Chapter 15
Molecular Assembly of Excitatory Synapses

Hirohide Iwasaki, Shinji Tanaka, and Shigeo Okabe

Abstract Excitatory synapses formed on dendrites are a key component of the functional neuronal network. Molecules present within excitatory synapses and their assembly mechanisms have been studied using multiple research strategies, including biochemistry, cell biology, imaging, and molecular genetics. These efforts have clarified the precise time courses and mechanisms of the synaptic molecular assembly, synaptic junction formation, and postsynaptic structure specialization. Using this knowledge and molecular manipulations, key molecules that regulate excitatory synapse formation have been identified. However, an integrated view of the molecular interactions that regulate excitatory synapse development has not yet been constructed. The difficulty in the integration of a wide range of experimental findings into a coherent model should be eliminated by the development of new imaging and computational approaches designed to examine excitatory synapses.

Keywords Glutamate receptors • Postsynaptic density • Dendritic spine • Actin cytoskeleton • Synapse maturation • Synapse organizer

15.1 The Structure of Excitatory Synapses

15.1.1 The Electron Microscopy of Excitatory Synapses

The ultrastructure of synapses in the central nervous system (CNS) was first described in the 1950s through electron microscopy (EM) studies (Palade and Palay 1954; Palay 1958). The chemical synapses revealed through electron microscopy comprised presynaptic nerve endings and postsynaptic elements separated by an extracellular space (synaptic cleft) with a width of 10–20 nm (Fig. 15.1).

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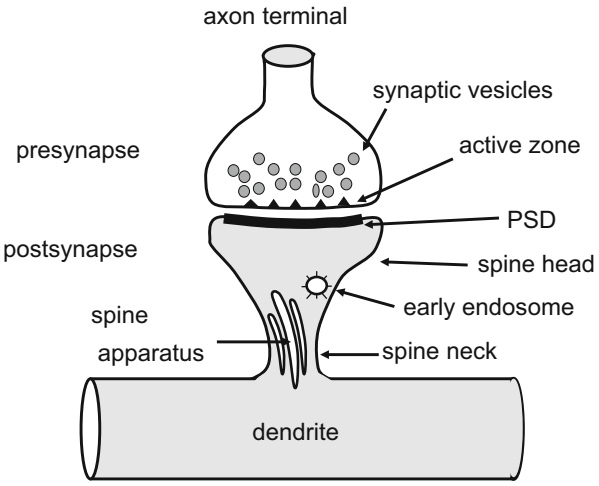

Fig. 15.1 The structure of excitatory spine synapses. Synapses are formed between axon terminals (presynaptic component) and dendrites (postsynaptic component). The axon terminal is filled with synaptic vesicles. The active zone and the PSD are both membrane thickenings located at either presynaptic or postsynaptic membranes. The cytoplasm of spines contains membrane organelles, such as early endosomes and the spine apparatus. Clathrin-coated pits and vesicles are major components of early endosomes and are important in endocytosis of membrane receptors in spines. The spine apparatus is a unique sER-related structure

The membranes on both the sides of the synaptic cleft exhibit densities on their cytoplasmic surfaces. The active zone corresponds to the presynaptic part of the membrane density and is important in the exocytosis of synaptic vesicles. Two types of membrane thickening have been described in the postsynaptic element (Gray 1959). The first type exhibits prominent postsynaptic membrane thickening (postsynaptic density, PSD) and is referred to as a type 1 synapse. In the synapses of the second type, the membrane density thicknesses on the presynaptic and postsynaptic sites are similar and this type of synapses was called type 2 synapses. Later studies revealed that type 1 synapses correspond to glutamatergic excitatory synapses (Petes et al. 1991). Type 2 synapses are inhibitory synapses with their neurotransmitters gamma-aminobutyric acid (GABA) and glycine. Therefore, the typical PSD is a component of glutamatergic excitatory synapses in the CNS.

Type 1 glutamatergic synapses often form on dendritic spines. The preferential formation of glutamatergic synapses on dendritic spines was confirmed in multiple types of neurons in the CNS [e.g., pyramidal neurons in the neocortex (Spacek and Hartmann 1983), pyramidal neurons in the hippocampus (Harris and Stevens 1989), and Purkinje neurons in the cerebellum (Harris and Stevens 1988)]. The PSD and spine can regulate signal processing in the postsynaptic cytoplasm, and the sizes of the two structures are correlated (Arellano et al. 2007; Harris and Stevens 1989). It should be emphasized that many neuron types receive glutamatergic synapses not on spines but rather on dendritic shafts. Together with motor neurons in the spinal cord, a majority of interneurons in the neocortex and hippocampus are classified as

neuron types that form excitatory glutamatergic synapses on dendritic shafts. The basic structures of the PSDs in these neuron types are similar to those of neurons possessing dendritic spines, although excitatory synapse formation on the dendritic shafts of interneurons requires unique protrusive dendritic activity and active synaptic junction translocation (Kawabata et al. 2012).

15.1.2 The Structure of the PSD

The PSD, an essential component of the glutamatergic excitatory synapse, is a dense, submembranous, and filamentous structure with a diameter of 200–500 nm and thickness of 30–60 nm (Harris et al. 1992; Spacek and Harris 1998). The PSD comprises both the membrane and cytoplasmic proteins that play roles in molecular assembly and signal transduction in postsynaptic sites. Neurotransmitter receptors such as the AMPA-type and NMDA-type glutamate receptors are major protein components of the PSD (Cheng et al. 2006; Moon et al. 1994). Cell adhesion molecules can also be detected in the PSD (Jordan et al. 2004). Molecules important for intracellular signal transduction, such as protein kinases and phosphatases, are also present in the PSD (Pocklington et al. 2006). The recruitment and accumulation of neurotransmitter receptors, cell adhesion molecules, and intracellular signaling molecules at the PSD are mediated by interactions of these molecules with PSD scaffolding proteins. These interactions are often regulated by synaptic activity.

The PSD can be isolated biochemically, and EM analyses confirmed the morphology of an isolated PSD as a circular disk with a diameter of 300–400 nm (Petersen et al. 2003). Isolated PSDs can be visualized by making metal replicas after rapid freezing and freeze-drying (Fig. 15.2). This sample preparation technique revealed morphological features of the two surfaces of the PSDs. The cytoplasmic surface of the PSD exhibits irregular protrusions, whereas a dense layer of small particles was present on the cleft surface. These differences are assumed to result from the differential organization of molecules on the two surfaces. A more detailed nanostructure analysis of the PSD can be achieved via EM tomography of the PSD (Lucic et al. 2005). EM tomographic analysis of rapidly frozen and freeze-substituted PSD preparations revealed vertically oriented filaments in the core of the PSD structure; these filaments were labeled with an antibody against the predominant PSD scaffolding protein PSD-95 (Chen et al. 2008). Periodic filamentous structures linking the presynaptic and postsynaptic membranes were also identified during a cryo-EM analysis of vitreous sections (Zuber et al. 2005). Cryo-EM tomography of fully hydrated samples provides the ideal imaging conditions for detecting molecular organization within the PSD. Using cryo-EM, the architecture of native PSDs in cultured hippocampal neurons could be successfully visualized, and PSD structures reconstructed with this technique displayed filaments running parallel to the plasma membrane, together with shorter connecting molecules perpendicular to the filaments

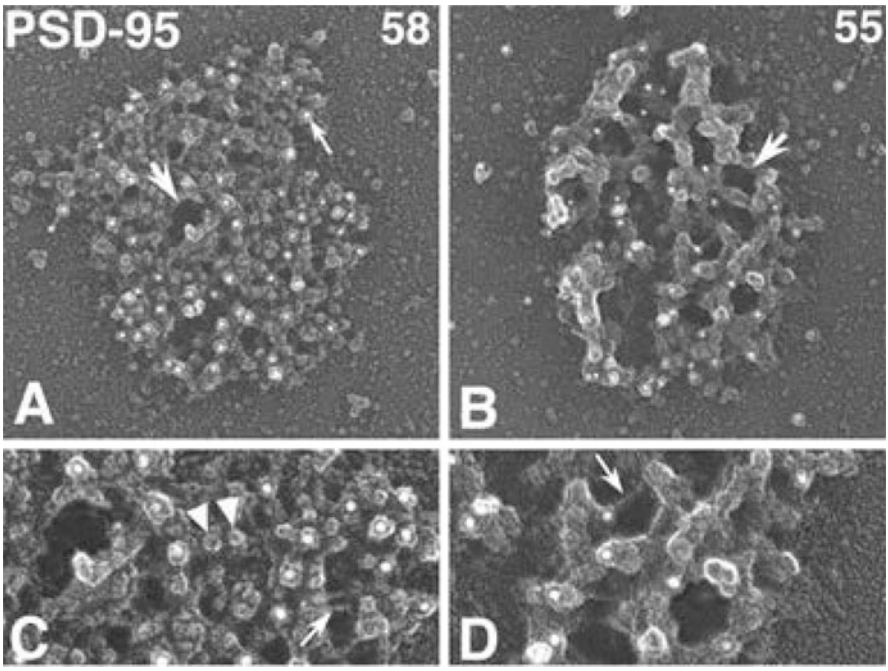

Fig. 15.2 Electron microscopic images of cleft and cytoplasmic surfaces of detergent-extracted PSDs labeled with a gold particle-conjugated anti-PSD-95 antibody (Petersen et al. 2003). **A** and **C** cleft surface of PSD at lower (**A**) and higher (**C**) magnifications. The large *arrow* in **A** is the opening in the central mesh structure. The small *arrow* in **A** indicates an immunogold particle for PSD-95. The *arrowheads* in **C** indicate granular particles. The *arrow* in **C** indicates underlying thin filaments within the central mesh. **B** and **D** cytoplasmic surface of PSD at lower (**B**) and higher (**D**) magnifications. The *arrow* in **B** indicates the opening in the central mesh structure. The *arrow* in **D** indicates underlying filaments of the central mesh. Numbers indicate the counts of gold particles

(Fernandez-Busnadiego et al. 2011; Lucic et al. 2007). The identification of the molecules corresponding to these parallel and perpendicular filaments may lead to an understanding of the general architecture of the PSD.

15.1.3 The Structure of Dendritic Spines

The dendritic spine is a tiny protrusion from dendritic shaft (Fig. 15.3) (Yuste 2010). Dendritic spine does not exhibit a uniform morphology and can be classified into three major categories: thin, mushroom, and stubby. The spine head is an enlarged structure at the end of the protrusion. The PSD is usually located within this spine head. The spine neck is a membranous tube with a width of 50–300 nm that connects the spine head and the dendritic shaft. The spine volume and surface area vary widely even within a single cell type. In the case of hippocampal

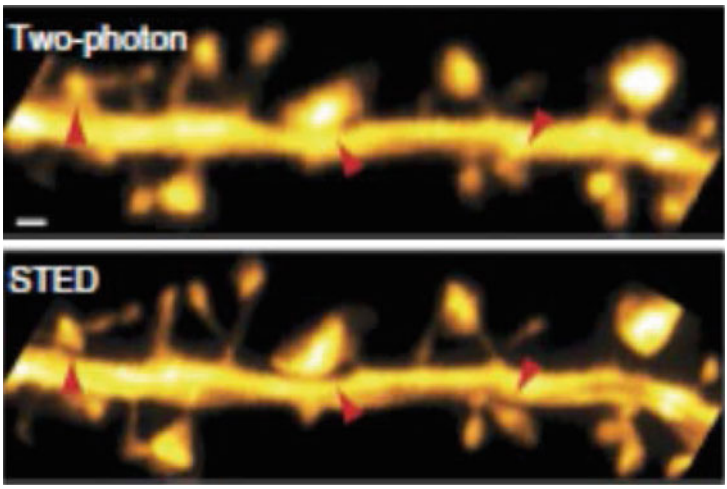

Fig. 15.3 The comparison of spine morphology imaged using two-photon excitation laser scanning and STED microscopy (Tonnesen et al. 2014). *Red arrows* indicate apparent stubby spines in two-photon images but resolved spine necks in STED images

pyramidal neurons in the stratum radiatum of area CA1, the spine volumes and surface areas range from 0.004 to 0.56 μm^3 and from 0.13 to 4.4 μm^2, respectively (Harris and Stevens 1989). Differences in the spine volumes and surface areas may reflect the previous histories of individual spines and their states of synaptic transmission. The spine volume correlates strongly with the area of the PSD (Arellano et al. 2007) and the number of functional AMPA-type glutamate receptors as estimated using the two-photon glutamate uncaging technique (Matsuzaki et al. 2001). This correlation indicates the possibility of parallel changes in spine volumes and postsynaptic function in response to synaptic activity.

Until recently, precise measurements of spine morphology were possible only through EM-based serial reconstruction. However, this situation was changed by the introduction of new super-resolution imaging technologies. Stimulated emission depletion (STED) microscopy, which can detect spine morphologies at a resolution of approximately 50 nm, has revealed the presence of thin spine necks in spines that had been previously classified as stubby using diffraction-limited imaging modalities (Fig. 15.3) (Tonnesen et al. 2014). Precise measurements of spine morphology based on super-resolution imaging represent a powerful approach for the efficient analysis of a large population of spines in live samples.

15.1.4 Spine Cytoplasm

15.1.4.1 Actin Cytoskeleton

The spine cytoplasm exhibits unique morphological features. Both the spine heads and necks are filled with a meshwork of actin filaments (Fischer et al. 1998; Hirokawa 1989). Detailed analyses of the orientations of this filamentous actin have been prohibited by the small scale of the spines. It is assumed that the actin filaments within spines exhibit a polarized orientation with their barbed ends located at the distal edge of spines, similar to the polarized orientation of filamentous actin in nonneuronal filopodia. Spines initiate from filopodia-like precursors, but detailed structural analyses of filamentous actin in spine precursors revealed a network-like organization of actin filaments distinct from the tight actin filament bundles present in nonneuronal filopodia (Korobova and Svitkina 2010; Okabe and Hirokawa 1989). In mature spines, actin filaments in the spine neck also exhibit a network-like appearance. From these observations, it has been postulated that tight actin filament bundling is not necessary for spine morphogenesis (Hotulainen and Hoogenraad 2010). The dynamics of actin filaments in spine synapses will be discussed in Sect. 15.3.4.

15.1.4.2 Smooth Endoplasmic Reticulum (sER)

sER is present in most of the dendritic spines on cerebellar Purkinje cells (Harris and Stevens 1988). This may be related to the fact that the release of calcium from IP3 receptors plays an essential role in synaptic plasticity in Purkinje cells (Miyata et al. 2000). Immuno-EM revealed the preferential enrichment of IP3 receptors in sER, suggesting that the sER within spines is a major source of calcium for IP3-dependent signaling (Walton et al. 1991). The fraction of dendritic spines that contains sER is much lower in hippocampal and cortical pyramidal neurons (Fig. 15.4) (Spacek and Harris 1997). The spine apparatus is a unique sER-related structure. This structure is present in a subset of spines in the hippocampus and is composed of sER-like membrane stacks connected by densely stained linkers (Deller et al. 2000). Synaptopodin is an actin-binding protein present in the spine apparatus, and synaptopodin knockout mice fail to form spine apparatuses (Deller et al. 2003). The knockout mice also exhibit deficits in LTP and impaired spatial learning. These results indicate that the spine apparatus plays important roles in the regulation of synaptic transmission. The spine apparatus is thought to be involved in either calcium release from internal stores or local protein synthesis. The latter possibility is supported by the fact that the spine apparatus has been shown to interact with polyribosomes (Steward and Reeves 1988). Direct evidence to support the roles of the spine apparatus in either calcium release or local protein synthesis has not yet been obtained.

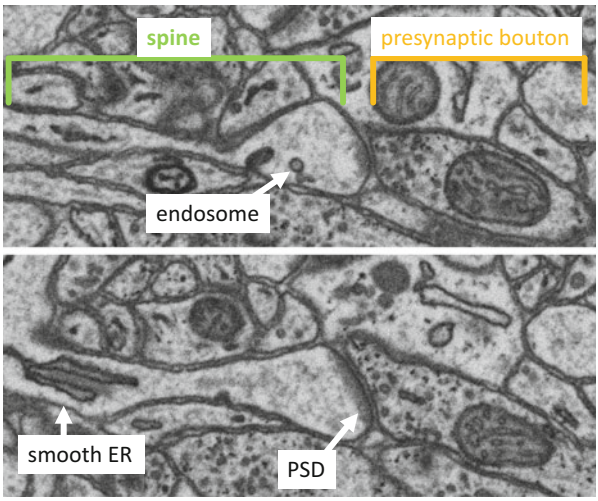

Fig. 15.4 The electron microscopic images of a mouse neocortical spine and its cytoplasmic specializations, such as PSDs, endosomes, and smooth ERs

15.1.4.3 Endocytotic Membranes

Typical clathrin-coated pit structures can be identified in a fraction of dendritic spines. Clathrin-coated pits are located in the vicinity of PSDs and are thought to be involved in AMPA receptor endocytosis (Racz et al. 2004). Active AMPA receptor endocytosis and its upregulation after the induction of synaptic plasticity have been reported (Petrini et al. 2009; Wang et al. 2008). Early endosomes and sorting endosomes have been reported to be present in a fraction of dendritic spines (Fig. 15.4) (Park et al. 2006). EM reconstruction analysis revealed that the number of endosomal-sorting complexes is much lower than the number of dendritic spines (only 10 % of the number of spines per unit dendrite length) (Cooney et al. 2002). This observation indicates that multiple spines in proximity share a common endosomal-sorting machinery for the delivery and recycling of endocytosed materials.

15.2 The Molecular Composition of the PSD

15.2.1 The Functional Roles of PSD Molecules

PSD-enriched fractions have been utilized for protein identification with a variety of biochemical methods. High-sensitivity mass spectrometry is a very powerful technique for protein species identification, and more than 400 proteins have been successfully identified (Husi et al. 2000; Jordan et al. 2004; Yoshimura et al. 2004). The identification of interaction partners using yeast two-hybrid screening also helped to increase the number of candidate proteins present within the PSD. Here

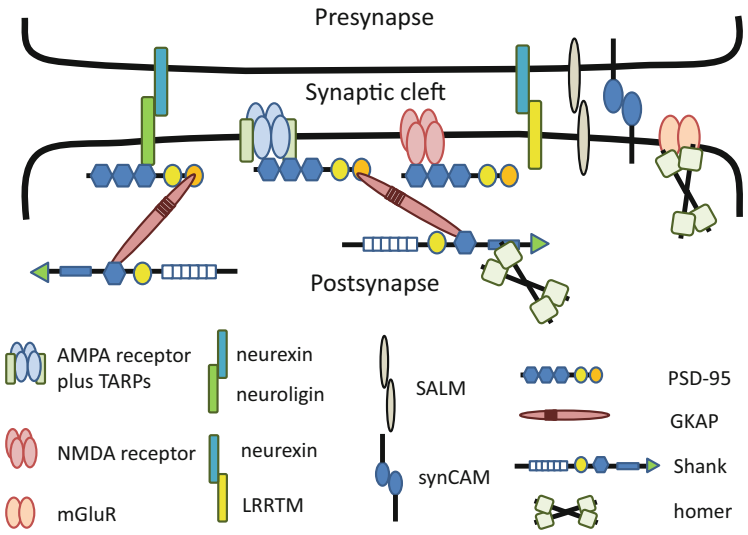

Fig. 15.5 Molecular assembly within spines. AMPA receptors and NMDA receptors are abundant membrane proteins within the PSD. Metabotropic glutamate receptors (mGluRs) are located at the periphery of the postsynaptic membrane. Postsynaptic neuroligin and LRRTM molecules form heterophilic binding with presynaptic neurexins. Homophilic interactions of SALM and synCAM cell adhesion molecules cross-bridge between presynaptic and postsynaptic membranes. PSD-95 interacts directly with NMDA receptors and indirectly with AMPA receptors via TARPs. Neuroligins also interact with PSD-95. GKAP interacts with both PSD-95 and Shank molecules. Shank and mGluRs are binding partners of another PSD scaffolding molecule, Homer, which forms homotetramers

we describe the properties of several major constituents of the PSD, including glutamate receptors, scaffolding molecules, and cell adhesion molecules (Fig. 15.5).

The purified PSD fraction is enriched for both AMPA-type and NMDA-type glutamate receptors (Cheng et al. 2006), and this enrichment has been confirmed by immunoelectron microscopy (Nusser 1999; Nusser et al. 1998; Petralia et al. 1994a, b; Tanaka et al. 2005). AMPA and NMDA receptors are essential functional elements of fast synaptic transmission. In the immature nervous system, a fraction of synapses exhibit relative AMPA receptor scarcity when compared with more mature synapses. The former synapses are "silent" in terms of normal synaptic transmission, but can be activated through NMDA receptor-dependent processes.

PSD scaffolding molecules accumulate within synapses and form molecular networks within PSDs. Predominant PSD scaffolding molecules include PSD-95, GKAP, Shank, and Homer. The guanylate kinase-like domain of PSD-95 directly binds to GKAP (Kim et al. 1997). The C-terminus of GKAP, in turn, interacts with the PDZ domain of Shank (Naisbitt et al. 1999). Shank also interacts with Homer via its proline-rich region (Tu et al. 1999). Accordingly, simple one-to-one interactions might exist between these four scaffolding proteins. PSD-95 belongs to the

membrane-associated guanylate kinase (MAGUK) protein family (Cho et al. 1992) and binds both NMDA receptors and AMPA receptors. Interactions of PSD-95 with the NR2 subunits of NMDA receptors are direct (Kornau et al. 1995) and those with AMPA receptors are indirect and occur via transmembrane AMPA receptor regulatory proteins (TARPs), which are auxiliary components of the native AMPA receptor complex (Tomita et al. 2005). A variety of cell adhesion molecules, including neuroligins, can interact with PSD-95 (Irie et al. 1997; Meyer et al. 2004). Group I metabotropic glutamate receptors (mGluRs) can interact with another PSD scaffolding protein, Homer (Brakeman et al. 1997).

Synapses are specialized sites of cell-to-cell contact. Both the formation of synaptic contacts and maintenance of assembled synaptic structures are regulated by the cell adhesion molecules present in synapses. Neuroligins are synaptic cell adhesion molecules that mainly localize at postsynaptic membranes. The roles of neuroligins in both synapse formation and assembled synaptic junction regulation have been extensively studied. There are five isoforms of neuroligin in humans (NL1, NL2, NL3, NL4X, and NL4Y) and four isoforms in mice (NL1, NL2, NL3, and NL4) (Varoqueaux et al. 2006). In the rodent brain, NL1 and NL2 are present in the excitatory postsynaptic membrane and inhibitory postsynaptic membrane, respectively, whereas NL3 is present in both the excitatory and inhibitory synapses. Neuroligin binds to presynaptic receptor neurexins, and this interaction induces the differentiation of both presynaptic and postsynaptic structures (Sudhof 2008). Presynaptic neurexins have multiple binding partners, including neuroligin and leucine-rich repeat transmembrane neuronal (LRRTM) (Ko et al. 2009), and can also interact indirectly with delta2 receptors via Cbln1 (Ito-Ishida et al. 2012; Matsuda et al. 2010). The postsynaptic cell adhesion molecule LRRTM, which binds to neurexin, can induce presynaptic differentiation (Linhoff et al. 2009). Other synapse organizers include synaptic cell adhesion molecules (synCAMs) (Biederer et al. 2002), netrin-G ligand (NGL) (Kim et al. 2006; Woo et al. 2009b), synaptic adhesion-like molecules (SALMs) (Ko et al. 2006; Mah et al. 2010; Wang et al. 2006), TrkC (Takahashi et al. 2011), protein tyrosine phosphatases (including LAR, PTPδ, and PTPσ) (Takahashi et al. 2011; Woo et al. 2009a; Yoshida et al. 2011), slit and NTRK-like family member (Slitrk) (Takahashi et al. 2012), calsyntenin 3 (Pettem et al. 2013), and IgSF9b (Woo et al. 2013). Some of these synapse organizers can selectively promote either excitatory or inhibitory synapses.

15.2.2 The Molecular Contents of Scaffolding Proteins in the PSD

Quantification of proteins in biochemically purified PSD preparations can effectively estimate the relative abundances of multiple proteins. One problem associated with this method is the possibility that the purification steps induce the

extraction of weakly associated proteins and/or nonspecific binding of proteins from other cellular compartments. To avoid this possibility, it is important to develop a method to quantify the local protein contents in single synapses. One possible method for obtaining measurements of local protein contents is the use of green fluorescent protein (GFP)-tagged PSD scaffolding proteins (Okabe 2013; Okabe et al. 1999, 2001). If one could estimate the number of GFP-tagged proteins in a single synapse, the number of native proteins could be deduced from the ratio of endogenous and GFP-tagged proteins as determined by immunolabeling (Sugiyama et al. 2005). GFP-based measurements of scaffolding protein contents in cultured hippocampal neurons revealed that a single postsynaptic site contained an average of 273 PSD-95 family proteins, 171 GKAP proteins, 310 Shank family proteins, and 343 Homer family proteins (Fig. 15.6). The estimated MAGUK protein content per synapse agrees well with the number of PSD-95 proteins determined from scanning transmission EM measurements of the average PSD mass and quantitative immunoblotting (Chen et al. 2005). This GFP-based quantification method also revealed similar concentrations of the four PSD scaffolding proteins per synapse, suggesting a relatively simple stoichiometry. The total mass of the four scaffolding proteins corresponds to 120 MDa, or 10 % of the total PSD mass, suggesting the importance of the four scaffolding proteins in the PSD structural framework (Okabe 2007).

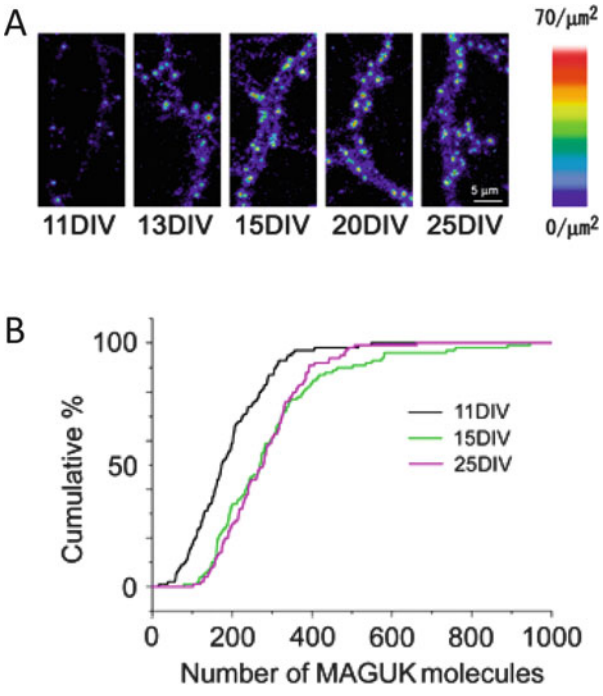

Fig. 15.6 Developmental changes in scaffolding protein numbers at single postsynaptic sites (Sugiyama et al. 2005). (**A**) Developmental changes in the Shank family protein. The *color* code indicates molecular density. (**B**) Developmental shift in the number of MAGUK proteins (PSD-95 family proteins) at single postsynaptic sites. Profiles range from 11 to 25 days in vitro (DIV)

15.3 Molecular Assembly and Synapse Development

15.3.1 Formation and Maturation of Dendritic Filopodia

Immature neurons express highly motile filopodia that protrude from dendritic shafts (Portera-Cailliau et al. 2003). Most dendritic filopodia are transient, and only a small proportion may stabilize and begin to form synaptic contacts (Okabe et al. 2001). There is an ongoing debate whether dendritic filopodia are a single entity or a mixture of protrusions with different properties and final fates (Portera-Cailliau et al. 2003). At minimum, a fraction of dendritic filopodia can serve as a spine synapse precursors. Actin filaments within dendritic filopodia are not organized into bundles but form meshworks of branched and linear actin filaments (Korobova and Svitkina 2010). In contrast, conventional filopodia in fibroblasts contain tightly bundled actin filaments (Okabe and Hirokawa 1989; Svitkina et al. 2003). This difference in actin organization may be of importance to specifying the sites of new actin polymerization within filopodia and could support more complex motility of these dendritic filopodia (Hotulainen et al. 2009).

Spine synapses may arise from interactions of motile filopodia with axons. Another possible route of excitatory synapse formation is direct contact between axons and dendritic shafts and the subsequent induction of dendritic spines at the sites of contact. The first model stresses the importance of active environmental scanning by filopodia (filopodial model) (Fiala et al. 1998), and the second model (Miller–Peters model) is based on EM observations and the categorization of nascent synapses in vivo (Miller and Peters 1981). When the order of appearance of the dendritic filopodia and postsynaptic molecular assembly was analyzed in a dissociated culture of neurons, filopodial formation generally preceded the acquisition of postsynaptic molecular assembly (Friedman et al. 2000; Okabe et al. 2001; Ziv and Smith 1996). However, imaging experiments of slice preparations revealed the presence of an alternative pathway in which protrusive dendrite activity occurs at the site of postsynaptic differentiation, with the subsequent stabilization of these structures as spine synapses (Marrs et al. 2001). These experiments involving dissociated neurons and slice preparations suggest the presence of two alternative spine differentiation developmental pathways. To detect the order of spine and synapse differentiation in a native tissue environment, an in vivo two-photon microscopic analysis of synapse development should be performed. Given the technical difficulties, reliable monitoring of synapse and spine formation in the early postnatal neocortex has not yet been accomplished. The adult mouse neocortex is a less challenging target for in vivo spine imaging, and in vivo imaging experiments combined with retrospective serial section EM have revealed that newly formed dendritic protrusions required maintenance without axonal interaction for 2 days before the gradual differentiation of synaptic junctions (Knott et al. 2006). This observation supports the filopodial model of neocortical synapse formation. It has not yet been demonstrated whether the same order of differentiation applies to synapses formed during early developmental stages.

15.3.2 The Recruitment of Cell Adhesion Molecules and Scaffolding Molecules to Synaptic Contacts

Without proper target recognition, appropriate synaptic contacts cannot be formed. Target recognition is mediated by molecular cues present on presynaptic and postsynaptic cell membranes. Cell adhesion molecules are considered the most important molecular cues for synaptic target recognition (Benson and Huntley 2012). Both homophilic and heterophilic interactions between cell adhesion molecules have been shown to be involved in synapse formation. Interactions between cell adhesion molecules are important to both the structural differentiation of synapses and determination of synapse subtypes and specificities.

Using assays that induced artificial synaptic structures between naïve neurons and nonneuronal cells expressing cell adhesion molecules, several cell adhesion molecules were identified as synapse organizers, molecules that can induce either presynaptic or postsynaptic structures in neurons (Krueger et al. 2012; Tallafuss et al. 2010). Neuroligins were the first identified synapse organizers (Scheiffele et al. 2000). These are heterophilic postsynaptic cell adhesion molecules that interact with presynaptic partner neurexins. Neurexins expressed on nonneuronal cells can induce postsynaptic differentiation in dendrites within a few days (Graf et al. 2004). The artificial postsynaptic sites contained a variety of postsynaptic molecules, including PSD-95, GKAP, and NMDA receptors (Graf et al. 2004; Nam and Chen 2005). Surface AMPA receptors are also recruited to the sites of neuroligin clusters via interactions with PSD-95 (Mondin et al. 2011). These results are consistent with the idea that postsynaptic neuroligins control molecular assembly at the postsynaptic sites via interactions with presynaptic neurexins. Interactions between neurexin and neuroligin at nascent synapses will trigger the simultaneous differentiation of both presynaptic and postsynaptic structures, thus synchronizing the differentiation process. Cultured immature cortical neurons exhibit the rapid recruitment of fluorescent protein-tagged NL1 clusters to sites of axodendritic contact (Barrow et al. 2009). Mobile NL1 clusters were also present in both dendritic shafts and filopodia in these immature neurons, suggesting vesicle-mediated NL1 transport. Rapid vesicle-mediated transport and local recruitment of neuroligins may represent a general strategy by which immature dendrites deliver sufficient amounts of synaptic cell adhesion molecules to local sites of contact with incoming axons.

The molecular interactions that enable neuroligin-induced postsynaptic differentiation have been studied using fluorescently tagged postsynaptic molecules (Giannone et al. 2013; Mondin et al. 2011). Neuroligins contain a C-terminal PDZ domain-binding motif that can bind to the PDZ domain of PSD-95. NL1 clusters that had been induced by antibodies against the extracellular epitope HA tag could not effectively induce subsequent intracellular PSD-95 clustering, in contrast to NL1 clusters induced by cross-linked neurexin 1β. This result indicates that neurexin binding facilitates interactions of clustered NL1 with PSD-95. Tyrosine phosphorylation of NL1 reduces its affinity for gephyrin, a scaffolding protein

found in inhibitory synapses, and allows the preferential binding of NL1 to PSD-95. This tyrosine phosphorylation-regulated competition between PSD-95 and gephyrin may underlie the effective clustering of PSD-95 mediated by NL1–neurexin interactions.

15.3.3 The Recruitment of Glutamate Receptors to Synaptic Contacts

Imaging of cultured immature hippocampal neurons revealed the presence of two distinct populations of postsynaptic structures (Gerrow et al. 2006). One was mobile non-synaptic complex of multiple scaffolding proteins, including PSD-95, GKAP, and Shank, but not NL1. The other was stationary and also contained PSD-95, GKAP, and Shank as well as NL1. Several imaging studies reported the presence of mobile postsynaptic packets containing key receptor and scaffolding molecules and postulated their importance in terms of the supply of molecules needed for postsynaptic functions (Barrow et al. 2009; Washbourne et al. 2002, 2004). These studies also indicate key roles of neuroligin and similar synaptic cell adhesion molecules in the initial synapse differentiation.

The subsequent recruitment of glutamate receptors might be an important role played by neuroligins and associated scaffolding molecules within synapses. A clustered complex of NL1 and PSD-95 was shown to be effective in the recruitment of mobile AMPA receptors to dendritic surfaces (Mondin et al. 2011). It was also shown that AMPA receptor recruitment was mediated by the AMPA receptor auxiliary subunit transmembrane AMPA receptor regulatory proteins (TARPs) (Opazo et al. 2010). These experiments suggest that the accumulation of AMPA receptors at nascent synapses is mediated by intracellular interactions between NL1, PSD-95, and an AMPA receptor complex that includes TARPs. When NL1 is overexpressed in cultured neurons, the relative contents of AMPA and NMDA receptors, which are estimated from excitatory postsynaptic currents at different membrane potentials, shift toward a higher NMDA receptor content, suggesting a more direct impact of the NL1 abundance on NMDA receptor recruitment to excitatory synapses (Budreck et al. 2013). This observation can be explained by the direct interaction of the extracellular domain of NL1 with the GluN1 subunit of the NMDA receptor. Enhanced NMDA receptor clustering at synapses, which was mediated by the overexpression of NL1, was shown to be independent of the presence of PSD-95. This further supported the presence of interaction domains distinct from the C-terminal PDZ domain-binding motif of NL1. In summary, the intracellular and extracellular motifs of neuroligin molecules coordinate during nascent postsynaptic molecular assembly, which includes both AMPA receptors (Giannone et al. 2013; Mondin et al. 2011) and NMDA receptors (Bard et al. 2010; Budreck et al. 2013). The molecular assembly induced by other synapse organizers likely utilizes strategies similar to those identified in neuroligin-dependent

postsynaptic organization. Further experimental evidence is required to confirm this point.

Glutamate receptor recruitment to synapses should be regulated by the transport of glutamate receptor-containing vesicles. In the case of NMDA receptors, NMDA receptor-containing vesicle transport was successfully visualized in cultured immature neurons (Washbourne et al. 2002). NMDA receptor recruitment to newly generated synapses is a rapid process, but the appearance of AMPA receptors on nascent synapses is delayed and may be initiated by the activation of NMDA receptors. AMPA receptor recruitment to synapses may be regulated by a process analogous to that of long-term potentiation (Ashby and Isaac 2011; Isaac et al. 1995). AMPA receptors may either be exposed to the surfaces of dendritic shafts and subsequently translocate along the plasma membrane into spines (Triller and Choquet 2008) or might be directly transported into spines via AMPA receptor-containing vesicles with subsequent exposure to the spine surface via local exocytosis (Patterson et al. 2010). In both surface receptor recruitment and local exocytosis, interactions of the C-termini of AMPA receptors with scaffolding molecules and regulation by posttranslational modifications play important roles (Bats et al. 2007; Kim et al. 2007; Opazo et al. 2010; Steiner et al. 2008; Xu et al. 2008).

15.3.4 Actin Organization and Dynamics in Spines

Dendritic spine morphology changes continuously (Majewska and Sur 2003; Majewska et al. 2006). Newly generated spines tend to be smaller and have less prominent heads. Older spines tend to be larger, with prominent heads (Yasumatsu et al. 2008). Spine morphology and life spans can be studied at better spatial and temporal resolutions in dissociated cultured neurons. Such studies have revealed rapid morphological changes in spines on the order of minutes, as well as the dependence of spine structural changes on the actin cytoskeleton (Fischer et al. 1998). Actin polymerization also drives structural changes in the PSDs (Blanpied et al. 2008). Partial PSD scaffold disassembly can be induced by actin depolymerization (Kuriu et al. 2006). Thus, both spine morphology and PSD molecular assembly are regulated by actin dynamics.

Spine maturation and stability may be closely related to the dynamic state of actin polymers. As described in Sect. 15.1.4.1, the orientations of actin filaments in dendritic protrusions are less organized than those in the filopodia of nonneuronal cells (Korobova and Svitkina 2010) (Fig. 15.7). To clarify the precise organization and dynamics of actin polymers, it is necessary to develop techniques that can monitor the state of actin polymers in the small volume of spine cytoplasm. Two-photon activation of photoactivatable (PA)-GFP-labeled actin provided evidence of retrograde actin flow in the spine head (Honkura et al. 2008). This finding indicates the addition of new actin monomers at the distal and peripheral domains of the spine head and subsequent filament treadmilling. Super-resolution imaging (PALM/STORM) of single actin molecules confirmed the presence of retrograde

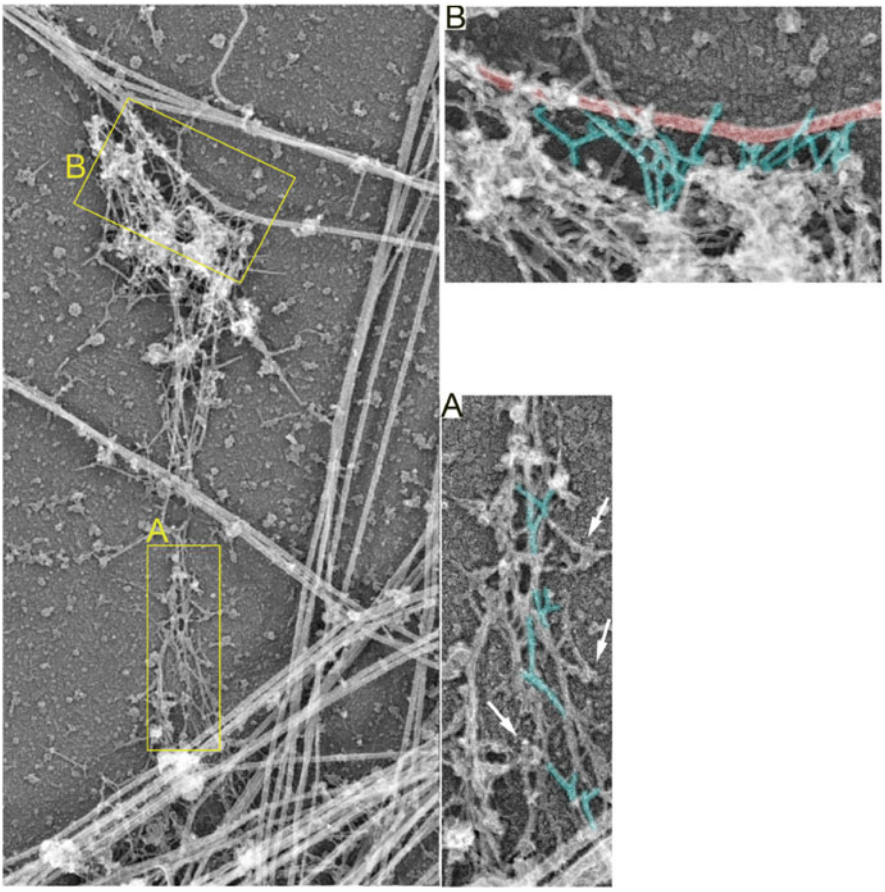

Fig. 15.7 Platinum replica electron microscopic images of a dendritic spine (Korobova and Svitkina 2010). Detergent extraction revealed cytoskeletal organization within the cytoplasm. *Yellow* boxes (**A**) (spine neck) and (**B**) (spine head) correspond to enlargements of images (**A**) and (**B**). Image B shows associations of actin filaments within a spine head (*cyan*) with an axonal microtubule (*red*). Image A illustrates branched actin filaments (*cyan*) in the spine neck. *Arrows* indicate putative filament ends

actin flow in the spine heads (Frost et al. 2010; Tatavarty et al. 2009). PALM imaging revealed that the velocities of individual actin molecules were heterogeneous and specifically enhanced in the vicinity of PSDs (Fig. 15.8). The heterogeneous actin movement and relatively short distance of the net actin flow are consistent with the idea that short actin filaments, with a less aligned orientation, form the main actin network within the spines.

Further subdomain-specific actin organization and dynamics were proposed following the super-resolution imaging of actin regulatory molecules (Chazeau et al. 2014). The formation of a branched actin network is driven by the Arp2/3

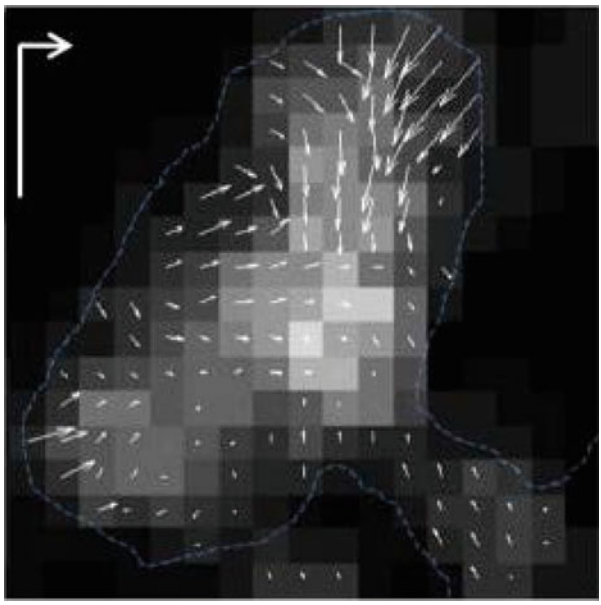

Fig. 15.8 PALM imaging of actin molecules in a spine (Frost et al. 2010). *Arrows* indicate the directions and relative velocities of single actin molecules tagged with photoactivatable fluorescent protein mEos2. The *gray* scale indicates the density of moving single particles. *Vertical scale bar*, 200 nm and *horizontal vector*, 100 nm/s

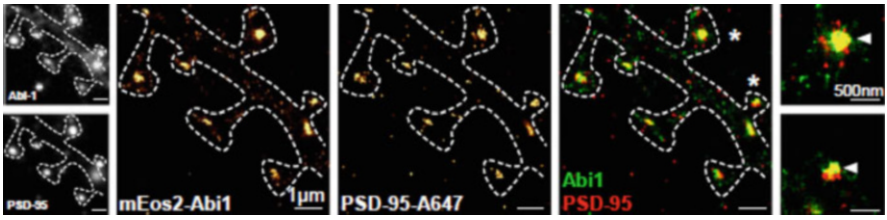

Fig. 15.9 Dual color super-resolution PALM images of mEos2-tagged Abi1 (a component of WAVE complex) and dSTORM images of endogenous PSD-95 labeled with Alexa647 (Chazeau et al. 2014). *Insets at left* are diffraction-limited fluorescence images. *Insets at right* are merged images of Abi1 and PSD-95. Higher magnification of the spines marked by *asterisks* are provided (*arrowhead*)

complex, following activation by WAVE (Takenawa and Suetsugu 2007). Conversely, the formation of aligned F-actin bundles is driven by the nucleation of single linear filaments through interactions with formins (Kovar et al. 2006). If the formation of an actin meshwork occurs uniformly at the distal submembranous domains of spines, WAVE complex proteins should be located at both the PSD and non-PSD subdomains of spines. However, PALM imaging revealed specific confinement of the WAVE complex in the vicinity of the PSD, indicating the formation of an actin meshwork specifically at the interface between the PSD and adjacent cytoplasm (Figs. 15.9 and 15.10). On the other hand, actin regulators that associate

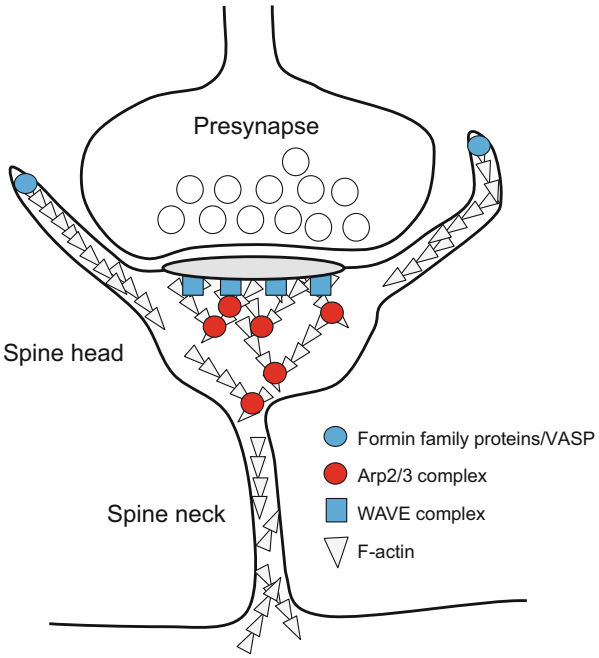

Fig. 15.10 A proposed model of actin filament organization and distribution of actin regulators in the spine cytoplasm. The WAVE complex is in the vicinity of the PSD and drives formation of an actin meshwork by activating the Arp2/3 complex. Actin regulators that associate with bundled actin filaments, such as formin family proteins and VASP, are preferentially associated with fingerlike protrusions from the spine head and regulate their dynamics. The barbed ends of F-actin correspond to fast-growing ends of these polymers

with bundled actin filaments, such as VASP and formin-like protein-2, were preferentially associated with fingerlike protrusions from the spine head. These observations indicate the nanoscale confinement of actin regulators within spines and the distinct roles of these regulators in spine morphology.

15.3.5 Spine Stability and Molecular Dynamics

Studies involving new optical techniques have revealed the precise regulation of actin dynamics within spines; however, the relationship between molecular dynamics and spine stability has not yet been clarified. The actin meshwork within spines is highly dynamic, with a half-life in minutes, at least in cultured neurons and organotypic slices (Honkura et al. 2008; Star et al. 2002). On the other hand, in the mouse neocortex, spines on pyramidal neurons can be maintained for more than several months (Grutzendler et al. 2002; Zuo et al. 2005). Thus, the system that

regulates spine morphology should be designed to support long-term structural stabilization based on highly dynamic polymers. The finding that the PSD can function as a site of WAVE recruitment may provide information to fill the gap between spine stability and actin turnover, as the size of the PSD or, more specifically, the presence of specific WAVE-interacting partners within the PSD can regulate the speed of actin meshwork generation within spines (Chazeau et al. 2014). This model suggests that spines with prominent PSDs should have larger amounts of actin polymers within a meshwork-like architecture. Indeed, there is a gradual increase in the concentration of PSD scaffolding molecules during development (Sugiyama et al. 2005). This increase may underlie the transition in spine actin organization, which in turn regulates the overall shapes of spines. This model also explains how initial cell-to-cell contacts and PSD scaffold assembly can shift spine actin polymers from simple bundles to meshwork-like organizations (Okabe et al. 2001).

If actin organization in spines is regulated by the PSD, the next question asks how PSDs and spine stability are related. In vivo imaging of neocortical spine turnover revealed the life spans of spines. Most spines in the mature neocortex are stable, as only 5 % of spines are formed and eliminated (Grutzendler et al. 2002; Zuo et al. 2005). A comparison of the morphologies of stable and dynamic spines revealed that larger spines tend to be more stable (Holtmaat et al. 2005). In vivo imaging of spines, together with fluorescently tagged PSD-95, revealed that spines containing PSD-95 clusters were more stable (Fig. 15.11 (Cane et al. 2014; Isshiki et al. 2014). However, the relationship between spine stability and the amount of PSD-95 within spines is not straightforward (Cane et al. 2014). PSD-95-containing spines generally exhibited increased stability, but newly generated spines rarely

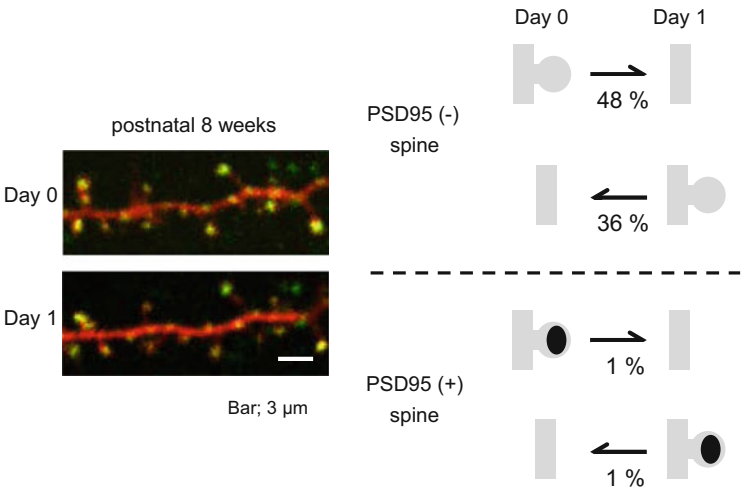

Fig. 15.11 In vivo imaging of mouse neocortical pyramidal neurons expressing DsRed and PSD-95–GFP at postnatal 8 weeks (*left*), at an interval of 24 h. Fractions of spine gain and loss in either PSD-95-negative or PSD-95-positive spines are shown

converted into persistent spines even if they had acquired PSD-95 assembly. This finding may indicate the presence of additional stabilization factors. On the other hand, a reduction in the PSD-95 content in spines could be detected well before the occurrence of spine pruning, indicating that a change in the PSD-95 content predicts the spine fate. These reports are consistent with the idea that the PSD scaffolds are important in spine stabilization.

Although in vivo imaging indicates the possibility that PSD scaffolds are important to spine stability, an in-depth understanding will require additional experimental evidence for a relationship between PSD scaffolds and actin dynamics. A recent analysis of WAVE-interacting partners identified several postsynaptic molecules, such as NL1 (Chen et al. 2014). It should be important to test whether actin dynamics and spine stability can be affected by mutations in the WAVE-interacting motifs of PSD molecules. Cortactin, which is enriched in PSDs, interacts with the major PSD scaffolding molecule, Shank, and can initiate branched actin polymer formation by recruiting the Arp2/3 complex (Hering and Sheng 2003; Iki et al. 2005). Although cortactin knockdown experiments revealed a strong spine phenotype, the detailed analyses of cortactin function within spine actin organization have not yet been performed. The regulation of actin meshwork generation by WAVE and cortactin at the interface between PSD scaffolds and the adjacent cytoplasm will be critical to our understanding of actin dynamics in spines.

15.4 Future Prospects

In the previous chapters, we described the quantitative properties of excitatory postsynaptic specialization and the developmental time course. An integrated view of the molecular interactions that regulate excitatory synapse development should be proposed using the accumulated data from synaptic molecules and their dynamics. However, this task remains difficult, and there are few proposed models of postsynaptic molecular assembly. Although biophysical models of molecular dynamics within spines have been constructed, their main focus was an explanation of glutamate receptor behaviors in the resting and activity-dependent states (Czondor et al. 2012; Earnshaw and Bressloff 2006). Theoretical models of actin polymer organization have been developed in several biological systems (Pollard et al. 2000). The relationship between local actin meshwork assembly and force generation has been extensively studied using the actin tail formation model system in the intracellular bacterial pathogen *Listeria monocytogenes* (Cameron et al. 2000). From these analyses, Mogilner and Oster proposed a "tethered ratchet" model in which the sequential events of actin branching, dissociation of new filaments from the load surface, and filament bending contribute to force generation (Mogilner and Oster 2003). At the interface between the PSD and adjacent spine cytoplasm, branched actin network formation may occur based on a similar molecular mechanism. In spines, the PSD structure is mechanically fixed by interactions

with the presynaptic membrane. The elastic force created by actin polymerization may be transmitted to the spine plasma membrane outside of the PSD and contribute to changes in spine morphology. The overall organization of the actin meshwork should also depend on the rate of actin filament capping and cofilin-induced severing (Calabrese et al. 2014; Pontrello et al. 2012). To construct a realistic model, the application of a particulate-based model that simulates the behaviors of actin and actin-related proteins within spines may be required (Inoue et al. 2011). Quantitative optical measurements of multiple actin-related molecules will be required to achieve this, as comprehensive data regarding the dynamics of actin-related molecules in spines remain lacking. Modeling of actin dynamics in both extended space and time presents another challenge that will require new strategies for the integration of microscopic Brownian dynamic modeling (Yamaoka et al. 2012) and macroscopic modeling using ordinary differential equations, partial differential equations, or stochastic differential equations (Gardel et al. 2004). Imaging and computational technologies are developing rapidly, and we expect that the modeling of molecular dynamics within dendritic spines may be realized in the near future.

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