules was quantified using the same model. For Fluorescence Loss In Photobleaching (FLIP) experiments, fluorescence decay was quantified using mono-exponential curve fitting.

**2.10 Serial electron micrograph acquisition and three-dimensional reconstructions**

Serial electron micrographs (EM) were obtained from the middle of the stratum radiatum in the CA1 area or adult male rat hippocampus as previously described (Spacek and Harris 1997). In brief, samples were dissected after an intracardial perfusion performed with 2% paraformaldehyde, 2.5% glutaraldehyde and 2 mM CaCl₂ in 0.1 M cacodylate buffer, pH7.35 (37°C) under deep anaesthesia, and then contrasted, embedded and sectioned for transmission EM and imaged with a Jeol 1200EX. Serial micrographs were aligned by cross-correlation analysis in IMod (Boulder Laboratory, University of Colorado). Structures of interest were outlined, and segmented for three-
dimensional reconstruction and morphological analysis in Amira (Visage Imaging, Inc.) using previously described identification criteria (Spacek and Harris 1997). Quantifications were performed by normalizing the total surface of ER membranes by that of the plasma membrane in 500nm long dendritic segments taken at, or at least 1.5 away from branch points or by measuring the average ER-tubule diameter as a function of the cell surface area along dendrite main-axis.

2.11 Modelling of nascent AMPA receptor diffusion in dendrites

Having established that dendritic branch points likely represent the entry-point of nascent dendritically translated membrane proteins into the ER, we build a mathematical model to further evaluate the extent of nascent GluR1 and GluR2 diffusion from a given synthetic source.

Synthesis rates and accumulation of nascent GluR1 and GluR2. Because of the combined effect of protein initiation, elongation and folding, it is quite difficult to estimate realistic synthesis rates of a given membrane protein a priori (Hershey 1991). Nevertheless, rough estimation of nascent GluR1 and GluR2 accumulation rates were extrapolated from protein elongation rates measured in vitro (~6 amino acids/sec per active ribosome) (Hershey 1991), the absolute numbers of ER-bound (thus active) ribosomes present at dendritic branch points in our EM snapshots, the relative abundance of GluR1 and GluR2 mRNA in hippocampal neuron dendrites deduced from the literature (Tsuzuki, Lambolez et al. 2001; Grooms, Noh et al. 2006; Poon and
Chen 2008), and the relative abundance of secreted/transmembrane proteins found in the murin transcriptome (http://locate.imb.uq.edu.au/) (Sprenger, Lynn Fink et al. 2008).

Single cell PCR measurement indicated that GluR1 and GluR2 mRNAs represent approximately 1/240 and 1/500 of total cellular mRNAs in cultured hippocampal pyramidal neurons, respectively (Tsuzuki, Lambolez et al. 2001). Assuming that GluR2 mRNAs display the same relative dendritic enrichment than GluR1 (~3.2) (Grooms, Noh et al. 2006; Poon and Chen 2008), GluR1 and GluR2 mRNAs can be expected to represent ~1/75 and 1/150 of total dendritic mRNAs, respectively.

Among the 58128 protein sequences present in the RIKEN FANTOM4 mouse protein sequence set, 4231 correspond to secreted (signal-peptide containing) proteins, and 14088 correspond to membrane (transmembrane domain containing) proteins (http://locate.imb.uq.edu.au/) (Sprenger, Lynn Fink et al. 2008). Although this estimation doesn’t take into account neither mitochondrial transmembrane proteins and cytoplasmic proteins processed by ER-bound ribosomes nor neuron specific transcriptional programs, one can estimate that ~31.5% of murin mRNAs will be processed by ER-bound ribosomes in a generic cell. Assuming that mRNA dendritic enrichment preserves these proportions, one can thus estimate that GluR1 and GluR2 mRNAs represent ~1/23.6 and ~1/47.2 of dendritic mRNAs processed by ER-bound ribosomes, respectively. Finally, assuming optimal and equal translation initiation rates, and an average size of 700 codons for all dendritic mRNAs, the ~100 ER-bound
ribosomes found at a single generic dendritic branch point could in principle assemble 600 amino acids per seconds, thus 51 proteins and therefore ~2.2 GluR1 and ~1.1 GluR2 per minute. These rates were considered to be rate limiting for GluR1 and GluR2 folding and post-translational modifications, whose potential effects on receptor diffusion were not considered in the present model.

*Computation of nascent receptor densities along dendrites.* A reference dendritic tree was obtained by imaging a 30 DIV neuron expressing a red cell-fill and segmented by centring 7μm long segments (the sized of bleached area used for FRAP) on dendritic segments. Apparent diffusion coefficients estimated for branch points or straight spiny dendrites were assigned to the resulting structure. Spatiotemporal variations of the density of nascent GluR1 and GluR2 produced at the rates described above at a distal branch point (chosen as a synthetic origin) was then computed in Matlab accordingly using a modified version of equation (8) presented above, allowing the use of distinct apparent diffusion coefficients assigned to specific dendritic regions. Color-coded densities of nascent GluR1 and GluR2 at different time points are shown as the result for the demonstration.

**2.12 Neuron morphology and spine density analysis**

Fluorescent images were input into Metamorph (Universal Imaging Corporation) and neurite outgrowth module was used to generate the camera lucida drawings. Total dendrite length, neurite process number and average dendrite length were also
measured in this module. For the spine density measurements, all of the protrusions on the longest two dendrites (primary and secondary dendrites) in the hippocampal neurons including spines and filapodia were counted. Spine density = total protrusion number / total dendrite length.

2.13 Statistics

Throughout the paper, data are given in mean±SEM. Number of cells and independent experiments used for quantifications are indicated in the text and in the figure legends. Statistical significance was assayed in StatView (Abacus Concepts) using 2-tails unpaired or paired t-tests when comparing pairs of dataset obtained in cell populations or in individual cells, respectively, or Anova when comparing more than two data sets.
3. Constrained Diffusion in the Dendritic Endoplasmic Reticulum and Local Confinement of Nascent AMPA Receptor Trafficking

3.1 Abstract

Regulated trafficking of neurotransmitter receptors and other integral membrane proteins is a hallmark of neuronal development and plasticity. Following their synthesis, integral membrane proteins dwell in the ER for variable periods that are typically rate limiting for plasma membrane delivery. Such prolonged occupancy suggests that lateral diffusion within the dendritic ER may determine the spatial range of receptor secretory trafficking within dendritic segments. Photobleaching and photoactivation approaches combined to electron microscopy allowed us to show that although rapidly diffusing within the continuous network of the somato-dendritic ER, membrane proteins such as nascent AMPA receptors are confined by an increased ER complexity near dendritic branch points and near dendritic spines. The spatial range of ER membrane protein mobility becomes progressively confined over neuronal development and is rapidly restricted through type I mGluR signaling. Thus, constrained lateral mobility within the ER provides a novel mechanism for compartmentalized trafficking of nascent receptors throughout dendrites.

3.2 Introduction

The rapid tuning of neuronal network activity requires the modulation of specific synaptic contacts, implying postsynaptic regulations occurring on a local scale.
across dendritic segments (Kennedy and Ehlers 2006). Although activity patterns and signaling events required for plastic changes to occur at single or small clusters of synapses are now emerging (Matsuzaki, Honkura et al. 2004; Harvey, Yasuda et al. 2008; Losonczy, Makara et al. 2008), the mechanisms underlying this extreme cellular compartmentalization are still largely unknown.

Regulation of postsynaptic receptor movements to and from synapses, especially glutamate α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors, is central for the expression of synaptic plasticity and involves virtually every facet of receptor life-cycle (Kennedy and Ehlers 2006). Dendritic synthesis (Kacharmina, Job et al. 2000; Ju, Morishita et al. 2004; Mameli, Balland et al. 2007) and local secretory processing of AMPA receptors (Gerges, Backos et al. 2006) are thought to be important for synaptic physiology, and would provide an efficient mechanism for targeted modulations of specified synapses (Sutton and Schuman 2006). However, unlike cytoplasmic proteins, the modalities of membrane protein dendritic synthesis are still obscure.

Whereas local synthesis of cytoplasmic proteins only requires mRNA, ribosomes, tRNA, and metabolites, local translation and trafficking of membrane and secreted proteins requires the entire complement of secretory organelles. Although the entire cast of secretory organelles is present in dendrites (Hanus and Ehlers 2008), the concerted regulations required for a local production and delivery of secretory cargoes still
challenge our understanding of dendritic secretory functions. Dendritic early secretory trafficking is particularly puzzling as the dendritic endoplasmic reticulum (ER) likely constitutes a continuous network (Spacek and Harris 1997; Cooney, Hurlburt et al. 2002) within which nascent membrane and secreted proteins may diffuse over large distances. ER-exit is rate limiting for the production of numerous synaptic receptors (Roche, Tu et al. 1999; Scott, Blanpied et al. 2001; Mu, Otsuka et al. 2003; Jaskolski, Coussen et al. 2004), including AMPA receptors (Greger and Esteban 2007; Penn, Williams et al. 2008). Although distinct AMPA receptor subunits seem to dwell in the ER for minutes up to hours (Greger, Khatri et al. 2002), it is currently unknown how these strikingly differing ER-exit rates translate into spatial domains over which newly assembled receptors may diffuse away from their ER-bound synthetic source.

By combining fluorescence recovery after photobleaching (FRAP), photoactivation and serial electron micrographs 3D reconstructions, we show that although laterally moving within a continuous network, ER membrane proteins such as nascent AMPA receptors can be confined at specific locations by local convolution of ER membranes. This ER complexity increases the dwell-time of ER proteins near dendritic branch points and spines, defining a previously unrecognized form of cellular compartmentalization. The dendritic ER complexity and confinement of ER membrane proteins increase over neuronal development and are acutely regulated by type I
metabotropic glutamate receptors (mGluRs)/PKC signaling, indicating that synaptic activity restricts nascent receptors within dendritic subcompartments.

### 3.3 Results

Electron microscopy (EM) studies have demonstrated a heterogeneous and convoluted arrangement of dendritic ER membranes (Spacek and Harris 1997; Cooney, Hurlburt et al. 2002), two features that could strongly affect the lateral diffusion of ER membrane proteins. The rationale of this study was thus to compare the diffusion of ER retained-GluR1 and GluR2 (ER-GluR1 and ER-GluR2) to that of a freely diffusing ER membrane protein whose dynamics would merely reflect the fundamental constraints affecting ER membranes independently of any receptor specific regulations.

The molecular determinants controlling AMPA receptor exit from the ER are now well characterized (Greger and Esteban 2007; Penn, Williams et al. 2008). GFP-tagged ER-GluR1 and ER-GluR2 mimicking nascent receptors were generated by single-point mutagenesis of their glutamate-binding site (Fig. 3-1A). Receptors assembled from these subunits were still able to hetero-oligomerize (Fig. 3-2A), showing that ER-retention was not merely due to a failure to initiate folding and assembly. Importantly, ER-GluR1 still specifically interacted with over-expressed SAP97, which is thought to associate with newly assembled GluR1-containing receptors in the ER (Sans, Racca et al. 2001) (Fig. 3-2C).
**Figure 3-1** ER and cell-surface fluorescent probes.

A, Mutated amino acids in the ligand binding site of GluR1 and GluR2 (ER-GluR1; ER-GluR2) and in VSVGts COPII interacting motif (ER-VSVG) and to make them ER retained.

B, Surface staining of ER-GluR1-GFP and ER-GluR2-GFP to show their trafficking and surface expression is blocked in hippocampal neurons compared to WT-GluR1-GFP and WT-GluR2-GFP.

C, Surface staining of ER-VSVG-GFP to show its trafficking and surface expression is blocked compared to VSVGts when expressed in hippocampal neurons at 37°C.

D. PM-phluorin (PM-phluo) is mostly expressed at the cell surface. Application of extracellular solution with pH5.5 quenched most of the cell surface fluorescent signals.

E, Expression pattern for YFP-GT46 in hippocampal neurons which is used as a plasma membrane protein probe in the FRAP experiments.
Figure 3-1 ER and cell-surface fluorescent probes.
**Figure 3-2** Binding assays for ER-GluR1 and ER-GluR2.

A, Immuno-precipitation of ER-GluR1 and ER-GluR2 with their binding partners in HEK293T cells. ER retained AMPA receptor subunits are tagged by GFP and WT receptor subunits are tagged by HA.

B, Immuno-precipitation of ER-GluR1 and SAP97 in HEK293T cells.
Figure 3-2 Binding assays for ER-GluR1 and ER-GluR2.
The thermo-sensitive mutant of the vesicular stomatitis virus glycoprotein (VSVGts) has been widely used to study membrane protein mobility within the ER (Lippincott-Schwartz, Roberts et al. 2000), and appeared as an excellent candidate to monitor a freely diffusing ER membrane protein. However, because membrane protein diffusion is strongly temperature-dependent, we generated a GFP-tagged version of VSVG constitutively retained in the ER (ER-VSVG) by mutagenesis of its COP-II interacting domain (Nishimura and Balch 1997; Sevier, Weisz et al. 2000) (Fig. 3-1C).

Fast diffusion of ER-retained GluR1 and GluR2

The diffusion of ER-GluR1 and ER-GluR2 was compared to that of ER-VSVG and other ER and plasma membrane proteins using FRAP in cultured hippocampal neurons (Fig. 3-3C). SERCA2a diffusion has already been described (Fukatsu, Bannai et al. 2004) and was used as a reference. For FRAP on the plasma membrane, we used two artificial surface integral proteins, GT46-GFP, whose diffusion has also been described (Pralle, Keller et al. 2000; Kenworthy, Nichols et al. 2004), and the pH sensitive GFP (pHluorin) fused to the N-terminus of a type I protein backbone (PM-pHluorin) (Fig. 3-1D).

Although the distribution patterns of ER-GluR1 and ER-GluR2 were comparable to that of ER-VSVG and appeared globally diffuse throughout the entire dendritic volume (Fig. 3-1B, C), ER-GluR1 and ER-GluR2 diffusion was slower than that of ER-VSVG and GFP-SERCA2a (Fig. 3-3C) (9.2x10⁻²±9.4x10⁻³, 8.1x10⁻²±4.2x10⁻³, 3.8x10⁻²±3.5x10⁻³,
**Figure 3-3** Rapid diffusion of integral membrane proteins in the dendritic ER and at the dendritic surface.

A, FRAP analysis of ER-VSVG diffusion in dendrites. Low resolution picture and kymograph of ER-VSVG fluorescence (pseudocolored) showing bleaching and rapid recovery.

B, Normalized recovery plot (raw and fitted data).

C, Average halftimes and recovery fractions of ER-VSVG, ER-GluR1, ER-GluR2 and SERCA2a in the dendritic ER and GT-46 and PM-phluo (see text) at the dendritic surface (mean±SEM, n=64, 38, 28, 44, 18 and 22 cells, in 5, 3, 3, 3, 3 and 3 experiments, respectively). Anova, *: p<0.05, **:p<0.01.
Figure 3-3 Rapid diffusion of integral membrane proteins in the dendritic ER and at the dendritic surface.
6.9x10^{2±1.3x10^{2}} \mu m^2/s in n=64, 44, 38 and 28 cells for ER-VSVG, GFP-SERCA2a, ER-GluR1 and ER-GluR2, respectively). A trend towards lower diffusion was observed when comparing the diffusion of GFP-SERCA2a, ER-VSVG, ER-GluR2 and ER-GluR1, in this order. However, these differences were not significant when comparing the four datasets altogether. Although lower than that of ER-VSVG, the exchangeable fractions of ER-GluR1 and ER-GluR2 were rather high (0.85±0.02, 0.79±0.02 and 0.75±0.03% for ER-VSVG, ER-GluR1 and ER-GluR2, respectively), indicating that most of these receptors were exchangeable and mobile in the ER (Fig. 3-3C).

Apparent diffusion rates measured in the ER were consistent with previous reports (Fukatsu, Bannai et al. 2004) and were collectively comparable to that measured at the cell-surface (Fig. 3-3C). The diffusion of ER membrane proteins such as ER-retained AMPA receptors, appeared relatively rapid and comparable to that of freely moving plasma membrane proteins. Thus, lateral diffusion within the dendritic ER is an important aspect of nascent AMPA receptor dynamics as is the diffusion of mature receptors at the neuronal surface (Newpher and Ehlers 2008; Triller and Choquet 2008).

**Heterogeneity, dynamics and continuity of the dendritic ER**

Although ER-VSVG distribution appeared globally diffuse in the acquisition conditions used for FRAP (Fig. 3-1C), confocal imaging at higher resolution revealed subcellular heterogeneities of the dendritic ER (Fig. 3-4A). This distribution pattern was consistent
**Figure 3-4** Morphology and dynamics of the dendritic ER.

A, Single confocal sections of 17DIV cultured neurons expressing ER-VSVG or immuno-labeled for BIP at different magnifications. Scale bars 5μm.

B, Time-lapse sequence of dendritic ER spatial heterogeneities over minutes (Z-stack projections).

C, Fluorescence loss of dendritic ER-VSVG fluorescence upon continuous bleaching of the soma. Left: ER-VSVG expression and bleaching in the soma and time-lapse sequences (min:sec) of dendrites in a bleached (upper panel) and an unbleached neuron (lower panel). Right: fluorescence decay over time in the soma (lower grey trace) and in dendritic segments (red, blue and green) at various distances from the soma in a bleached neuron, fluorescence decay in the soma (in an unbleached cell, upper grey trace), and corresponding average decay halftimes in dendrites (mean±SEM, 11, 11 and 7 dendrites in 9 cells for “25”, “50” and “100μm”, respectively, 3 experiments). Anova, **: p<0.01.
Figure 3-4 Morphology and dynamics of the dendritic ER.
with previous descriptions of other ER membrane proteins in neurons (Bannai, Inoue et al. 2004; Fukatsu, Bannai et al. 2004). In addition to ER tubules in the soma, most neurons displayed clusters of increased fluorescence distributed along dendrites (Fig. 3-4A). These spatial heterogeneities were not resulting from the overexpression of ER-VSVG as they were also detected in untransfected neurons after immuno-staining of the ER chaperone protein BIP (Fig. 3-4A), and likely corresponded to convoluted ER structures seen on electron micrographs (Spacek and Harris 1997; Cooney, Hurlburt et al. 2002).

The dynamics of these ER fluorescent clusters were analyzed by time-lapse confocal imaging. Consistent with studies in fibroblasts (Lippincott-Schwartz, Roberts et al. 2000), the dendritic ER appeared quite dynamic. Although a submicron-scale morphing of ER membranes was occurring over seconds, convoluted structures seen along dendrites were overall stable and could be followed for up to one hour (Fig. 3-4B).

The continuity of the somato-dendritic ER has been inferred from live-cell imaging studies in fibroblasts (Lippincott-Schwartz, Roberts et al. 2000) and EM three-dimensional reconstructions of small dendritic segments (Spacek and Harris 1997; Cooney, Hurlburt et al. 2002) but has never been directly demonstrated. To address this, we performed “Fluorescence Loss In Photobleaching” (FLIP) experiments (Fig. 3-4C, D). The continuous bleaching of ER-VSVG in somata led to a significant loss of dendritic fluorescence. This loss occurred within minutes and was slower in most distal dendritic
segments, directly demonstrating a free exchange of ER molecules and thus the apparent continuity of the somato-dendritic ER.

Altogether, these data show that the dendritic ER is a heterogeneous and dynamic network into which lateral mobility potentially allows ER-membrane proteins to explore large portions of the neuronal volume. However, we hypothesized that despite this apparent continuity, spatial cues distributed along the dendritic ER may locally slow down the diffusion of ER membrane proteins.

*Constrained diffusion within the dendritic ER: developmental regulation and relation to dendritic spines*

The dimensions of bleached areas used for FRAP studies have a direct effect on the derived apparent diffusion rates (Feder, Brust-Mascher et al. 1996; Siggia, Lippincott-Schwartz et al. 2000). Indeed, because the volume of any object increases with its size faster than its surface area, the interface available for exchanges of bleached and non-bleached molecules counting for fluorescence recovery will be relatively smaller in objects of increased dimensions. Because of this boundary constraint, apparent recovery rates measured within a continuous structure will progressively decrease with the diameter of the bleached area. We hypothesized that any form of spatial complexity affecting diffusion would be materialized as an additional slowdown of diffusion rates appearing upon progressive increase of bleached surface areas.
As a proof of principle of this assumption, we compared in HELA cells the influence of the size of the bleached areas on the apparent diffusion rates measured for integral proteins in the plasma membrane, or in the ER, a much more complex structure due to the typical polyhedral ER arrangement found in fibroblasts (Fig. 3-5A). As predicted, recovery halftimes measured for ER-VSVG increased faster than those measured for PM-phluo at the cell-surface (Fig. 3-5B and C). In contrast to recovery rates, corresponding recovered fractions were not significantly affected (Fig. 3-5C), indicating that the apparent complexity of the ER was not due to an uneven distribution of ER-VSVG binding sites, which would have resulted in a progressive decrease of recovered fractions.

Thus, whereas recovery rates in small areas reflect the molecular properties of cellular membranes, a level at which spatial organization (such as lipid raft) cannot be resolved by conventional light microscopy (Kusumi, Nakada et al. 2005; Eggeling, Ringemann et al. 2009), recovery rates in larger areas reflect larger-scale spatial features further constraining molecule diffusion. Therefore, the apparent complexity of continuous cellular membranes (whatever the physical origin of this complexity) can be estimated by comparing apparent diffusion rates derived from FRAP in small (typically <1μm) and large areas (in this case >5μm).

Confocal imaging of neurons at 8-11 and above 17 days in vitro (DIV) revealed a higher heterogeneity of the dendritic ER in mature neurons (Fig. 3-6A), suggesting a
Figure 3-5 FRAP-based monitoring of the ER apparent complexity in HEla cells.

A, Fluorescence pictures (pseudocolored) of the plasma membrane (PM-phluorin, selectively fluorescent at neutral extracellular pH, blue box) and the ER (ER-VSVG-YFP, red box) at different magnifications. Scale bar 5μm.

B, Raw and fitted FRAP plots after bleaching of PM-phluorin (blue) or ER-VSVG-YFP (red) in 1μm or 9μm diameter disks.

C, Variation of recovery halftimes (t1/2) and recovery fractions (inset) as a function of the diameter of bleached areas (mean±SEM, after normalization by values obtained using 1μm disks). 12 to 13 measures for each data point, 3 and 2 experiments respectively for PM-phluorin and ER-VSVG-YFP. T-tests, *: p<0.05, **: p<0.01.
Figure 3-5 FRAP-based monitoring of the ER apparent complexity in HEla cells.
**Figure 3-6** Developmental regulation of the dendritic ER complexity and relation to dendritic spine density.

A, Confocal imaging (Z-stack projection) and variance (mean±SEM, n=13 cells, 1 experiment) of ER-VSVG fluorescence in dendrites of young (8 DIV, left) and mature (22 DIV, right) neurons. Scale bar 5μm. T-tests, **: p<0.01. B, Low resolution pictures and fitted FRAP plots in young (blue) and mature (red) neurons after bleaching of 1μm or 12μm dendritic segments. C, Variation of corresponding recovery halftimes (t1/2) and recovery fractions (inset) as a function of the length of bleached segments (mean±SEM, 14 to 15 measures for each data point, 4 experiments).

D, Low resolution pictures of a red cell-fill (cherry, left) and ER-VSVG (right) in aspiny and spiny dendrites. E, Upper panel: variations of fluorescence recovery t1/2 (left) as a function of dendritic spine density after sequential bleaching of 1μm (light grey) and 8μm (dark grey) segments, and corresponding apparent complexity index (right). Lower panel: mean recovery t1/2 halftimes (left), complexity index (middle) and recovery fractions (right) for spine densities under (L, blue) or above (H, red) 0.5 spines/μm (mean±SEM, 22 cells, 4 experiments). Light and dark colors correspond to FRAP experiments in small and large dendritic segments, respectively. T-tests, *: p<0.05, **: p<0.01. F, Confocal imaging (stack projection) of β-actin-cherry and ER-VSVG in neurons used for FRAP (upper panel) or in a distinct subpopulation of neurons (lower panel).
Figure 3-6 Developmental regulation of the dendritic ER complexity and relation to dendritic spine density.
developmental increase of the dendritic ER complexity. Directly supporting this, our FRAP-based assay showed that whereas the small-scale mobility of ER-VSVG was comparable in young and mature neurons, the extension of bleached segments in dendrites of comparable diameters led to a faster increase of recovery rates measured in mature neurons (Fig. 3-6B and C). As seen in fibroblasts, recovery extends were not significantly affected (Fig. 3-6C).

Previous EM studies documented a spatial correlation between dendritic spine density and maturity, and ER morphological complexity (Spacek and Harris 1997). The biophysical consequences of this observation remain unknown. We compared in dendrites of varying spine densities the diffusion rates derived from 1 and 8μm and a complexity index calculated as the ratio between the later and the former values (see Materials and Methods). In 16 DIV- 22 DIV neurons, small-scale diffusion rates remained constant contrasting with those derived from 8 μm segments, which increased with spine density (Fig. 3-6E). Consequently, ER complexity was increased in dendrites with a spine density above 0.5 spines/μm demonstrating an effect of spine density and potentially synaptic activity on ER complexity (Fig. 3-6E).

To address to what extent the previous results were due to ER entry into spines (Santamaria, Wils et al. 2006), we imaged neurons expressing ER-VSVG and βactin-cherry, a probe widely used to visualize dendritic spines (Helton, Otsuka et al. 2008) by confocal microscopy. In contrast to 17 DIV neurons which were used for the FRAP
experiments where only few spines contained ER membranes (11±1.2%, n=25 cells), ER membranes were detected in most spines in more mature neurons (89±2.1%, n=27 cells) (Fig. 3-6F). In agreement with EM studies (Spacek and Harris 1997), the increased ER complexity seen over development and in spiny dendrites was thus primarily due to the spatial organization of the ER in the dendritic shaft and was further elevated by entry into mature spines. We could thus conclude that although the absolute (sub-micron scale) mobility of ER-VSVG was comparable in young and old neurons and in dendrites with low or high spine densities, its actual diffusion over larger dendritic segments was markedly restricted in mature neurons, and especially in spiny dendrites.

Increased ER complexity at dendritic branch points and relation with secretory functions

To directly evaluate ER heterogeneity throughout dendrites, we performed FRAP measurements at various locations within individual neurons. Neither small-scale diffusion rates and recovery fractions nor ER apparent complexity differed between proximal and more distal dendritic regions (Fig. 3-7A, B). In contrast, a two-fold increase of ER complexity was detected at dendritic branch points. Importantly, this was not seen at the cell-surface using PM-phluo (Fig. 3-7C) indicating that branch points act as specific diffusion traps restricting ER protein mobility.

The restricted diffusion was further confirmed by photoactivation (Fig. 3-7D, E). In some instances, ER-VSVG molecules photoactivated in dendritic segments flanked
Figure 3-7 Subcellular heterogeneity of the dendritic ER complexity and constrained diffusion at dendritic branch points.

A, Low resolution pictures of ER-VSVG in a 15 DIV hippocampal neuron and corresponding FRAP plots obtained after sequential bleaching of 1\(\mu\)m (light colors) and 7\(\mu\)m dendritic segments (dark colors) in distal (DD, blue), proximal (PD, green) dendrites and at a dendritic branch point (BP, red). B, Corresponding average recovery t\(\frac{1}{2}\), recovery fractions and complexity indexes (mean±SEM, n=16 to 18 measurements for each data point, 3 experiments). Anova, **: p<0.01. C, Low resolution pictures and average complexity indexes measured for PM-phluorin (PM-phluo), (mean±SEM, n= 12 cells, 2 experiments) in proximal dendrites (green) and at branch points (red). Note in B and C the increased complexity seen for ER-VSVG but not PM-phluorin at branch points.

D, Cell-fill (cherry) and photoactivation of ER-VSVG and redistribution of activated molecules (pseudocolored) at branch points. Scale bar 5\(\mu\)m. E, Upper panel: cell-fill and kymographs of photoactivated ER-VSVG in straight dendritic segments (S, blue) and in segments flanked by branch points (BP, red). Lower panel: corresponding fluorescence decay plots and decay t\(\frac{1}{2}\) (mean±SEM, n=10 cells, 3 experiments) measured in matched dendritic lengths (white brackets in cherry pictures). Paired t-tests, *: p<0.05. Note the slower decay in dendritic segments flanked by (and encompassing) branch points.
Figure 3-7 Subcellular heterogeneity of the dendritic ER complexity and constrained diffusion at dendritic branch points.
by branch points rapidly accumulated and dwell at these locations (Fig. 3-7D). Conversely, average decay rates measured in paired 15-30μm dendritic segments flanked by (and encompassing) branchpoints were significantly slower than those measured in straight dendritic segments in the same cells (Fig. 3-7E).

Apparent ER complexity could arise from various forms of spatial heterogeneities. As an independent approach, we used serial electron micrograph three-dimensional reconstructions to unambiguously address whether ER membranes would display any form of specific spatial arrangement at branch points in vivo. Reconstruction of three dendritic segments over a total length of 75 μm and containing 9 branch points (Fig. 3-8, 3-9 and 3-10) demonstrated an increased anastomosis of ER cisternae and a higher prevalence of ER sheets with membrane bound ribosomes (Fig. 3-9B, C). These two characteristic features of the rough ER (RER) (Frey, Perkins et al. 2006) resulted in an overall higher morphological complexity, which could be quantified by measuring the surface over length ratio of 1 μm ER segments distributed along dendrites. Complex ER membrane arrangements were also observed in the dendritic shaft in areas of high spine density, regardless of ER entry into spines, directly supporting our FRAP data. These morphological variations certainly affect local membrane diffusion along dendrites, providing a structural basis for the biophysical properties described using our FRAP assay.
Figure 3-8 Electron micrographs in the adult rat hippocampus CA1 stratum radiatum.

The dendrites shown in Figure 3-9 and 3-10 are highlighted in blue and pink, respectively. Note the excellent preservation of cellular ultrastructures.
Figure 3-8 Electron micrographs in the adult rat hippocampus CA1 stratum radiatum.
Figure 3-9 Ultrastructural complexity of the dendritic endoplasmic reticulum in vivo.

A, Raw micrographs at various locations within a CA1 neuron dendrite. ER cisternae are colored in light green. Scale bar 500nm.

B, Three-dimensional reconstruction of electron micrographs at different magnifications, featuring the plasma membrane (grey), endoplasmic reticulum (ER, green), ER-bound ribosomes (red) and clathrin coated endocytic vesicles (blue). Numbers correspond to approximate locations of micrographs shown in (A). Scale bar 1μm. Note the extensive branching of the ER and the presence of ER sheets at dendritic branch points (arrows) contrasting with ER tubules (arrowheads) in straight dendritic segments. Note the morphological complexity of the ER in the dendritic shaft in areas of high spine density (crossed-arrow).

C, Same view as in (A) showing only the plasma membrane and the location of ER-bound ribosomes.
Figure 3-9 Ultrastructural complexity of the dendritic endoplasmic reticulum in vivo.
Figure 3-10 Ultrastructural complexity of the dendritic endoplasmic reticulum *in vivo*, another example.

A-B, Three-dimensional reconstruction of electron micrographs at different magnifications, featuring the plasma membrane (grey), endoplasmic reticulum (ER, green), ER-bound ribosomes (red) and clathrin coated endocytic vesicles (blue). Scale bar 1µm.

C, Same view as in (A) showing only the plasma membrane and location of ER-bound ribosomes.
Figure 3-10 Ultrastructural complexity of the dendritic endoplasmic reticulum \textit{in vivo}, another example.
As described previously, ER-bound polysomes were strikingly more frequent at branchpoints (Fig. 3-9B, C). Although mRNA encoding cytoplasmic proteins can also be associated with ER membranes (Stephens, Dodd et al. 2005), this suggests that branch points constitute the entry-point of locally translated secretory proteins into the secretory pathway.

To investigate whether the dendritic ER complexity corresponded to a specialization towards secretory functions, we analyzed its relationship with various components of the secretory pathway in cultured neurons (Fig. 3-11A). Sec23 immuno-labelling revealed a higher density of ER exit sites (ERES) at branch points. As previously described both in mammal (Horton, Racz et al. 2005) and insect (Ye, Zhang et al. 2007) neurons, the expression of Golgi fluorescent proteins showed the frequent localization of dendritic secretory outposts at these locations. Thus, the local convolution of the dendritic ER seen there likely represents another important feature for secretory functions and was thus expected to occur in regard to secretory organelles regardless of their location. Indeed, FRAP experiments performed in cells co-expressing ER-VSVG and Arf1-cherry, which marks these structures (Fig. 3-11A), showed a 3-fold increase of the complexity of ER segments opposed to Golgi outposts in straight dendritic portions (Fig. 3-11B).
Figure 3-11 Enrichment of secretory organelles at dendritic branch points and increased complexity in ER segments close to secretory outposts.

A, Confocal imaging (stack projection) of ER-VSVG (left), Sec23 immuno-reactivity (IR, middle), Galactosyl-transferase-GFP (GT-GFP, upper right panel) and Arf1-cherry (Arf1-chy, lower right panel) showing the distribution of ER spatial cues, ER exit sites and Golgi outposts in dendrites (arrows). Branch points are marked by asterisks. Scale bar 5μm.

B, Low-resolution pictures of ER-VSVG and Arf1-chy in dendrites without (-, blue) or with (+, red) secretory outposts and corresponding recovery t1/2, recovery fractions and complexity index after consecutive bleaching of 1μm (light colors) and 7μm (dark colors) areas (mean±SEM, n=20 dendrites in 10 cells). Paired t-tests, *p<0.05, **: p<0.01.
Figure 3-11 Enrichment of secretory organelles at dendritic branch points and increased complexity in ER segments close to secretory outposts.
Actual ER-retained AMPA receptor diffusion and acute regulations by type I mGluR / PKC signaling

The previous results show that the actual diffusion of nascent receptors within the ER cannot be reliably appreciated using conventional FRAP measurements. We thus re-analyzed the diffusion of ER-GluR1 and ER-GluR2 using the approach used for ER-VSVG. As seen for this molecule, the recovered fractions of ER-GluR1 and ER-GluR2 were not significantly affected by the length of bleached dendritic segments (not shown). However, to our surprise, the complexity index calculated for ER-GluR1 was significantly higher than that of ER-VSVG, whereas ER-GluR2 gave intermediate values (Fig. 3-12). The distribution patterns of these three proteins were comparable (Fig. 3-1B, C), indicating that the restricted diffusion of ER-GluR1 was resulting not only from the morphology of ER membranes but also from GluR1-specific regulations.

With the assumption that the fundamental constraints seen for ER-VSVG (neuronal maturity, spine density, dendritic tree complexity) would affect receptor diffusion with the same magnitude, we can use the measured or extrapolated diffusion coefficients to draw exploration maps (see Materials and Methods) that would reflect their actual spread throughout mature dendrites. This provided a clear visual readout of the increased confinement of ER-GluR1.

Activation of type I (mGluR1 and mGluR5, Gq coupled) mGluRs in hippocampal neurons has been shown to increase the dendritic accumulation of AMPA
**Figure 3-12** Constrained diffusion of ER-GluR1 and ER-GluR2.

Complexity index for ER-VSVG, ER-GluR1 and ER-GluR2 dendritic diffusion (mean±SEM, 19, 19 and 17 cells, respectively, 4 experiments). Anova, **: p<0.01.
Figure 3-12 Constrained diffusion of ER-GluR1 and ER-GluR2.
receptor subunits mRNAs (Grooms, Noh et al. 2006), dendritic ER-export (Aridor, Guzik et al. 2004), and the dendritic translation of exogenous (Kacharmina, Job et al. 2000; Ju, Morishita et al. 2004) and endogenous AMPA receptors (Mameli, Balland et al. 2007). In the cerebellum, activation of these receptors affect the ultrastructural morphology of ER membranes (Banno and Kohno 1998), suggesting that type I mGluRs regulate ER secretory function and, synergistically, its spatial organization. To investigate this, ER complexity was monitored using ER-VSVG in individual dendrites, in basal conditions and 30-45 minutes after the addition of 100µM DHPG, a selective type I mGluR agonist. This revealed a 50% increase of ER complexity (Fig. 3-13A), which occurred without changes of small-scale diffusion rate and extent, and was blocked by the PKC inhibitor bisindolylmaleimide (Fig. 3-13A). In some neurons, the DHPG effect could be detected at the morphological level when ER-VSVG was imaged at higher resolution, (Fig. 3-13B). Despite the differences seen for their apparent large-scale diffusion, DHPG increased the apparent complexity measured for ER-GluR1 (Fig. 3-13C) with the same magnitude than for ER-VSVG. Type I mGluRs receptors being predominantly postsynaptic in the hippocampus (Lujan, Nusser et al. 1996), these results indicate that presynaptic glutamate release directly increases the complexity of the postsynaptic ER and thereby confines nascent receptor diffusion through Gq/PKC signaling.

mGluR and NMDA receptor activation induces distinct forms of long term depression (LTD) of excitatory synaptic transmission in several brain structures
**Figure 3-13** Acute regulation of dendritic ER complexity by type I mGluR / PKC signaling.

A, ER-VSVG recovery halftimes (t½), recovery fractions and complexity index after sequential bleaching of 1μm (light colors) and 7μm long (dark colors) dendritic segments in individual cells before and 30-45min after the addition of 100μM DHPG in the absence (blue) or in the presence of PKC inhibitor (PKCi, red). Post/pre normalization (mean±SEM, 42 and 16 cells, in 6 and 4 experiments, for DHPG and DHPG+PKCi, respectively). Paired t-tests, *: p<0.05.

B, Variation of ER-VSVG distribution pattern after the addition of DHPG (projection of confocal picture stacks).

C, Post/pre variations of ER complexity measured for ER-VSVG and ER-GluR1 after DHPG treatment (mean±SEM, 42 and 19 cells in 6 and 4 experiments, respectively) and for ER-VSVG 15-30min after NMDA treatment (n=34 cells, 4 experiments). Paired t-tests, *: p<0.05.
Figure 3-13 Acute regulation of dendritic ER complexity by type I mGluR / PKC signaling.
including the hippocampus. Interestingly, mGluR-LTD in the ventral tegmental area, relies on membrane protein synthesis, whereas NMDA-LTD does not (Mameli, Balland et al. 2007). Although calcium imaging indicated that NMDA application triggered strong postsynaptic responses in our culture system (data not shown), ER diffusional properties remained unaffected after cell exposure to a NMDA-LTD induction protocol (Beattie, Carroll et al. 2000) (Fig. 3-13C). Thus, in contrast to DHPG, NMDA didn’t induce any acute changes of ER complexity, strengthening the notion that the mGluR–dependent regulation of ER complexity was not a common feature of LTD, but was directly tuned to secretory functions.

3.4 Discussion

The present study represents the first direct demonstration that the local convolution of ER membranes restricts the diffusion of freely moving membrane protein at specific locations. Other studies have demonstrated that dendritic spines act as diffusion sinks restricting cytoplasmic protein diffusion throughout dendrites (Santamaria, Wils et al. 2006), documenting another instance of compartmentalization occurring without direct immobilization. Although in the present case, constrained diffusion was not due to ER entry into spines, our results extend this notion to nascent secretory proteins, opening new venues to investigate to what extent the variety of dendritic geometries found in vivo affects the compartmentalization of conserved cellular functions.
In *Drosophila* oocytes, rapid ER-export is required to counteract the diffusion of nascent secretory cargo within the ER, and directly determines cargo processing and subsequent compartmentalized secretion by satellite secretory platforms (Herpers and Rabouille 2004). The higher confinement of ER proteins that we observed in regard to dendritic secretory organelles further indicates that ER conformation itself is an important parameter affecting local ER-export and secretory processing.

The distribution patterns and time-course of their N-glycan maturation indicated that nascent GluR1 and GluR2 reach the Trans-Golgi with time constants of ~3 and 12 hours, respectively (Greger, Khatri et al. 2002), suggesting strikingly different ER-export rates. Combined to a faster ER-export, the increased confinement of GluR1 that we described here likely facilitates the compartmentalized production of corresponding receptors. The causes of this increased confinement are not yet clear but might be linked to the GluR1 specific interaction with SAP97, which is thought to associate with nascent receptors at the level of the ER (Sans, Racca et al. 2001).

Membrane curvature strongly affects phospholipid and membrane protein diffusion and determines their partition and interaction within cellular membranes (Laradji and Kumar 2006; Manneville, Casella et al. 2008). It is thus tempting to speculate that the increased convolution of the dendritic ER near dendritic branch points and spines may facilitate the formation of specialized lipid/protein platforms, thereby locally affecting ER secretory and signaling functions.
The maintenance of ER membrane in dendritic spines relies on F-actin interacting proteins such as Myosin Va (Takagishi, Oda et al. 1996; Miyata, Finch et al. 2000) and synaptopodin (Deller, Korte et al. 2003). The molecular basis for the dendritic ER complexity still needs to be delineated but thus likely involve cytoskeleton interacting proteins. Supporting this notion, pharmacological disruptions of the cytoskeleton lead to significant changes of the dendritic ER complexity (data not shown). Various fluorescent protein chimera allowing to monitor microtubule nucleation and elongation have been characterized (Perez, Diamantopoulos et al. 1999; Stepanova, Slemmer et al. 2003). Real time imaging may reveal some interesting correlations between microtubule dynamics at branch points and the increased ER complexity and secretory organelle enrichment seen there. Finally, ER proteins such as CLIMP63 (Klopfenstein, Kappeler et al. 1998; Vedrenne, Klopfenstein et al. 2005) and members of the Reticulon family (Voeltz, Prinz et al. 2006), which control ER tubule elongation and branching, respectively, represent potentially interesting candidates to investigate to what extent altering ER complexity would affect neuronal functions.
4. The Functions of ER Protein CLIMP63 in Dendritic ER Complexity Regulation, Dendrite Outgrowth and Spine Formation

4.1 Abstract

Microtubules and actin filaments are important for dendrite and spine formation. The cytoskeleton functions not only as an intracellular frame work to keep cells in shape, but also regulates the spatial organization and function of secretory organelles in neuronal dendrites and spines. However, the mechanisms of how the spatial organization of secretory organelles controls the morphogenesis of neurons are not clear though it is shown the appearance of these organelles is necessary for normal neuronal development and function. Increasing body of literatures point to cytoskeletal associated proteins functioning as spatial organizers and interactors of secretory organelles. In this study, I identified an ER protein CLIMP63 as a novel microtubule-associated protein regulating dendritic ER spatial organization, neuronal dendrite elongation and spine formation. Enhancement of ER membrane association with microtubules mediated by CLIMP63 stabilized neuronal processes undergoing neurite extension and dissociation of ER membrane from microtubules mediated by CLIMP63 microtubule-binding-deficient mutant caused spine growth. It is shown in this study that CLIMP63 regulates the dendritic ER morphology by a PKC dependent mechanism and potentially controls the intracellular Ca\(^{2+}\) signaling and secretory function, which illuminate the function of dendritic secretory organelles in neuronal morphogenesis.
4.2 Introduction

The formation of axon and dendrites, neuronal polarization, spines and synapses is a prerequisite for neurons to integrate and propagate information within the brain. Both microtubules and actin filaments are required for axon and dendrite specification (Jan and Jan, 2003). Microtubules are generated in the microtubule organizing center and are stabilized by Microtubule Associated Protein (MAP) capping and then ultimately transported into the growing dendrite via molecular motors (Kirschner and Mitchison 1986; McAllister 2000). Microtubules are generally dynamic and are in an equilibrium state of constant polymerization and depolymerization at dendritic branch points. Microtubule polymerization and subsequent invasion into filopodia are necessary for the stabilization of filopodia that will become dendrites. MAPs such as MAP1A and MAP2 play roles in neuritogenesis by promoting microtubule assembly and stabilization (Calvert 1995; Vandecandelaere, Pedrotti et al. 1996; Harada, Teng et al. 2002). The phosphorylation status of MAP2 can regulate the conformation of microtubule bundles and control the dendrite extension (Hely, Graham et al. 2001). Actin filaments also function as a framework for filopodia of the dendritic growth cone. It is primarily localized to the outer shell of dendrites as well as in dendritic spines and plays a key role in spine formation (Tada and Sheng 2006). Microtubules and actin filaments in dendrites and axons not only act as the intracellular framework for neuronal morphogenesis and maintenance but also as trafficking machinery to transport membranes and proteins
(Goldstein and Yang 2000; Bridgman 2004). In addition, microtubule dependent Golgi localization in dendrites is critical for the formation of neuronal polarity and dendritic branches (Horton, Racz et al. 2005; Ye, Zhang et al. 2007). Although the function of microtubules and actin filaments in the secretory organelle morphology maintenance is intensively studied as discussed in the introduction section, how the cytoskeleton mediated spatial organization of secretory organelle in dendrites regulates postsynaptic function is not clear yet. It is known that ER morphology is maintained by microtubules, actin filaments and their binding proteins. Microtubule motors can either drag the ER membranes along underlying microtubules or drive the sliding of membrane-associated microtubules, whereas microtubule polymerization can promote the movement of an ER tubule bound to the dynamic microtubule tip (Waterman-Storer and Salmon 1998). Both plus-end-directed motors of the kinesin family and the minus-end-directed dynein motor have been implicated in microtubule-based membrane movements (Lane and Allan 1999). The ER localized in dendrites is important for dendritic arbor morphogenesis (Lohmann, Myhr et al. 2002; Lohmann and Wong 2005), neuron migration (Shim, Wang et al. 2008) and ER association with actin filaments is important for synaptic function in purkinje cells (Miyata, Finch et al. 2000). Therefore, I manipulated dendritic ER morphology by microtubule associated protein and studied the function of ER spatial organization in neuronal function.
I characterized a microtubule associated protein CLIMP63 and its function in regulating dendritic ER spatial organization and postsynaptic function. In previous studies it has been shown that stable attachment of ER membranes to microtubules is essential for maintaining the ER network morphology. CLIMP63 has revealed a stable interaction between the ER membrane and microtubules. CLIMP63 is a type II ER membrane protein which was identified in a search for organelle specific markers of the early secretory pathway (Schweizer, Ericsson et al. 1993; Schweizer, Rohrer et al. 1995). It is located in the ER shown by immuno-gold electron microscopy and is excluded from the outer nuclear membrane by forming large immobile oligomers in the reticular ER (Schweizer, Ericsson et al. 1993; Klopfenstein, Klumperman et al. 2001). Purified CLIMP63 binds to microtubules and when overexpressed it rearranges the ER along bundled microtubules, suggesting that CLIMP63 functions in anchoring ER membranes to the microtubules (Klopfenstein, Kappeler et al. 1998). Fibroblasts that overexpress CLIMP63 dominant negative microtubule binding deficient mutants exhibit a poorly extended ER without changing microtubule cytoskeleton (Vedrenne, Klopfenstein et al. 2005). These discoveries suggest a CLIMP63 binding property to microtubules rearranges the ER membranes. Overexpression of wild-type CLIMP63 increases the number of interacting points between the ER membrane and microtubules and therefore induces their coalignment, whereas overexpression of mutated CLIMP63 that does not bind to microtubule disrupts the anchoring of the ER to microtubules. CLIMP63 is
required to stabilize the extended ER network in a microtubule dependent manner. Interestingly, CLIMP63 binding to microtubules is cell cycle and phosphorylation dependent. During mitosis CLIMP63 binding to microtubule is inhibited by phosphorylation and ER network is less developed and switch from microtubule to actin dependent organization (McCullough and Lucocq 2005). Depolymerizing microtubule or microtubule dependent motor activity disruption caused ER membrane collapse is strikingly similar to the effect of CLIMP63 microtubule binding deficient mutants. Therefore, during the dynamic ER membrane extension formation on microtubule, CLIMP63 may act in synergy with kinesin motors to stabilize the ER tubules along the microtubule which would otherwise retract to the cell center because of membrane tension generated by the extensive tubule formation (Upadhyaya and Sheetz 2004; Koster, Cacciuto et al. 2005).

I propose that ER organization and function is important for the two phases of neuronal development, the dendritic outgrowth and the spine formation. I characterized CLIMP63 expression in rat brain and found that the dendritic ER spatial organization, dendritic morphology and spine density were regulated by CLIMP63. Overexpression of CLIMP63-WT tightly associated dendritic ER with microtubules and made the ER membrane smooth and less complex, which was shown by live-cell imaging and quantitative FRAP analysis. This dendritic ER morphology change is correlated with an observation of decreased spine density and increased dendritic length. On the contrary,
overexpression of CLIMP63-ΔMBD a microtubule-binding deficient mutant caused the dendritic ER dissociated with microtubules and the ER membranes had a more heterogeneous and complex structure. Strikingly, this increased dendritic ER complexity was correlated with higher spine densities and decreased dendritic length. These findings directly point to a cytoskeleton dependent ER morphology change and its significant consequences in neuronal morphogenesis and postsynaptic function. Although all the results shown in this section are preliminary, the underlying molecular mechanisms would provide us enormous insights into the function of satellite secretory organelles. Neuronal activity and signaling pathways regulating ER morphology can also modify its secretory function and intracellular Ca\textsuperscript{2+}, which in turn control dendrite and spine formation.

4.3 Results

The dendritic ER is important for normal postsynaptic function. The rationale of this study is to move forward to examine functional consequences of dendritic ER spatial organization. In order to manipulate the spatial organization of dendritic ER, I used a microtubule associated protein CLIMP63 which has been reported to regulate ER membrane structures (Klopfenstein, Kappeler et al. 1998). CLIMP63 protein distribution in brains has not been studied before. I characterized its expression and phosphorylation in cultured rat hippocampal and cortical neurons. After confirming its expression in the rat brain and its function in regulating ER morphology in fibroblasts, I used WT or
dominant negative forms of CLIMP63 to manipulate ER spatial organization in neurons and studied its function in dendritic morphogenesis and spine formation. I also showed that CLIMP63 regulation of dendritic ER spatial organization is PKC signaling dependent.

**CLIMP63 mRNA and protein expression in brains**

CLIMP63 is originally cloned from human cells in a study trying to identify ER to Golgi trafficking proteins (Schweizer, Ericsson et al. 1993). The protein sequences for human, rat and mouse CLIMP63 are aligned, and their cytoplasmic microtubule binding domains are highly consensus (Fig. 4-1A). In Allen brain atlas the CLIMP63 mRNA is highly expressed in hippocampus and cerebellum (Fig. 4-1B). CLIMP63 protein is expressed in rat hippocampal neurons (Fig. 4-1C). It is highly expressed in the cell body, dendrites and also occasionally in spines (Fig. 4-1C). Endogenous CLIMP63 immunofluorescence shows a tubular distribution with puncta structures along the tubules, which is consistent with the previous finding that CLIMP63 is microtubule associated. CLIMP63 can also be detected by immuno-blots reaction in rat cortical neurons (Fig. 4-2A). These discoveries demonstrate a broad distribution of CLIMP63 in different brain regions. The expression profile of CLIMP63 in rat brains during development will be characterized in the future experiments. Different forms of human CLIMP63 DNA constructs including WT and dominant negatives were used to
**Figure 4-1** CLIMP63 sequences for different species and CLIMP63 expression in brains.

A, The protein sequences (cytoplasmic domain) for CLIMP63 also known as Cytoskeleton-Associated Protein 4 (CKAP4) in different species: *Homo sapiens* (hm), *Rattus norvegicus* (rat), *Mus musculus* (ms). Potential phosphorylation site serine3, 17, 19, 101 are shown in red.

B, CLIMP63 mRNA distribution in different regions in mouse brain. Note its expression in hippocampus, cerebellum and Olfactory bulb. (Adapted from Allen Brain Atlas)

C, CLIMP63 protein expression in dissociated rat hippocampal neurons detected by immunofluorescence. Scale bar 10 μm.
Figure 4-1 CLIMP63 sequences for different species and CLIMP63 expression in brains.
Figure 4-2 CLIMP63 expression and phosphorylation in rat cortical neurons.

A, CLIMP63 expression in dissociated rat cortical neurons. CLIMP63 protein was detected in cortical neurons by immunoblot with anti-CLIMP63-cytoplasmic-domain antibody (CLIMP63-cyto) and anti-CLIMP63-ER-lumen-domain antibody (CLIMP63-lumen). 5μg and 10μg proteins were loaded in lane 1 and 2.

B, Detection of endogenous and overexpressed CLIMP63 phosphorylation in HEK293T cells and cortical neurons. Lane 1-6 in the blots: immunoblot of endogenous and overexpressed CLIMP63 by phospho-specific antibodies generated against S3, 17 and 19 in HEK293T cells. CLIMP63-mcherry was expressed in HEK293T cells and before loading, the protein samples were treated with (lane 4-6, triplicate) or without (lane 1-3, triplicate) λ-phosphatase. The phosphorylation for both endogenous and overexpressed CLIMP63 was detected (lane 1-3) and the signal decreased when samples were treated with phosphatase (lane 4-6). Note the molecular weight difference between endogenous and overexpressed proteins (arrows point to endogenous CLIMP63). Immunoblot detection of endogenous CLIMP63 in cortical neurons by using S3, S17 and S19 phoso-specific antibodies (lane 7).
Figure 4-2 CLIMP63 expression and phosphorylation in rat cortical neurons.
manipulate the dendritic ER morphology based on the expression of CLIMP63 in rat hippocampus and the highly conserved sequences of human and rat CLIMP63.

Phosphorylation of CLIMP63 in rat brains

It is shown that CLIMP63 is phosphorylated during mitosis and this phosphorylation disrupts its binding to microtubule based on in vitro binding essays (Vedrenne et al., 2005). CLIMP63 has four consensus phosphorylation sites in its cytoplasmic microtubule binding domains, three of which (Serine3, Serine19, and Serine101) are protein kinase C (PKC) sites, whereas Serine17 is a casein kinase II (CKII) site. Interestingly, S3, S17 and S19 are critical for the microtubule binding. In order to test the phosphorylation of CLIMP63 in rat brains, polyclonal phospho-specific antibodies against S3, S17 and S19 were developed. Single band of phosphorylated CLIMP63 can be detected in cortical neurons (Fig. 4-2B). Although the immuno-blots results shown are preliminary and the specificity of CLIMP63 phospho-specific antibodies needs to be further tested, this is the first study to identify CLIMP63 phosphorylation in rat brains and it opens many possibilities for future studies, such as signaling pathways that can regulate CLIMP63 phosphorylation, activity regulated phosphorylation during neuronal development and its consequences in neuronal ER morphology and postsynaptic functions.

CLIMP63 mutant expression caused the ER morphology change in fibroblasts
Previous studies have demonstrated that CLIMP63 binding to microtubules is critical for the ER morphology maintenance in fibroblasts (Klopfenstein, Kappeler et al. 1998). Either strengthening or disrupting this binding caused abnormal ER membrane structures. When CLIMP63-WT was overexpressed in Cos7 cells, ER membranes, shown by ER chaperone protein BIP staining, wired around, formed a tubular structure and the typical three-way junctions were no longer observed in the cells (Fig. 4-3A). When CLIMP63-ΔMBD microtubule-binding deficient mutant was overexpressed, ER membranes had a meshwork structure with more three-way junctions (Fig. 4-3A), and they did not completely shrink to the peri-nuclear region as reported (Klopfenstein, Kappeler et al. 1998). It is also observed in previous studies that CLIMP63 is phosphorylated during mitosis (Vedrenne, Klopfenstein et al. 2005). I used CLIMP63 phosho mutants as dominant negatives to manipulate ER morphology. In order to confirm CLIMP63 phosho mutants’ effect on ER morphology I expressed CLIMP63-3E (Serine3, 17, 19-Glutamate) a phospho-mimic mutant in Cos7 cells and found the overexpression of CLIMP63-3E induced the ER membrane morphology change similar to the CLIMP63-ΔMBD mutant (Fig. 4-3B), indicating that phosphorylation of CLIMP63 caused its dissociation from microtubules. Interestingly, overexpression of CLIMP63-3A (Serine3, 17, 19-Alanine) a phospho-deficient mutant caused the ER membrane bundling in tubules and this effect is similar to CLIMP63-WT.
Figure 4-3 CLIMP63 WT ΔMBD 3A 3E mutations cause the ER morphology change in Cos7 cells.

CLIMP63-WT, CLIMP63-ΔMBD (A), CLIMP63-3A and CLIMP63-3E (B) were expressed in Cos7 cells. The morphology of Cos7 cells was shown by citrine cell fill in left column; ER morphology was shown by BIP immunofluorescence in the middle column; the expression of CLIMP63 in Cos7 cells was confirmed by immunofluorescence in the right column. Scale bar 10 μm.
Figure 4-3 CLIMP63 WT and ΔMBD 3A 3E mutations cause the ER morphology change in Cos7 cells.
overexpression (*Fig. 4-3B*). The overexpression of different forms of CLIMP63 dominant negatives can modify the morphology of ER membranes and was used to manipulate dendritic ER structures in hippocampal neurons.

*Regulation of dendritic ER spatial complexity by CLIMP63*

It is shown in Chapter 3 that the spatial organization of dendritic ER restricted early secretory receptor trafficking and potentially confined its final distribution in neurons. In order to study the postsynaptic functions of dendritic ER, I used CLIMP63 to manipulate dendritic ER structure and confirmed the spatial organization change directly by immunofluorescence and quantitative FRAP, which has been discussed in Chapter 3. It has been shown in Chapter 3 that dendritic ER spatial complexity is regulated by type I mGluR dependent PKC activation. Intriguingly, CLIMP63 has four phosphorylation sites in its microtubule binding domain (*Fig. 4-1A*) and three of them serine3, serine17 and serine19 are critical for its binding to microtubules, therefore, it suggests an type I mGluR (based on findings in Chapter 3) and PKC activation dependent CLIMP63 phosphorylation mechanism which can regulate dendritic ER complexity. I proposed that PKC activation upon mGluRs causes CLIMP63 phosphorylation and its dissociation from microtubule which upregulates ER complexity. CLIMP63-WT, CLIMP63-3A, and CLIMP63-3E were coexpressed with ER-VSVG-GFP, and ER complexity was measured in these three different conditions by
FRAP method. Consistent with the prediction, CLIMP63-3E increased dendritic ER complexity compared to that of CLIMP63-WT and CLIMP63-3A overexpressing neurons (Fig. 4-4B). More interestingly, when CLIMP63-WT overexpression neurons were treated PKC activator PMA, the dendritic ER complexity has a trend of increase, but this effect was not observed in CLIMP63-3A overexpressing neurons (Fig. 4-4B), which suggests PKC dependent phosphorylation of CLIMP63 is critical for dendritic ER morphology regulation. When CLIMP63-3E is overexpressed in neurons, it is observed directly by immunostaining of ER protein BIP that dendritic ER membrane became more heterogenous and formed puncta structures (Fig. 4-4A) which is similar to what we observed with complex ER membranes in previous study. CLIMP63-WT and CLIMP63-3A made ER smooth and homogenous in dendrites (Fig. 4-4A). It was also observed that CLIMP63 signals appeared in spines in CLIMP63-3E expressing neurons with higher frequency than CLIMP63-WT and CLIMP63-3A expressing cells (data not shown). Therefore, CLIMP63 dependent dendritic ER morphology change and the spine entering may regulate postsynaptic functions together.

CLIMP63 dependent regulation of synaptic properties

Both microtubule and actin filament cytoskeletons regulate cell morphology. In neuronal dendrites actin filaments are mainly distributed in spines and are critical for spine formation (Tada and Sheng 2006). CLIMP63-ΔMBD mutant caused
**Figure 4-4** Alteration of dendritic ER spatial complexity by CLIMP63 expression.

A, Dendrite morphology (citrine, left column), dendritic ER morphology (BIP immunofluorescence, middle column) and the expression of CLIMP63-WT, 3A and 3E (CLIMP63 immunofluorescence, right column).

B, ER-VSVG recovery halftimes (t1/2), recovery fractions and complexity indexes after sequential bleaching of 1μm and 12μm long dendritic segments in individual cells expressing CLIMP63-WT, 3A and 3E and in CLIMP63-WT, 3A expressing cells after addition of 100 nM PMA for 30 min (mean±SEM, 19, 29, 26, 9, 6 cells, in 4 experiments, for CLIMP63-WT, 3A, 3E, WT plus PMA, and 3A plus PMA, respectively).
Figure 4-4 Alteration of dendritic ER spatial complexity by CLIMP63.
the dissociation of ER membrane from microtubules and potentially actin filaments regulate ER morphology in a more dominant manner in this condition (Georges, Hadzimichalis et al. 2008). Given the fact that the ER membranes also appear in spines (Spacek and Harris 1997) and Ca\(^{2+}\) from ER in spines is important for purkinje cell LTD in cerebellum (Miyata, Finch et al. 2000), when the dendritic ER binding to microtubule is disrupted, actin filaments may cause ER membrane moving into spines on a myosin Va based mechanism (Miyata, Finch et al. 2000) and the relocated ER may in turn regulates postsynaptic functions.

In order to test how the spatial organization of ER regulates spine formation and postsynaptic function, CLIMP63-WT and CLIMP63-ΔMBD were expressed in hippocampal neurons. CLIMP63-WT expression in neurons caused a significant loss of spines (Fig. 4-5), but on the contrary, CLIMP63-ΔMBD expression increased the spine density significantly compared to control neurons (Fig. 4-5). It was also observed that CLIMP63 signal appeared in dendritic spines with much higher chances when CLIMP63-ΔMBD is overexpressed (data not shown). Both of these observations point to an actin based mechanism that regulates ER morphology, positioning and functioning.

*The functions of CLIMP63 in dendritic growth*

The ER localized in dendrites is important for dendritic arbor morphogenesis (Lohmann, Myhr et al. 2002; Lohmann and Wong 2005) and neuron migration in mouse neocortex
**Figure 4-5** Modification of dendritic spine density by alteration of CLIMP63 functions.

A, Dendrite morphology (citrine, left column) and the expression of CLIMP63-WT and CLIMP63-ΔMBD (CLIMP63 immunofluorescence, right column).

B, Quantification of spine density in CLIMP63-WT, CLIMP63-ΔMBD and only citrine expressing control cells (mean±SEM, 37, 65 and 50 in 2 experiments). Unpaired t-tests, *: p<0.05.
Figure 4-5 Modification of dendritic spine density by alteration of CLIMP63 functions.
(Shim, Wang et al. 2008). During early neuronal development, ER may regulate the neurite generation and elongation. Therefore in order to test the function of dendritic ER spatial organization in dendritic morphogenesis I overexpressed CLIMP63-WT and CLIMP63-ΔMBD in dissociated hippocampal neurons and examined dendritic outgrowth. Interestingly CLIMP63-WT increased but CLIMP63-ΔMBD decreased the average dendritic length (Fig. 4-6B). The representative camera lucida drawings of neurons expressing CLIMP63-WT and CLIMP63-ΔMBD are also shown (Fig. 4-6A).

This discovery is consistent with CLIMP63’s function in the ER morphology regulation. ER membranes have two different dynamic modes on microtubules. Microtubule motors can either drag the ER membranes along underlying microtubules or drive the sliding of membrane-associated microtubules, whereas microtubule polymerization can promote the movement of an ER tubule bound to the dynamic microtubule tip (Waterman-Storer and Salmon 1998). During neurite outgrowth microtubule polymerization is enhanced and microtubules extend into neurites (Calvert 1995; Vandecandelaere, Pedrotti et al. 1996; Harada, Teng et al. 2002). The experiment results indicate that CLIMP63-WT may strengthen the binding between ER membrane and microtubules and anchor the ER membrane to the microtubules in the neurites and stabilizes them. Microtubules and actin filaments may function at different neuronal stage to regulate dendrite and spine formation.
**Figure 4-6** Modification of dendritic outgrowth by alteration of CLIMP63 functions.

A, Dendrite morphology (citrine, left column), the expression of CLIMP63-WT and CLIMP63-ΔMBD (CLIMP63 immunofluorescence, middle column), and the skeleton of the dendritic tree (camera lucida drawings, right column).

B, Quantification of average dendritic length in CLIMP63-WT, CLIMP63-ΔMBD and only citrine expressing control cells (mean±SEM, 19, 24 and 17 in 3 experiments).
Figure 4-6 Modification of dendritic outgrowth by alteration of CLIMP63 functions.
4.4 Discussion

Although the results shown here in this chapter are preliminary, this is the first study on the microtubule associated proteins regulating ER morphology and its postsynaptic function. The geometry of cells and spatial organization of cellular organelles reveal more and more important functions (Horton, Racz et al. 2005; Ye, Zhang et al. 2007). Considering the complex morphology of neurons and the precisely regulated intracellular localization and structure of its secretory organelles, it is extremely interesting to understand the detailed mechanisms and functions of the organelle organization in neurons.

CLIMP63 and microtubule interaction dependent ER morphology regulation was studied in fibroblasts, however, CLIMP63 expression and function in brains is not clear. In this study, I characterized its expression in neurons and used CLIMP63 as a tool to manipulate dendritic ER morphology and studied the effects of dendritic ER structure on neuronal function (Fig. 4-7). The experiment results indicate microtubules and actin filaments in neuronal dendrites and spines may regulate ER morphology interdependently and competitively. When the ER association to microtubules is enhanced by CLIMP63-WT overexpression, dendrite outgrowth and elongation is promoted (Fig. 4-6), which is consistent with the microtubule polymerization’s effect on neurite outgrowth (Calvert 1995; Vandecandelaere, Pedrotti et al. 1996; Harada, Teng et al. 2002). However, when ER’s association to microtubules is disrupted, actin filaments
Figure 4-7 Bidirectional regulation of ER spatial complexity and postsynaptic functions. Our data suggest dendritic ER complexity is regulated by CLIMP63 bidirectionally. When CLIMP63 is phosphorylated by PKC, it dissociates from microtubules and the ER membranes anchoring to microtubules is disrupted. When activated PKC level is low, more CLIMP63 stay in the unphosphorylated status and the ER membrane binds more tightly to microtubules. ER membrane association with microtubules regulates its spatial complexity. When their binding is strong, ER tubules extend along microtubule and have a simple tubular structure. However, increased PKC activation and CLIMP63 phosphorylation dissociate ER membrane from microtubules and it has a more branched and complex structure, which could be dependent on actin filaments. This bidirectional regulation of ER spatial complexity influences neurite outgrowth and spine formation.
Figure 4-7 Bidirectional regulation of ER spatial complexity and postsynaptic functions.
could be the major regulator for ER morphology, which could make ER more complex and increase spine density. Interestingly, dendrite elongation, branching and spine formation happen at different neuronal development stages sequentially. CLIMP63 temporal expression profile in different brain regions could regulate this programmed neuronal development process. EM study on CLIMP63 expression profile will be useful.

Intriguingly, on top of CLIMP63 temporal expression, phosphorylation of CLIMP63 adds another layer of regulation to the dendritic ER morphology and neuronal function. Besides the intrinsic regulation of CLIMP63, extracellular signals which activate intracellular PKC can manipulate CLIMP63 phosphorylation status and regulate the postsynaptic function by changing ER spatial organization. Type I mGluR activation is one of the good candidates for this extracellular signals. In Chapter 3, I have discussed type I mGluR and PKC’s function in regulating dendritic ER complexity and restricted secretory cargo protein movement in the dendritic ER. mGluR and PKC activation may also regulate CLIMP63 phosphorylation and dendritic ER morphology, dependent or independent of protein synthesis. It will be interesting to see whether CLIMP63-ΔMBD can occlude ER complexity change induced by DHPG (mGluR activation).

This CLIMP63 mediated manipulation of ER spatial organization can also be used to study other postsynaptic functions. The dominant negative forms or RNAi knocking of CLIMP63 method will be used. Postsynaptic mEPSCs will be examined, which provides interesting information about postsynaptic receptor properties.
Postsynaptic AMPA receptor and NMDA receptor composition will be examined in this experiment and it will provide us insights for the ER spatial organization’s function in postsynaptic receptor property regulation. The dendritic ER spatial organization and postsynaptic receptor composition change may occlude or inhibit the induction of LTP and this experiment will link the intracellular organelle directly to the neuronal function and neuronal circuits. Fusion peptides blocking CLIMP63 binding to microtubules will be generated and they can be used as powerful tools to study the function of CLIMP63 and ER organization in plasticity.

Dendritic ER spatial organization change may have two independent functions, one is regulating intracellular Ca\(^{2+}\), and the other is regulating the efficiency of secretory pathway in neurons. Ca\(^{2+}\) levels in neurons can be regulated by the release of Ca\(^{2+}\) from intracellular stores, as well as influx through Ca\(^{2+}\) channels. Release from stores principally involves Calcium-Induced Calcium Release (CICR) or activation by ligands that lead to production IP\(_3\), which acts on internal stores such as the ER. The ER is positioned adjacent to the plasma membrane to sense the local changes in the environment (Spacek and Harris 1997; Berridge 1998; Bootman, Lipp et al. 2001). Intracellular Ca\(^{2+}\) stores may act locally to influence dendrite dynamics (Lohmann, Myhr et al. 2002). Two patterns of spontaneous Ca\(^{2+}\) increase have been observed in dendrites of developing chick RGC: global increases in Ca\(^{2+}\) levels induced by action potentials and local increases in Ca\(^{2+}\) mediated by CICR. Blockage of local, but not global, activity
causes rapid dendritic retraction, supporting the idea that Ca\textsuperscript{2+} release from intracellular stores can regulate dendritic branch stability. Local Ca\textsuperscript{2+} signaling may also play a role in regulating filopodia dynamics (Lohmann and Wong 2005). Whether these mechanisms are involved in selectively maintaining the dendrites remains to be examined. It will be extremely interesting to look at intracellular Ca\textsuperscript{2+} dynamics by regulating ER spatial organization by CLIMP63, and it will be very useful for us to understand how the Ca\textsuperscript{2+} signaling regulate neuronal function. It is shown in previous studies that ERES number in dendrites is increased during neuronal development (Aridor, Guzik et al. 2004). ERES number and vesicle export rate could be examined in conditions with different ER spatial complexity. These proposed future experiments will help us distinguish the two independent roles of dendritic ER in regulating postsynaptic neuronal functions.
5. Conclusions and Future Directions

5.1 Regulation of Dendritic ER Spatial Organization

The appearance of ER in dendrites of hippocampal neurons and its structural continuity have been observed for years (Spacek and Harris 1997; Gardiol, Racca et al. 1999) and it also occasionally enters mushroom spines and makes contacts with PSDs (Spacek and Harris 1997). Although in cerebellum Purkinje cells the ER in dendrites and spines are thought to be important for intracellular Ca\(^{2+}\) release which is critical for LTD induction (Miyata, Finch et al. 2000), how the signaling is controlled by ion channels distributed in ER is not clear. IP\(_3\) receptors, ryanodine receptors and SERCA Ca\(^{2+}\) channels are critical to modulate the Ca\(^{2+}\) release, and they are constantly retained in the ER. The structure of ER membrane including its membrane volume, folding and tubule branching can significantly influence the distribution of these ion channels and in turn the Ca\(^{2+}\) dynamics such as its raise and decay kinetics which can activate different downstream signaling proteins.

The ER is also important for integral membrane protein and secretory factor synthesis and modification. For integral membrane proteins in multimers such as AMPA receptors, each subunit needs to be correctly folded and assembled into tetramers before export from the ER (Greger and Esteban 2007). Given the halftime for their dwelling in the ER varies from minutes to hours, the structure of dendritic ER membrane can control
the nascent receptor dwelling region, concentration and potentially their relaxed or confined distribution on the cell surface.

In this study dendritic ER structure was visualized by ER retained fluorescent probes which revealed dendritic ER dynamically. These fluorescent molecule tagged ER retained nascent membrane proteins not only allowed us to examine the dendritic ER morphology, but also made it possible to study protein dynamics inside the ER membrane. Interestingly these nascent membrane proteins are highly mobile in dendritic ER with a comparable diffusion rate to that of plasma membrane proteins and there are no obvious diffusion barriers in the somato-dendritic ER. Diffusion on the cell surface is considered as one of the critical mechanisms to transport newly synthesized proteins (Adesnik, Nicoll et al. 2005). Given the rate of protein movement in the dendritic ER is comparable to that on the cell surface, diffusion is an important aspect of protein dynamics in the ER and the somato-dendritic ER is a platform for newly synthesized protein transport.

Electron microscopy has the highest resolution for intracellular structure visualization, but it does not allow us to examine the membrane structure in live-cells. A quantitative FRAP based method was developed to study the dendritic ER membrane spatial organization and protein diffusion. Intriguingly it is found the ER is more complex and protein diffusion in the ER is more restricted at dendritic branch points and in dendritic regions with Golgi apparatus. These restricted secretory cargoes may
have more chance to be transport to close-by Golgi, locally processed and targeted to cell
surface. The discovery of “complex ER-Golgi” units in dendrites and dendritic branch
points raised the question of dendrite Golgi biogenesis. In neurons, ERES extend far into
the dendrites (Horton and Ehlers 2003; Aridor, Guzik et al. 2004). Therefore, it is possible
that the dendritic Golgi form de novo around ERES. To test this hypothesis, the
examination of the spatial relationship between dendritic ERES and dendritic Golgi and
the microtubule dependent Golgi outposts localization will be required.

Based on the discovery of correlation between ER spatial complexity and Golgi
appearance (so as defined “complex ER-Golgi”), the possibility of local control over
secretion and protein trafficking remain intriguing. In Drosophila oocytes, mRNA for
proper polarity establishment is precisely targeted and translated in particular regions.
For example, proper polarized secretion of protein Gurken requires rapid ER exit of the
newly synthesized protein before it diffused within the ER membrane away from the
spot of translation (Herpers and Rabouille 2004). When an ER export motif in Gurken
was mutated, its dwell time in the ER increased, it diffused with the ER membrane, and
the secretion became distributed than local (Herpers and Rabouille 2004). Given the
dwell time for a number of important neuronal membrane proteins is on the scale of
hours and the forward trafficking is regulated by ER export and retention signals (Scott,
Blanpied et al. 2001; Mu, Otsuka et al. 2003; Greger and Esteban 2007) it will be
important to determine the function of dendritic “complex ER-Golgi” units in local versus global secretion.

5.2 The Molecular Mechanisms for Dendritic ER Spatial Organization Regulation

As discussed in the introduction section, microtubules and actin filaments are critical to maintain ER morphology (Terasaki, Chen et al. 1986; Du, Ferro-Novick et al. 2004). Microtubules and actin filaments associated proteins such as kinectin (Santama, Er et al. 2004), CLIMP63 (Klopfenstein, Kappeler et al. 1998), p22 (Andrade, Zhao et al. 2004) and myosin Va (Bridgman 1999) also play important roles in the ER morphology regulation. The preliminary data (not shown) indicate that dendritic ER complexity is regulated by cytoskeletons. Latrunculin treatment disrupting actin filaments decreased dendritic ER complexity which suggests actin filaments make the ER more complex in neurons (data not shown). Microtubules’ regulation on dendritic ER complexity need to be further tested, given the neuronal dendritic microtubules are highly stable and nocodazole did not have obvious effect on dendritic ER complexity (data not shown). In neurons, microtubules are mainly in dendritic shaft and actin filaments in spines, therefore they may play different roles in regulating ER organization in separated regions. Most interestingly, preliminary data have shown that ER membrane location in dendrites and spines is regulated by microtubule binding protein association or dissociation from microtubules and it is possible that under different neuronal
development stages and neuronal activation status, microtubules and actin filaments have interdependent and competitive roles in ER morphology regulation.

It is shown in Chapter 4 that the overexpression of different CLIMP63 phosphorylation mutants regulated dendritic ER spatial organization in neurons potentially via an mGluR and PKC dependent pathway. CLIMP63 phosphorylation needs to be tested under direct mGluR and PKC activation in neurons by using phospho-specific antibodies generated against each potential phosphorylation site. The studies on underlying signaling pathway to regulate dendritic ER complexity provide useful information about the mechanisms of dendritic ER spatial organization in regulating postsynaptic functions.

There are other non-cytoskeleton associated proteins which can regulate ER morphology. The myelin-associated membrane protein reticulon-4(RTN4)/Nogo has been extensively studied with regards to its neurite outgrowth inhibitory function, both in limiting plasticity in the healthy adult brain and regeneration during central nervous system injury. Surprisingly, Nogo and other reticulon paralogues are mainly localized to the ER, and are likely to have a role in modulating the morphology and functions of the ER (Voeltz, Prinz et al. 2006; Teng and Tang 2008). Overexpression and RNAi knocking down strategies can be used to test RTN4’s function in dendritic ER spatial organization. RTN4 is reported to be important for ER membrane curvature generation (Voeltz, Prinz et al. 2006) and potentially it can make the dendritic ER more complex. It will be
extremely interesting to study RTN4’s intracellular function as a structure protein on ER morphology regulation in neurite outgrowth in contrast to its function on the cell surface as a signaling molecule.

5.3 The Function of Dendritic ER Spatial Organization in Regulating Intracellular Ca\(^{2+}\) and Postsynaptic Signaling

Ca\(^{2+}\) levels in neurons can be regulated by the release of Ca\(^{2+}\) from intracellular stores, as well as influx through Ca\(^{2+}\) channels. Release from stores principally involves Calcium-Induced Calcium Release (CICR) or activation by ligands that lead to production of IP\(_3\), which acts on internal stores such as the ER. The ER is positioned adjacent to the plasma membrane to sense the local changes in the environment (Spacek and Harris 1997; Berridge 1998; Bootman, Lipp et al. 2001). Intracellular Ca\(^{2+}\) stores may act locally to regulate dendrite dynamics (Lohmann, Myhr et al. 2002). Global increases in Ca\(^{2+}\) levels induced by action potentials and local increases in Ca\(^{2+}\) mediated by CICR have been observed (Lohmann and Wong 2005). Blockage of local, but not global activity causes rapid dendritic retraction, supporting the idea that Ca\(^{2+}\) release from intracellular stores can regulate dendrite stability. Local Ca\(^{2+}\) signaling may also play a role in regulating filopodia dynamics (Lohmann and Wong 2005). CLIMP63-3E mutant up-regulated dendritic ER complexity and the entry of ER membrane into spine were also observed. Given CLIMP63-3E’s function in up-regulating spine density in neurons, it will be extremely interesting to look at intracellular Ca\(^{2+}\) dynamics by regulating ER
spatial organization via CLIMP63 and very useful to understand the function of Ca\textsuperscript{2+}
dynamics in regulating spine generation and postsynaptic function.

It is known that the ER membranes make contacts with mitochondria membranes by mitofusin 2 (de Brito and Scorrano 2008). It is also shown that the ER-mitochondria Ca\textsuperscript{2+} transfer regulates apoptosis (Pinton, Giorgi et al. 2008) and glutamate release can be modulated by mitochondria Ca\textsuperscript{2+} release (Reyes and Parpura 2008). Most interestingly, mitochondria are important for morphogenesis and plasticity of spines and synapses (Li, Okamoto et al. 2004; Macaskill, Rinholm et al. 2009). Although the translocation of mitochondria to synaptic regions is shown (Li, Okamoto et al. 2004; Macaskill, Rinholm et al. 2009) the function of mitochondria in synaptic plasticity is not clear yet. It is possible that the tethering and Ca\textsuperscript{2+} transfer between ER and mitochondria are important for normal synaptic function. The dendritic ER is more complex in spiny regions than that in aspiny regions as discussed in Chapter 3, therefore it is interesting to look at the relationship between dendritic ER spatial organization and mitochondria localization and signaling. In other words, the dendritic ER spatial complexity may not only regulate Ca\textsuperscript{2+} release from ER but also Ca\textsuperscript{2+} release from other related resources.

5.4 The Function of Dendritic ER Spatial Organization in Regulating Secretory Trafficking and Targeting of Surface Proteins

Integral membrane protein diffusion is more restricted in more complex dendritic ER membranes and the ER spatial complexity is correlated to Golgi
appearance in dendrites which is shown by the quantitative FRAP method discussed in Chapter 3. Although all the evidence points to a local control of secretory trafficking, it is still not yet directly proved. It is extremely interesting to study membrane protein final distribution on the cell surface in neurons with different ER complexity. Although this is technically challenging, a few strategies could be tested. In order to examine the local control of secretory function, the newly synthesized proteins should be translated in dendritic regions instead of somatic region. Therefore, 3’ UTR of CaMKII could be used to locate the mRNA of the membrane proteins into the dendrites and local stimulation can start the translation in dendritic regions (Sutton, Taylor et al. 2007). CLIMP63 or other structure proteins can be used to manipulate ER complexity and combined with the initiation of local protein translation, it would be possible to study how the ER spatial organization regulates local membrane protein trafficking and their final distribution on the cell surface. According to the model, increased dendritic ER spatial complexity should cause a more restricted protein distribution on the cell surface.

Other strategies can also be used to study the local secretory pathway. ERES numbers and ER exit efficiencies could be tested by immunofluorescence and live-cell imaging combined with CLIMP63 mediated ER complexity change. It is proposed that complex local ER, ERES and local Golgi at specific dendritic regions form highly efficient protein and membrane processing units (or microdomains) which are critical for neuronal function.
5.5 The Function of Dendritic ER in Neuronal Development

The preliminary data have shown that when ER binding to microtubules is enhanced by CLIMP63-WT, ER tubules associated tightly with microtubules, the ER structure was less complex and neurites had higher average length. Microtubule polymerization and subsequent invasion into filopodia are necessary for the stabilization of filopodia that will become dendrites. MAPs such as MAP1A and MAP2 play roles in neuritogenesis by promoting microtubule assembly and stabilization (Calvert 1995; Vandecandelaere, Pedrotti et al. 1996; Harada, Teng et al. 2002). In addition, the ER localized in dendrites is important for dendritic arbor morphogenesis (Lohmann, Myhr et al. 2002; Lohmann and Wong 2005) and neuron migration (Shim, Wang et al. 2008). It will be interesting to study the function of ER spatial organization in neurite morphogenesis, but the function of microtubules and ER morphology needs to be separated. The caveat of the present study is that CLIMP63-WT changes the morphology of both microtubules and ER membranes. Therefore it is difficult to use this protein as a strategy to study ER’s function in neurite outgrowth. However as we discussed in earlier sections, many other ER structure proteins which do not have side effects on microtubule bundling can be used to manipulate dendritic ER spatial organization and help us reveal ER’s function in early neuronal development.
5.6 The Function of Dendritic ER Spatial Organization in Synaptic Plasticity

Synaptic input such as mGluR activation by DHPG increased dendritic ER complexity, and CLIMP63 dependent ER complexity up-regulation also promoted spine outgrowth. It is difficult to distinguish the temporal sequences for ER complexity change and spine morphogenesis from these experiments. There could be a positive feedback loop between dendritic ER spatial structure arrangement and spine morphogenesis/postsynaptic receptor composition regulation.

“Input specificity” is a distinct feature of LTP. Isolated groups of synapses on the same postsynaptic cell can be regulated independently (Bonhoeffer, Staiger et al. 1989; Steward and Schuman 2001). Synaptic strength enhancement at one set of synapses would not influence the strength of other sets of synapses if the two groups of synapses are activated by different afferents (Andersen, Sundberg et al. 1977; Bliss and Collingridge 1993; Steward and Schuman 2001; Lynch 2004). However how specific is specific? Although LTP is input-specific at the level of individual synapse, there are also data showing in mouse hippocampal pyramidal cells that LTP at individual synapses reduces the threshold for potentiation at neighboring synapses (Harvey and Svoboda 2007). Given dendritic ER is important for intracellular Ca\textsuperscript{2+} regulation, local protein synthesis and constrained protein trafficking of which are all critical for LTP expression, it will be interesting to study how the spatial organization of dendritic ER regulates the input-specificity of LTP. When the dendritic ER is more complex, Ca\textsuperscript{2+} signal and newly
synthesized protein trafficking could be more restricted in a specific dendritic or synaptic regions which could help to maintain the properties of local compartments and make each synapse more isolated from each other. Synaptic stimulation on one synapse could locally regulate ER complexity and control the level of specificity and integration of information on the same dendritic branch.

5.7 Dendritic ER Function and Diseases

The lack of information on the basic questions of localization and function of neuronal secretory organelles hindered the understanding of neurodegenerative diseases. Previous studies have suggested the role of Huntington’s disease protein huntingtin in intracellular trafficking and axon transport along microtubules (Li and Li 2004; Gunawardena and Goldstein 2005; Smith, Brundin et al. 2005), and the knocking down of huntingtin is found to induce a aberrant poorly extended ER network in mouse neuroblastoma and human glioblastoma cells, however, leaving microtubules intact (Omi, Hachiya et al. 2005). Intriguingly, the involvement of huntingtin protein in ER structure maintenance may point to a role of ER organization in the development of Huntington’s disease.

In Alzheimer’s disease pathological secretion of Aβ peptides depresses excitatory synaptic transmission (Kamenetz, Tomita et al. 2003). The normal secretion of Aβ requires the secretory machinery and studies about organization and function of satellite secretory pathways in the dendrite may give a better understanding of this disease.
Fragile X syndrome is the most commonly inherited form of cognitive deficiency in humans. X-linked FMR1 gene inactivation leads to the absence of the fragile X-mental retardation protein (FMRP) which is a selective RNA-binding proteins and regulates the translocation of a population of mRNA in response to activation of type I mGluR (Bassell GJ and Warren ST, 2008). In the absence of FMRP the mRNA translocation is dys-regulated and leads to altered synaptic function. Given the important role of dendritic ER in protein translation, studying the function of dendritic ER will be helpful to understand this disease and develop new therapeutic treatments.
References


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