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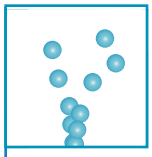
DENDRITIC SPINES: THE LOCUS OF STRUCTURAL AND FUNCTIONAL PLASTICITY

Carlo Sala and Menahem Segal

Consiglio Nazionale delle Ricerca Institute of Neuroscience and Department of Medical Biotechnology and Translational Medicine, University of Milan, Milan, Italy; Neuromuscular Diseases and Neuroimmunology, Neurological Institute Foundation Carlo Besta, Milan, Italy; and Department of Neurobiology, The Weizmann Institute, Rehovot, Israel



Sala C, Segal M. Dendritic Spines: The Locus of Structural and Functional Plasticity. *Physiol Rev* 94: 141–188, 2014; doi:10.1152/physrev.00012.2013.—The introduction of high-resolution time lapse imaging and molecular biological tools has changed dramatically the rate of progress towards the understanding of the complex structure-function relations in synapses of central spiny neurons. Standing issues, including the sequence of molecular and structural processes leading to formation, morphological change, and longevity of dendritic spines, as well as the functions of dendritic spines in neurological/psychiatric diseases are being addressed in a growing number of recent studies. There are still unsettled issues with respect to spine formation and plasticity: Are spines formed first, followed by synapse formation, or are synapses formed first, followed by emergence of a spine? What are the immediate and long-lasting changes in spine properties following exposure to plasticity-producing stimulation? Is spine volume/shape indicative of its function? These and other issues are addressed in this review, which highlights the complexity of molecular pathways involved in regulation of spine structure and function, and which contributes to the understanding of central synaptic interactions in health and disease.

I. INTRODUCTION

Ever since their first detection by Ramon y Cajal (408), dendritic spines have been postulated to underlie the neuronal locus of plasticity, where short-term alterations in synaptic strength are assumed to be converted to long-lasting memories that are embedded in stable morphological changes. Initially, dendritic spines were visualized using Golgi impregnation. This was followed in the late 1950s by electron microscopic (EM) reconstruction of neuronal tissue at the nanometer resolution. The EM picture introduced us to the postsynaptic density, the presynaptic terminal and its vesicles, and other organelles found in (e.g., spine apparatus) or absent from (e.g., mitochondria) the dendritic spine. Still, the search for morphological changes in dendritic spine following plasticity-producing stimulation was tedious, and the results were not always consistent. In one of the earliest EM studies, Fifkova and Van Harreveld (128) found that within 2–6 min after a long (30 s) tetanic stimulation of the perforant path, spines in the dentate gyrus increased their size by 15%, followed by a further increase by 38% at 10–60 min after the stimulation. On the other hand, Desmond and Levi (101) found that the postsynaptic density (PSD) surface area per synapse increased markedly, but they did not report on a change in spine head volume. Geinisman (141) reported that following long-term potentiation (LTP) there is an increase in number of perforated synapses, with the PSD breaking down to two independent components, thus increasing the size of the overall synaptic contact. In a similar series of studies, using advanced morphometric three-dimensional reconstructed EM images, Stewart and colleagues (484) found that following chemical LTP, which presumably activates all synapses in the sliced hippocampus, there is an increase in the number of perforated synapses without a change in volume of the spines. In other studies by the same group (395) with anesthetized rats, a significant increase in thin and mushroom spine volumes was found, but without a change in synapse density. In further studies, they (322) describe an increase in perforated synapses in the dentate gyrus some 24 h after the tetanic stimulation in awake rats. In sharp contrast, Sorra and Harris (474), measuring three-dimensional reconstructed spines from serial section EM pictures, could not find any significant effect of LTP on morphological properties of spines. The different reported effects of LTP on spine properties are partly due to methodological differences, including the nature of stimulation used for generating plasticity, the brain area chosen for study, the condition of the tissue (in vivo vs. in vitro), the time after the

stimulation, and the methods of analysis. These are not trivial considerations, as there are reports that handling of the sliced tissue (e.g., cooling, blocking activity with drugs) may have considerable effect on dendritic spine density (238), regardless of the response to a tetanic stimulation, and probably irrespective of the physiological state of the tissue.

A different approach to the study of the role of dendritic spines in synaptic plasticity examined the effects of enriched environment on morphological and functional attributes of neural tissue, including dendritic spines. In one of the earlier studies, Globus et al. (147) found that allowing rodents to develop in an enriched environment resulted in an increase in dendritic spine density. In these early studies it was not possible to separate between direct effects on dendritic spines and global effects on the neural tissue, including an increase in blood supply, glia proliferation, and an increase in volume of the cortex. The power of the novel time lapse in vivo imaging for addressing the same issue studied by Globus et al. is exemplified in a recent study of the effects of enriched environment on dendritic spine density (219). The authors demonstrate that not only spine density is increased following life in an enriched environment, but there is also an increase in transient spines, and in turnover rate of spines. These observations are highly relevant to the assumed role of spines in storage of memories (see sects. VI and VII).

While the morphometric studies that compared populations of fixed spines have yielded important information, in that they indicated that dendritic spines undergo some morphological changes following an intense experience, be it a tetanic stimulation, growth conditions, hibernation, and hormonal and emotional variations, they were not so informative as to the exact changes that a given spine can undergo following such an intense experience, and the molecular mechanisms leading to these changes.

The ability to follow individual dendritic spines over time, using in vivo time-lapse imaging, first introduced two decades ago (160, 343), allowed a rapid progress in the description and analysis of changes that may take place in dendritic spines in response to plasticity-producing stimulation. The advantages of the time lapse imaging over the more traditional counting and measuring of different populations of spines are obvious; repeated-measures statistics are stronger than comparisons among different samples, especially in cases of great heterogeneity of spine shapes. On the other hand, the time lapse imaging is inherently restricted to a smaller simultaneous samples of spines likely to be located on the same segments of the dendrite, which may not represent the population of spines at large, and is restricted to light microscopic resolution, which is blind to possible changes in subsynaptic organelles.

Regardless of the pitfalls of the time-lapse live imaging, there was a tremendous increase in the amount of information accumulated in the past decade about the mechanisms and roles of dendritic spines in neuronal plasticity, as evidenced by the large number of review articles published in the year of 2012 (53, 121, 129, 258, 306, 364, 386, 416, 427, 517 to list a few).

Despite the rapid accumulation of information on the molecular and functional attributes of dendritic spine regulation, some standing issues are still unsettled: Is spine formation triggered by incoming afferents or by an intrinsic process independent of afferent activity; does it develop from a filopodium that shrinks or from a dendritic shaft synapse that elongates to become a spine? Indications for both of these possibilities have been reported, but it is not entirely clear what is the dominant mode of spine formation, if there is any. Obviously, young neurons are endowed with filopodia, so it is likely that the initial formation of spines is caused by conversion from filopodia, whereas in adult neurons, where there is also a significant generation of novel spines, they are not preceded by filopodia. Exceptions to this generalization can also be found; when neurons fail to receive input, spines tend to shoot out protrusions, probably in search of glutamate excitation (415).

Likewise, once a spine is formed, how long does it take to become functional, and to react to presynaptic stimulation? There are reports of different time scales, from minutes to days and weeks, but the verdict is not out yet. Along the same line, once a spine is formed, how stable it is, and what might cause it to disappear/shrink, and at what rate? Finally, given the large number of different families of molecules assumed to be necessary for the formation of spines, are all spines equipped with the same molecular cascades, or are different spines endowed with different molecules, which may cause different morphologies and perhaps functions?

A. What Are the Functional Demands on "Plastic" Spines?

The key issue in spine research is the functional correlates of the great heterogeneity in spine morphologies. Why are there short and long, stubby and mushroom spines? Do they serve different functions, or are only the "mature" ones functional, and the other types "reserve" contacts, to be used upon request? On the functional end, the attempt to narrow down the wealth of different memory mechanisms into a minimal number of simple electrophysiological and molecular "building blocks" within dendritic spines is at best only a crude approximation. At present, there are a variety of memory processes: working, procedural, declarative, semantic, and perceptual, and they are associated with different brain areas and different time scales and may utilize different molecular cascades (326). In fact, despite extensive studies utilizing pharmacology and genetic manipulation of specific genes related to plasticity, there is still no clear link between different plasticity processes and spine morphology. It is plausible that different types of memories obey different electrophysiological rules and may leave different morphological fingerprints in the brain.

At the cellular level, there are different plasticity-producing stimulation patterns that are likely to use different molecular cascades and morphological changes. For example, while the dominant mode of producing LTP in brain areas such as the hippocampus involves activation of the *N*-methyl-D-aspartate (NMDA) receptor (168, 186), there is a distinct non-NMDA mediated LTP, which is assumed to activate release of calcium from stores (156, 410). Is it possible that these two ways to generate LTP are funneled into the same morphological changes in the spine? Also, spike timing dependent plasticity (STDP) (121), metaplasticity, and chemical LTP (448) are all assumed to involve changes in distributions of glutamate receptor subtypes following a change in intracellular calcium concentration and affect synaptic responses for extended periods of time. Are all these plastic processes sharing the same locus of change? Finally, there are strong indications that plastic changes take place also at the presynaptic terminal, and involve changes in release probability (113, 116), as well as appearance and disappearance of synaptic boutons (34). Furthermore, some plastic changes involve nonsynaptic mechanisms, including changes in glia properties (352) and dendritic excitability (131). These may not necessarily involve direct changes in spine morphology.

B. Longevity of Memories

Different types of memories have different rules and time course; motor memory (speech, bicycle ride, piano playing) is in general persistent, while working memory is highly labile. Can we assume that both types are "stored" in dendritic spines? Recent studies on motor learning in vivo addressed these issues, have been reviewed extensively (193, 243), and are discussed below (see sects. VI and VII).

One interesting diversion from the traditional view of memories being stores in stable dendritic spines are recent indications that ongoing phosphorylation state of a molecule (e.g., PKMzeta, Ref. 429) controls apparently stable memories. If so, are stable spines necessary for stable memories, or are these morphological changes an epiphenomenon, and some specific molecules are the ones to carry the "memories"?

II. METHODOLOGICAL CONSIDERATIONS

A. Labeling of Neurons

One of the mysteries of the Golgi stain is its selectivity. Over a century after its discovery, it is still not known why so few neurons stain with the Golgi method, and if there is something unique about the ones that do stain, that may bias the evaluation of changes produced by behavioral/chemical manipulations in the brain. A similar issue is not addressed systematically; with the use of transfection/infection methodologies, a favorite method for imaging individual neurons in vivo as well as in cultured brain slices and dissociated neuronal cultures, only a small fraction of neurons take up the plasmid and express the protein of interest, e.g., green fluorescent protein (GFP). Furthermore, once the neuron takes up the plasmid, and if the promoter is ubiquitous, it is likely to recruit the protein-generating cellular machinery for producing large amounts of the transfected molecules. Intuitively, this should suppress the neuronal responses to other demands for proteins. However, this issue has not been addressed systematically, except to show that adjacent, transfected and nontransfected neurons share the same electrical properties (458, 504).

B. Estimation of Spine Dimensions From Fluorescent Images

A large number of recent publications use time-lapse imaging to estimate changes in spine dimensions following an acute exposure to plasticity-producing stimulation, e.g., caged glutamate. Some of these studies demonstrate immediate and striking, up to threefold, increases in spine volume (311). However, these changes may result partially from changes in fluorescence intensity rather than size of the imaged spine. The earlier studies believe to have accounted for this change in fluorescence intensity, and a tentative correlation between fluorescence intensity and spine size has been documented [see Holtmaat et al. (194), 10 spines imaged and reconstructed at the EM level], but this issue is not addressed systematically, and there is a clear increase in size of the fluorescent spot upon an increase in fluorescence intensity. One way to deal with these two possible sources of change, without being committed to one or the other, is to express the change as a "normalized fluorescence intensity" (583). Obviously, even if the measured change is in fluorescence intensity rather than size of the spine, it is a dramatic change that should be analyzed.

GFP-tagged molecules are used routinely in the study of localization, dynamics, and mobility of molecules following induction of changes in response to electrical or chemical stimulation. For example, GFP-tagged PSD-95 (e.g., Ref. 110), marks the postsynaptic side of the synapse. The postsynaptic density, as seen in EM pictures, is far smaller than the corresponding fluorescent image of GFP-PSD-95 (427), indicating that the fluorescent image provides by far an overestimation of organelle size, and is most likely affected by the amount of light emitted by the fluorophore. This has been confirmed in recent imaging methods using super-resolution microscopy (see sect. X and Refs. 298, 345).

C. Dendritic Spine Density

What is the "normal" dendritic spine density? The numbers range from 0.2 to 3.5 spines per 1 μm of dendrite (the latter is a count in human postmortem cortex, Ref. 35). Obviously, this number is dependent on age, cell type, and position along the dendrite. However, it is also dependent on the method of measurement. The most accurate count is based on three-

dimensional EM reconstructed dendrite which yield a larger density than a light microscope, single plane Golgi stained neuron. Likewise, the spine shape is also dependent on all these parameters, so it is of utmost importance to consider the baseline spine density before dwelling into changes produced by one treatment or another.

1. Direct versus indirect effects on spine morphology

There are several ways to generate changes in spine morphology. In general, one can activate individual spines, either by electrical stimulation or by flash photolysis of caged glutamate. The latter stimulation also activates nonsynaptic glutamate receptors, which may or may not reside on spine heads, but also on dendritic shaft. On the other hand, the entire network can be activated by protocols such as "chemical" LTP or long-term depression (LTD), which will cause obvious changes in a large proportion of the imaged spines, but not be specific to a given spine, and in fact can be caused by changes in dendritic excitability (131).

2. Spatiotemporal resolution

The detection of dynamic changes in spine parameters, including volume changes, transient rise in $[Ca^{2+}]_i$, spine motility as well as reactivity to flash photolysis of caged molecules, is dependent on the spatiotemporal resolution of the imaging system and on the concentration of the calcium dye employed. By the same token, flash photolysis of caged molecules, a popular vehicle to study properties of individually activated dendritic spines, can vary from nanosecond flash to several milliseconds. Obviously, the sphere of diffusion of the activated compound will vary as a function of the duration of flash so that the action of the photoreleased molecule may not be as localized as one wishes.

These are several cautionary notes that a critical evaluation of the vast literature on dendritic spines need to consider. Obviously, the more standard the methods are, the more generalized and reliable are the results.

III. ELECTROPHYSIOLOGICAL PROPERTIES OF DENDRITIC SPINES

The early theoretical view of dendrites and spines considered them as being passive cylindrical compartments. Based on cable theory, research addressed issues such as the electrotonic distance of the synapse from the soma (405). Already in the 1980s, Perkel, Rall, and Shepherd considered dendritic spines as having excitable membrane (325, 387, 456), and subsequently the theoretical research on dendritic spines gained momentum and began to be relevant to the rapidly accumulating experimental evidence for the presence of voltage-gated currents in dendritic spines.

With the increased use of molecular and high-resolution imaging tools for localized receptors and ion channels in different compartments of neurons, the knowledge about the electrical properties of dendritic spines is becoming more relevant than ever before. Furthermore, the ability to alter the morphology of dendritic spines using insertion and deletion of specific genes also calls for a better understanding of the electrophysiological consequences of these alterations.

A. Voltage-Gated Sodium Channels in Spines

While theoretical considerations assumed that the spine neck is not a barrier to the transfer of the synaptic charge to the parent dendrite (449), experimental evidence for this assumption is rather scarce, for the simple reason that recording of electrical activity from spine head has not yet been achieved due to the small size of this organelle. Still, the estimation of the axial resistance of the spine neck is crucial in the attempt to extract the relevance of the physical distance of the spine head from the parent dendrite. Estimations of the spine neck resistance range from 1.2 GΩ (158), which is critical for the ability to generate large currents at the spine head, to ~4–50 MΩ (490). The latter estimate is based on the measurement of diffusion of fluorescent molecules from spine head to the parent dendrite. A more recent estimate of spine neck resistance is based on voltage measurements in the dendrite adjacent to the spine and equalization of the magnitude of calcium transient in the spine head and the electrical responses in the dendrite, and amounted to ~500 MΩ (167). This latter study indicates that the spine head can generate a large local voltage transient, sufficient to sensitize adjacent spines. The functional implications of this will be dealt with below (see sect. VII). Interestingly, this recent study did not address the functional relevance of large heterogeneity of spine neck parameters.

The presence of voltage-gated Na channels in dendritic spines has been demonstrated using live imaging methods. Using the Na dye SBFI, Rose et al. (418) measured Na transients in dendritic spines of CA1 neurons in a slice, suggested to amount to a modest change of 4 mM for a train of 20 action potentials. Obviously, these were early days of imaging and the resolution was not so high. Later studies using second harmonic generation (360) were able to image membrane potential changes in dendritic spines, and reported a 10% change in fluorescence of FM4–64 for 100 mV. More recently, using voltage-sensitive dyes injected into individual neurons in layer 5 of the neocortex, Palmer and Stuart (377) compared back-propagating action potentials (bAP) with synaptic inputs. They found that bAP can invade the spines without significant loss in magnitude, and they estimated the size of the response to vary from 80 to 20 mV, depending on the distance from the soma, as expected for apical dendrites. Interestingly, they found that hyperpolarization of the soma can increase the size of the synaptic input, indicating that there is no involvement of voltage-gated channels in the amplification of the synaptic input. Furthermore, they estimated spine neck resistance to be on the order of 500 MΩ, which will reduce the synaptic response at the soma by <15%. More recently, Acker et al. (3)

imaged single voxels at high temporal resolution using voltage-sensitive dyes in cortical slices, to find a 16% change in fluorescence/100 mV in individual dendritic spines. They confirmed the presence of voltage-sensitive fast changes in fluorescence, correlated with somatic action potentials. Interestingly, they did not report any difference between spines of different lengths and sizes. This indicates that the length of the spine neck is not relevant to the transfer of voltage signals from the parent dendrite to the spine head, but it does not predict the transfer from the head to the dendrite. Another, rather indirect means for localizing voltage-gated sodium channels in spine heads was used by Yuste and colleagues (19) who measured the voltage response to flash photolysis of caged glutamate, to find that blockade of Na channels with tetrodotoxin (TTX) reduced markedly the responses at the soma, indicating that the spine Na channels act to boost the glutamate responses. This suggestion contradicts that of Palmer and Stuart (see above).

Finally, while there is accumulating evidence, using voltage-sensitive dyes and Na dye for voltage-gated Na channels in the spine head, the morphological evidence for this is rather scarce. Using quantitative EM immunocytochemistry, Lorincz and Nusser (288) were unable to detect the presence of Nav channels in dendritic spines. These channels were found in somata and proximal dendrites, still at much lower density than in the axon initial segment, but were conspicuously absent from dendritic spines. Thus it is still unclear which are the channels that are activated, at very short latencies, by the bAP.

B. Potassium Channels in Spines

Of the variety of voltage-gated potassium channels residing on central neurons (212), special attention is devoted to the Kv4.2 channel that carries the transient I_A current which is assumed to reside primarily on remote dendrites and spines (13). In a recent EM mapping study, Kerti et al. (228) found this channel to distribute evenly on dendrites and spines, but with no preference to remote dendrites. An interesting association between Kv4.2 and an actin binding protein filamin has been demonstrated (388), and it was suggested that filamin doubles the current density generated through the Kv4.2 channels in transfected cell lines. However, a recent mapping study could not localize filamin within dendritic spines of cultured hippocampal neurons (357).

Another, voltage-independent/calcium-dependent potassium channel that is found in dendritic spines is the small potassium (SK2) type. It is activated by a rise in intracellular calcium, is selectively blocked by apamin, and resides at high concentrations near postsynaptic density within dendritic spines (44). Blockade of SK channels or their genetic deletion increases excitability of dendrites and spines and enhances their ability to undergo LTP (7). This channel is subject to modulation by the M1 muscarinic receptor, which reduces the sensitivity of this channel to intracellular calcium concentration, causes an increase in the size of the excitatory postsynaptic potential (EPSP), and may underlie the effects of muscarinic drugs on learning and memory (145). Interestingly, the channel is activated by an influx of calcium through the NMDA receptor. In the process of LTP, this channel is assumed to be removed from the plasma membrane, and thus increase excitability of the dendritic spine (145).

Another voltage-insensitive potassium channel assumed to be selectively associated with the postsynaptic density is the G protein-coupled K channel (GIRK, Ref. 294). It complexes with protein kinase A, protein kinase C and phospholipase C, as well as protein phosphatase 1, protein phosphatase 2A, and regulators of G protein signaling, all of which modulate its activity. It is assumed to be activated by the metabotropic GABA receptor (GABA-B) and by serotonin to hyperpolarize the spine membrane and cause depotentiation of the excitatory synapse. Interestingly, morphine enhances GIRK channel activity in the hippocampus (351). While GIRK channels are not assumed to selectively localize in dendritic spines, their interactions with excitatory neurotransmission has important implications for dendritic spine plasticity.

C. Calcium Handling in Spines

Compared with NaV channels, much more is known about the presence and functions of calcium channels in dendritic spines. Intracellular calcium concentration ($[Ca^{2+}]_i$) is maintained at a low (50–100 nM) concentration in resting conditions and can rise, following stimulation, by several orders of magnitude. This rise in $[Ca^{2+}]_i$ activates cascades of kinases and phosphatases, resulting changes in cellular functions (see sect. VI). The dendritic spine is endowed with several species of voltage-gated calcium channels. These include channels of the L [Ca(v)1.2 and Ca(v)1.3], T, and R [Ca(v)2.3] types (43). The former channel is distributed in the soma and dendrites as well, but is concentrated in dendritic spine postsynaptic density and distal dendrites (277), but the T and R channels are probably responsible for the rise of $[Ca^{2+}]_i$ following back-propagating action potentials (43). These channels are localized primarily on spines of pyramidal neurons and selectively activate the SK channel, which is instrumental in rapid restoration of membrane potential following voltage-gated depolarization of the spine head (44). Interestingly, this selective action of the Ca(v)2.3 channel indicates that there are calcium microdomains within the dendritic spine head, as small as they may be. Other types of calcium channels are present in other cell types; cerebellar Purkinje cells are endowed with P-type channels, while thalamic neurons contain an abundance of T-type channels (89). These channels differ in their kinetics, voltage dependence, and drug sensitivity (see Ref. 517 for review).

Another category of routes for calcium entry into spines are the ligand-gated channels. These include two subtypes: ionotropic and metabotropic receptors. The most abundant one is

the glutamate receptor. Spine heads contain ionotropic glutamate receptors of the AMPA and the NMDA types, as well as metabotropic glutamate receptors. The NMDA receptors, when activated, allow both Na^+ and Ca^{2+} to influx into the spine. The NMDAR is voltage/Mg sensitive and, upon activation, is likely to be responsible for most of the influx of calcium into the spine head, in as much as blockade of the NMDA receptors suppresses the calcium transient recorded upon glutamate activation of the synapse (44, 428). In fact, it has been proposed that some spines may be endowed only with NMDA receptors, making them "silent synapses" at resting potential, and, when activated, cause an influx of calcium, leading to the recruitment of AMPA receptors into the spine heads (301). Spine heads contain several other receptor species, including the metabotropic glutamate receptor (mGluR), likely to cause activation of phospholipase C followed by release of calcium from stores (123, 151), and the nicotinic cholinergic receptor (119).

Two other possible modes of calcium entry into spines are the transient receptor potential (TRP) channel and the store-operated calcium entry (SOCE) channel. The TRPs constitute a family of channels that is subdivided into several species (355). There is accumulating evidence that TRPC3, a nonselective cation channel, allows calcium into cells, and it is assumed to mediate the effects of brain-derived neurotrophic factor (BDNF) on spine formation and synaptic activity (11, 348). It is further suggested that the effects of BDNF to cause trafficking of AMPA receptors into the synapse, an essential element in synaptic plasticity, are mediated partially by the TRP channel (348). NMDA-mediated calcium influx may also involve TRP channels, at least in olfactory bulb neurons (485). Another member of the TRP channel family, the TRPC6 has also been associated with excitatory synapses, and assumed to regulate their formation (585) via a CaMKIV-CREB pathway. Thus overexpression of TRPC6 results in a higher density of spines, higher rates of miniature excitatory postsynaptic currents (mEPSCs), and a better performance in a water-maze task.

The role of calcium stores in dendritic spine function and plasticity is still debated. Neuronal calcium stores are of two types, the inositol 1,4,5-trisphosphate (IP_3) receptor and endoplasmic reticulum store, studied extensively in cerebellar Purkinje cells (151) but is also ubiquitous in other brain regions as well, including the hippocampus and cortex. The IP_3 receptor is assumed to mediate the action of acetylcholine (ACh) and other neuromodulators, to cause release of $[Ca^{2+}]_i$ from stores and to cause a subsequent change in AMPA and NMDA receptor functions (123). Interestingly, while IP_3 receptors have been localized in dendritic spines of GABAergic cerebellar Purkinje cells and spiny striatal cells where they have been studied extensively (196), they are probably present in dendritic shaft but not in spines of pyramidal neurons (453), even though their function in synaptic plasticity has been extensively documented (123, 410). One possible mode of involvement of endoplasmic reticulum in spine calcium-induced calcium release from IP_3 stores has been proposed recently (192); synaptic activation of glutamate receptors could evoke a delayed calcium surge in large spines that was blocked by mGluR antagonists and heparin (indicating IP_3 stores). The role of these delayed calcium surges in synaptic plasticity is not entirely clear.

The more controversial store is the one associated with the ryanodine receptor. This store has been studied extensively in muscle fibers but is also found in neurons throughout the brain (529). There are three species of ryanodine receptors (RyR1–3) that are differentially localized in dendrites and spines of central neurons. Its main attribute is that it is activated by calcium influx into the cell, meaning that the influx of calcium ions through the plasma membrane is amplified by release of calcium from RyR-associated calcium stores. Caffeine and low concentrations of ryanodine (0.5–1 μM) are agonists for this store, and high concentrations of ryanodine (100 μM) or cyclopiazonic acid (CPA) are antagonists of the store. Initial studies (254, 299) were unable to detect an effect of CPA on subthreshold synaptically evoked rise of $[Ca^{2+}]_i$ in CA1 neurons of the hippocampus, a response that was shown to be mediated by activation of the NMDA receptor. In contrast, Emptage et al. (112) demonstrated that release from stores is responsible for most of the rise of $[Ca^{2+}]_i$ that is seen following synaptic activation in CA1 neurons of cultured hippocampal slices. Interestingly, they found that blockade of calcium-induced calcium release from stores does not affect back-propagating action potential-induced calcium rise. This observation contrasts with that of Sabatini and co-workers (299, 428) who found that the decay time constant of the elevated $[Ca^{2+}]_i$ caused by back-propagating action potential is prolonged by CPA. Admittedly, Sabatini and co-workers (299, 428) found that this is a heterogeneous effect, likely due to heterogeneity of the concentration of RyRs in dendritic spines.

The issue remained controversial when Brunig et al. (61) reported that they could not detect any effect of caffeine on spine motility, unlike the effects of NMDA or AMPA receptor activation. Furthermore, Harvey and Svoboda (172) could not confirm an involvement of RyR in tetanic stimulation-induced spine head expansion. These observations contrast with the effects of caffeine on $[Ca^{2+}]_i$ and spine morphology reported before (251), and the description of the presence of calcium stores in dendritic spines (170). A more recent study proposes that the RyR2 and -3 isoforms mediate the action of BDNF on dendritic spines and on cognitive tasks associated with the hippocampus (5).

A possible molecular and structural substrate associated with the RyR is the spine apparatus, which is enriched with synaptopodin, an actin binding peptide that was originally detected in the kidney, and later in the brain (338). It has been proposed that synaptopodin is colocalized with the presence of the RyR

in dendritic spines of rat hippocampus (534). The association of synaptopodin with RyR may resolve the disagreements about the proposed involvement of RyR in synaptic plasticity, as only some large spines contain the spine apparatus and synaptopodin, while the others may not be affected by activators of the RyR (253).

Strikingly, recent work by Stutzmann and Mattson (486) associates Alzheimer's disease (AD) neuropathology with an overexpression of RyRs in dendrites and particularly in spines. Furthermore, caffeine caused a large facilitation of reactivity to tetanic stimulation only in neurons from young 3xTg mouse hippocampus. This supersensitivity to activation of the RyR was noticed before the emergence of neuropathology in these mice, indicating that activation of RyR stores may lead rather than lag behind the pathology of AD. These results indicate that the association of RyR with dendritic spines, and their role in release of calcium from stores in relation to synaptic plasticity, may be more critical than originally suggested, especially in the development of AD.

Once $[Ca^{2+}]_i$ rises in the dendritic spine, it is removed rapidly from the cytoplasm. Several routes for removal of excess calcium are found in dendritic spines, including extrusion via Ca^{2+}-ATPase pump and Na^{+}-Ca^{2+} exchangers (62). Calcium can also be pumped into intracellular calcium stores that are present in the endoplasmic reticulum by activation of endoplasmic reticulum Ca^{2+}-ATPase. Interestingly, one of the main sinks for accumulation of excess intracellular calcium is the mitochondria, which is conspicuously absent from the dendritic spine (473). The absence of mitochondria, the relative sparse endoplasmic reticulum, and the small volume of the spine led to the suggestion that the spine can raise $[Ca^{2+}]_i$ momentarily to very high levels, much above those of the parent dendrite, so as to effectively activate local calcium-dependent phosphorylation/dephosphorylation of key proteins in the signal transduction cascades (88).

D. The Spine Neck: Barrier or Bridge?

As mentioned above, earlier studies (490) measured the dissipation of a fluorescent dye from the spine head to the parent dendrite, to estimate the spine neck resistance to be <50 MΩ. In a later study, using photoactivatable GFP, Bloodgood and Sabatini (43) found large variations in the diffusional coupling between spine head to the parent dendrite, which are not explained only by spine neck length. In fact, the diffusional coupling was found to be regulated by pre- and postsynaptic activity, such that active neurons restrict the coupling between their spines and the parent dendrites. Further studies by Grunditz et al. (158) demonstrated that postsynaptic depolarization is sufficient to restrict the diffusion between the spine and dendrite. In fact, spine neck regulates the local depolarization of the spine head, to allow variable synaptically evoked calcium transients in the spine head (149).

The functional significance of spine neck length has three facets: first is the question of what chemical, if any, is transmitted between the spine head and the parent dendrite, and what might it signal to the dendrite. Second is the question of whether there are functional differences between short and long spines with respect to magnitude/quality of synaptic currents. Third is the question of what regulates spine neck length and its function as a variable barrier.

In as much as there are no differences between short and long spines in voltage responses to bAP arriving from the soma, Araya et al. (18) reported that shorter spines produce larger somatic EPSCs in response to flash photolysis of caged glutamate. In support of this is the recent suggestion (22) that the spine neck morphology may regulate the diffusion of glutamate receptors from the dendrite into the synapse, which may underlie the neck-length dependent variations in synaptic responses, and the suggestion that a synapse on filopodia is not as effective, and certainly not as stable as a synapse on a spine. On the other hand, Harris and Stevens (171), in an extensive EM reconstruction study, found a correlation between spine head size and PSD dimension, but could not find a correlation between PSD size and spine neck length. PSD size is correlated with magnitude of the synaptic response as has been shown by several groups. What then is the function of the synapse on long spines if they do not impact the soma? Spine neck length can vary as a function of exposure to plasticity-producing stimulation (228), so their functional integration into the dendritic signaling is likely to result from their exposure to enhanced stimulation.

What is the signal that is transmitted between spine head and the parent dendrite? Because of the fast kinetics of decay of elevated calcium, and the fact that the spine neck is a rather narrow (~0.1–0.2 μm) and long (1–2 μm) tube, it was assumed that the path between the spine head and the dendritic shaft is not used for dissipation of excess calcium from the spine head (428). However, flash photolysis of caged calcium in dendritic spines (249) indicates that spine neck length can be considered a variable resistor for the diffusion of calcium from the spine head to the parent dendrite, such that short spine necks allow elevated calcium to diffuse from the spine head into the parent dendrite.

Small molecules can carry the message sent from activated spines to neighboring ones. One of them is Ras. It is generated by uncaging of glutamate near a dendritic spine. It has been associated with LTP of reactivity of the spine to glutamate and can spread into the parent dendrite and adjacent spines up to 10 μm away from the activated one (173). This rise of active Ras is likely to underlie the enhanced sensitivity of spines adjacent to the activated one to subsequent activation. RhoA is another such molecule that becomes persistently active following intense stimulation and can spread to adjacent spines (341).

IV. MOLECULAR CONSTRUCTION OF SPINES

The identification of the molecules and molecular families involved in spine formation and morphology started in the late 1990s when it became possible to transfect neuronal cultures with cDNAs expressing proteins potentially important for spine morphogenesis. Since then, several hundreds of proteins/hormones/growth factors have been proposed to act as spine morphogenic factors **(TABLE 1)**. For most of these proteins their activity was demonstrated by transfecting wild-type or mutant species in hippocampal/cortical neurons in dissociated cultures or organotypic brain slices. For some of them, the animal model (knockout, knock-in, or transgene) has been developed so as to reinforce and extend the in vitro findings. Interestingly, the presence of functional mitochondria in dendrites seems to be required for the correct formation, maturation, and plasticity of dendritic spines as suggested by recent experiments in neurons deleted or transfected with mutant proteins, such as PGC-1α, Drp1, OPA1, Sig-1Rs, PINK1/PARK6, and Centaurin-α1, that regulate formation, morphology, function, and dendritic localization of mitochondria (77, 280, 492, 515, 574). In this review we classify the spine morphogenic factors depending on the molecular composition/function, and we emphasize the molecules that have been demonstrated to have a spine morphogenic activity in both culture and brain tissue.

A. Actin Binding and Cytoskeletal Proteins

The cytoskeleton of dendritic spines is composed mainly of filamentous (F)-actin that forms parallel filaments in the neck of the spines and lattice and twisted filaments in the spine head. Thus it is not surprising that all the molecules that regulate growth and shrinkage of dendritic spines will eventually modify positively or negatively actin polymerization, and even the overexpression of actin alone in neurons could increase dendritic spine density (215).

A number of actin binding proteins have been implicated in spine morphogenesis **(FIGURE 1)**. Among them, Drebrin A was the first to be proposed as a regulator of spine morphology when overexpressed in cultured neurons (42, 176, 330). In cultured cortical neurons, the overexpression of Drebrin cDNA (Drebrin A, the adult isoform) positively modifies the length of dendritic spines (176) while in hippocampal cultures mature spines became less stable. It is suggested that Drebin activity is dependent mainly on its F-actin interaction but also on its binding to some actin-regulatory proteins, such as profilin, myosin, gelsolin, and Ras (42, 303). Although these are consistent in vitro observations, Drebrin knockout mice have morphologically normal dendritic spines but show altered homeostatic plasticity, suggesting that Drebrin regulates a subset of actin involved in synaptic plasticity (17).

Among the other actin-binding proteins shown to be morphogenic for spines are myosins II and VI, NESH, spinophilin/neurabin II, synaptopodin, acidic calponin, cortactin, SPIN90, VASP, and WAVE. These proteins regulate actin polymerization directly in the dendritic spines where they accumulate. On the other hand, none of them is specific for dendritic spines because these proteins regulate actin polymerization in many other cellular functions and subcellular compartments.

Among the molecular motors that bind and contract actin filaments, myosin IIB and myosin VI (Myo6) have been found to regulate actin polymerization in spines. These motors are formed by a hexameric protein complexes with two heavy chains and four light chains. Myosin II (in particular the IIB or the nonmuscle *MyH10* isoform) is highly localized at synapses and regulates dendritic spine morphology and synaptic strength (426, 582). In culture, blocking myosin IIB with blebbistatin or with shRNA against the heavy chain inhibits mature spine formation and induces filopodia-like protrusions (426). Also, spine maturation into a mushroom shape requires the contractile activity of myosin IIB (189). Interestingly, the myosin II regulatory light chain (MLC) can be phosphorylated and activated by GIT1/PAK complex (see sect. IV*B*) to induce dendritic spines formation (582). Another myosin II expressed in neurons and in spines is the muscle sarcomeric *MyH7B* isoform, knocking down of which alters spine head morphology (422). The comparison of *MyH10* and *MyH7B* knock down indicates that the two myosin II might have a cooperative mechanism to control spine morphology and synapse function (422).

The role of myosin VI (Myo6) in dendritic spines morphogenesis has been demonstrated both in vitro and in vivo (371). Myo6 also regulates AMPA receptor internalization because Myo6-deficient mouse (called Snell's waltzer) exhibits reduced synapse number, abnormal short dendritic spines, profound astrogliosis, and deficit in the stimulation-induced internalization of AMPAR (371). These data suggest that a limited number of specific actin-based motors control the structure and function of spines in addition to the polymerization state of actin.

Abi-1, Abi-2, NESH/Abi-3, members of the c-Abl tyrosine interactor (Abi) protein family, are linked to PSD scaffold proteins and spine F-actin (378, 401). In the PSD Abi-1 binds to ProSAP2/Shank3, a scaffold protein of the PSD (see sect. IV*B*) and is translocated to nuclei from synapses where it can activate gene transcription of E-box-containing genes upon NMDA receptor stimulation. Functional studies in hippocampal cultured neurons have demonstrated that Abi-1 knocking down by RNAi changes both dendrite morphology, by increasing the branching, and spine structure that became more similar to an immature shape. Thus Abi-1 can regulate dendrite and synapse formation working as a synapto-nuclear messenger specifically controlling gene transcription (401). However, Abi-1 can also activate Rac1 and F-actin

Table 1. *List of major proteins involved in molecular construction of spines*

Name	Main Funtion	Experimental Model	Methods	Reference Nos.
		Actin binding and cytoskeletal proteins		
Abi-1	Actin binding protein	C	RNAi	378, 401
Abi-2	Actin binding protein	M	KO	155
Abi-3/NESH	Actin binding protein	C	Over/RNAi	26
αN-Catenin	Actin binding protein	C	Over	1
Arp2/3	Actin binding protein	M	KO	231
Calponin	Actin binding protein	C	Over	407
Cortactin	Actin binding protein	C	Over/RNAi	185
Drebrin A	Actin binding protein	C	Over/KO	17, 42, 176, 303
MLC	Actin binding protein	C	RNAi	582
Myosin IIB	Actin binding protein	C	Blebbistatin/RNAi	189, 422, 426
Myosin VI	Actin binding protein	M	KO	371
Neurabin I	Actin binding protein	C OBS	Over	369, 590
Neurabin II Spinophilin	Actin binding protein	M	KO	122
Profilin I/II	Actin binding protein	C OBS	Over/RNAi	4, 323
SPIN90	Actin binding protein	C M	Over/RNAi/KO	81, 236, 275
Synaptopodin	Actin binding protein	C	RNAi	368, 534
VASP	Actin binding protein	C	RNAi	282
WAVE1	Actin binding protein	C M	RNAi KO	237
WAVE3	Actin binding protein	C	Over	391
CP	Actin capping protein	C	RNAi	120
Eps8	Actin capping protein	C M	Over/RNAi/KO	320, 480
EB3, p140Cap/SNIP	Microtubule binding protein	C	RNAi	159, 210
MAP1B	Microtubule binding protein	M	RNAi	510
Actin	Cytoskeleton	OBS	Over	215
		Small GTPase and associated proteins		
ARF6	GTPase	C M	Over/RNAi/KO	83, 329, 404
Cdc42	GTPase	OBS M	KO	501, 522
Rac1	GTPase	C OBS M	DN/CA/PI/KO	52, 496, 501, 502, 522
Rap1	GTPase	C	CA	557
Rap2	GTPase	M	Transgene	425
Rem2	GTPase	C	RNAi	143
RhoA	GTPase	OBS	CA	501
Rif	GTPase	C	DN/CA	195
Rnd1	GTPase	C	Over/RNAi	206
ARHGEF6/αPIX	RhoGEF	OBS M	RNAi/KO	359, 406
ARHGEF7/βPIX	RhoGEF	C OBS	RNAi	441
Dock180	RhoGEF	C	Over/RNAi	232
GEFT	RhoGEF	C	Over	60
Kalirin-7	RhoGEF	C OBS M	Over/RNAi/KO	64, 217, 295, 296, 383, 385, 556, 558, 570, 571
Lfc/GEF-H1	RhoGEF	C	Over/RNAi	222, 424
Tiam1	RhoGEF	C	Over/RNAi	328, 507, 508, 581
Vav	RhoGEF	C OBS M	KO	161
α1-Chimerin	RhoGAP	C	Over/RNAi	526
oligophrenin1	RhoGAP	OBS M	RNAi/KO	229, 230
p190RhoGAP	RhoGAP	M	KO	450
p250GAP	RhoGAP	C OBS	RNAi	166, 202, 349
RhoGAP2	RhoGAP	C	Over	524
SRGAP2	RhoGAP	C	Over/RNAi	71

Continued

Table 1.—*Continued*

Name	Main Funtion	Experimental Model	Methods	Reference Nos.
EPAC1/2	RapGEF	C M	Over/RNAi/KO	477, 550, 565
PDZGEF1	RapGEF	C	Over/RNAi	274
Rap1GAP	RapGAP	C	Over	313
SPAR	RapGAP	C	Over	190, 376
RasGRF1	RasGEF	C	Over/RNAi	274
SynGAP	RasGAP	C M	KO	528
EFA6A	ArfGEF	C M	Over/RNAi/KO	83
Centaurin α1	ArfGAP	C	Over/RNAi	492
SrGAP3	RacGAP	M	KO	114, 539
PAK	Kinase	M	Transgene	180
PAK1	Kinase	OBS C	DN/CA/RNAi	48, 175
PAK3	Kinase	C OBS M	Over/CA/DN/RNAi	40, 46, 48, 50, 103, 256, 317, 359, 506
		Cell surface receptors and adhesion molecules		
α3 Integrin	Adhesion protein	M	KO	227
α5 Integrin	Adhesion protein	C	Over/RNAi	543
Arcadlin	Adhesion protein	M C	KO	568
DSCAM	Adhesion protein	M	KO	312
IL1RAPL1	Adhesion protein	C M	Over/KO	382, 523
N-cadherin	Adhesion protein	C OBS	Over/RNAi	96, 106, 316, 367, 430, 568
Neuroligin1,2,3,4	Adhesion protein	C M	Over/RNAi/KO	21, 45, 78, 79, 87, 208, 493
SALM2	Adhesion protein	C	Over/RNAi	242
Syndecan-2	Adhesion protein	C	Over/RNAi	117, 118, 283
Telencephalin	Adhesion protein	C M	KO	404
APP	Membrane protein	M	Transgene	270
TSPAN7	Membrane protein	C	Over/RNAi	31
Vezatin	Membrane protein	C M	KO	96
beta2-nAChR	Receptor	C M	Over/KO	289
GABAAR	Receptor	M	KO	183
GluA2	Receptor	C	Over/RNAi	380, 430
GluN1	Receptor	OBS M	RNAi/KO	10, 521
GluN2B	Receptor	M	KO	59
Npn-2	Receptor	M	KO	512
NgR1	Receptor	C M	Over/KO	6, 272
PGC-1α	Receptor	C	Over/RNAi	77
		Receptor tyrosine kinases and other kinases		
EphB1/2/3	TK receptor	C M	Over/RNAi/KO	118, 184, 203, 224, 225, 331, 383
EphA4/ephrin-A3	TK receptor	C OBS M	Over/RNAi/KO	55, 66, 339, 587
ErbB2/B4	TK receptor	M	KO	30
ErbB4	TK receptor	OBS	Over/RNAi	278
p75NTR	TK receptor	M OBS	KO	576
TrkB	TK receptor	M	KO	68, 535
CaMKII	Kinase	C OBS	Over/RNAi	218, 366
CaMKIIalfa	Kinase	M	KO	562
CDKL5	Kinase	C	Over/RNAi	414
DCLK1	Kinase	C M	RNAi/KO	460
DGKf	Kinase	C M	Over/RNAi	233
LIMK-1	Kinase	M	KO	318
MARK4	Kinase	C	Over	573

Continued

Table 1.—*Continued*

Name	Main Funtion	Experimental Model	Methods	Reference Nos.
NDR1/2	Kinase	C OBS	RNAi	520
PAR1b	Kinase	C	RNAi	177
PI3K	Kinase	C	Activation	92
PKMz	Kinase	C	Over	417
Plk2	Kinase	C M	KO	274
Wnt7a; Dvl1	Kinase	C M	KO	84
Postsynaptic scaffold proteins and adaptor proteins				
CASK	Scaffold	C	Over/RNAi	69
CTTNBP2	Scaffold	C	RNAi	75, 76
cypin	Scaffold	C	Over/RNAi	516
DISC1	Scaffold	C	Over/RNAi	181
GIT1	Scaffold	C M	RNAi/KO	321, 582
Homer1a	Scaffold	C	Over	432
Homer1b	Scaffold	C OBS	Over	179, 433
intersectin-s	Scaffold	C	RNAi	505
IQGAP1	Scaffold	M	KO	137
N-WASP	Scaffold	C	Over/RNAi	544
PICK1	Scaffold	C	Over/RNAi	347
PSD-95	Scaffold	C OBS M	Over/KO	111, 354, 398, 483, 533
Preso	Scaffold	C	Over/RNAi	273
SAP102	Scaffold	C	Over/RNAi	72
Shank1	Scaffold	C M	Over/KO	200, 433
Shank2	Scaffold	M	KO	444, 549
Shank3	Scaffold	C M	Over/RNAi/KO	108, 421, 530, 541
TANC1/2	Scaffold	C M	Over/KO	164
WAVE1	Scaffold	M	KO	468
afadin	Adaptor	M	KO	33
IRSp53	Adaptor	C M	Over/RNAi/KO	82, 234, 443
Numb	Adaptor	C	Over/RNAi	356
PAR-3	Scaffold	C	RNAi	581
PAR-6	Scaffold	C	Over/RNAi	580
Micro RNA (miRNA), mRNA binding protein, and transcription factors				
miR-29a/b	miRNA	C	Over	284
miR-125a	miRNA	C	Antisense	335
miR-125b	miRNA	C	Over/sponge	109
miR-132	miRNA	M	Over/sponge/transgene	109, 166, 202, 315, 381
miR-134	miRNA	C	Over/antisense	445
miR-138	miRNA	C	Antisense	463
miR-185	miRNA	M	KO	559
Satb1	DNA binding protein	C from M	KO	28
hnRNPK	RNA binding protein	C	Over/RNAi	402
Staufen2 (Stau2)	RNA binding protein	C	Over/RNAi	148
TLS	RNA binding protein	M	KO	134
Cux1/Cux2	Transcription factors	C M	KO	91
FoxO6	Transcription factor	M	KO	436

C, primary neuronal cultures; OBS, organotypic brain slices; M, genetically modified mice; Over, overexpression; DN, dominant negative; CA, constitutively active; PI, pharmacological inhibition.

polymerization upon calcium/calmodulin-dependent protein kinase II (CaMKII)-α phosphorylation at serine-88 induced by glutamate stimulation (378). Abi-2 is also strongly expressed in the brain, and its knockout in mice impairs spine density, by reducing the number of mushroom shaped spines. These morphological alterations correlate with negatively altered both short- and long-term memory formation (155).

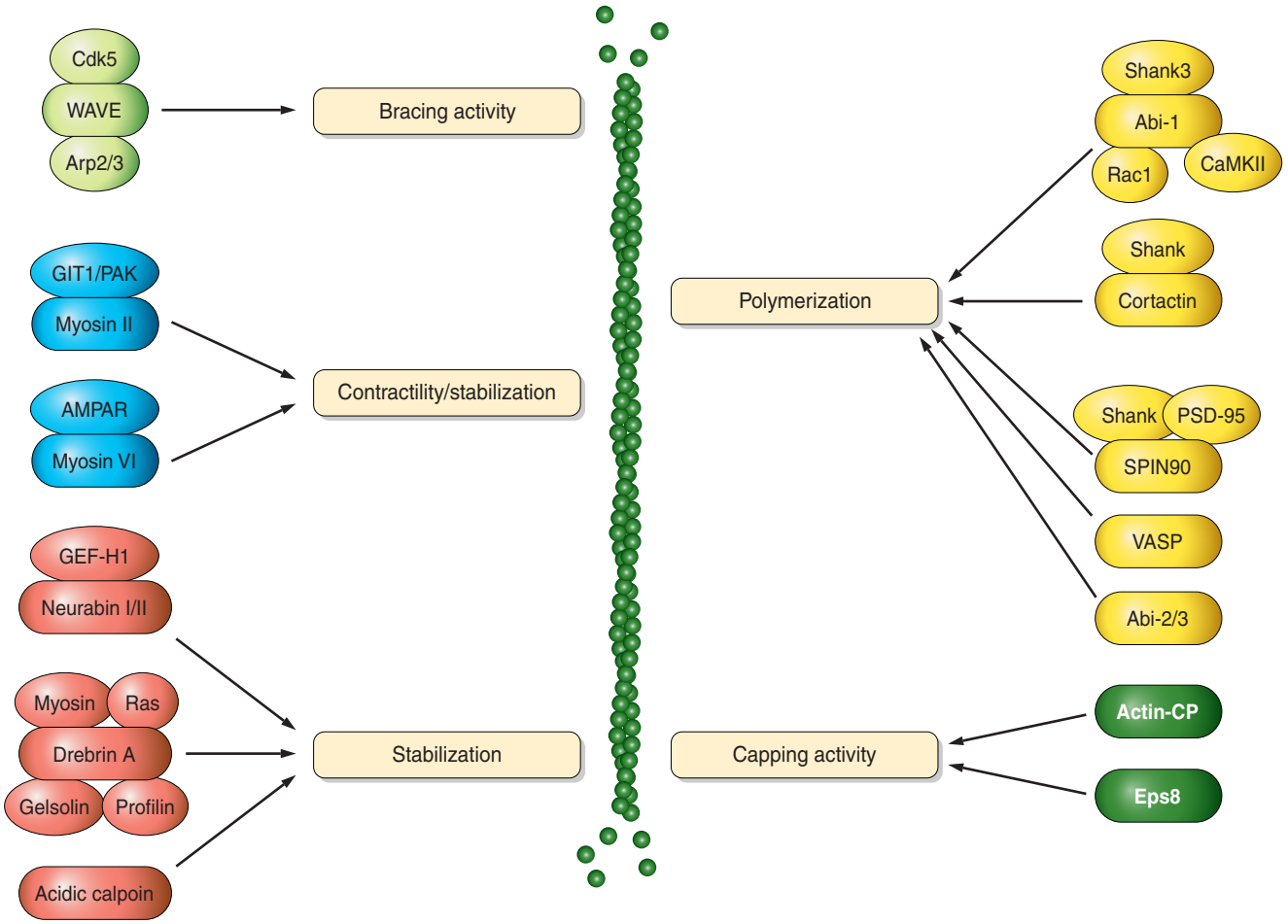


FIGURE 1. Actin binding proteins. The scheme shows the principal actin binding proteins (in ovals) and the main associated proteins (in the ellipse) divided for the principal activity on actin filaments (one filament in green).

The manipulation of NESH/Abi-3 expression has multiple effects: overexpression of NESH/Abi-3 in cultured neurons reduces the amount of mushroom-type spines and synapse density but increases the thin filopodia, while siRNA knockdown also reduces mushroom spine numbers but increases spine density (26). Whether NESH/Abi-3 activity is regulated by synaptic stimulation was not determined as yet.

Neurabin I and neurabin II (or Spinophilin) are both F-actin-binding proteins with a common domain organization forming NH_2-terminal actin binding domain, PP1 binding domains, PDZ and a COOH-terminal coiled-coil domain. The two proteins are able to associate in homo- and heterodimers with the coiled-coil domain and to cross-link F-actin. Immature cultured neurons overexpressing neurabin I have more dendritic filopodia, while older neurons have enlarged spines (369). The overexpression of the NH_2-terminal actin-binding domain is sufficient to increase actin polymerization, spine motility, density, and length (590). Similarly, spinophilin is predominantly localized in dendritic spines (as the name suggests) of pyramidal neurons and is necessary for the development of dendritic spines because knockout mice show more filopodia and immature spines compared with wild-type mice of the same age and express defects in glutamatergic transmission (122). Molecularly both proteins associate in synapses with a similar complex of molecules, including Lfc, a Rho GEF that regulates the Rho-dependent organization of F-actin in spines (424).

Cortactin is another synaptic actin binding protein. Its knockdown by siRNA eliminates dendritic spines in hippocampal neurons, while its overexpression causes elongation of spines. Because the localization of cortactin in synapses is regulated by NMDA receptor activation, cortactin can also be considered as a regulator of activity-dependent spine morphogenesis (185). The synaptic proteins cortactin-binding protein 2 (CTTNBP2) binds to and recruits cortactin to spines; in the absence of CTTNBP2, the number of dendritic spines is reduced, and cortactin and other synaptic proteins are less associated with spines heads (75, 76).

Acidic calponin is another actin binding protein the expression of which is upregulated during dendritic spine plasticity following pilocarpine-induced seizures and which consequently regulates spine morphology and den-

sity through the reorganization and stabilization of actin filaments (407).

SPIN90/WISH [SH3 protein interacting with Nck, 90 kDa/Wiskott-Aldrich syndrome protein (WASP) interacting SH3 protein] is another scaffold regulator of actin polymerization in dendritic spines. Overexpression of SPIN90 in cultured neurons increases the number and length of dendritic filopodia/spines via an N-WASP-independent mechanism, while knockdown of its expression and in knockout mice dendritic spine density and maturation is reduced (81, 236, 275). SPIN90 binds to PSD-95 and Shank and might work as their downstream effector on spine morphogenesis (81, 236, 275).

The multifunctional actin-binding protein vasodilator-stimulated phosphoprotein (VASP) is a major regulator of actin polymerization. In cultures VASP knockdown leads to a significant and specific decrease in the density of spines and synapses. Not surprisingly, the actin binding domains of VASP, Ena/VASP homology 1 and 2 (EVH1, EVH2), are required for VASP accumulation near spine actin (282). While VASP is considered an actin anti-capping protein, the actin-capping protein CP, a regulator of actin filament growth, has also been described to have a similar effect to that of VASP; CP knock-down in cultured neurons reduces spine and synapse development and maturation (120). Similarly, Eps8, another actin capping protein, is important for spine morphogenesis (320, 480).

The WAVE (Wiskott-Aldrich syndrome protein family Verprolin homologous proteinis) families of proteins are key regulator of actin polymerization through their ability to activate the actin-related protein (Arp2/3) complex. Loss of WAVE1 in cultured neurons and in vivo induces a decrease in mature dendritic spines (237, 468). On the other hand, WAVE3 overexpression in cultured neurons decreases spine numbers, but the remaining are eventually more mature (391). WAVE1 activity on spine actin is dependent on Cdk5 phosphorylation that inhibits its ability to recruit mitochondria in dendrites (488) and to regulate the actin polymerizing Arp2/3 complex (237). The Arp2/3 complex is critical for maintaining actin polymerization in spines because the postnatal knockout of the Arp2/3 subunit ArpC3 leads to progressive loss of dendritic spines and abolishes the activity-dependent enlargement of spines heads (231).

Although polymerized actin is still the main component of the spine cytoskeleton (220), recent evidence suggests that microtubules actively participate in the shaping of dendritic spine morphology. EB3 is microtubule tip-tracking protein that enters spines with the microtubules and modulates spine morphology by interacting with p140Cap/SNIP, a regulator of Src tyrosine kinase. Thus EB3-labeled growing microtubule ends control p140Cap recruitment to spines as well as modulate cortactin function and dendritic spine actin dynamics (159, 210). Localization of p140Cap to spines is also dependent on the presence of partitioning-defective 1b (PAR1b), a cell polarity-regulating serine/threonine protein kinase. Thus PAR1b knock-down neurons exhibit reduced mushroom-like dendritic spines and more filopodia-like protrusions, without changes in total number of protrusions (177).

Microtubule-associated protein 1B (MAP1B) is highly expressed during the beginning of neuronal development and is present in dendritic spines. In MAP1B knockout mice, the density of mature dendritic spines is reduced but is compensated by an increase of immature filopodia-like protrusions. In these mice, changes in spine morphology are associated with a decrease in Rac1 activity and an increase in RhoA activity and decrease in phosphorylated cofilin. Thus MAP1B directly or indirectly regulates the actin cytoskeleton (510).

B. Small GTPase and Associated Proteins

Actin polymerization is controlled by extracellular and intracellular signals that all come together on small GTPase of the Rho family. The switch between the active GTP-bound and inactive GDP-bound states can change the activity of specific actin binding proteins which will promote or suppress the polymerization of actin filament.

For example, the actin-based ruffling motion of lamellipodia, which is regulated by small GTPases in many cell types, is also present in the peripheral submembrane region of dendritic spines (252).

The three members of the Rho family of small GTPases mostly involved in spine morphogenesis are RhoA, Rac1, and Cdc42 (52, 353, 363, 496, 501, 502, 522), distinguished in two opposite activities; RhoA inhibits, whereas Rac and Cdc42 promote the growth and/or stability of dendritic spines **(FIGURE 2*A*)**. However, this classification is too simplistic because the effects of each individual GTPase on the final spine morphology depend also on the relative level of expression and activity of the three GTPase in each spine.

Rho family GTPases are regulated by a number of upstream and downstream molecules, and several nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) regulate Rho GTPases in spines.

Eight Rho-GEF proteins have thus far been described to play a role in regulating spine morphology. Among them, only one which seems to be specific for RhoA is GEF-H1/ARHGEF2/Lfc [Lbc (lymphoid blast crisis)'s first cousin] (222, 424). Lfc translocates upon synaptic stimulation in a calcium-dependent manner from microtubules to spines where it binds to neurabin II and is associated with AMPA receptor complex. However, the effect on spine morphology is contradictory also because the shRNA inhibition of Lfc in cultured neurons seems to reduce RhoA activity but increase Rac1 activity (222, 424).

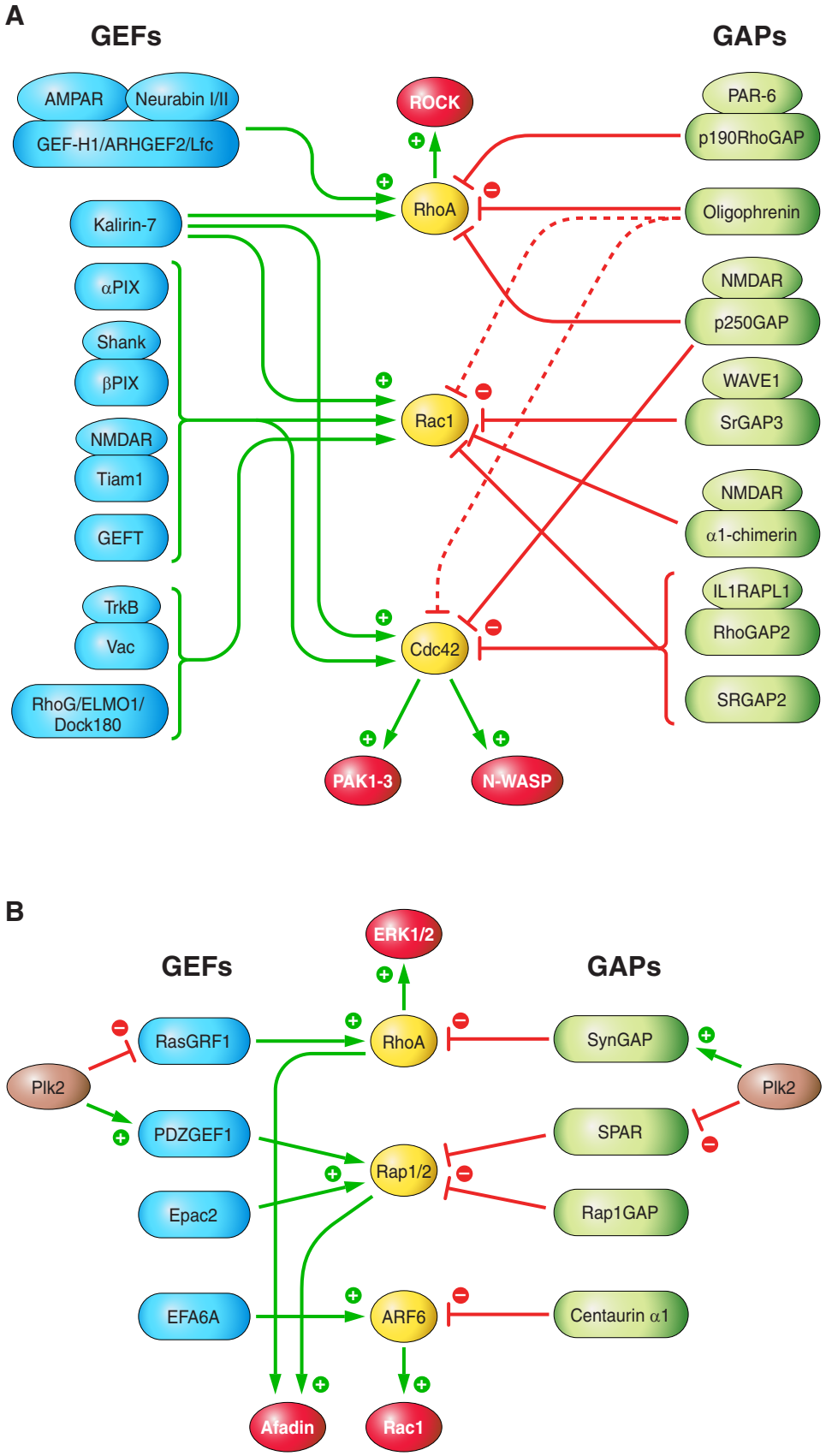


FIGURE 2. Small GTPase. The summary diagram shows how small GTPase of the Rho family (*A*) and of the RAS family (*B*) are controlled in regulating actin in spines. The GEF and GAP proteins (in blue and green ovals) and main associated proteins (in ellipses) are indicated.

A major and most studied spine RhoGEF is Kalirin-7, which can activate all three RhoGTPase. Kalirin-7 is highly concentrated in the PSD where it binds to PSD-95 and other scaffold proteins. Several studies (including in vivo experiments) demonstrate that Kalirin-7 promotes spine formation especially in cortical neurons (64, 295, 296, 385, 558). The activity of Kalirin-7 is also regulated by the phosphorylaion of CaMKII and CdK5 (296, 558).

ARHGEF6/αPIX and ARHGEF7/βPIX [α/β-p21-activated kinase (PAK) interacting exchange factor] are both GEF for Rac1 and Cdc42 in dendritic spines. Mutations in the ARHGEF6 gene are found in patients afflicted by a nonsyndromic form of X-linked intellectual disability, and its role in spine maturation has been demonstrated also in the KO mice (261, 359, 406, 582). βPIX is recruited at synapses though its specific interaction with COOH-terminal unique sequence at the scaffold protein Shank (see below) (379), and its activity is regulated by CaM-kinase kinase/CaM-kinase complex and synaptic activity (441).

The Rac1/CdC42 GEF Tiam1 is also involved in dendrite and spine development, and it has been demonstrated in vitro to be associated with NMDA receptors and to be a downstream signaling of the EphB receptor-dependent spine development (see below) (507, 508). The activity of Tiam1 is also regulated by the scaffold polarity protein PAR-3 that is able to spatially restrict Tiam1 activity to dendritic spines (581).

The last three RhoGEF described to play a role in spine are GEFT, Vav, and Dock180. GEFT is diffusely expressed in the brain and induces both neurite outgrowth and dendritic spine enlargement, acting on the Rac and Cdc42-PAK pathways (60). With the use of KO mice it has been demonstrated that Vav mediates BDNF-stimulated Rac-GTP production and spine head enlargement in CA3-CA1synapses as well as theta burst-stimulated LTP (161). Dock180 has been identified as the only RhoGEF, among the 70 tested, to be able to promote spine morphogenesis in a complex formed by RhoG/ELMO1/Dock180 signaling module (232).

Activated GTP-bound Rho GTPases are inhibited by specific GTPase activating proteins (RhoGAP), five of which seem to be important for dendritic spine actin remodeling. An extensively studied synaptic RhoGAP is oligophrenin because when its gene is mutated, a form of syndromic X-linked intellectual disability emerges (41). Both in vitro and in vivo (in the KO mice), oligophrenin suppression reduces spine length and maturation supposedly by over activation of RhoA although Rac1 and Cdc42 are also substrates of oligophrenin (153, 229).

p250GAP is an NMDA receptor-associated RhoGAP, yet the knock-down of which increases spine maturation by modulating RhoA (202, 349) or Rac1 activity (202), probably depending on the type of neurons studied. The p190RhoGAP is another GAP specific for RhoA that is regulated by Arg kinase and partition scaffold protein PAR-6 and is important for maintaining the number and morphology of mature spines in adult mice (450, 580).

A peculiar RhoGAP specific for Rac1 is the non-kinase phorbol ester receptor α1-chimerin, a protein that when overexpressed in cultured hippocampal neurons inhibits the formation of new spines and removes the existing ones by binding to synaptic GluNA2 subunit of the NMDA receptors (526). Two opposite effects on spines have been observed for two other synaptic RhoGAPs called SRGAP2 and RhoGAP2/ARGEF22. When overexpressed, SRGAP2 promotes spine maturation and limits spine density (71), while RhoGAP2 increases spine density (524) and both specifically stimulate the GTPase activity of Rac1. These data indicate that the general conclusion that RhoA inhibits, while Rac and Cdc42 stimulate the growth and/or stability of dendritic spines is too simplistic, and a more complex analysis of the relative contribution of each RhoGTPase to spine actin polymerization is needed.

Indeed, a recent study examined the spine activation of RhoA and Cdc42 using a florescent methodology and two-photon glutamate uncaging. Activation of both RhoA and Cdc42 was similar; an initial fast phase lasting 5 min which was followed by persistent activation enduring more than half an hour. However, the two GTPase have a different mobility: RhoA diffused and spread outside the spine up to 5 μm along the dendrite while active Cdc42 remained concentrated in the stimulated spine. Interestingly, inhibition of the RhoA-ROCK pathway blocked the initial spine growth, and the Cdc42-Pak pathway was essential for the maintenance of the change in spine plasticity (341).

The major synaptic downstream effectors of RhoGTPase are the p21-activated kinases PAK1 and PAK3 that when activated promote formation and/or growth of spines. RNAi-mediated knock-down of PAK3 or transfection of dominant-negative PAK3 mutant induces abnormally long dendritic spines that lack synapses and a loss of mature spine synapses (46, 359). Similarly, PAK1 activity promotes spine maturation (48, 175).

However, in PAK3 knockout mice, spines are morphologically normal, but long-term synaptic plasticity is reduced and memory consolidation is impaired (317). Alterations in synaptic plasticity and a reduction in spine density have been found in cortical but not hippocampal neurons of transgenic mice overexpressing dominant negative PAK (for both PAK1 and PAK3) (180).

More recently, Dubos et al. (103) showed that inhibition of PAK3 resulted in an activity-dependent growth of new and unstable spines and the impairment of plasticity-mediated spine stabilization and formation of persistent spines (103). Thus PAK3 activity seems to regulate negatively spine growth (103). The role of PAK3 in mediating activity-dependent rear-

rangement of synaptic connectivity associated with learning is clearly suggested by the finding that in human, PAK3 mutation causes intellectual disability (8, 256). Lim-kinase 1 (LIMK-1) is a downstream molecule of PAK and Rho-associated kinase ROCK. Knockout mice for LIMK-1 manifest altered spine morphology, synaptic plasticity, and reduced spatial learning (318).

A link between Cdc42 and actin remodeling is Wiskott-Aldrich syndrome protein (N-WASP), which binds and activates the Arp2/3 complex and promotes actin branching and polymerization. N-WASP is localized in spines, and the knockdown of endogenous N-WASP or its inhibition with wiskostatin or the overexpression of an N-WASP mutant, which is unable to bind Arp2/3, induces a significant decrease in the number of spines and excitatory synapses in cultured neurons (544).

Finally, a specific synaptic scaffold protein, called G protein-coupled receptor kinase-interacting protein 1 (GIT1), is involved the functional assembly of αPIX and PAK3. The importance of GIT1, that also contains an ADP-ribosylation factor (Arf) GAP domain, is demonstrated by the finding that its knockout both in cultured neurons and in transgenic mice reduces dendritic spine number (321, 582).

Ras family of GTPases and the related Rap GTPases are also involved in regulating spine morphogenesis **(FIGURE 2*B*)**. The involvement of Ras and the downstream mitogen-activated protein kinase signaling pathways in regulating spine morphogenesis has been demonstrated by the observation that spine density in cortical pyramidal neurons is increased in transgenic mice expressing active Ha-Ras (138). In contrast, transgenic mice overexpressing constitutively active Rap2 (Rap2V12) in forebrain displayed fewer and shorter dendritic spines in CA1 hippocampal neurons (425).

A major regulator of Ras activity in spines is the RasGAP protein SynGAP that plays a major inhibitory role by developmentally repressing excitability synapse and dendritic spine formation during the critical period (86, 528). On the other hand, neurons overexpressing the postsynaptic RapGAPs SPAR (190, 375, 376, 476) and Rap1GAP (313) have spines with altered morphology and larger head. Thus these results suggest that Ras and Rap, in the postsynaptic compartment, have an opposite effect in controlling synapse strength, spine morphology, and synaptic plasticity. As far as synaptic plasticity is concerned, Ras is a positive effector of LTP, while Rap1 and Rap2 regulate LTD and depotentiation (589). Interestingly, the activities of Ras and Rap are coordinated by polo-like kinase 2 (Plk2), a homeostatic suppressor of overexcitation, that controls the phosphorylation-dependent ubiquitin-proteasome degradation of the Ras activator RasGRF1 and Rap inhibitor SPAR and the phosphorylation-activation of Ras inhibitor SynGAP and Rap activator PDZGEF1 (274).

It is still not clear how Ras and Rap and mitogen-activated protein kinase downstream pathways regulate dendritic spine formation (553). One effector could be Afadin, although it regulates dendritic spines mostly via cadherin function (33). Ras also binds to and interferes with drebrin activity on actin (42). Rap1 effect on spine morphology is also mediated by the adaptor protein AF-6, a scaffold protein associated with actin and other signaling proteins (557). However, both SynGAP and SPAR can regulate actin polymerization via cofilin function or directly by binding to actin (65, 376).

Among the Ras-related family of small GTPases, the ADP-ribosylation factor 6 (ARF6) and the related GEF (EFA6A) and GAP (Centaurin α1) for ARF6 seem to regulate spine development and maintenance by controlling the conversion of filopodia to spines, or vice versa (329), and the stability of both early and mature spines by inducing the internalization of telencephalin [TLN/intercellular adhesion molecule-5 (ICAM5)] specifically at the filopodia and activating Rac1 (83, 333, 404).

Finally, two other small GTPase have been involved in regulation of spine number and morphology, namely, Rem2 and Rnd1 (143, 206). Another GTPase, Rif, is regulating filopodia formation (195). However, little is known about how are these molecules regulated and their respective effectors. Also, thus far, the data were collected only with in vitro experiments.

C. Cell Surface Receptors, Extracellular Matrix, and Adhesion Molecules

Most of the recently discovered adhesion proteins important for the formation and development of brain synapses are also involved in dendritic spine formation. Among them Neuroligin, SALM, netrin-G ligand, and IL1RAPL1 have been found to induce synapses and spine formation both in vitro and in vivo. Interestingly, all of these proteins bind to PSD-95, a major excitatory scaffold protein (see below).

The overexpression of Neuroligin1 in cultured rat hippocampal neurons promotes spine morphogenesis, together with excitatory postsynaptic differentiation, while knock-down by shRNA of three members of the family, Neuroligin1–3, reduces dendritic spine number (78, 79).

Similar results have been described for members of other synaptic adhesion molecules. In cultured rat neurons, overexpression and the knock-down of netrin-G ligand 1 and 2 (NGL-1, NGL-2) and SALM2, three synaptic adhesion-like molecules (SALM) isoforms increase or decrease the number of PSD-95-positive dendritic protrusions, respectively (21, 235, 242) **(FIGURE 3A)**.

The overexpression of interleukin-1 receptor accessory protein-like 1 (IL1RAPL1) and 2 (members of a novel family of brain specific Toll/IL-1 receptors) induce the formation of new

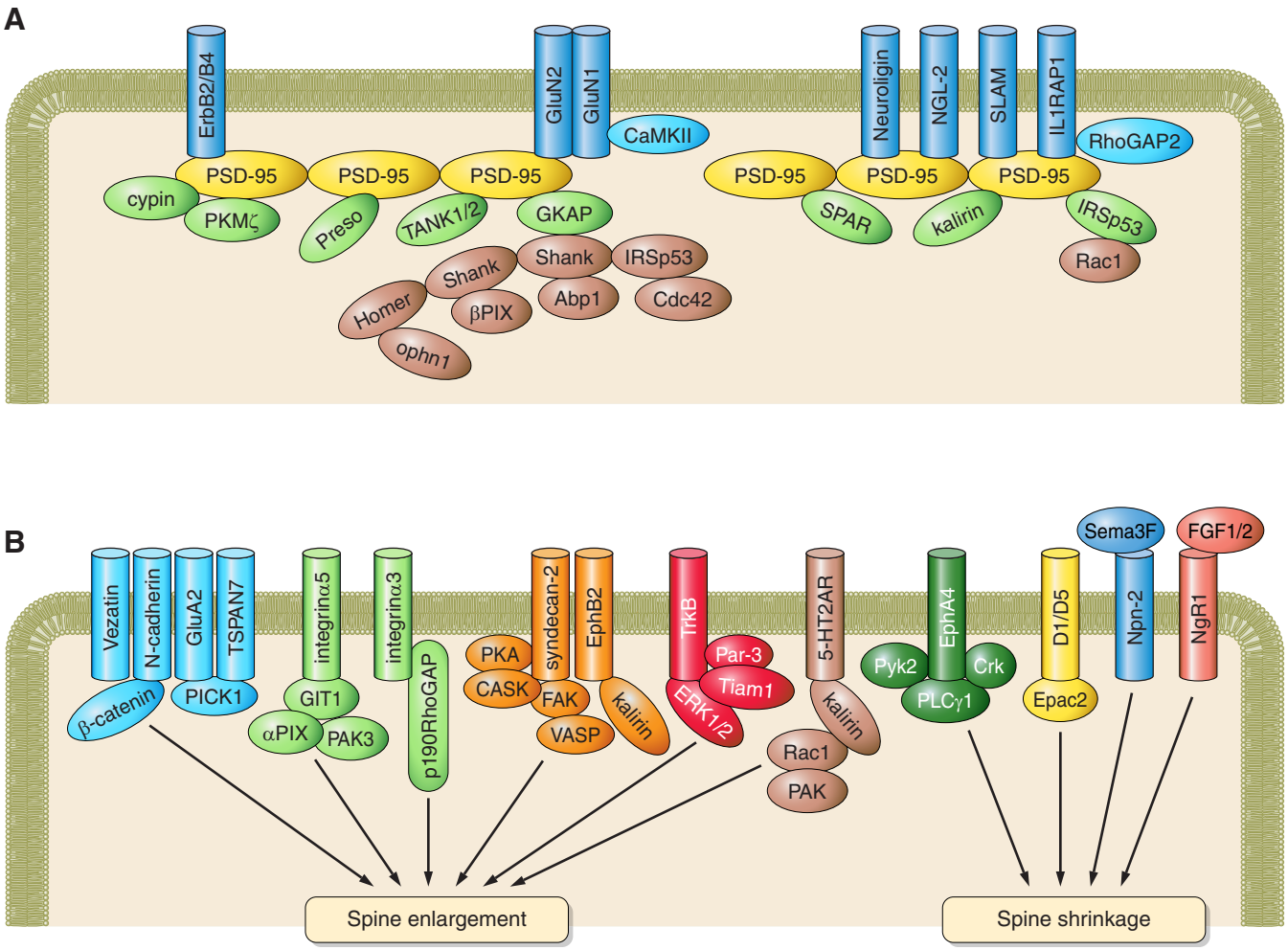


FIGURE 3. Synaptic scaffolds and membrane proteins. The cartoon shows the major PSD-95 associated proteins (*A*) and synaptic membrane proteins and associated complexes (*B*) involved in regulating dendritic spine formation.

spines, while in IL1RAP1 knockout mice, the number of dendritic spines and excitatory synapses in the CA1 region of the hippocampus is reduced (382).

The activity of these molecules in dendritic spines correlates with their ability to induce the maturation of excitatory synapses by binding and recruiting pre- and postsynaptic partners. With the use of mutants of these proteins and overexpression in cultured neurons, it has been demonstrated that either the extracellular or the intracellular domains are sufficient to increase the number of dendritic spines. For example, the inhibition of Neuroligin1-dependent shedding of the extracellular domain makes the protein more capable of inducing dendritic spines but also the intracellular PDZ binding domain is required for this (489). For IL1RAPL1, on the other hand, the binding to RhoGAP2 (see above) at the intracellular domain is required for the ability of IL1RAPL1 to increase spine density when overexpressed in cultured neurons (524).

The cadherin-based homophilic cell adhesion system is also involved in spine morphogenesis, and it regulates pre- and postsynaptic apposition and synaptic plasticity (437). Neuronal cadherin (N-cadherin) seems to be required for depolarization-induced spine enlargement when neuronal activity induces N-cadherin conformational change and redistribution at synapses (367). N-cadherin can also regulate spine morphology via the recruitment to spines of β-catenin, which interacts with the actin cytoskeleton, when its tyrosine dephosphorylates by receptor tyrosine phosphatases that belong to the LAR (leukocyte common antigen-related) family, a protein that is also essential for the formation of spines and synapses (106). However, the main role of N-cadherin is in the long-term maintenance and stability of dendritic spines (316). The regulated endocytosis of N-cadherin via protocadherin arcadlin and TAO2b/MEK3/p38 activation links neuronal activity with changes in dendritic spine morphology (568).

Some cadherin-associated membrane proteins contribute to dendritic spine morphogenesis: Vezatin is an integral protein indirectly associated with E-cadherin-catenin complex (but in neurons preferentially with N-cadherin) and actin cytoskeleton, and its conditional knockout in mice leads to a reduced

proportion of mature dendritic spines that correlate with an increased proportion of stubby spines (96). Finally, N-cadherin mediates the ability of the GluA2 subunit of the AMPA receptor to induce dendritic spine formation. Indeed, the extracellular NH_2-terminal domain of subunit GluR2, named NTD domain, is sufficient to induce spine growth through the *cis*- or *trans*-interaction of synaptic N-cadherin (380, 430).

Among the integrin family of adhesion protein the $\alpha 5$ subunit regulates spine morphogenesis and synapse formation in hippocampal neurons because its knockdown or the overexpression of a dominant negative mutant reduces the number of spines and synapses; $\alpha 5$ downstream signaling is dependent on Src kinase, Rac, and the signaling adaptor GIT1 protein (543). Mice knocked out for the $\alpha 3$ subunit develop normal spines up to P42 when they gradually lose hippocampal synapses and spines because the missing activation of p190RhoGAP that negatively modulate RhoA function (227).

In one of the first published overexpression experiments in neurons, Ethell and Yamaguchi (117) demonstrated that cell surface heparan sulfate proteoglycan syndecan-2 plays a critical role in spine development because its transient transfection accelerates spine maturation. Two years later, the same group demonstrated that syndecan-2 induces spine formation and clustering at synapses after being phosphorylated by EphB2 receptor tyrosine kinase (118). More recently it has been demonstrated that syndecan-2 downstream signaling leading to spinogenesis involves neurofibromin-mediated activation of PKA and Enabled (Ena)/vasodilator-stimulated (VASP) (283).

The role of extracellular matrix proteins in spine formation, maturation, and plasticity has been first indicated by Oray et al. (370a) who suggested that tPA may affect dendritic spine maturity much like monocular deprivation. More recently, other matrix components, like chondroitin sulfate proteoglycans (97a) and reelin (356a), have been shown to play a significant role in maturation of dendritic spines of cortical and hippocampal neurons.

Because molecules involved in axon guidance cues also have key roles in synapse formation and function, it is not surprising that some of these proteins are also involved in dendritic spine formation. Among them a clear role has been demonstrated for Sema3F and its holoreceptor components Npn-2, which are negative regulators of spine development and synaptic structure. Genetic ablation of either Sema3F or Npn-2 induces an increase of aberrantly shaped spines in granule cell dentate gyrus and pyramidal neurons of cortical layer V, two areas where Sema3F or Npn-2 are highly expressed (512). Nogo Receptor 1 (NgR1), a member of the Nogo-66 receptor family that controls axonal grown cone collapse and myelin-dependent grown inhibition after injury, has been recently described to control activity-dependent spine morphogenesis and turnover. Mice knockout for NgR1 showed altered spine morphology in apical dendrites of hippocampal CA1 neurons and higher spines turnover in somatosensory neurons (6, 272). NgR1 activity on spines seems to be mediated by a direct binding to FGF1 and FGF2 (272). Thus both Sema3F/Npn-2 and FGF1-FGF2/NgR1 signaling complexes are among the few molecular cascades that inhibit spine overgrowth and suppress dynamic changes during development.

D. Receptor Tyrosine Kinases and Other Kinases

Several receptor tyrosine kinases have been implicated in regulating synapse formation **(FIGURE 3*B*)**. Among them, the erythropoietin-producing hepatocellular carcinoma (Eph) receptors form a peculiar family of receptor tyrosine kinases (RTKs) with unique activity in brain synapses. Indeed, Eph receptors and ephrin ligands mediate both cell-to-cell adhesion and signaling in both directions by forming multimeric clusters that link juxtaposed cell membrane. EphB receptors are directly associated with NMDA receptors at the postsynaptic membrane, mediating NMDA receptor clustering upon activation by ephrin ligand (95). Among the different EphB receptor subtypes, the EphB2 seems to be specifically involved in spine morphogenesis, and its role has been demonstrated both in vitro and in vivo (118, 184, 203, 224, 225, 331, 383). EphB2 phosphorylates Syndecan-2 (see above); interacts with the GEF proteins intersectin, Numb, kalirin, and focal adhesion kinase (FAK)/Src/Grb2/paxillin; and activates Cdc42/N-WASP-actin, Rac-Pak-actin, and RhoA-actin pathways, respectively (203, 225, 331, 356, 383, 459, 505). Interestingly, the interaction of presynaptic ephrins with postsynaptic Eph receptors might coordinate the reciprocal interaction between presynaptic and postsynaptic functional size during structural changes accompanying synaptic plasticity (464).

As opposed to EphB, the activation of EphA4 by ephrin-A3, expressed also by astrocytes, induces spine retraction and spine modifications as observed in pyramidal neurons of EphA4 knockout mice (66, 339). Two pathways have been identified downstream of EphA4 activation: one involves inhibition of the scaffold protein Crk associated substrate (Cas), of the focal adhesion kinase (FAK) and of the proline-rich tyrosine kinase 2 (Pyk2) with the impairment of $\beta 1$-integrin activity (55). The second involves activation of phospholipase C$\gamma 1$ (PLC$\gamma 1$) that induces spine retraction by modulating the association of the actin depolymerizing/severing factor cofilin with the plasma membrane (587). Thus EphB2 and EphA4 activation have opposite effects on spine morphogenesis and synaptic activity, while some amounts of the ephrin specific ligands may contribute to balancing their activity.

Neuregulin-1 (NRG1) signaling through tyrosine kinase receptor ErbB4 participates in regulating dendritic spine maturation, since synaptic activity induces the recruitment of ErbB4 into the synapse that selectively enhances AMPA synaptic currents and increases dendritic spine size (278). This was con-

firmed in vivo by the observation that knockout of ErbB2/B4 impairs dendritic spine maturation and alters the postsynaptic scaffold proteins interaction with glutamate receptors. These morphological alterations in ErbB2/B4-deficient mice are associated with behavioral manifestation of increased aggression and reduced prepulse inhibition (30).

Other than the intracellular kinases that are directly downstream signaling of the small GTPase (see above), few other intracellular kinases have been demonstrated to be important for dendritic spine morphogenesis. Not surprisingly, the CaMKII, the most abundant synaptic kinase, is one of them. CaMKII regulates synaptic plasticity by phosphorylating several substrates. Confirming earlier in vitro experiments (218), the kinase activity appears to be required for the tetanic stimulation to induce dendritic spine enlargement because it was absent in homozygous CaMKIIα(K42R) knock-in mice that express normal quantity of a kinase-dead CaMKIIα (562). On the other hand, there are indications that CaMKIIβ, the other CaMKII isoform, can increase spine volume by bundling F-actin through a direct stoichiometric interaction in the absence of the kinase domain (366). CaMKII also mediates the activity of Wnt7a-Dvl1 on dendritic spines. Wnt7a-Dvl1-deficient mice showed defects in spine morphogenesis and mossy fiber-CA3 synaptic transmission in the hippocampus, and CaMKII inhibition abolishes the effects of Wnt7a on spine growth and excitatory synaptic strength (84).

Two lipid kinases have been recently demonstrated to be important for dendritic spine morphogenesis. Diacylglycerol kinases (DGKs) are the principal kinases that terminate diacylglycerol (DAG) by phosphorylating DAG into phosphatidic acid (PA). Among the several DGKs isoforms, DGKζ interacts with PSD-95, localizes at excitatory synapses, and regulates spine maintenance but not formation. Although these data are strongly supported by the observation that mice knockout for DGKζ show reduced spine density and excitatory synaptic transmission, the molecular mechanisms underlying these actions are not known (233).

The second lipid kinase is the phosphoinositide-3-kinase (PI3K). This kinase is mainly acting when a stimulated tyrosine kinase receptor induces the recruitment and activation of the PI3K complex (formed by the catalytic and inhibitory subunits) to the receptor. Activated PI3K leads to the production of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) from phosphatidylinositol 4,5-bisphosphate. PIP_3 acts as a second messenger by activating the Akt kinase, of which downstream targets include glycogen synthase kinase 3 (GSK3) and the mammalian target of rapamycin (mTOR). This signaling cascade is involved in regulating dendritic development (211), synaptic plasticity (259), and perhaps spine morphogenesis (92, 259). Interestingly, a recent study demonstrated that a PI3K-activating transduction peptide is sufficient to induce synaptogenesis and spinogenesis in both cultures of rat hippocampal neurons and in CA1 hippocampal neurons in vivo (92). PI3K activation was effective also in adult neurons, suggesting that functional synaptogenesis can be induced in mature mammalian brains by the specific activation of PI3K (92).

Among the kinases implicated in long-term maintenance of neuronal plasticity and memory, protein kinase M ζ (PKMζ) is an atypical isoform of protein kinase C (PKC), which when overexpressed on primary cultures of cortical neurons modifies spine morphology by reducing their length without changing their density. The regulation of spine morphology by PKMζ may explain its role in long-term synaptic plasticity (417).

Two families of kinases, NDR1/2 and DCLK1/2, involved in regulating cellular morphogenesis in eukaryotes and cellular migration, respectively, have been recently found to play a role in dendrites and dendritic spine morphogenesis. Genetic manipulations of NDR1/2 have demonstrated that it positively regulates spine formation by activating Rabin8, a GDP/GTP exchange factor (GEF) of Rab8 GTPase, while DCLK1/2 negatively regulates spine formation by disassembling postsynaptic density complex (460, 520).

E. Postsynaptic Scaffold Proteins and Adaptor Proteins

The PSD is an assembly of several scaffold proteins that link synaptic receptors and membrane proteins to spine cytoskeleton and signaling molecules. Among the PSD scaffold proteins, the PSD-95 and the Shank families of proteins play a critical role in spine morphogenesis.

The four members of the PSD-95 family (PSD-95/SAP90, PSD-93/chapsyn-110, SAP102, and SAP97) have a common structure formed by three PDZ domains, a SRC homology (SH3) domain, and a guanylate kinase-like (GK) domain (455).

SAP97 is longer because it has also a LIN2/LIN7 (L27) domain at the NH_2-terminal before the first PDZ domain. PSD-95 family is part of the protein superfamily called MAGUK (membrane-associated guanylate kinase), which is marked by the existence in all members of one GK and at least one PDZ domain.

As described before, PSD-95 (actually the most abundant PSD scaffold protein) is an important player in synapse and dendritic spine formation because it is the scaffold protein that interactes with neuroligin, NGL, SALM, and IL1RAPL family of proteins, which in the postsynaptic membrane mediate the trans-synaptic adhesion with specific membrane-associated presynaptic proteins to induce synapse formation. The classical example is the trans-synaptic neuroligin-neurexin interaction that regulates pre- and postsynaptic differentiation (79, 154, 399). Actually, at least in cultured neurons, the numerical

balance between inhibitory and excitatory synapses is proportional to the amount of PSD-95 expression (142, 399).

On the cytoplasmic side, PSD-95 interacts also with a broad range of signaling molecules linking together cytoplasmic signal-transducing enzymes to membrane receptors and assembling signal coupling in the PSD. Thus, at least in cultured hippocampal neurons, the overexpression of PSD-95 increases the number of dendritic spines and induces the maturation of excitatory synapses (111, 398). This is done by recruiting transmembrane proteins but also intracellular signaling, scaffold and adaptor proteins such as kalirin-7 (383), SPAR (376), IRSp53 (82), Preso (273), TANC1/2 (164), nNOS (354), Shank2 (483), and cypin (516) that are directly or indirectly involved in spine formation. In vivo, deletion of PSD-95 partially reduces spine number in the striatum and in the hippocampus (533).

SAP102 is the other member of family potentially involved in spine morphogenesis. A specific NH_2-terminal domain, the expression of which is regulated by alternative splicing, regulates SAP102 interaction with GluN2B and promotes the formation of synapses and long spines, suggesting that SAP102 mediates NMDA receptor ability to induce dendritic spine formation (72). Finally, like PSD-95, CASK, a member of the MAGUK family of proteins, also promotes spine formation by linking synaptic adhesion molecules to the actin cytoskeleton, and its activity is regulated by SUMOylation (69).

Several in vitro and in vivo studies indicate that Shank family of PSD scaffold proteins is a major regulator of dendritic spine morphology. The Shank family is composed of three genes (*Shank1–3*) that codify for a multi-domain proteins containing an NH_2-terminal ankyrin repeat, followed by a SH3, a PDZ domain, a long proline-rich region, and a sterile alpha motif domain (SAM) at the COOH terminus (454).

The protein-protein interaction domains present in Shank link at least two glutamate receptor subtypes: the NMDA receptors/PSD-95/GKAP complex, through the PDZ domain that binds to the COOH-terminal of SAPAP/GKAP, another scaffold protein of the PSD, and the type 1 mGluRs/Homer interaction at the proline-rich domain.

Shank1 was the first to be associated with spine morphogenesis by the demonstration that co-overexpression of Shank1 and Homer1b (the long isoform of Homer1 gene) in hippocampal neurons induces the maturation of spines during development and the volume enlargement of mature spines, without altering their number. Overexpressed Shank1 and Homer1b are also able to accumulate in the PSD other synaptic proteins like GKAP/SPAP and GluN1and the F-actin (179, 433).

Homer are scaffold proteins, structurally formed of an NH_2-terminal Ena/VASP homology 1 (EVH1) domain and a COOH-terminal coiled-coil domain that mediates tetramerization with other Homer proteins (179). The EVH1 domain specifically binds to a PPXXF sequence that has been found in Shank1–3 proteins, mGluR1/5, inositol-1,4,5-trisphosphate (IP_3) receptor, ryanodine receptor, and in some members of the TRPC family of ion channels (555, 575). Interestingly, the short-splice variant of Homer1 (Homer1a) that is deleted from the coiled-coil domain and cannot tetramerize is a natural dominant negative, and importantly, synaptic activity modulates the expression of Homer1a (14, 432, 555).

As opposed to Shank1, the overexpression of Shank3 is able to induce the formation of new spines at least in cerebellar granule cells, while Shank3 knock-down by RNAi expression in hippocampal neurons reduces the total number and the mature-like spines. Shank overexpression has a global effect on synapse maturation because it induces also the maturation of the presynaptic compartment (108, 420, 433).

The ability of Shank and Homer to regulate spine maturation can be explained by the association of Shank with cortactin (346), Abp1 (403), αPIX (379), and Cdc42-binding protein IRSp53 (472) while Homer binds to oligophrenin-1 (153). Interestingly, Shank1 and Homer1b in the PSD form a mesh-like matrix structure that serves as a structural platform to assemble other synaptic proteins that contribute to the enlargement of dendritic spines (179). Thus neurons overexpressing Homer1b mutants, unable to tetramerize, or the dominant negative Homer1a splice variant have a reduced number of smaller spines, and a reduced number of synaptic AMPA and NMDA receptors (178, 432). Finally, Shank1 and Homer1b cooperation on spine enlargement can be explained also by the demonstration that their overexpression in hippocampal neurons recruits the entire endoplasmic reticulum compartment to dendritic spines (435).

The role of Shank1 (see below) in regulating dendritic spine shape has been demonstrated also in vivo in the knockout mice that showed alteration in PSD protein scaffold composition, smaller dendritic spines, and weaker excitatory synaptic transmission (200).

In contrast to Shank, the overexpression of PICK1 in hippocampal neurons reduces spine size, and its knockdown increases spine size. PICK1 is a synaptic scaffold protein that binds AMPAR subunits GluA2/3 that contain a PDZ and BAR domain proteins (165). The PICK1 effect appears to be mediated by its direct binding and inhibition of Arp2/3 complex activity (347).

IQGAP1 is a member of a family of scaffold proteins structurally formed by a calponin homology domain that bind actin, a WW motif allowing the association with ERK1/2, the calmodulin binding IQ domain, and a RasGAP-related domain that binds to Cdc42 and Rac1. Mice knockout for IQGAP1 show a specific reduction of dendritic spine number in specific brain

regions associated with cognition and emotion, such as the hippocampus and lateral amygdala. In these brain areas IQGAP1 directly interacts with GluN2A and contributes to ERK signaling in response to GluN2A-dependent NMDAR stimulation, linking this pathway to spine morphogenesis (137).

Insulin receptor substrate 53 (IRSp53) is PSD adaptor protein that links Rac1 to WAVE2 and binds to Shank and PSD-95. Overexpression of IRSp53 in cultured neurons increases the number of dendritic spines while its knockdown reduces the number, length, and width of spine. IRSp53 might play a role in linking PSD-95 to activated Rac1/Cdc42 and spine actin because IRSp53 ability to bind WAVE2 with its SH3 domain is required for inducing spine morphogenesis (82). However, dendritic spine morphology is normal in mice knocked out for IRSp53 although several synaptic functions, such as NMDA receptor-mediated transmission, LTP, and learning and memory are altered in these mice (234, 443).

F. Micro RNA, mRNA Binding Protein, and Transcription Factors

The identification of microRNAs (miRNAs) as noncoding RNAs that mediate posttranscriptional gene silencing and their implication in neuronal development and neurodegeneration have stimulated the search for specific miRNA that play a role in dendritic spines morphogenesis and their respective molecular targets. Among the miRNAs selectively expressed in the brain, at least five have been demonstrated to possess a functional role in controlling synaptogenesis and spine morphology: miR-134, miR-138, miR-132, miR-125b, and miR-29a/b. Interestingly, several miRNA target genes codify for proteins previously demonstrated to be involved in spine morphogenesis.

The first identified miRNA, miR-134, negatively regulates the size of dendritic spines and excitatory synaptic transmission by inhibiting the translation of Limk1 that controls spine development (see above). Interestingly, BDNF increases Limk1 expression by relieving the miR-134 translational inhibition via the TrkB/mTOR signaling pathway, which inactivates a not well defined miR-134-associated silencing complex (445).

With the use of a functional screen, miR-138 was found to be highly expressed in the brain and specifically localized in dendrites. miR-138 negatively regulates the size of dendritic spines in rat hippocampal neurons by inhibiting the expression of the enzyme acyl protein thioesterase 1 (APT1), which controls the depalmitoylation of synaptic proteins such as the α13 subunits of G proteins ($G\alpha_{13}$). In addition to $G\alpha_{13}$, other synaptic proteins could be a substrate of APT1, although the spine size reduction can be caused by the elevated level of palmitoylated $G\alpha_{13}$, in the absence of APT1, which might increase the activity of RhoA (463).

Rho-family GTPase activating protein p250GAP is the target of miR132. The expression of miR132 is regulated by synaptic activity and CREB during periods of active synaptogenesis. By reducing the expression of p250GAP, miR132 increases spine formation. This is done by modulating synapse-specific Kalirin7-Rac1 signaling (166, 202) (see also below). The role of miR132 in spine morphogenesis has also been demonstrated in vivo in transgenic mice that express miR132 in forebrain neurons. Hippocampal neurons in these mice showed a marked increase in dendritic spine density, but these morphological changes are associated with significant deficits in novel object recognition and with the decreased expression of the Rett Syndrome protein MeCP2 (166). miR-132 controls synaptic plasticity and spine maturation in visual cortex (315), while in olfactory bulb (OB) neurons regulates not only dendritic complexity and spine density but also the survival of newborn neurons, suggesting that this miRNA regulates a set of genes involved in neurogenesis and structural plasticity (381). Finally, miR-132, together with miR-125b, is associated with the Fragile-X mental retardation protein (FMRP) that regulates their activity. Overexpression of miR-125b reduces dendritic spine number and maturation, an opposite effect to that of miR-132 overepression, and inhibits the expression of GluN2A (109). miR-125a controls PSD-95 expression and spine maturation by forming an inhibitory complex with AGO2 that can be disassembled by mGluR-dependent dephosphorylation of FMRP (335).

Finally, miR-29a/b, like miR-125b, also reduces mushroom-shaped dendritic spines with an associated increase in dendritic filopodia when overexpressed in hippocampal neurons; in this case, miR-125b probably downregulates the expression of Arpc3, a component of the ARP2/3 actin nucleation complex (284).

Local translation of specific mRNAs in proximity to dendritic spines has been implicated in synapse remodeling and synaptic plasticity. Not surprisingly, proteins involved in controlling mRNA targeting and translation in dendrites are directly involved in regulating spine morphogenesis. In vitro and in vivo experiments have demonstrated that the absence of TLS (translocated in liposarcoma), Staufen2, and heterogeneous nuclear ribonucleoprotein K (hnRNPK) selectively impair mRNA targeting of proteins that regulates the number of mature dendritic spines (134, 135, 148, 402). Translation of TLS transported dendritic mRNA is controlled by metabotropic glutamate receptor (mGluR5) activation, and some mRNA for cytoskeleton proteins, such as β-actin, are specifically guided by Staufen2. hnRNPK interact with Abi-1 forming in a multiprotein complex reciprocal regulating spine formation and synaptic maturation (402). Another major dendritic protein involved in controlling dendritic mRNA translation is the Fragile-X syndrome protein FMRP (see below).

Neuronal development is controlled by the activity of specific subsets of transcription factors (TFs) that coordinate the ex-

pression of proteins involved in neuronal morphogenesis. Recently a subclass-specific TFs have been found to control dendritic spines and the maturation and strength of the synapses. Cux1 and Cux2 encode the vertebrate homologs of the *Drosophila* homeobox transcription factor Cut, which controls the dendrite morphology of postmitotic populations in the peripheral nervous system (PNS) (157, 214, 247). Both Cux1 and Cux2 are also expressed in postmitotic brain neurons where they coordinate the expression of Xlr3b and Xlr4b, chromatin remodeling genes previously implicated in cognitive defects. Indeed, knockout mice for Cux2 show a specific reduction of dendritic spine numbers in cortical layers II-IV neurons (91). Similarly, knockout of FoxO6, a neuron-specific member of the FoxO family of transcription factors, impairs the number of dendritic spines in hippocampal neurons by altering the expression of genes involved in synaptic function associated with learning and memory (436).

Satb1 is a genome organizer protein highly expressed in mature cerebral neurons. In postnatal cortical pyramidal neurons of Satb1 knockout mice, the density of dendritic spines is reduced probably because the transcription control of some immediate early genes (IEGs) (*Fos*, *Fosb*, *Egr1*, *Egr2*, *Arc*, and *Bdnf*) is altered; Satb1 binds to genomic loci of these IEGs (28).

G. Steroid Hormones

The first indication that dendritic spine density changes during the estrous cycle of female rats was demonstrated more than 20 years ago (551). The density of dendritic spines was correlated with the fluctuations of both estradiol and progesterone levels occurring during the estrous cycle of females, and the ovariectomy of female rats induced a decrease in dendritic spine density in the hippocampus, which could be rescued by estradiol therapy (152). Interestingly, this estradiol cyclic regulation of spine synapse density was found only in the hippocampus and not in the neocortex or in the cerebellum (400), although it has been reported that estradiol promotes in vitro and in vivo the formation of dendritic spines in Purkinje neuron (431).

The original suggestion that circulating hormones are directly controlling spine density is now questioned by the observations that synapse density was not influenced by application of estradiol to hippocampal slice cultures irrespective of the sex of the animals providing the tissue (125, 257). On the other hand, in hippocampal slice cultures, the inhibition of aromatase activity (the enzyme responsible for 17β-estradiol synthesis) resulted in loss of spine synapses that could be rescued by the application of exogenous estradiol (257, 586). Thus these data suggest that locally produced estradiol rather than estradiol originating from the reproductive organs regulates dendritic spine synapse density.

The level of estradiol synthesis in the hippocampus is also controlled by gonadotropin releasing hormone (GnRH). Although GnRH is the key regulator of reproduction, by controlling the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the pituitary and thus steroid hormone synthesis in the gonads, it can also influence estradiol synthesis directly (209). Hippocampal slice culture treatment with GnRH induced the release of estradiol into the medium and increased dendritic spine density in a dose-dependent manner (400). These data suggest that estradiol regulates dendritic spines and synapse function also via a paracrine mechanism.

On the other hand, several studies demonstrated recently that dendritic spine densities are clearly different between male and female mice in vivo. For example, the density of spines in males is similar to their density in ovariectomized females, while the hippocampus of intact cyclic females could contain double that of males (93, 125, 462). Parental estradiol treatment of ovariectomized mice caused an increase in the number of mature mushroom spines (279). This sex-specific difference in spine number correlates with the plasma levels of estradiol that is up to five times higher in females at the stage of proestrus than in males, while there is no difference between males and ovariectomized females (125). It should be added that the estradiol concentration in hippocampus of males and ovariectomized females is extremely low (125). These studies indicate that circulating estradiol rather than locally synthetized estradiol regulates spine density.

Whatever its origin, estradiol has two time constants of effects on spines: a rapid (hours) and slow (days, discussed above) one. A 2-h exposure is sufficient to significantly increase the total density of spines in stratum radiatum of the CA1 region of the hippocampus (see review in Ref. 370). This rapid effect cannot be explained by the classical transcriptional genomic pathway of the canonical intracellular estradiol receptors. Thus a putative membrane estrogen receptor or a different, more rapid pathway for the intracellular estrogen receptor should be considered for the nongenomic response.

The most probable candidates for extranuclear, dendritic, and synaptic membrane estrogen receptors (ER) should be ERα and ERβ (191). Both are also classical nuclear receptors, but they may work with different mechanisms at synapses, driving either kinases, metabotropic glutamate receptor (mGluR), or G protein activation (54, 475).

ERα has been localized in spines of hippocampal pyramidal and granule neurons by immunoelectron microscopic and Western blots analysis in the PSD where it is accumulated upon NMDA stimulation (191, 336).

ERβ is also abundantly expressed in cortical neurons (327) and has been shown to be required for correct memory and hippocampal synaptic plasticity because mice knockout for ERβ display impaired learning (287). Finally, in cultures, the rapid activation of ERβ with the specific agonist WAY-

200070 increased spine density and PSD-95 accumulation in membrane regions (478). At the mRNA and protein levels, no differences were found between sexes for these receptors (423). Thus both ERα and ERβ are functionally present in dendritic compartments and can be rapidly activated to modulate synapse function and spine morphogenesis.

Downstream pathways that are responsible for rapid spine cytoskeletal rearrangements have been partially identified. Blockade of the ERK pathway pharmacologically or by reducing intracellular [Ca^{2+}] abolishes the effect of estradiol on the spine density (340).

In cultured cortical neurons, direct activation of ERβ by WAY-200070 induces the phosphorylation of PAK and ERK1/2(478) while activation of ERβ induced the phosphorylation and activation of WAVE1 (439). Estradiol can also activate FAK and actin polymerization by triggering c-Src/PI3K dependent phosphorylation on Tyr397 (438). In developing hypothalamus, estradiol promotes spine formation by a rapid, nongenomic activation of PI3 kinase in presynaptic neurons that increase the release of glutamate. This triggers higher glutamate receptor number and mitogen-activated protein kinases activation in postsynaptic neurons and protein synthesis-dependent formation of dendritic spines (447).

Finally, another possible mechanism of action of estrogen is through the regulation of GABAergic transmission. Estradiol application in vitro, acting probably on both ERα and Erβ, appears to suppress GABAergic transmission, increasing excitability in hippocampal cells and facilitating the formation of new dendritic spines (342). On the other hand, in mice treated with the specific ERβ ligand LY3201, GABAergic synapse became stronger through the higher expression of GAD in cortex and hippocampus, and this correlates with a reduction of number of spines in hippocampal neurons, suggesting that that ERα and ERβ have opposite effects on synapse plasticity (498).

Among the other steroid hormones, glucocorticoid activity is also important for dendritic spine plasticity. Chronic glucocorticoid treatment leads to loss of stable spines, suggesting that glucocorticoids play a role in the development and maintenance of dendritic spines in cortical neurons (286). More recently it has been demonstrated that corticosterone reduces the expression levels of caldesmon (CaD), a ubiquitous actin-linked regulatory protein that binds to and stabilizes actin filaments, causing dendritic spines to become less stable. Corticosterone-mediated suppression of CaD expression levels is caused by inhibition of serum response factor-dependent transcription. Because CaD enlarges the spine-head size by stabilizing F-actin dynamics, CaD is a critical target in the corticosterone-induced damaging effects on dendritic spine morphogenesis (500).

In contrast, it has been demonstrated that stress-responsive glucocorticoid receptor (GR) is present on dendritic spines and regulates local signaling of the actin cytoskeleton. Corticosterone rapidly (60 min) increases the number of large spines and total levels of phosphorylated ERK1/2 proteins. Thus acute corticosteroids treatment promotes spine formation via multiple kinase networks including PKA and mitogen-activated protein kinase (246). In summary, corticosteroids might mediate both acute stress induced spine morphogenesis and synapse formation and chronic stress mediated spines elimination.

V. DEVELOPMENT OF DENDRITIC SPINES

A. Is Spine Formation Affected by Active Synaptic Interaction?

The rules that govern the formation of dendritic spines in the developing brain, as well as the question of whether these mechanisms are different from those that regulate spine formation in the adult brain are still not resolved. Two observations are common: in the young brain there are few spines and many filopodia which disappear in the mature neuron (126, 473). Dendritic filopodia, unlike growth cone-associated filopodia, are extremely sensitive to ambient activity; they form and are more motile if action potentials are blocked with TTX, or if synaptic activity is blocked (397), indicating that they may play a role in the formation of connections among neurons, and in the synchronized activity typical of young networks. On the other hand, most of the synapses in the young brain reside on the dendritic shaft (412). Do spines form from filopodia, which are motile and transient by nature, or are spines formed from existing shaft synapses on dendrites? An intermediate possibility is that dendritic filopodia shoots out to sense a source of glutamate release in an axon (e.g., Ref. 415), whereas a shaft synapse is formed at the same location and develops into a spine (169, 174). Removing the synapse away from the shaft, by the production of a spine, may allow the cell to generate more synapses with en-passage axons, and be able to scale down the efficacy of the synapse, such that a spine synapse will have a lower impact on the parent neuron. Synaptic connections leading to spontaneous network bursts have been seen also in young cultured and in vivo neurons (3–4 days old) before the formation of spines, and these are likely to result from shaft synapses (271). Likewise, the conversion of filopodia to spines is not likely to occur in mature neuron, when filopodia are rare, and spines can form within minutes to hours, as illustrated in cycling female rats (552) and during the recovery from hibernation (394, 396) as well as in living adult mice (561, 563). Interestingly, exposure of neurons to TTX, which blocks action potential discharges, causes filopodia to grow off existing spines even in mature neurons (415), indicating that the neuron is extremely sensitive to changes in ambient activity, to the extent that even if it does not support the existence of filopodia, they are generated

within minutes after the demand emerges. On the other hand, Reilly et al. (412) imaged developing neurons in culture and were unable to observe a shaft synapse that converts to a spine synapse over 31 h of monitoring. Thus, even though both shaft synapses and filopodia are abundant in juvenile neurons, the eventual spine synapse does not seem to develop from a shaft synapse. Still, young neurons are far more responsive to glutamate challenge and develop dendritic spines than adult neurons (591; see also Ref. 264), and the reasons for this are not entirely clear. Likewise, the fate of the shaft synapse as well as the stimulus that triggers the formation and location of a spine synapse need further exploration.

VI. SHORT-TERM MORPHOLOGICAL AND FUNCTIONAL PLASTICITY OF SPINES

A. Formation of New Spines

Early time-lapse imaging of dendrites following LTP-inducing protocol described the formation of new spines in cultured hippocampal slices (115). The new spines were detected within 30 min after the induction of LTP in the slice and amounted to an addition of ~15% to the existing population of spines. Interestingly, the authors did not report on a change in volume of the existing spines on these dendrites following LTP induction. Similar observations were made by Shi et al. (458) who described NMDA receptor-dependent formation of new filopodia in response to tetanic stimulation in cultured hippocampal slices. In neither of these studies was there any indication that the growth of the new spine or filopodia is associated with formation of new synapses, although the electrophysiological responses recorded in the slice showed the typical rapid growth of synaptic responses associated with LTP generation. These issues were addressed in a study with dissociated hippocampal neurons (150, 373) where new spines were found to be attached to synaptophysin-containing terminals. Still, the issue of when do nascent dendritic spines become functional was subject to further explorations. Bonhoeffer and colleagues (344) combined confocal microscopy with EM studies of cultured hippocampal slices to suggest that physical contacts with presynaptic fibers are made within tens of minutes after enhanced activation, but mature spines with presence of both pre- and postsynaptic elements, indicating a functional synapse, are formed only 15–19 h after the plasticity-evoking stimulation. In a similar approach, Zito et al. (591), also using cultured hippocampal slices, were able to record responses to flash photolysed glutamate from young (<2 h) spines, responses that in many respects were indistinguishable from mature spines of the same volume. This indicates that new spine already contain AMPA receptors, confirming earlier work with dissociated cultures (58, 150, 365). More recently, Kwon and Sabatini (264), working in acute cortical slices of young (P8–12) mice, found that new spines could emerge within <2 min after glutamate uncaging near the dendritic shaft of layer 2–3 pyramidal neurons. These new spines are functional in the sense that they respond to presynaptic stimulation, much the same way as old dendritic spines. Interestingly, in this experiment glutamate uncaging did not cause expansion of existing spine heads (see below).

On the other extreme is the study of Knott et al. (241) done with time-lapse imaging of in vivo neocortex, suggesting that only 4 days after their formation dendritic spines become innervated and mature.

Altogether, these results do not provide a clear universal time course for the relations between spine formation and its functions. The time ranges between minutes, or as soon as a spine is formed, to hours and days. The reason for this broad time spectrum is not entirely clear, and it may have to do with the age of the tissue, the stimulation, and detection protocols. A recent observation addressed this age possibility by showing that nascent spines that are exposed repeatedly to flash photolysis of caged glutamate (LTP protocol) have a higher propensity to survive in mature form, compared with unstimulated ones (188). This stabilization is dependent on activation of the NMDA receptor and its linked CaMKII, but it is not clear that these novel mature spines are at all innervated.

B. Spine Expansion

Individual dendritic spines that are activated by electrical stimulation or by photoconversion of caged glutamate can undergo a nearly immediate (<1 min) expansion of their head volume by over threefold (311). This was accompanied by a rapid (2 min) increase in synaptic responses to cage glutamate, likely to be mediated by lateral diffusion of glutamate receptors into PSD, which can take place within a fraction of a second (182a). Interestingly, the authors proposed that only small spines can expand, while the large ones are resistant to the effects of caged glutamate and do not expand. The initial expansion was dependent on activation of NMDA receptors in the spine head while a long-lasting expansion was dependent on activation of kinases (566). Later studies reported that the change in spine volume was smaller than in the original report [Yang et al., 40% (566); Lai et al., 40% (267)] and may develop over a longer time period [20 min (267, 583) or longer (442)]. Regardless of the magnitude of the immediate effects, the ability of spines to change their volume over a short time is impressive indeed. Furthermore, spine expansion is associated with an increase in the density of glutamate receptors in the spines, as seen following induction of LTP (e.g., Refs. 188, 310). The question is if these volume changes are a necessary condition for the electrophysiological change or are just a by-product of the functional change. A corollary issue is if all types of plasticity (e.g., LTP, STDP, LTD) involve a volume change in the affected spines. The differences between the different reports on the latency and magnitude of spine head expansion may be related to the insertion of glutamate receptors into the spine heads such that spines will inflate only when glutamate receptors are added into their heads (248, 250).

A clear dissociation between spine expansion and LTP has been demonstrated by Yang et al. (566), where blockade of protein kinase A did not block the initial spine expansion, but its maintenance, whereas it did block generation of LTP. Morphological factors, such as spine neck length and initial head dimension, may cause spine expansion to be fast (172, 276, 311), or rather slow (267). In a recent study by Lynch and co-workers (255), they suggest that a difference between young (2 wk) and adult (8 wk) neurons recorded in acute hippocampal slices may underlie this heterogeneity, in that the young mice spines can show an immediate, twofold expansion, whereas spines in the adult slices are less prone to expansion, and when they do, they lack the immediate surge in spine volume, and inflate to roughly 40% above their normal size.

The role of the expanded spine in synaptic plasticity has been alluded to also in the study by Matsuo et al. (310), who found that newly synthesized GFP-GluR1 molecules tend to accumulate in large mushroom spines, and not in thin spines or stubby ones, 1 day after fear conditioning. This effect is transient and dissipates 2 days later. This observation is similar to a previous EM study by Ostroff et al. (372), showing that polyribosomes accumulate in dendritic spines 2 h after a tetanic stimulation. These spines also have larger postsynaptic density, indicating that protein synthesis contributes to the maintenance of spine inflation, and to the enhanced synaptic transmission in these spines.

Several exceptions to the correlation between spine expansion and magnitude of synaptic responses have recently been described. One of the most studied plastic change in synaptic properties is the homeostatic scaling (518). In a recent study, conducted with cultured slices of the hippocampus exposed to TTX, Soares et al. (467) describe a significant increase in mEPSC amplitude and frequency, without a change in spine size or density. They ascribe the functional change in synaptic currents to a change in the balance between different glutamate receptor subtypes.

A similar diversion from the general correlation between spine size and synaptic current has been described in a phenomenon called distance-dependent scaling (DDS) (465). It has been known that synapses on remote dendrites generate much larger synaptic currents than synapses on proximal dendrites. Smith et al. (465) could not find a correlation between the large DDS of synaptic current and spine size. In a recent study, Shipman et al. (461) proposed that this scaling is associated with a change in GluA2 subunit density on the remote spines, illustrating that a change in synaptic current is not necessarily associated with spine volume.

Finally, the spine volume variations have important implications with respect to the involvement of intracellular calcium concentration in synaptic plasticity. This was alluded to in a recent theoretical study (362), suggesting that changes in spine volume may result in different magnitudes of surges of spine $[Ca^{2+}]_i$ which will affect the stability of synaptic plasticity.

VII. LONG-TERM CHANGES AND DENDRITIC SPINES

The longevity of dendritic spines has been the subject of intense investigation since the introduction of chronic time-lapse imaging of spines in the intact neocortex. In one of the earliest studies, Trachtenberg et al. (511) found a large fraction (50%) of stable spines in mouse barrel cortex. They further found that plasticity increases the turnover of spines, in line with a later suggestion that sensory deprivation prevents spine loss, found during normal development of the neocortex (592). In contrast, small retinal damage caused a threefold increase in spine turnover in the affected visual cortex (226), leaving the issue of whether sensory deprivation facilitates or suppresses spine turnover rate still open. In a different study, Holtmaat et al. (194) found that thick spines persist for a month, whereas thin spines are transient, with a slower turnover of spines in the visual compared with the somatosensory cortex. This corroborates the suggestion that thick spines are maintaining "memory," as suggested in the in vitro studies (above). Furthermore, a long-term change in spine generation and elimination dynamics resulting in a reorganization of synaptic connectivity has been reported to take place in the mouse neocortex following extensive motor learning. These changes lasted many days after the training (561, 563). Some possible differences in the proportion of stable spines found between different studies may result from differences in type of cranial window produced to image the spines, as results with cranial window where the skull was completely removed produced different results from those where the skull was thinned down, but not removed, maintaining a healthier cortex (560).

While the general finding is that spine density/size increase with long-term memory, and that large spine heads indicate "memory" (223), there are notable exceptions. In two recent studies, fear conditioning is actually associated with a reduction in spine density. In one of these studies (440), the authors analyzed hippocampal neurons where GFP-GluR1 was generated by a *c-fos* promoter, indicating that these neurons are activated by the learning experience. Compared with home-caged mice or mice exposed to the context alone, fear-conditioned animals had fewer dendritic spines on the activated neurons. In the other study (267), the authors used time-lapse microscopy in living mice, focusing on dendritic spines of layer 5 pyramidal neurons of frontal association cortex. In these mice, fear conditioning was associated with elimination of dendritic spines, whereas fear extinction involved reappearance of spines, in close proximity to the ones that were eliminated by fear conditioning.

A. Spine Pruning and LTD

LTD has been considered as a parallel and opposite process to the more common LTP. Both processes involve a rise in

$[Ca^{2+}]_i$, probably to different magnitudes and duration. There are several protocols for induction of LTD, including a low-frequency (1 Hz) stimulation, as well as an exposure to metabotropic receptor agonist. The electrophysiological and biochemical rules that govern LTD have been studied extensively, but the morphological basis of LTD is much less explored. While there is a general consensus that LTP or enhanced synaptic activity is correlated with an increase in spine size/density, there is less information on the possible parallel reduction in dendritic spine density/shape with LTD, which causes a reduction in reactivity to afferent stimulation. In an early study, Nagerl et al. (361), using an organotypic hippocampal slice, found that spines retract, in an NMDA-dependent manner, with low-frequency stimulation, which causes LTD. Interestingly, the ability to retract spines is drastically reduced with age of the culture, as is spontaneous formation and retraction of spines. Altogether, the number of retracted spines is rather low (2–3 spines/100 μm of dendrite). In another study, Kamikubo et al. (221) found that regardless of the means for induction of LTD, it is associated with synapse loss. Interestingly, the synapse loss was measured weeks after the induction of LTD, and it required three exposures to the LTD generating stimulation. Thus it is not clear if the morphological change is in any way related casually to the electrophysiological LTD.

In another recent study using LTD induction protocol in cultured slices of the hippocampus, a significant retraction of presynaptic boutons was described, even more striking than the earlier report on spine shrinkage (34). Altogether, these studies indicate that LTD is associated with a reduction in functional synapses with LTD, even though the actual proportion of reduced synapses does not match the marked electrophysiological changes that are seen following induction of LTD.

Does a neuron lose its synapses when spines are pruned, or does it retain the synapses on the dendritic shaft? Since a mature synapse contains an array of adhesion molecules linking the pre- and postsynaptic membranes, and the synapse is engulfed by glial cells, it is unlikely that the presynaptic terminal detaches from the postsynaptic side, especially since the synapse is tough enough so as to resist mechanical dissociation (as in the process of preparation of synaptosomes). However, recent EM study suggests that cortical neurons do lose their connection with afferent inputs (241). In contrast, there is a marked decrease in spine density in hibernating animals that is counteracted by an increase in shaft synapses (396, 536). When the animal rises from hibernation, the spines reappear, and the animal remembers tasks learned before hibernation, indicating that persistence of spines is not a prerequisite for memories (85). In fact, when trained 24 h after arousal from hibernation, animals remember better than controls (545). Likewise, female rats in estrus cycle, when their hippocampal spine density is down by 30%, are not less capable of remembering items learned during earlier time in the cycle. It is therefore unlikely that neurons lose their afferents once their spines disappear. On the other hand, when the cell loses its afferents, as is the case in the cultured hippocampal slice, the denervated spines disappear, only to reappear when new axons innervate the vacated region on the dendritic shaft (132).

In a similar study, conducted with cultured slices, chronic blockade of AMPA receptors led to disappearance of spines, but this was compensated by the appearance of shaft synapses, and by an increase in efficacy of synaptic transmission (309). Thus reduced synaptic activity, as is the case with LTD (above), should not necessarily lead to disappearance of synapse, even though the spines may dissolve.

Another interesting disparity is the observation that long-term sensory deprivation prevents rather than enhances spine pruning (592). Still, input deprivation produced four synapses away from the monitored neuron in the barrel cortex is different from a local ongoing blockade of activity by TTX, especially since the extrinsic sensory afferents constitute only a fraction of the excitatory input to the cortical neuron. In another study, blockade of activity with TTX increased rather than decreased the number of functional synapses, as evidenced by the staining with presynaptic proteins, but these studies were conducted with 2- to 3-day-old hippocampal slices, at an age where few if any spines are functional and the excitatory synapses are made on the dendritic shaft, so the rules that govern the structure-function relations may be different at this young age (271).

B. Elementary Units of Plasticity

What is the elementary unit of plasticity? Is it the individual dendritic spine or a cluster of them? Are they all placed on the same dendritic segments, or can they synchronize on different dendritic branches? As indicated (173), activated Ras can diffuse away from a spine into adjacent ones. In fact, adjacent spines on a dendritic branch are frequently activated synchronously in a network, which indicates that they probably react to common inputs (495). This synchronization contributes to the strengthening of the network "memory" and confirms earlier rules suggesting a need for cooperativity to produce LTP (546). Clusters of spines are formed on adjacent locations on dendrites in motor learning tasks, and these are longer lasting than individual spines grown in association with the learned task (133). Thus spine clustering may be an efficient way to maintain spines that are formed during learning, and this may have functional significance. In similar studies on sensory cortex, Makino and Malinow (300) described functional clustering of adjacent spines in response to sensory stimulation. Interestingly, sensory deprivation causes a nonselective enhancement of reactivity of spines, not specific to clusters of spines/dendrites. This homeostatic upregulation is similar to silencing-induced upregulation of synaptic activity, seen originally in cultured neurons (518). In a more recent study, learning of motor skills caused an increase in parallel fiber multiple

synapse boutons, contacting adjacent spines on the same dendrite (274a). Interestingly, spines adjacent to those innervated by a large bouton appeared to shrink, indicating that concerted weakening of adjacent spines may constitute a homeostatic balancing of synaptic strength. Interestingly, in young neurons, prior to formation of dendritic spines, there are already clustered bouts of activity in adjacent synapses (238a) that are dependent on sodium spikes. This indicates that synchronized activity of adjacent synapses is important for the formation and strengthening of developing networks.

VIII. MOLECULES IN SYNAPTIC PLASTICITY

Neuronal activity profoundly influences spine morphology. Calcium influx through NMDA receptor activation has been shown to play a critical role in regulating spine size and density because several molecular events that control actin polymerization in spines are Ca^{2+} dependent (311, 588). Brain-derived neurotrophic factor (BDNF) has also been suggested to play both a major neurotrophic role in controlling spine development but also stimulation-dependent spine grown. Both NMDA receptor and TrkB, the receptor for BDNF, activate signaling molecules present in the spines or recruited upon stimulation. Some of these recruited molecules play an essential role in synapse and dendritic spine plasticity.

The importance of NMDA receptor is supported also by genetic experiments. Deletion of the GluN1 subunit in cortical layer 2/3 pyramidal neurons leads to a decrease in spine density, but the remaining spines have a larger head size as well as an increase in the miniature excitatory postsynaptic current amplitude and frequency (521). In brain slices, knock-down of GluN1 increases the motility of spines and over time leads to their loss. Interestingly the C-tail of the receptor is required for the long-term stabilization of synapses and spines and not only for the ionotropic properties of NMDA receptors (10). However, the NMDA receptor composition and the different signaling downstream of its activation are important for the effect on spine morphology. For example, mice lacking GluN2B in pyramidal neurons of cortex and CA1 region of the hippocampus show substantial alterations in the NMDAR-dependent LTD and also exhibit decreased dendritic spine density (59). In medium spiny striatal neurons, the blockade of GluN2A containing receptor with antagonist NVP-AAM077 induces a significant increase of spine head width while treatment with D1 receptor agonist (SKF38393), which induces a decrease of GluN2A-containing NMDA receptors, also increase spine head width. On the other hand, the GluN2B antagonist (ifenprodil) abolishes the spine morphological effect of the D1 agonist. Thus NMDA receptor composition at the corticostriatal synapses plays an important role in determining the structure and function of dendritic spines (527).

The most important molecule responding to a rise in $[Ca^{2+}]$ upon NMDA receptor activation is the calcium-sensing calmodulin (CaM) that activates the serine/threonine kinases CaMKI, CaMKII, and CaMKIV. These kinases can phosphorylate several proteins implicated in spine morphology and plasticity including Kalirin-7 and other signaling and scaffolding proteins (470, 542, 558). Recently it has been demonstrated that the CaMKII phosphorylation of Rpt6 proteasomal subunit and proteasome activation is required for NMDA receptor-dependent, activity-induced spine outgrowth (163).

Other neurotransmitters might modulate morphology and plasticity of dendritic spine. Among them is serotonin. Activation of the $5\text{-}HT_{2A}$ receptors in pyramidal neurons increases spine size via a Kalirin-7-Rac1-PAK-dependent pathways. These data might have an important implication in neuropsychiatric disorders for the link between serotonergic signaling, altered in multiple neuropsychiatric disorders, and dendritic spine morphogenesis (217).

As mentioned before, dopamine is also a relevant neurotransmitter in its ability to modulate dendritic spines plasticity. The number of dendritic spines is reduced in striatal medium spiny neurons of rats treated for 3 wk with 6-hydroxydopamine, the neurotoxin that selectively kills dopaminergic and noradrenergic neurons (471, 540). Woolfrey et al. (550) showed that D1/D5 receptors activation with selective agonist SKF-38393 induced spine shrinkage by activation of the RapGEF protein Epac2.

The cholinergic system has also been implicated in dendritic spine morphology. Cortical pyramidal neurons of layer V have lower spine density after the specific elimination of the cholinergic system using saporine-conjugated antibodies against nicotinic receptors (192 IgG) (457). In vivo studies have demonstrated that $\beta 2$ subunit of the acetylcholine nicotine receptor knockout induces a decrease of spine density in the higher order association areas (29). Thus activation of $\beta 2$-containing nAChRs by nicotine in vivo or in organotypic slice culture rapidly increases the number of spines by inducing intracellular calcium rise and activation of CaMKII (289).

Finally, some genetic evidence suggests that inhibitory synapses also control dendritic spine development; mice lacking the $GABA_A$ receptor $\alpha 1$ subunit showed an increased density of dendritic filopodia during the second and third postnatal weeks and a parallel decrease in density of mature mushroom spines (169). These studies suggest that correct balancing between excitatory and inhibitory synaptic transmission is important for regulating outgrowth of filopodia and spine maturation (183). Thus these data underline the relevance of the main neurotransmitters signaling in modulating dendritic spine plasticity.

Among the neurotrophic factors, BDNF and the high-affinity receptor TrkB (tropomycin-related kinase B) have been strongly implicated in synaptic and dendritic spine formation

and plasticity (291, 293). Several studies have demonstrated the ability of BDNF to change dendritic spine density and morphology in a variety of neuronal populations (12, 291, 293, 499, 519). Not surprisingly, TrkB knockout mice have significantly fewer dendritic spines on CA1 hippocampal neurons (292). In contrast, hippocampal pyramidal neurons of mice carrying loss-of-function mutation of the pan-neurotrophin receptor $p75^{NTR}$ have a higher spine density, suggesting a possible antagonistic action of $p75^{NTR}$ on TrkB receptor (576). The activation and phosphorylation of ERK1/2 is necessary for BDNF to increase dendritic spine functions, but TrkB receptor can also interact and phosphorylate Tiam1, the Rac1 GEF protein (9, 328). Interestingly, BDNF seems to have a specific local activity because its mRNA is transported and translated in dendrites in an activity-dependent manner (12, 531).

As mentioned above, synaptic stimulation induces changes in spine morphology probably by recruiting some spine-specific actin regulatory proteins. This is the case with the two actin-binding proteins profilin II and N-catenin that are accumulated in spines by either synaptic NMDA receptor activation or LTP induction. Profilin II translocates into dendritic spines following the raise of postsynaptic Ca^{2+} levels and by the association with Ena/VASP family proteins and remains concentrated in spines for hours. By locally reducing actin dynamics, profilin II can stabilize spine morphology and reduce motility upon stimulation (4). In the lateral amygdala, the enhancement of associatively induced synaptic responses to fear learning is also molecularly mediated by the recruitment of profilin II to spines and the associated enlargement of PSD (268). These results suggest that profilin II links the synaptic activation of glutamate receptors with the functional stabilization of spine morphology to a mature shape. A major role in regulating dendritic and spine morphology of both profilin I and II have been supported also by genetic studies (323).

αN-catenin is a cadherin-associated protein that accumulates in activated synapses and links the adhesion molecules to actin filaments in association with β-catenin. In cultures, transfected αN-catenin promotes the maturation of dendritic spines by inhibiting their motility and stabilizing their shape. Thus endogenous αN-catenin may mediate neural activity-dependent spine stabilization by suppressing their motility and turnover when recruited at synapses (1).

Two other actin binding proteins Drebrin and Synaptopodin are recruited to spines and promote actin assembly and spine stabilization upon LTP induction or NMDA stimulation (136, 368, 494). As mentioned before, Synaptopodin regulates also Ca^{2+} concentration in spine and spine structure because the genetic deletion of synaptopodin alters synaptic plasticity probably because dendritic spines became devoid of spine apparatus (100).

As mentioned before, LTD is accompanied by a marked shrinkage of spines. Spine shrinkage requires activation of NMDA receptors and calcineurin and is mediated by the actin-severing protein cofilin (588). Calcineurin and phosphatidylinositol 3-kinase (PI3K) mediate dephosphorylation and promote cofilin translocation to dendritic spines, which is partially mediated by β-arrestin-2 (392).

Finally, activity-dependent remodeling of synapses is also mediated by protein kinase Polo-like kinase 2 (Plk2, also known as SNK). Synaptic activity induces Plk2 expression and targeting to dendritic spines where it phosphorylates and induces degradation of RapGAP protein SPAR that causes loss of mature dendritic spines (375).

IX. DISEASE-RELATED CHANGES IN DENDRITIC SPINES

Most of the neurological disorders characterized by alteration in cognition, emotion, and memory loss are often caused by altered synaptic connectivity and plasticity (for reviews, see Refs. 223, 384). Not surprisingly, anatomo-pathological analysis of brains of patients affected by Alzheimer's disease, schizophrenia, intellectual disabilities, and autism spectrum disorders often shows alteration in dendritic spine morphology (see **FIGURE 4**).

A. Alzheimer's Disease

Several studies suggest that synapse and dendritic spine dysfunctions are preceding and contributing to the eventual neuronal death and neurodegeneration in Alzheimer's disease (AD) (307, 572). This is also supported by many postmortem tissue samples from patients with AD that consistently showed prominent synapse and dendritic spine loss in the hippocampus and throughout the cortex, the principal areas affected by AD-related pathology (99, 240, 537).

Indeed, the number of synapses and spines eliminated is often greater than the expected level for the amount of lost neurons and better correlates with the cognitive decline, suggesting that the primary synaptic failure is a prominent pathogenic cause of AD (99, 537).

The identification of specific genes associated with the pathogenesis of AD will lead to better understanding the molecular mechanisms underlying synapse and spine alterations. Mutations in three major genes implicated in beta amyloid (Aβ) metabolism (APP, PSEN1, and PSEN2) have been associated with familial AD with an autosomal dominant form of inheritance and early onset of the disease (37, 38). These mutations cause a well-documented increase in production of pathogenic Aβ oligomers that at the end are responsible for inducing spine alterations, and reducing spine density (265, 452).

The role of these mutations has been demonstrated using several transgenic mouse models of AD that have been generated

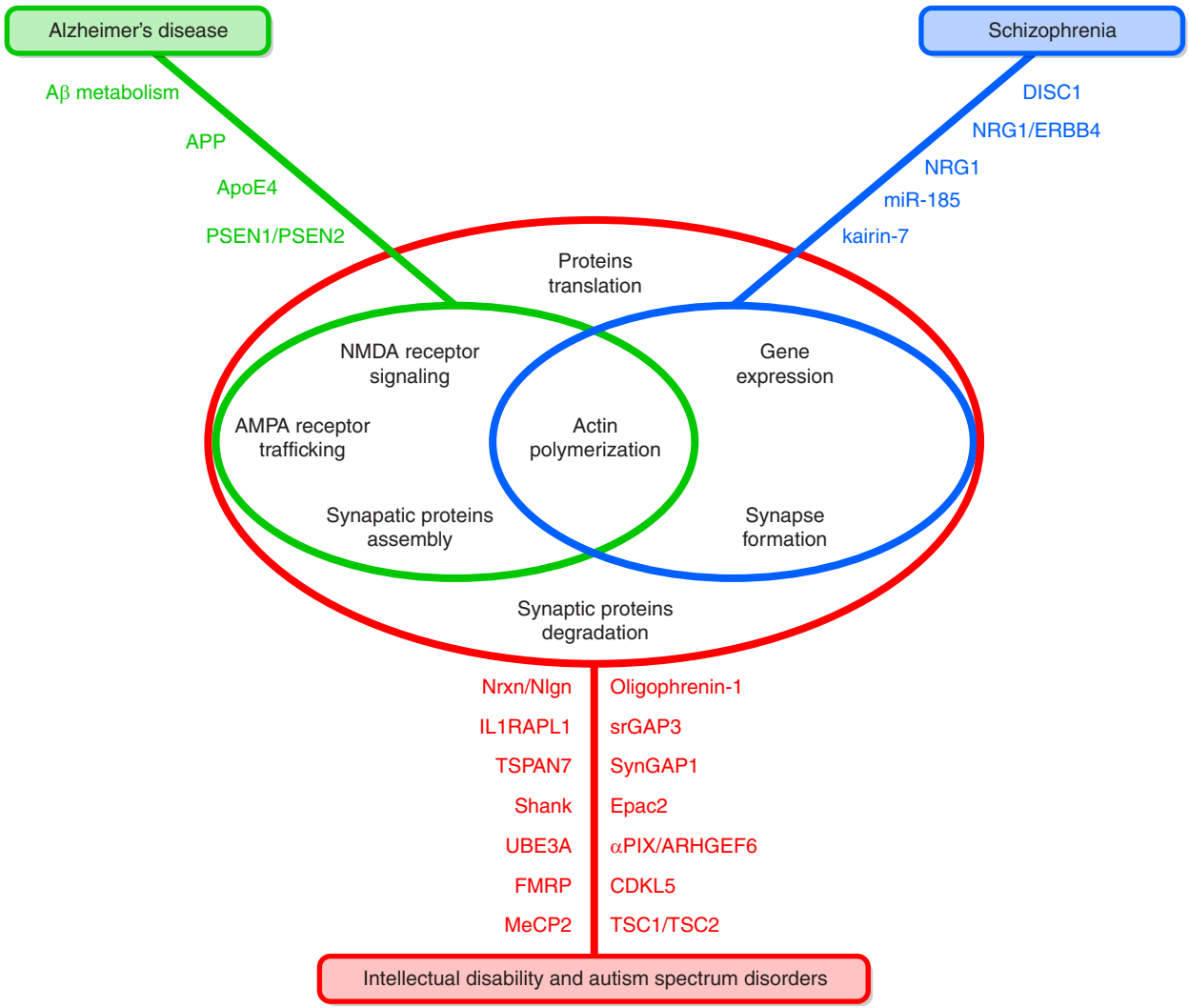


FIGURE 4. Disease proteins disregulating spine morphology. The diagram shows the list of major proteins, codified by genes mutated in the indicated neurological diseases (Alzheimer's disease in green, schizophrenia in blue, and intellectual disability and autism spectrum disorders in red), which are involved in altering spine morphologies through different molecular mechanisms listed in the center of the figure.

(23). These mouse models show behavioral deficits in reference and working memory but also dendritic and synaptic alterations, similar to what is found in human patients. For example, the transgene Tg2576 mouse model that expresses mutant human APP shows reduced spine density in hippocampal neurons from CA1 and dentate gyrus, even before the development of amyloid plaques, suggesting that soluble pathological Aβ oligomers initiate synapse alterations before neurodegeneration (207, 324). Similarly, APP/PS1 transgenic mice show alterations in spine density and morphology in dendrites in proximity of plaques but also a significant decrease in the frequency of large spines in neurons of plaque-free regions (239, 514).

Many other genes have been identified as high risk factors for the development of AD in the elderly. Among these genes, apolipoprotein E (ApoE) is the most important and studied risk factor. ApoE has three major isoforms: apoE2, E3, and E4. ApoE4 is associated with a high risk of developing the disease, while the other ApoEs seem to be neuroprotective. Transgenic mice for the human ApoE isoforms show that indeed ApoE4 transgene has reduced spine density in the dentate gyrus and in the cortex in an age-dependent manner, while mice expressing human ApoE2 and ApoE3 have normal numbers of spines. One study also showed that in the human brain there is an inverse correlation between ApoE4 expression and spine density in dentate gyrus neurons (105, 213). Interestingly, the transgene expression of ApoE2 in two different mouse models of AD (Tg2576 and PDAPP mice) can rescue spine density to normal levels (270). Thus all the mouse models of AD show alteration of spine morphology that correlates well with synaptic and behavioral dysfunctions. Consequently, measuring spine morphology is a good readout of the progression and severity of the AD pathology.

But which are the molecular mechanisms causing spine degeneration in AD? Recent results suggest that some signaling pathways regulating synaptic plasticity are involved. For ex-

ample, cofilin and drebrin, the actin-binding proteins with opposite effects on actin dynamics (see before), are both affected in AD. Cofilin phosphorylation and activity are upregulated by Aβ 1–42 peptide in a concentration-dependent manner, resulting in alteration in actin polymerization (240, 302). In striking contrast, drebrin, the postsynaptic protein that binds and stabilizes actin in spines, is severely reduced in the brains of patients with AD and in transgenic animal models of the disease (90, 147).

PAK is another important factor that regulates spine morphology, the activation of which is strikingly reduced in both AD patients and in mice models of AD (584). mRNA and protein levels of kalirin-7 were also found to be considerably reduced in the hippocampus of patients, indicating an alteration of the kalirin-7/Rac1/PAK pathway causes spine pathology in AD (570, 571).

More recently it has been shown that the partitioning defective-1 (PAR-1)/microtubule affinity-regulating kinase (MARK) family kinases is a potential mediator of Aβ toxicity in dendritic spines. MARK4 overexpression in cultured neurons mimics Aβ effect on synapses and dendritic spines, while a peptide that is specifically able to inhibit the activities of all PAR-1/MARK family members eliminates the toxic effects of Aβ oligomers on dendritic spines and synapses (573).

Most of the signaling molecules altered by Aβ described above are downstream of NMDA receptor, and overactivation of the NMDAR/calcineurin/GSK-3β pathways is specifically induced by Aβ treatment. Calcineurin is a calcium-sensitive phosphatase that regulates synaptic plasticity and is required for AMPA receptor internalization and LTD. Indeed, Aβ oligomer-induced AMPA receptor internalization and spine loss is prevented by calcineurin inhibition (197, 554).

Finally, it is important to note that AD patients have altered expression of many pre- and postsynaptic proteins and that, at least in cultured neurons, Aβ oligomers destabilize some synaptic protein complexes, like Shank and Homer platform; reduce the surface expression of NMDARs and AMPARs; and induce synapse and spine collapse (20, 197, 419, 466).

Identifying the molecular mechanisms of the Aβ toxicity in dendritic spines may be the focus of targeted pharmacological intervention and may open new therapeutic avenues towards the cure of AD.

B. Schizophrenia

Schizophrenia is a psychiatric disorder involving disturbing thoughts, emotions, cognition, and perception of reality, with an incidence of 0.5–1% of the population. It emerges in late adolescence or early adulthood. Postmortem studies in human brains have reported grey matter loss and altered spine density in specific brain regions such as the dorsolateral prefrontal cortex (DLPFC), an area that shows reduced activity during cognitive tasks in the affected individuals (146, 149, 497). Similarly, a reduction in grey matter volume in superior temporal gyrus as well as in spine density has been consistently reported (491, 569). Likewise, a reduction in hippocampal volume associated with reduced spine density on subicular and CA3 dendrites has been found (245, 482). Altogether these results propose a strong association between loss of grey matter, reduced spine density, and functional hypoactivity in specific brain regions of patients affected by schizophrenia (244).

The discovery of specific gene mutations associated with schizophrenia accelerated the search for the molecular basis of spine alteration observed in schizophrenic patients. Among the hundreds of gene variants that have been associated with schizophrenia, up to now few of them have shown constant associations with schizophrenia: DISC1, ERBB4, and NRG1 are the three most studied in relation of synapse and spine function. However, rare but very penetrant CNVs have been recently found in many genes encoding synaptic proteins, but these genomic studies need to be confirmed by functional experiments (538). Also, mutations in some synaptic genes could be associated either with schizophrenia or intellectual disabilities and autism, suggesting that some pathogenic mechanisms are shared by the two neurological diseases (139).

The disrupted in schizophrenia 1 (DISC1) gene was identified in a Scottish pedigree with a disruption in the DISC1 open reading frame (479). Subsequently, DISC1 polymorphisms, SNPs, and frame shift mutations affecting DISC1 protein function have been linked to schizophrenia in other families (285, 446). More recently, DISC1 functions have been associated with neurodevelopmental disorders that clinically manifest schizophrenia plus mood or schizoaffective disorder (57).

In cultured cortical neurons, DISC1 knockdown reduces spine area (181), and in mice that carry DISC1 mutations that mimic a well-defined schizophrenia risk allele, spine numbers in dentate gyrus granule cells are reduced (262). However, in another transgenic mouse expressing an inducible human DISC1 mutant, spine density in cortical pyramidal neurons was increased when the expression of mutant DISC1 was induced prenatally, while combined prenatal and postnatal expression of mutant DISC1 increased spine density specifically in dentate granule cells (24). Thus DISC1 controls spine morphology in a specific developmental and mutation-dependent manner.

Molecularly, DISC1 is a scaffold protein interacting with a regulator of spine morphogenesis such as kalirin-7, and regulates kalirin-7 binding to PSD-95 and access of to Rac1 in response to NMDA receptor activation (181). Strikingly, kalirin knockout mice express strongly reduced spine density in the frontal cortex and present schizophrenic-like behavior including impairments in working memory, prepulse inhibition, and altered sociability (64, 556). Even more remarkably, the expression of kalirin-7 mRNA was reduced in the DLPFC

(187), and several missense mutations of the kalirin-7 gene were identified in association with schizophrenia (260).

Polymorphisms in NRG1 and a rare CNV for the NRG1 receptor ERBB4 have been strongly associated with schizophrenia (314, 451, 538). As mentioned above, mice knocked out for these genes show alteration in spine density, and these morphological alteration are associated with several schizophrenia-relevant behavioral phenotypes that can be treated with clozapine (30, 278, 314).

The 22q11.2 microdeletion syndrome accounts for 1–2% of schizophrenia cases, although only 20–25% of individuals affected by this syndrome manifest schizophrenia beyond other symptoms including severe intellectual disability. Morphologically, all mice models for the syndrome [Df(16)$A^{+/-}$ and the knockout for single genes present in this region ZDHHC8 and DGCR8] show reduced hippocampal spine density and sizes (337, 481). ZDHHC8 is a palmitoyl transferase that contributes to PSD-95 palmitoylation and localization to synapses. Dgcr8, instead, regulates miRNA processing and neuronal development. Interestingly, Gogos and co-workers (559) found that in the Df(16)$A^{+/-}$ mouse the altered spine development is caused by a specific reduction of a micro RNA, miR-185, localized in the deleted chromosomal region, that mediate the repression of a newly discovered Golgi apparatus resident protein termed Mirta22. The modes by which this protein regulates dendritic spine formation remain to be elucidated.

C. Intellectual Disability and Autism Spectrum Disorders

Intellectual disability (ID) and autism spectrum disorders (ASD) are severe neurodevelopmental disorders characterized, respectively, by low intelligence quotient (IQ) and impairment of social interactions and adaptation. The two clinical manifestations are often present in the same individual, and indeed, ~75% of people with ASD also show a nonverbal IQ below 70, a classical sign of ID.

This group of neurodevelopmental diseases affects 1–2% of children, with diagnosis usually occurring below 3 years of age (409, 509). Numerous mutated genes for synaptic proteins have been associated with both ID and ASD, and functional studies have shown that these genes are regulating synapse and dendritic spine formation and plasticity (532).

Neuropathological data for spine morphological alteration in ID and ASD patients have been collected using Golgi-impregnated postmortem human brain tissue. Two major phenotypes have been described. The reduced number of spines but normal morphology is the phenotype often associated with ID, whereas an increase in immature elongated spine density accounts more for ASD and fragile X syndrome (127, 201, 205). These data support the suggestion that developmental retardation of neuronal connectivity constitutes the hallmark of brain dysfunction in these patients.

The genetic search for mutations in ID and ASD patients is still under way, and several reports have identified rare mutations and CNVs in many synaptic genes. We will summarize the function of some of these genes in relation to dendritic spine alteration. Not surprisingly, most of these genes code for proteins that are involved in synapse formation like adhesion and scaffold molecules or in regulating actin polymerization through small GTPase pathways (47, 525).

We mentioned before the relevance of the Neuroligin-Neurexin protein complex for synapse and dendritic spine formation. A strong association of mutations in Nrxn1, Nlgn1, Nlgn3, and Nlgn4 genes were found in patients with ASD (reviewed in Refs. 94, 98, 487). However, in addition to the strong in vitro evidence for the ability of Neuroligin proteins to regulate spine number and formation, the knockout mice and transgenic mice mimicking the human mutations do not show alterations in spine morphology (or dendritic spine morphology analysis were not reported) except for the α-neurexin, double or triple knockout of the three isoforms, that showed a modest reduction of dendritic spine number in cortex (45, 87, 104, 208, 493). Thus in vivo mutation or deletion of one Neuroligin and Neurexin perturbs functionally of the synapses, but the overall structure remains normal.

Mutations of the interleukin-1 receptor accessory protein-like 1 (IL1RAPL1) gene are linked to autism and ID (67). In addition to the role of IL1RAPL1 in regulating dendritic spine formation through the interaction with PSD-95 and RhoGAP2 protein (382, 524) (see also above), the extracelluar domain of IL1RAPL1 is able to induce presynaptic differentiation by binding to PTPδ, suggesting that IL1RAPL1 has a function similar to other synaptic adhesion molecules (524).

The tetraspanin 7 (TSPAN7) protein is codified by the *TM4SF2* gene and is a member of the membrane protein tetraspanin superfamily. It is formed by four transmembrane domains linked by a short extracellular loop (EC1), a very short intracellular loop (IL), a longer extracellular loop (EC2), and short NH_2- and COOH-terminal cytoplasmic tails. Various mutations in the *TM4SF2* gene (2, 579) have been directly associated with nonsyndromic ID. A possible function of TSPAN7 in promoting filopodia and dendritic spine formation by binding to PICK1and regulating AMPA receptor trafficking to synapses has been proposed (31).

Several genes linked to ID and autism code for proteins implicated in the Rac1/Cdc42/PAK and Rho pathways, like the upstream regulators of Rac/Cdc42 such as αPIX/ARHGEF6 and srGAP3/MEGAP (Slit Robo Rho GTPase-activating protein 3), or downstream effectors such as PAK3 and LIMK and the RhoGAP protein oligophrenin-1. In this review we have

already illustrated the function of most of these genes in regulating actin cytoskeleton in spines (see above).

A translocation breakpoint on chromosome 3p25, which causes the functional inactivation of srGAP3, has been described in a patient with severe intellectual disabilities (114).

SrGAP3 inhibits Rac by forming a complex with the scaffold protein WAVE1 (469). Knockout mice for SrGAP3 show various neuroanatomical changes, including enlarged dendritic spines, but also schizophrenia-related behavioral phenotype, suggesting that mutations of SrGAP3 may contribute to various neurodevelopmental diseases (539).

Implication of the Ras/Rap pathways in ID is currently suggested by the identification of mutations in the SynGAP1 gene and RAPGEF4, encoding the synaptically localized RapGEF Epac2 (25, 162). Interestingly, in the SynGAP1 mouse model, dendritic spine synapses develop prematurely during the early postnatal period, and this early spine maturation enhances excitability in the developing hippocampus, associated with classical behavioral abnormalities. SynGAP1 function is critical during development because inducing SynGAP1 mutations past the critical developmental period does not alter spine morphology (86). Epac2 missense mutations (mimicking human mutations) in cultured neurons increase dendritic spine number (Epac2-T809S) and volume (Epac2-V646F) (550), but in two independent in vivo studies, Epac2-deficient mice showed reduced dendritic spine motility and density (or normal density) in cortical neurons (477, 565).

The fmr1 gene encodes the fragile X mental retardation protein (FMRP). The absence of FMRP, caused by the abnormal expansion of the CGG sequence in the gene, causes the fragile X (FX) syndrome, a major cause of congenital ID which is more frequent in males than in females. Several studies have shown that FX patients have neurons with an increased number of longer and thinner dendritic spines (27). Fmr1 knockout mice modeling FX syndrome have recapitulated some of the immature spine morphologies observed in the brains of FX humans (204).

FMRP is an RNA-binding protein that regulates, in an activity-dependent manner, both the transport and translation of a subset of neuronal mRNAs to synapses (15, 16, 27, 32, 97, 124). The deregulated translation of mRNAs associated with FMRP might explain why in FX syndrome there are alterations in dendritic spine and synapse morphology and functions (32). Indeed, the translation of several synaptic proteins, such as PSD-95, SAPAP1–3, Shank1, Shank3, IRSp53, MAP1B, mGluR5, GluN1, GluN2B, and GluA1, is altered in synaptosomes of fmr1 KO mice (39, 109, 199, 266, 281, 290, 334, 350, 577, 578). Additionally, some data link FMRP function to GTPase signaling. In *Drosophila*, the homologous FMRP regulates dendritic development by controlling actin cytoskeleton via the translational suppression of both Rac1 and profilin (411). Mice knockout for Fmr1 have impairment in LTP that can be rescued by BDNF treatment or by the stimulation of a Ras/PI3K pathway, indicating a potential role of the Ras signaling in this process and alterations in the connection between GTPases and actin polymerization in absence of FMRP (198).

The Phelan-McDermid syndrome (PMS; also named 22q13.3 deletion syndrome) is characterized by severe intellectual disability and autistic-like behavior (51, 305, 390, 548). The small deletion of chromosome 22 involves almost always a region that encodes the PSD scaffold protein Shank3. Deletion of one of the two Shank3 genes is assumed to cause the neurological pathology of PMS. De novo mutations in SHANK3 (107, 140, 332) and also in SHANK2 have been identified in individuals with ID and autism (36). The importance of Shank3 and Shank2 haploinsufficiency has been demonstrated in recent studies using knockout mice (56, 389, 530, 541). Cultured neurons knocked down for Shank3 and heterozygous or homozygous knockout mice for Shank3 have alterations in synaptic function and dendritic spine morphology (108, 530, 541). Thus these studies demonstrate that Shank3 is a major regulator of both synaptic morphology and function.

Mutations in tuberous sclerosis proteins 1 and 2 (TSC1, TSC2) or the phosphatase and tensin homolog (PTEN) genes are frequently diagnosed with autism, and each of these proteins regulates synaptic structure (63, 130, 503). Indeed, PTEN deficiency shows dendritic hypertrophy and elevated spine density while TSC1 or TSC2 ablation induces an enlargement of dendritic spines (263, 503).

The Angelman syndrome gene UBE3A, located at the maternal duplications of chromosome 15q11-q13, encodes an E3 ubiquitin ligase whose maternal deficiency reduces dendritic spine density and length in cerebellar, hippocampal, and cortical pyramidal neurons (102, 567). The multiple and not completely clear function of UBE3A suggest a potential link between autism and altered synaptic structure (297).

Rett syndrome is caused, in the majority of the patients, by mutations on the MECP2 gene, which encodes methyl-CpG-binding protein-2 (MeCP2), a protein that regulates gene transcription by binding to methylated DNA. Straight confirmation of MeCP2 contribution in the control of synapses is suggested by postmortem analysis of brains of female patients that showed lower spine density in the hippocampal CA1 pyramidal neurons (70, 216). Similarly, in mice deficient in MeCP2, spine dynamics is severely impaired early during development (269). CDKL5 is another gene that can be mutated in Rett syndrome. Interestingly, CDKL5 positively regulates dendritic spine morphology and synapse activity by phosphorylating NGL-1 and stabilizing its association in synapse with PSD95 (414).

Finally, it is important to note that when pharmacological and genetic manipulations successfully rescue both functional and behavioral phenotypes in many mouse model of intellectual disability and autism, there was always a parallel recovery of spine alterations, indicating the importance of these microstructural perturbations in the pathogenesis of these neurodevelopment diseases (269, 393, 413, 513).

X. PERSPECTIVES

This review attempts to outline and organize the bewildering number of molecules associated with the construction, maintenance, plasticity, and elimination of dendritic spines. The striking heterogeneity of spine morphologies on different types of neurons, as well as among adjacent spines in the same dendritic branch, remained puzzling: could it be that different molecular pathways act in different neurons/spines? Why is it that there are so many parallel molecular pathways for the formation/maturation of spines? Recent studies indicate that the functions of dendritic spines may be regulated by additional receptors residing on the spine head and/or neck. Among them is the GABAergic innervation that targets not only dendritic shafts but also selective spines and can suppress the calcium elevation in spines in response to synaptic stimulation (80). Another recent modulator of synaptic function is the nicotinic cholinergic receptor, found on dendritic spines of cortical neurons, which may enhance the activation of the NMDA receptor, and facilitate excitatory sensory transmission (564). Finally, some dendritic spines express receptors for corticotropin releasing factor, which may also modulate their ability to respond to afferent stimulation (74). These studies indicate that the functions of individual dendritic spines may be modulated by a host of factors, which challenges even more the understanding the role of dendritic spines in neuronal plasticity.

While the apparently simple dendritic spine appears to be more complex than ever realized, the tools for its investigation are improving as well. In fact, the major breakthroughs in the study of dendritic spines paralleled the development of imaging and molecular tools. Such was the case with the introduction of the high sensitivity cooled CCD, the confocal microscope which allowed three-dimensional reconstruction and time-lapse imaging of individual live spines and the two-photon microscope which allowed the investigation of dendritic spines in the cortex in vivo. The new generation of super-resolution microscopy, which breaks the limits of optical spatial resolution by an order of magnitude, include the sptPALM (304), the uPAINT (144), the STED (547), the STORM (182), and the DH-QPM (308). These tools are beginning to be introduced in the study of the distribution and dynamics of receptor molecules in dendritic spines. Initial observations (345) describe clusters of glutamate receptors in relations to PSD-95. Further experiments promise to revolutionize the understanding of the interrelations among molecular families in the spine and their relevance to plasticity-induced changes in spine morphology and function.

Several other imaging and molecular tools are being explored in the study of spine plasticity. Among them is the introduction of highly sensitive genetic calcium sensors (GCaMP6; Ref. 73) that can be used for imaging activity over extended periods of time, without the need to introduce dyes into the imaged cells. Selective activation of individual spines can be achieved by optogenetic activation of presynaptic fibers terminating on individual dendritic spines (374).

In conclusion, the combined use of high-resolution live microscopy, with the ability to stimulate and monitor individual molecular changes in clusters of spines in vivo over long periods of time, and the introduction of System Biology ability to monitor the simultaneous expression of hundreds of genes, will undoubtedly contribute to the understanding of the universal rules that govern the heterogeneous construction and function of central dendritic spines. We are almost there.

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Address for reprint requests and other correspondence: M. Segal, Dept. of Neurobiology, The Weizmann Institute, Rehovot 76100, Israel (e-mail: Menahem.segal@weizmann.ac.il).

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Carlo Sala and Menahem Segal

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