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Signaling Between the Actin Cytoskeleton and the Postsynaptic Density of Dendritic Spines

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ABSTRACT: The dendritic spine may be considered a fusion of a specialized actin-based structure akin to filopodia and lamellopodia, with an excitatory postsynaptic density containing glutamate receptors and signal-transducing machinery. This specialized neuronal microdomain is the site of the majority of excitatory synaptic contacts in the mammalian brain. Regulation of spine morphology, composition, and stability are likely to contribute to long-lasting changes in synaptic efficacy. Thus, understanding the function and regulation of dendritic spines is a fundamental problem ranging from molecular through behavioral neurobiology. A complete understanding of dendritic spines will require a knowledge of all the molecular components and how these components interact. Here we wish to accomplish two goals: to catalog many of the known components of hippocampal dendritic spines and suggest how these may contribute to spine function; and to compare dendritic spines with other actin-based structures, namely lamellopodia, filopodia, microvilli, and stereocilia, to gain some insight into possible common vs. specialized mechanisms of regulation of the shape, motility, and longevity of these actin-based structures. *Hippocampus 2000;10:527–541.* © 2000 Wiley-Liss, Inc.

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PROPOSED FUNCTIONS OF SPINES

The spine is suggested to act as a biochemical compartment which isolates subsynaptic activity-driven calcium ion flux from the rest of the dendrite and therefore also from other nearby synapses (Muller and Connor, 1991; Koester and Sakmann, 1998; Mainen et al., 1999). The restriction of the calcium pulse to the spine is believed to allow localized signaling cascades, which act on spine constituents to induce local modifications of spine structure and composition. Thus the history of activity through the synapse could be stored as a change in the morphology or existence of the spine, or of its molecular composition, i.e., the spine could act as a local substrate to store temporally patterned activity as changes in spine constituents or morphology.

Recent dynamic imaging of spines indicates that they can be relatively stable entities over the time frame of hours (Korkotian and Segal, 1999) but undergo rapid changes in shape over seconds, displaying side-to-side and contractile wiggling movements, both of which are dependent on the actin cytoskeleton (Fischer et al., 1998; Dunaevsky et al., 1999). Manipulations of electrical activity have an effect on spine number as well as on the rapid actin-dependent movements of spines (Halpain et al., 1998; Engert and Bonhoeffer, 1999; Kirov and Harris, 1999; Maletic-Savatic et al., 1999; McKinney et al., 1999; Toni et al., 1999). Further, increased activity can induce rapid changes in AMPA receptor levels that are localized specifically to the activated synapses (Shi et al., 1999). This localized increase in receptor levels has been suggested to occur due to local insertion of receptor, specifically at the activated synapse, through the calcium-induced activation of calcium-calmodulin-dependent kinase II (CaMKII; Hayashi et al., 2000) and increased exocytosis of dendritic Golgi-derived vesicles (Maletic-Savatic and Malinow, 1998). Effects of actin polymerization inhibitors on synaptic physiology indicate that postsynaptic actin filaments are involved in the maintenance of AMPA receptor-mediated transmission and its enhancement following tetanization (Kim and Lisman, 1999). Actin filaments are also required to maintain postsynaptic AMPA receptor clusters (Allison et al., 1998). These results suggest that AMPA receptors may be stabilized by association with the actin cytoskeleton or, based on recent evidence for rapid AMPA receptor cycling at synapses (Luscher et al., 1999; Noel et al., 1999), that AMPA receptor-containing vesicles may traffic partially along actin filaments. Other components of dendritic spines, including NMDA receptors (Rao and Craig, 1997), CaMKII (Shen and Meyer, 1999), and cortactin (Naisbitt et al., 1999), can undergo activity-regulated changes in synaptic attachment, although it is not yet known whether these effects occur only globally or at the level of individual spines. In summary, considering these three modes of activity-dependent change in spines (in number, shape, and molecular composition), all may be based to some extent on the actin cytoskeleton of the spine.

THE SPINE IS AN ACTIN-RICH STRUCTURE

In attempting to determine what molecules serve to differentiate the spine from the dendrite, an obvious dif-

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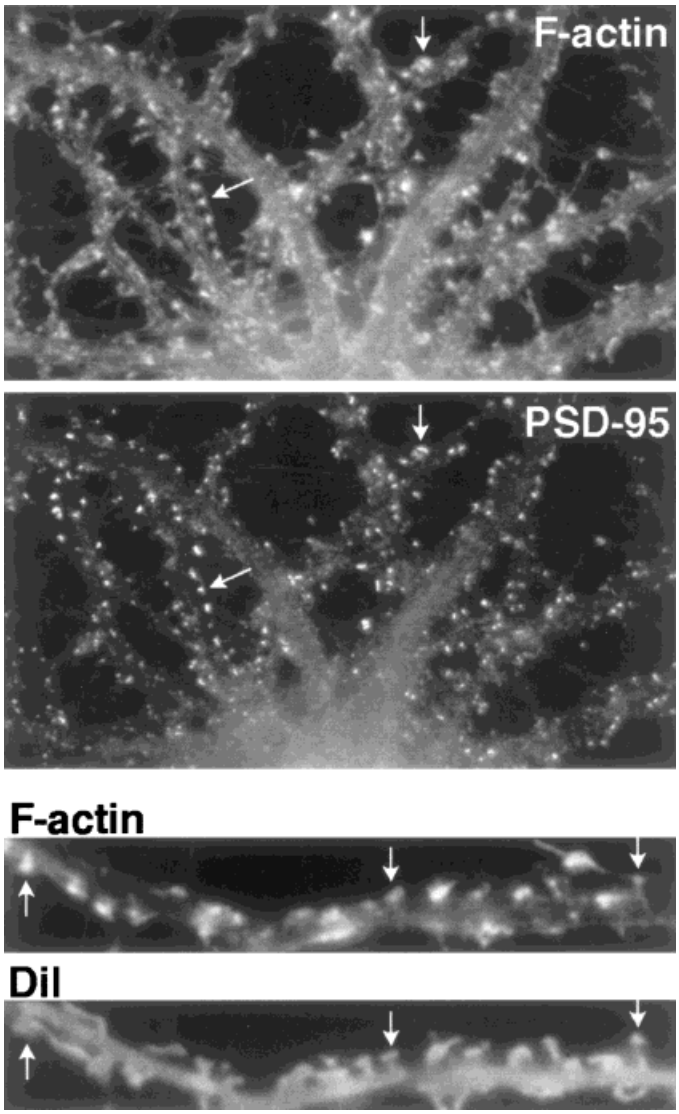

FIGURE 1. Upper: Dendritic spines are visualized on a cultured hippocampal neuron by double-labeling with rhodamine-phalloidin for filamentous actin (F-actin) and immunofluorescence for the excitatory postsynaptic scaffolding molecule PSD-95. Dendritic spine heads appear offset from the dendrite shafts, are rich in F-actin, and contain concentrations of PSD-95. Lower: Visualization of dendritic spines by application of the lipophilic dye DiI to subsets of neurons, imaging of the labeled dendritic profile, and subsequent fixation, permeabilization, rhodamine-phalloidin staining, and imaging of the same dendrite region for F-actin. DiI reveals the spine neck and spine head profile. The spine heads are enriched in F-actin. Images courtesy of Daniel W. Allison.

ference in the two compartments is apparent in the cytoskeletal content. Light and electron microscopic analyses show an overall enrichment of actin in the spine compared to the rest of the dendrite (Matus et al., 1982; Cohen et al., 1985). Labeling with fluorescent phalloidin to label only F-actin reveals a striking punctate staining at spiny synaptic sites (Fig. 1) (Drenckhahn et al., 1984; Wyszynski et al., 1997). Using antibodies specific for actin isoforms, β and γ actin appear to be specifically enriched in spines (Kaech et al., 1997; Micheva et al., 1998). Electron microscopic analyses show that the actin microfilaments are organized into a meshwork in the head of the spine and in longitudinal bundles within the spine neck (Fig. 2F) (Fifkova and Delay, 1982; Landis and Reese, 1983; Hirokawa, 1989). Decoration with the S1 fragment of myosin to reveal filament polarity showed that some actin filaments in the spine head radiated from the membrane and were attached to the postsynaptic density (PSD) or the plasma membrane by their barbed or fast-growing ends (Fifkova and Delay, 1982; Markham and Fifkova, 1986). Other actin filaments were parallel to the PSD and attached to it by fine, periodically occurring strands. Actin filaments in the spine head were extensively branched. In spines containing a spine apparatus, filaments were attached to the spine apparatus, and tended to radiate out from it with mixed polarity. In the spine neck, in contrast, bundles of longitudinally oriented actin filaments with their long axis parallel to that of the spine were seen (Fifkova and Delay, 1982; Landis and Reese, 1983; Hirokawa, 1989). In the neck of mushroom-shaped spines, S1 decoration showed bundles of actin filaments of uniform polarity with their barbed fast-growing ends towards the spine head, but it was unclear whether bundles of mixed polarity were also present (Fig. 2F) (Fifkova and Delay, 1982). In "sessile" or stubby spines, which had no clear demarcation between head and neck, the actin filament bundles were sometime seen to extend from the PSD, where the barbed end was attached, and into the dendrite shaft (Fig. 2E) (Markham and Fifkova, 1986).

Rapidly changing actin microfilament structures at the cell periphery are implicated in the growth and motility of many cell types (Welch et al., 1997; Carlier, 1998). Thus lamellopodia at the leading edge of fibroblasts, and filopodia and lamellopodia on neuronal growth cones, both sites of growth and movement, are also rich in actin microfilaments which are necessary for their activity. Paradoxically, organized arrays of actin microfilaments can also act as the primary cytoskeletal support of stable membrane protrusions from the cell periphery, such as hair cell stereocilia and epithelial microvilli (Bartles, 2000). The dendritic spine is both stable for long periods of time and capable of rapid changes in form, to the extent of branching to form multiple appositions or disappearing entirely in response to differences in activity levels. Thus the actin cytoskeleton of the spine must combine the stable characteristics of the cytoskeleton of stereocilia and microvilli with the dynamism of actin in lamellopodia and filopodia.

ACTIN NETWORKS AND THE MORPHOGENESIS OF LAMELLOPODIA, FILOPODIA, MICROVILLI, AND STEREOCILIA

Lamellopodia contain an actin filament meshwork very similar in appearance to that described within the spine head (Fig. 2A), while filopodia have longitudinally oriented actin bundles like those in the spine neck (Fig. 2B). In both of these specializations the actin microfilaments are subject to retrograde flow away from the direction of growth simultaneously with actin polymerization

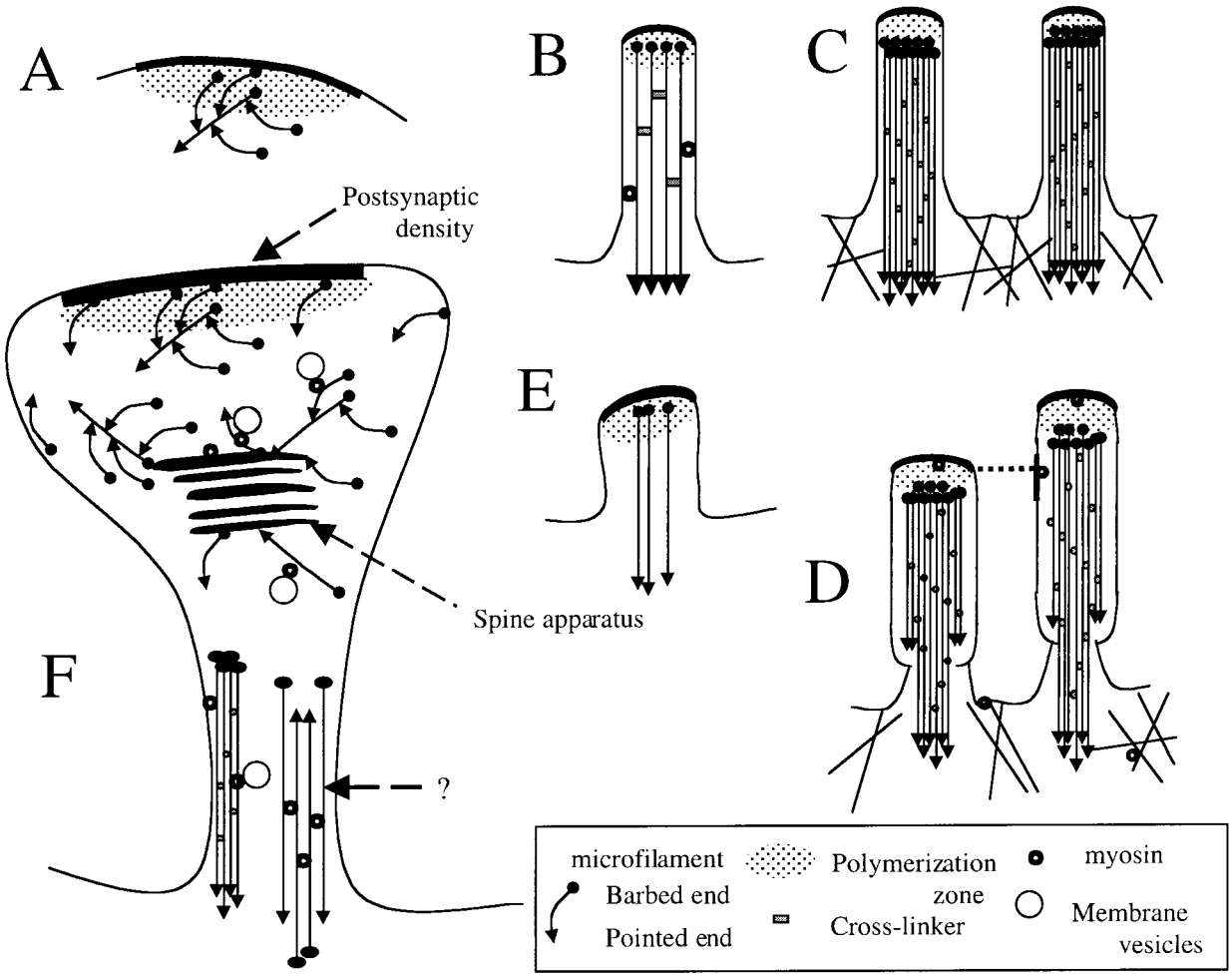

FIGURE 2. Comparison of actin cytoskeleton in spines with other actin-rich organelles. A: Lamellopodium. A branched actin network radiates from the leading edge of the lamellopodium, with many microfilament barbed ends in the zone that favors polymerization. B: Filopodium. Actin microfilaments are bundled with their barbed ends attached to the tip of the filopodium, in a zone that favors polymerization. The retrograde movement of the bundle down the filopodial membrane towards the growth cone may be achieved by the action of unconventional myosin motors. C: Microvilli. Uniformly polarized microfilaments are bundled closely by the cross-linkers villin, fimbrin/plastin, and espin. The bundle is embedded at its base in a meshwork of microfilaments. D: Stereocilia. The microfilament bundle is again closely packed and bundled with the barbed ends attached to the tip. Submembrane specializations at the tip and along the sides of microvilli are the attachment points for extracellular fibrils that connect adjacent microvilli. Unconventional myosins at the tip and side plaques as well as in the cuticular web at the base of the stereocilia are important in morphogenesis and function of the sterocilia. E: Sessile spine. Microfilaments with their barbed ends attached near the postsynaptic density can extend down into the dendrite shaft. F: Mushroom spine. Microfilament arrangements in the neck and spine head are different. The branched networks seen in the spine head resemble the lamellopodial arrangement, while the uniformly polarized bundles in the neck are similar to filopodia and microvilli. It is not known if bipolar filaments are present in the neck that might contribute to actomyosin-based contractility. Microfilaments radiate both to and from the spine apparatus. Myosin motors may be involved in membrane trafficking to and within the spine, as in stereocilia.

at the leading edge of the membrane (Welch et al., 1997). Net polymerization results in forward membrane protrusion with respect to the substrate, whereas if the retrograde flow rate matches polymerization, the cell is stationary with respect to the substrate. The dynamic actin networks of lamellopodia and filopodia are necessary for their function as devices which couple sensing of environmental cues to directed movement and growth of the cell. Microvilli and stereocilia also have longitudinally oriented actin filament bundles like filopodia, but here the bundles are larger, extensively cross-linked, closely packed, and stable, and the length of the bundle is strictly regulated within a specific size range in a cell-specific manner (Tilney et al., 1992; Bartles, 2000; DeRosier and Tilney, 2000). The stable actin network of these two structures is important for their functions, in the case of the microvilli to increase absorptive surface, and for stereocilia to serve as the mechanosensory element of hair cells. In comparing the characteristics of these different actin networks with those in the dendritic spine, it may be useful initially to think of the spine neck as similar to a microvillus or a stationary filopodium and the spine head as a relatively stationary lamellopodium.

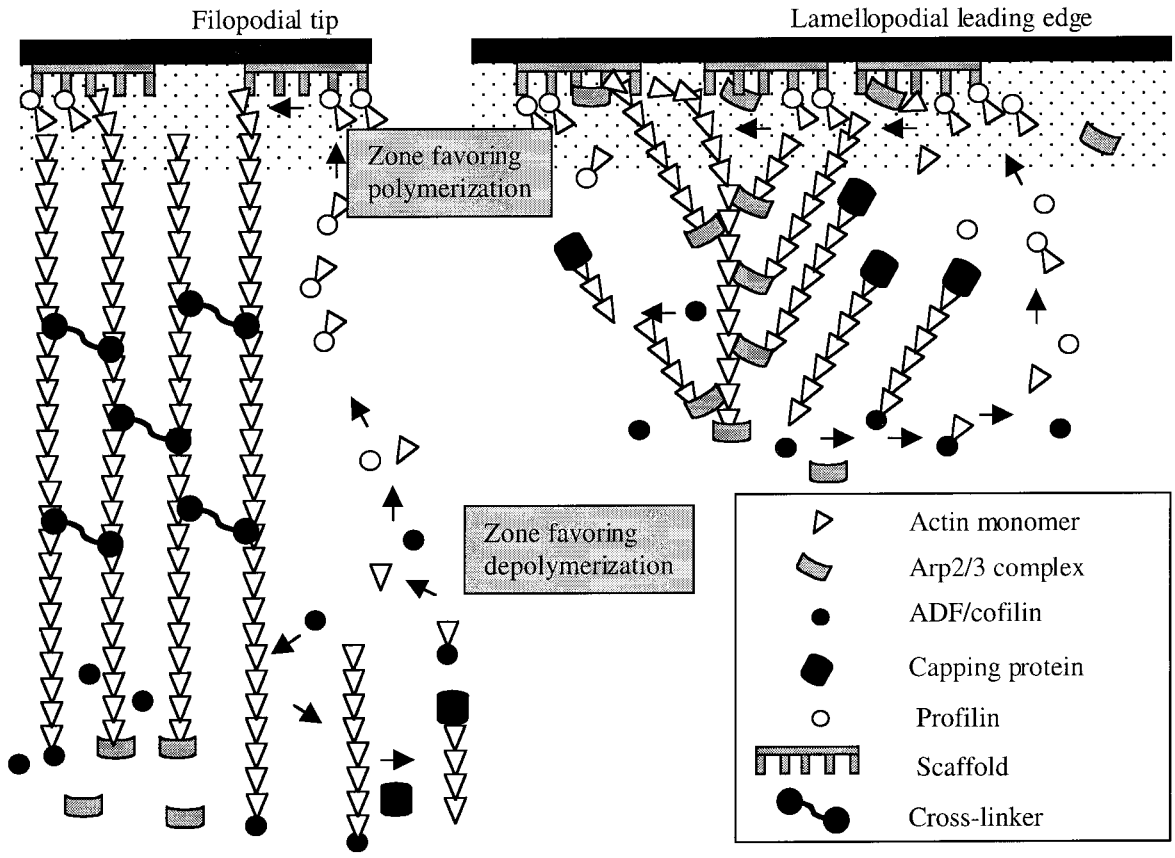

FIGURE 3. Models for actin microfilament polymerization/depolymerization in filopodia and lamellopodia (based on Svitkina and Borisy, 1999; Borisy and Svitkina, 2000).

Actin microfilament polymerization is regulated by a variety of proteins. Among these, the microfilament nucleating Arp 2/3 complex, the actin depolymerizing factor ADF/cofilin, the actin capping protein, and the actin monomer binding protein profilin are believed to be core components involved in actin filament dynamics in all systems (Loisel et al., 1999; Machesky and Cooper, 1999; Machesky and Gould, 1999; Borisy and Svitkina, 2000). Arp 2/3 functions mainly in filament nucleation, profilin in promoting extension from the barbed end, and ADF/cofilin and actin-capping protein in releasing actin monomers from filaments outside the favored zone to allow treadmilling of the network (Fig. 3).

Additional proteins that interact with these core components, and are differently regulated and localized in lamellopodia and filopodia, are believed to differentiate the filament dynamics that lead to protrusive activity in these organelles (Fig. 3) (Svitkina and Borisy, 1999). For both lamellopodia and filopodia, signaling from membrane receptors responding to growth factors and extracellular matrix (ECM) components directs growth through action on the Rho-family of small GTPases (Hall, 1998). Different sets of ligands activate different Rho-family GTPases and lead to the assembly of different actin arrangements. Activated Rho injected into fibroblasts induces stress fibers, activated Rac induces lamellopodia, and active Cdc42 induces filopodial protrusion. The active GTPases localize to the membrane and bind scaffolding proteins such as members of the Wiskott-Aldrich syndrome protein (WASP) family or the enabled/vasodilator-stimulated phosphoprotein (ena/VASP) family (Mullins, 2000). These scaffolding proteins have multiple binding sites for actin, profilin-actin, microfilaments, and the microfilament nucleating Arp2/3 complex, and their activation at the membrane results in filament nucleation and assembly.

Lamellopodia

In lamellopodia, the microfilament network is extensively branched and interconnected (Svitkina and Borisy, 1999) (Figs. 2A, 3). Constitutively activated Rac GTPase can induce lamellopodial formation when transfected into fibroblasts (Ridley et al., 1992). Active Rac is believed to bind a scaffolding protein concentrated at the leading edge of the lamellopodium. VASP is concentrated at the leading edge and may be the scaffolding protein involved (Rottner et al., 1999). This complex then activates the Arp2/3 complex, which binds to the side of existing actin filaments and nucleates the formation of new filaments there (Mullins et al., 1998). The new filament has its pointed end capped by the Arp 2/3 complex, and the barbed end grows rapidly due to association of actin monomers. This model of actin polymerization in lamellopodia was called "dendritic nucleation" because of the proposed role of side-bound nucleation sites in creating branched networks of microfilaments. Further branching requires additional Arp 2/3

binding to the sides of existing filaments and the nucleation of new filaments. Thus Arp2/3 is detected throughout the extent of the polymerizing filament network (Svitkina and Borisy, 1999). The model suggests that the entire branched network of microfilaments treadmills together, in contrast to former models involving treadmilling of individual filaments.

Filopodia

While little is known of the molecular constituents of dendritic filopodia, axonal growth cone filopodia and those on fibroblasts have been comparatively well studied (Figs. 2B, 3). In axonal growth cone filopodia, the core filament bundle consists of ~20 microfilaments which have their barbed ends attached to an electron-dense submembrane patch at the tip of the filopodium (Lewis and Bridgman, 1992). The bundle can be extremely long and extends into the body of the growth cone. Cdc42, a small GTPase of the Rho family, has been shown to induce de novo filopodial formation in fibroblasts via activation of N-WASP, a member of the WASP family (Miki et al., 1998). At the tip of the filopodium is a concentration of β1 integrin, members of the ezrin-radixin-moesin (ERM) family which link actin filaments to the membrane, and accumulations of phosphotryosine (Wu and Goldberg, 1993; Wu et al., 1996; Grabham and Goldberg, 1997; Goldberg et al., 2000). Also at the tip is a concentration of Mena (mammalian enabled), an ena/VASP family member that is localized at the filopodial tips of both axonal and dendritic growth cones and is suggested to favor polymerization at this site by its scaffolding activity for Arp2/3 and other core actin-binding proteins (Lanier et al., 1999; Goldberg et al., 2000). All of these components of the tip may affect the rate of actin polymerization and therefore of protrusion of the filopodium. Arp2/3 is believed to be needed for nucleation of the filaments in initiating filopodial formation, but cannot be necessary for further elongation of the filaments in the bundle since it is not localized along the length of filopodia (Svitkina and Borisy, 1999). An actin-filament cross-linking protein such as filamin, fascin, or α-actinin must bundle filaments in neuronal filopodia. Filamin is recruited into filopodia induced in fibroblasts by either Cdc42 or RalA, and is necessary for the formation of these filopodia (Ohta et al., 1999). The rates of filopodial growth and retraction appear to depend primarily on the rate of actin assembly into microfilaments, which occurs at the tip (Mallavarapu and Mitchison, 1999). An additional factor that affects the rate of movement is retrograde flow of the actin bundle, which may be regulated by myosin motors (Lin et al., 1996) and membrane attachments at the side of the actin bundle.

Microvilli

In the more stable microvillus specializations, as in filopodia, the core consists of a bundle of actin filaments of uniform polarity, all with their barbed ends toward the membrane facing a patch of submembrane electron-dense material (Fig. 2C) (DeRosier and Tilney, 2000). Three proteins are associated with the growing bundles: villin, fimbrin, and a small espin (Ezzell et al., 1989; Fath and Burgess, 1995; Bartles et al., 1998; Bartles, 2000). All three have actin-binding and -bundling ability in vitro. Villin and fimbrin have a calcium-sensitive actin-bundling ability, whereas espin is calcium-insensitive. Villin is first associated with the growing bundle, followed by fimbrin and then espin. Villin knockout mice still have microvilli, but these microvilli do not show the morphological changes in response to damaging stimuli shown by normal mice (Pinson et al., 1998; Ferrary et al., 1999). It is suggested that the calcium-insensitive espins hold together the microvilli actin bundles in the absence of villin.

Hair Cell Stereocilia

Stereocilia projections of hair cells of the inner ear sensory epithelium function in mechanoelectric transduction of sound-induced stimuli (Tilney et al., 1992). There is a gradient across the sensory epithelium in length of the stereocilia array, and strict control of the length and arrangement of the stereocilia is essential for hearing. Microfilaments in stereocilia are extremely compacted and cross-linked, probably by fimbrin/plastin and a novel form of espin (Bartles et al., 1998; Bartles, 2000). The study of some human hereditary deafness conditions has implicated unconventional myosins in the morphogenesis of stereocilia. The shaker 2 mutation is in myosin-XV and results in extremely short stereocilia (Probst et al., 1998), the myosin-VI mutation results in a disordered array of stereocilia which develop to normal lengths but eventually fuse together (Self et al., 1999). Recently a mouse transgenic missing a particular integrin subunit, α8, was found to lack stereocilia on hair cells in the utricle but not in other compartments of the inner ear (Littlewood Evans and Muller, 2000). This defect was asociated with the lack of localization of focal adhesion kinase (FAK) to the luminal surface of the hair cell in this compartment, suggesting that ECM signaling through this integrin and FAK to the actin cytoskeleton may be important in generating stereocilia. Another hereditary deafness condition, DFNA1, was found to be a mutation in a member of the formin gene family called Diaphanous in *Drosophila* (Lynch et al., 1997). Formin family proteins are effectors of Rho GTPase, contain multiple profilin binding sites, and can promote actin polymerization from a membrane attachment site (Tanaka, 2000). These mutation analyses suggest that control of the hair cell actin cytoskeleton through membrane receptors, Rho family GTPase activation, and effects on activation and localization of unconventional myosins as well as on actin polymerization are involved in generating the stereocilia.

COMPARISON OF DENDRITIC SPINES WITH OTHER ACTIN-RICH STRUCTURES

How does the spine compare with other actin-rich structures such as lamellopodia, filopodia, stereocilia, and microvilli (Fig. 3)? The actin filament arrangement in spines appears to combine elements of several of these structures. Filament bundles in larger spine necks may be like those in the filopodia or microvilli. Filaments radiating from the PSD into the dendrites of sessile spines resemble the filopodial arrangement of actin filaments. The

branched and networked filaments in the mature spine head resemble the arrangement seen in lamellopodia, with the barbed end attachment to the PSD suggesting that the PSD could act as a nucleation site for further growth of the network or to destabilize it. The leading edge of the lamellopodium, and the filopodial tip, both contain concentrations of ECM receptors (such as integrins) and polymerization scaffolding proteins (such as ERM and ena/VASP proteins), which correlate with the presence of an electron-dense submembrane thickening in these regions. Although these structures are transient on a much shorter time-scale than the PSD, the PSD and these membrane protein clusters may serve as similar foci for signaling from outside the cell to the actin cytoskeleton. Microvilli and stereocilia contain similar submembrane specializations at their tip into which the barbed ends of microfilament arrays are inserted. The tip region of stereocilia also contains concentrations of mechanosensitive calcium channels essential for hair cell function (Lumpkin and Hudspeth, 1995). Thus, all these organelles contain an actin cytoskeleton polarized around a membrane-associated "sensory" element: the leading edge of the lamellopodium, the tips of filopodia and stereocilia, and the PSD of the excitatory spine synapse.

Dendritic spines may also share some actin-regulatory proteins with these other organelles. Rho family GTPase signaling appears to be a key factor in forming lamellopodia and filopodia, and may also play a role in the genesis of microvilli and stereocilia. This form of signaling has also been implicated in regulating spine formation (see below); the upstream factors that activate Rho family GTPase signaling during spine formation are still to be identified. The scaffold proteins described above that enable actin filament polymerization at membrane attachment zones in lamellopodia and filopodia are similar in their ability to bind multiple regulators of the actin cytoskeleton, including membrane-associated proteins, as well as actin itself. It is unclear whether some proteins of the PSD share this ability, or whether the scaffolding proteins seen in other systems are present and serve a similar function in spines. The stability of the microvillar-type specializations may partly be attributed to the cross-linking proteins that hold the microfilaments together in tightly packed bundles. Fimbrin/plastin, one of the cross-linkers, is found in both microvilli and stereocilia, as is one of the espin family of proteins. A number of proteins that have in vitro actin cross-linking ability are known to be in spines and/or associated with the PSD; their role in spines is to be determined.

MOLECULAR CONSTITUENTS OF SPINE SYNAPSES

Due to the application of the yeast two-hybrid technique and the analysis of PSD-enriched subcellular fractions, there has been an explosive increase in the number of spine synapse-associated proteins identified over the last few years. In order to attempt an analysis of the roles of these proteins in spine formation and modification, we first compiled a list of those proteins for which there are strong data as to postsynaptic localization, grouped according to their putative functions at the synapse. Below, we describe the evidence for a role of these proteins in the formation and function of spine synapses.

Actin-Binding Proteins of the Spine Synapse

Many of the spine-enriched actin-binding proteins also function in the filopodia and lamellopodia of nonneuronal cells and neuronal growth cones, but are generally not found at nonspiny excitatory synapses. There are no currently known actin-binding proteins that are completely unique to spines; many of these proteins are found at adherens junctions and actin filament networks in several cell types. Further, it is very likely that actin-regulating protein families discovered in other cell types are present and possibly concentrated in spines. Thus we might expect to find that Arp2/3, ADP/cofilin, profilin, capping protein, WASP and ena/VASP family proteins, ankyrins, and other actin-regulatory proteins act in spines to change spine morphology in response to synaptic stimuli.

Of these common actin-regulating proteins, Rho family small GTPases do indeed regulate actin dynamics in dendrites and dendritic spines as they do in fibroblasts. Using a Purkinje cell-specific promoter, a transgenic mouse line expressing constitutively active Rac1 in Purkinje cells showed an increase in the number and decrease in the size of spines, as well as an increase in the appearance of spines with multiple synapses (Luo et al., 1996; Nakayama and Luo, this issue). In retinal ganglion cells, constitutively active Rac or dominant-negative Rho increased the rate and extent of motility of fine dendritic protrusions, whereas dominant-negative Rac or constitutively active Rho decreased it (Wong and Wong, 2000).

The mediators of Rho family GTPase function in dendrites are presently unknown; they may include members of the WASP and ena/VASP families, or maybe some of the synaptic scaffolding proteins already identified (see below). Of the other actin-binding proteins of spines, proteins such as α-actinin and drebrin seem likely, from their proposed functions in other cell types, to be the microfilament cross-linkers and regulators that rearrange the actin cytoskeleton, rather than part of the membrane-associated scaffold that polarizes actin rearrangements. Direct evidence for their activity in changing the spine cytoskeleton is currently limited to studies of the protein drebrin.

Drebrin is a developmentally regulated actin-binding protein that is expressed mainly in neurons in two alternatively spliced isoforms (Shirao et al., 1988). It binds to actin filaments with a 1:5 stoichiometry and strongly inhibits actin-binding of tropomyosin and α-actinin, the cross-linking activity of α-actinin, and actomyosin interaction. Drebrin also binds to profilin and forms a complex with myosins I, II, and V (Ishikawa et al., 1994; Hayashi et al., 1996). Overexpression of drebrin in fibroblasts induces actin rearrangement from stress fibers into a meshwork and the growth of dendrite-like protrusions (Shirao et al., 1992). Drebrin is strongly localized to spines in nervous tissue, and immunogold analyses show that it is distributed throughout the spine cytoskeleton, and not particularly concentrated in the PSD (Hayashi et al., 1996). The overexpression of drebrin in cultured cortical neurons for a

2-week period significantly increased spine length but had no effect on spine density (Hayashi and Shirao, 1999). It is unclear which of drebrin's many interactions with microfilament regulatory proteins is responsible for this effect.

α-actinin 2 is an actin-binding protein that also binds to the NMDA receptor NR1 and NR2 subunits (Wyszynski et al., 1997). It is present at spiny postsynaptic sites on pyramidal neurons but not at shaft excitatory postsynaptic sites on inhibitory interneurons (Rao et al., 1998; Wyszynski et al., 1998). Immunogold labeling shows that it is present at the PSD as well as in the body of the spine, and is associated with the spine apparatus. The actin-bundling activity of nonmuscle actinins in vitro is decreased by calcium and increased by PIP2, while NR1-α-actinin-binding is directly antagonized by calcium/calmodulin. All these features make α-actinin a good candidate for mediating synaptic activity effects on the actin cytoskeleton.

Myosins may be involved both in regulating actin microfilaments (Morales and Fifkova, 1989) and, together with synaptopodin (Mundel et al., 1997; Deller et al., 2000), in localizing membranous organelles in spines, where myosin V is present (Espreafico et al., 1992). This idea is supported by the effects of myosin mutations on stereocilia formation (as described previously), and more directly by the absence of smooth endoplasmic reticulum in Purkinje spines of the mouse myosin V mutant (Takagishi et al., 1996). Myosin light chain phosphatase as well as myosin light chain are Rho GTPase targets, and possible mediators of Rho catalyzed changes in the actin cytoskeleton (Hall, 1998). Activating Rho should therefore dramatically change the interactions of actin with the myosins present in spines.

Adducin, cortactin, and spectrin are actin-binding proteins of the submembrane cytoskeleton. Adducin phosphorylated in its MARCKS-domain (by PKC) is enriched in dendritic spines of hippocampal neurons in culture, while dephosphorylated adducin is uniformly distributed (Matsuoka et al., 1998). This phosphorylated form of adducin has a much lower ability to cap actin microfilaments at their barbed end and to recruit spectrin to actin filaments, so that phosphorylation may be a way to destabilize the actin cytoskeleton in spines. Adducin is also phosphorylated by a Rho-activated kinase in fibroblasts, resulting in its accumulation at the leading edge; this phosphorylation was essential for migration (Fukata et al., 1999). Cortactin is another actin-binding protein that localizes to the leading edge of fibroblasts upon Rac GTPase activation, but is otherwise partitioned into the cytosol (Weed et al., 1998). Cortactin shows an increase in synaptic localization in cultured hippocampal neurons upon glutamate stimulation (Naisbitt et al., 1999). An activity-dependent shift of these actin-binding proteins to spine membrane or cytosol could cause dramatic changes in the actin cytoskeleton of the spine. Many of the spine actin-binding proteins, such as α-actinin, cortactin, spinophilin, and spectrin, can bind directly to structural proteins of the PSD (see below) as well as to the actin cytoskeleton. They are thus close to sites of receptor activation and susceptible to posttranslational modifications that could change their effects on the actin cytoskeleton.

Spinophilin/neurabin II is an actin-binding protein that appears highly enriched in dendritic spines (Allen et al., 1997; Satoh et al., 1998). It is capable of forming actin bundles in vitro, and has three SH3 domains that presumably mediate protein interactions and a predicted C-terminal coiled coil region that could induce dimerization. Further, it has the ability to bind to protein phosphatase 1 (PP1), another protein that is concentrated in spines, and inhibit its phosphatase activity. Finally, it has a PDZ domain (see below) that interacts with rat Mint/lin10, a protein involved in basolateral protein targeting in vulval precursor cells in the nematode *Caemorhabdites elegans* (Ide et al., 1998).

Thus far we have considered ways in which the PSD could control the actin cytoskeleton, but there are also ways in which components of the actin cytoskeleton could regulate PSD elements. α-actinin-2 binds the NMDA receptor competitively with calcium/calmodulin (Wyszynski et al., 1997). This interaction could serve to regulate NMDA receptor localization or activity. Depolymerization of spine actin with latrunculin A caused a loss of α-actinin from spines, but most NMDA receptors remained associated with the PSD, suggesting that α-actinin does not play a major role in anchoring the NMDA receptor at the synapse (Allison et al., 1998, 2000). However, extensive coexpression studies suggest that α-actinin binding to the NMDA receptor may help to maintain the channel in a state of high open probability, and that calcium- and calmodulin-mediated release of α-actinin from the receptor underlies calcium-dependent inactivation of NMDA receptor-mediated currents (Zhang et al., 1998; Krupp et al., 1999). Spectrin also exhibits regulated binding to the NMDA receptor (Wechsler and Teichberg, 1998), another interaction that may regulate receptor function or localization. Cortactin binds Shank, a major synaptic scaffolding protein (see below), and spinophilin/neurabinII appears itself to function as a scaffolding protein. Spinophilin has been suggested to facilitate the dephosphorylation of AMPA receptor at synapses by anchoring the PP1-spinophilin complex to PSDs via binding of the spinophilin PDZ domain to a synaptic interactor (Hsieh-Wilson et al., 1999; Yan et al., 1999).

Structural Proteins of the PSD

The structural and scaffolding proteins of PSDs are interposed between the actin cytoskeleton and the transmembrane proteins that constitute the postsynaptic receptor ensemble. Most are concentrated both at spiny and nonspiny excitatory postsynaptic specializations and are therefore unlikely to have a function unique to spines, while a few have not been completely characterized as to distribution. Some structural proteins, such as catenins (Uchida et al., 1996; Benson and Tanaka, 1998), are also concentrated at inhibitory synapses, and a number (AF-6/s-afadin, catenins, CASK/lin2) function also at specialized junctions of epithelial cells.

Many of these scaffolding proteins contain or bind to PDZ domains, ~90-amino-acid protein domains that form a binding pocket for specific C-terminal interacting sequences (reviewed by Kennedy, 1997; Kornau et al., 1997; Craven and Bredt, 1998). In brief, many postsynaptic PDZ-containing proteins bind to the C-terminal regions of glutamate receptors and thus form the first tier of submembrane proteins beneath the postsynaptic membrane. Different PDZ proteins bind to different glutamate recep-

tors, thus permitting independent regulation of the localization of these receptors to postsynaptic sites. For example, the PSD-95 family (Kornau et al., 1995; Kim et al., 1996; Muller et al., 1996a) and S-SCAM (Hirao et al., 1998; Ide et al., 1999) bind to the NMDA receptor, while GRIP/ABP (Dong et al., 1997; Srivastava et al., 1998) and PICK1 (Dev et al., 1999; Xia et al., 1999) bind to the AMPA receptor. Several of these proteins have multiple PDZ domains that bind to different synaptic components, and most also self-associate, thus serving as scaffolds to assemble multiprotein complexes.

Some of the large PDZ proteins that bind to multiple components of the PSD can scaffold ion channels, enzymes, cell adhesion proteins, and structural proteins. For example, PSD-95 binds NMDA receptors (Kornau et al., 1995), potassium channels (Kim et al., 1995), neuronal nitric oxide synthase (Brenman et al., 1996), SynGAP (Chen et al., 1998; Kim et al., 1998), the cell adhesion protein neuroligin (Irie et al., 1997), and other structural proteins including CRIPT (Niethammer et al., 1998; Passafaro et al., 1999) and GKAP (Kim et al., 1997; Takeuchi et al., 1997). Based on the binding interactions, it seems probable that proteins such as PSD-95 function to localize interacting receptors and to link them to downstream signal-transducing proteins.

Sequential yeast two-hybrid screens have resulted in the discovery of assemblies of interacting proteins at the postsynaptic density. Shank appears to be one of the central scaffolding protein families (Boeckers et al., 1999; Naisbitt et al., 1999; Yao et al., 1999). Shank contains a PDZ domain, an SH3 domain, a SAM domain, a proline-rich region, and multiple ankyrin repeats. Shank binds to GKAP, Homer (Tu et al., 1999), and cortactin. Thus, through GKAP Shank is linked to PSD-95 and NMDA receptors, through Homer to metabotropic glutamate receptors, and through cortactin to the actin cytoskeleton. Like PSD-95 and Shank, the PDZ proteins S-SCAM (Hirao et al., 1998; Ide et al., 1999) and CASK/lin2 (Butz et al., 1998; Maximov et al., 1999; Hsueh et al., 1998, 2000) exhibit multiple interactions and may be central scaffolding proteins. Non-PDZ proteins of the AKAP superfamily (AKAP-79 and yotiao) also function as synaptic scaffolds linking NMDA receptors, protein kinase A, protein phosphatase 1, and calcineurin (Klauck et al., 1996; Lin et al., 1998; Feliciello et al., 1999; Westphal et al., 1999; Sik et al., 2000).

Although these scaffolding proteins do not directly bind actin and generally are not spine-specific, many do have indirect links with the actin cytoskeleton and thus are poised to participate in signaling between the PSD and the actin cytoskeleton of spines. As just mentioned, cortactin may mediate signaling between actin microfilaments and Shank scaffolds. Both AF-6/s-afadin and the catenins are components of epithelial cadherens junctions and bind to the actin cytoskeleton (Mandai et al., 1997; Buchert et al., 1999). S-SCAM binds δ-catenin (Ide et al., 1999) as well as the NMDA receptor, neuroligin, and GKAP. The Homer/Vesl proteins (Kato et al., 1998; Tu et al., 1998, 1999) contain an EVH (Ena/VASP homology) domain, as described above for the Ena/VASP family of proteins, and perhaps may regulate actin polymerization. The links between PSD proteins and the actin cytoskeleton may serve more of a signaling than an anchoring function. As an initial test of these linkages, Allison et al. (1998, 2000) found that the more directly actin-binding components α-actinin, drebrin, and CaMK II were dependent on the actin cytoskeleton for spine localization, but PSD-95, GKAP, and NMDA receptors were maintained at postsynaptic specializations in the absence of detectable F-actin.

Signaling Proteins of the PSD

The signaling proteins of the PSD include the glutamate receptors, which are the primary receptive component, other cell surface receptors which recognize and adhere to the presynaptic specialization, and the associated enzymes which can modify PSD components and generate second-messenger signaling cascades. Compared with the number of structural proteins, there are relatively few signaling proteins known to be concentrated in spines.

Cadherins (Fannon and Colman, 1996), ephrins and Eph receptors (Torres et al., 1998; Buchert et al., 1999), neuroligin (Irie et al., 1997; Song et al., 1999), densin 180 (Apperson et al., 1996), NCAM-180 (Muller et al., 1996b; Schuster et al., 1998; Uryu et al., 1999), integrins (Einheber et al., 1996), and syndecan2 (Ethell and Yamaguchi, 1999; Hsueh and Sheng, 1999) are all present at the PSD of spine synapses and may mediate adhesion to and signaling from the presynaptic terminal. The precise function of these proteins at the spine is an area of current investigation. Many of these proteins may function both in development of spines and during plasticity. For example, overexpression of syndecan2 in hippocampal neurons promoted the formation of head-bearing protrusions at a stage when control neurons expressed only filopodial type protrusions (Ethell and Yamaguchi, 1999). This effect was dependent on the presence of a PDZ-binding motif in syndecan2 that has been shown to bind to CASK/lin2 (Hsueh et al., 1998). In fibroblasts, syndecan2 transfection induces the formation of filopodia via activation of Cdc42 (Granes et al., 1999). These results suggest that a signaling pathway important in spine maturation involves syndecan2, PDZ scaffolds, Rho family GTPases, and the actin cytoskeleton of spines. Another family member, syndecan-3, which forms a complex with fyn and cortactin, has been implicated in LTP in hippocampal slices (Lauri et al., 1999).

The major functional input for dendritic spines is glutamate activation of AMPA- and NMDA-type ionotropic and group I metabotropic glutamate receptors. Whereas the NMDA receptor is present at all mature hippocampal spine synapses regardless of size, the AMPA receptor is concentrated in large spines but undetectable in many small spines (Nusser et al., 1998; Takumi et al., 1999; Racca et al., 2000). These results imply that different-sized spines indicate a functionally different kind of synapse. Synaptic clustering of NMDA and AMPA receptors can be regulated by long-term changes in global activity levels (reviewed by Craig, 1998; Turrigiano and Nelson, 1998). For both receptors, chronic activity blockade increases synaptic clustering of the receptors, probably as a means of homeostasis. Whereas only long-term regulation of targeting has been shown for NMDA receptors, AMPA receptors can also undergo very rapid activity-regulated endocytosis or synaptic insertion associated with long-term depression or long-term potentiation (Carroll et al., 1999; Luscher et al., 1999;

Shi et al., 1999; Hayashi et al., 2000; Man et al., 2000). The exact mechanisms of AMPA recycling are not yet clear, but appear to involve interactions with NSF (Nishimune et al., 1998; Song et al., 1998) and with PDZ proteins such as GRIP/ABP (Dong et al., 1997; Srivastava et al., 1998) and PICK1 (Dev et al., 1999; Xia et al., 1999).

Perhaps surprisingly, while glutamate receptors obviously play central roles in synaptic plasticity, their function appears to be unnecessary for basic aspects of synapse development. Synapses appear capable of developing quite normally under conditions of chronic blockade with tetrodotoxin and cocktails of receptor antagonists. Thus, in spite of the silent-synapse model of synapse development, NMDA receptor function is not required for AMPA receptors to cluster and function at synapses (Li et al., 1994; Okabe et al., 1998; Rao et al., 1998). However, activation of AMPA, NMDA, and metabotropic glutamate receptors can modulate synapse development, including the density of synaptic receptors (Craig, 1998; Turrigiano and Nelson, 1998; Gomperts et al., 2000). Glutamate receptor function can also modulate the density and size of dendritic spines, as described earlier. Another example of this is seen in the transgenic mouse lacking the NR3 subunit of the NMDA receptor; this lack resulted in increased calcium entry through NMDA receptor activation during development and increased spine size and number (Das et al., 1998).

The downstream mediators of synaptic signaling in spines are numerous and will not be described extensively here (see Kennedy, 1998). Some of the signaling components that are particularly concentrated in spine synapses include the phosphatases calcineurin (Halpain et al., 1998) and PP1 (Ouimet et al., 1995) and the kinase CaMKII (Kennedy et al., 1983; Jones et al., 1994; Kennedy, 1998). Although many other kinases and signal-transducing proteins are not specifically enriched in spines, they can certainly also function in spines. CaMKII is particularly interesting since it is an abundant postsynaptic density protein, a holoenzyme of α and β subunits, and is believed to play a crucial role in regulation of synaptic strength (Kennedy, 1998). Autophosphorylation of CaMKII on thr-286 leads to calcium-independent activity, and due to its holoenzyme nature, such phosphorylation could be propagated beyond the time-course of a stimulus, serving as a medium-term mode of information storage. Indeed, either overexpression of constitutively active CaMKII asp-286 or targeted replacement of thr-286 to ala-286 result in deficits in spatial learning and LTP (Mayford et al., 1996; Giese et al., 1998).

DEVELOPMENT OF POSTSYNAPTIC SPECIALIZATIONS IN HIPPOCAMPAL NEURONS IN CULTURE

In our laboratory, we have used the approach of studying spine synapse development and modification using a hippocampal culture model (Goslin et al., 1998). In this system, synapses develop over an extended period of 2–3 weeks. When synapses develop at contact sites, one possibility is that one side of the contact develops first and induces specialization in the other cell. Another possibility is that both pre- and postsynaptic assembly of synaptic components occurs independently to form prefabricated pre- and postsynaptic elements, and the synapse develops by aligning the two elements. In hippocampal neurons in culture, there is evidence for organization of the pre- and postsynaptic components of the synapse before contact actually occurs. Synaptic vesicle clusters can be seen in growing axons before they contact the somatodendritic domain (Kraszewski et al., 1995). These aggregates of vesicles appear to be capable of release and recycling (Antonov et al., 1999; Zakharenko et al., 1999). Further, we found that the NMDA-type glutamate receptor could be observed in aggregates before presynaptic contact, as could elements of a postsynaptic scaffold containing PSD-95 family proteins (Rao et al., 1998). Shortly thereafter, the PSD-95 family-containing scaffold became localized at sites apposed to the synaptic vesicle aggregate. The NMDA receptor, however, did not attach to the postsynaptic scaffold for another 1–2 weeks of development in vitro. This developmental pattern suggested, firstly, that postsynaptic proteins could aggregate without contact with a presynaptic specialization; secondly, that postsynaptic proteins of the PSD-95 family form a primary scaffold at the synapse without receptor attached to it; and finally, that the attachment of NMDA receptors to this scaffold might be regulated (Fig. 4).

In contrast, another glutamate receptor, the AMPA receptor, aggregated only at contact sites with presynaptic specializations in this culture system (Craig et al., 1993). In pyramidal cells, aggregates of the AMPA receptor were observed at spines only after 2 weeks in culture. These aggregates were not at this stage colocalized with the NMDA receptor aggregates, which were primarily nonsynaptic (Rao et al., 1998). This pattern suggested that the localization of these two types of glutamate receptor could be independently regulated.

Striking differences were also observed in the molecular composition of excitatory postsynaptic elements on pyramidal cell spines vs. on interneuron shafts (Allison et al., 1998; Rao et al., 1998). CaMKII and the actin-binding proteins α-actinin and drebrin clustered at spine synapses of pyramidal neurons but were not observed at shaft synapses on GABAergic interneurons. The excitatory synapses on interneurons did contain clusters of the NMDA receptor and PSD-95, and actually higher concentrations of AMPA receptor and the scaffolding protein GKAP. Similar differences between pyramidal spine and interneuron shaft synapses have been noted by other groups, including selective association of CaMKII (Kennedy et al., 1983; Jones et al., 1994; Kennedy, 1998) and calcineurin (Sik et al., 1998) with pyramidal spine synapses, and selective association of citron with interneuron shaft synapses (Furuyashiki et al., 1999; Zhang et al., 1999). These differences in molecular composition presumably contribute to the differences in morphology and signaling properties of these two classes of hippocampal glutamate synapses.

Further experiments examining the cytoskeletal anchoring of postsynaptic components also provided evidence for independent assembly of the AMPA vs. NMDA receptor complexes and for postsynaptic cell type-specific differences. Treatment of cultures with cytoskeleton-depolymerizing agents and detergent extraction

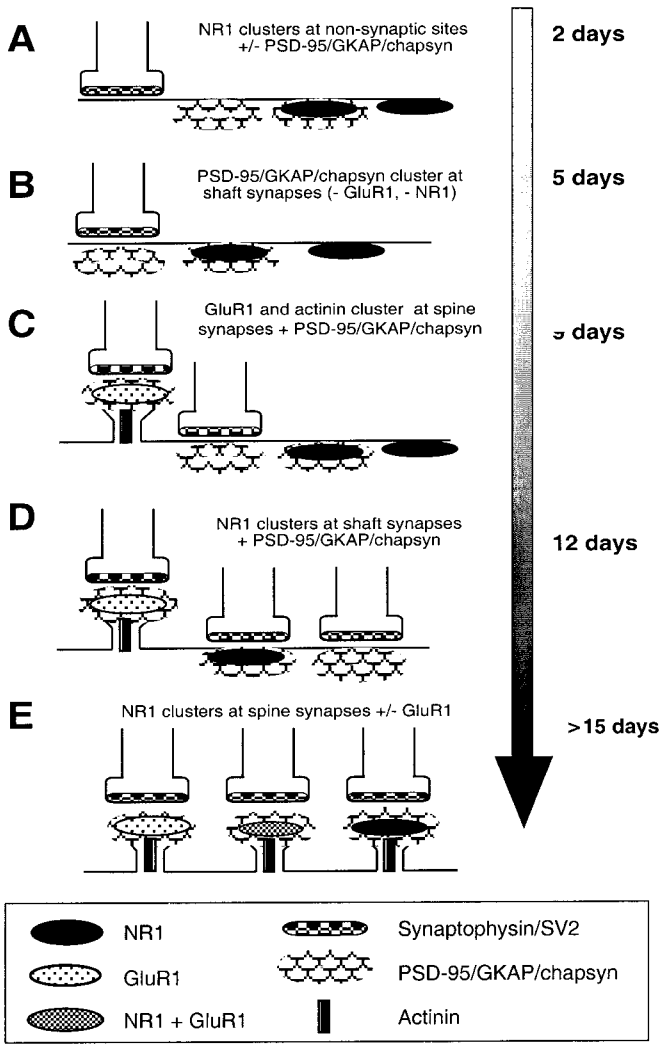

FIGURE 4. Summary of stages of development of excitatory postsynaptic sites on hippocampal pyramidal neurons, as indicated by the molecular markers used in this study. Timeline indicates the earliest time in culture at which each event was observed; many of these events occurred over a span of days to weeks. A: NMDA receptors were clustered from the earliest stages in development at nonsynaptic sites in the soma and proximal dendrite shaft; some clusters colocalized with PSD-95, GKAP, and chapsyn, and some did not. B: PSD-95, GKAP, and chapsyn formed clusters within the first week in culture at dendrite shaft synapses lacking clusters of either NMDA or AMPA receptors. C: α-actinin-2 formed synaptic clusters in the second week in culture, but only at spine synapses colocalizing with AMPA receptor but not NMDA receptor clusters. D: During the second and third weeks in culture, NMDA receptor clusters were predominantly at fine terminal branches of the dendritic tree, many of them colocalizing with PSD-95, GKAP, and chapsyn; some of these were synaptic. E: Finally, only begining at 3 weeks did NMDA receptor clusters become primarily localized at dendritic spines throughout the extent of the dendritic arborization, where they often colocalized with AMPA receptor clusters and always with α-actinin-2, PSD-95, GKAP, and chapsyn. Under other conditions, the NMDA receptor can cluster at synapses earlier. Taken from Rao et al. (1998; copyright 1998 by the Society for Neuroscience).

was used to examine the cytoskeletal anchoring of postsynaptic components (Allison et al., 1998, 2000). These treatments showed that the AMPA receptor at spine synapses had a strong dependence on the integrity of the actin cytoskeleton for its localization to synapses, as did CaMKIIα and actin-binding proteins such as α-actinin and drebrin. In contrast, both the NMDA receptor and associated proteins such as PSD-95 family members and GKAP were still anchored at spine synapses after complete loss of detectable filamentous actin. In addition, whereas AMPA receptors at pyramidal spines were dependent on actin for clustering and were detergent-extracted, AMPA receptors on interneurons were not, indicating different mechanisms for AMPA receptor clustering on the two neuron types.

Activity blockade did not disrupt the basics of synapse assembly in hippocampal cultures, but did modulate clustering of the NMDA receptor, actually increasing synaptic localization. Treatment of cultures with receptor antagonists was used to achieve chronic global blockade of glutamate receptors. In low-density cultures, chronic APV treatment resulted in a dramatic increase in the synaptic localization of the NMDA receptor at 3 weeks (Rao and Craig, 1997). This treatment did not affect clustering of the AMPA receptor at synapses, again suggesting independent regulation of the two receptors. Further, the localization of both the PSD-95 family and of presynaptic markers was not affected by the treatment, indicating that blockade of the NMDA receptor regulated only the synaptic targeting of the NMDA receptor. Thus, conditions which affect the level of global excitation of the NMDA receptor system resulted in homeostatic changes in the NMDA receptor level at excitatory synapses. This cellular phenomenon may be a means to reset the neuronal response to changing input levels during development and to regulate the ability of the neuron to undergo other forms of plasticity such as LTP or LTD (a form of metaplasticity). It is presently unclear whether in a subcellular context local activity differences between synapses would result in spine-specific changes in NMDA receptor levels; if they do, this may be a means of regulating the capacity of each spine for plasticity of synaptic efficacy, morphology, and longevity.

CONCLUSIONS

We have described some interactions between proteins of the postsynaptic density, the primary receptive element of the postsynaptic specialization, and the spine cytoskeleton, the actin-rich substructure on which the PSD is placed. Most of these interactions have been described in vitro using reconstituted systems. It will be important to determine if such interactions are involved in spine development and regulation in situ and what physiological situations call them into action. Multiple receptor-associated signaling pathways must combine within the spine compartment to summate input and pass it on to the dendrite shaft as well as store the history of input in the PSD and spine cytoplasm. It seems likely that spines may serve as biochemical compartments for many second-messengers pathways. The complexity of signal transduction

in spines is an issue that will require new approaches to assay the function of dynamic assemblies of proteins acting in concert. Modes of rapid progress may include the development of novel multichannel live-cell fluorescence probes and of phosphoprotein and protein interaction chips to be used in combination with more sophisticated gene-targeting approaches.

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