

# Organization of the Arp2/3 Complex in Hippocampal Spines

Bence Rácz<sup>1</sup> and Richard J. Weinberg<sup>2</sup>

<sup>1</sup>Department of Anatomy and Histology, Faculty of Veterinary Science, Szent István University, 1078 Budapest, Hungary, and <sup>2</sup>Department of Cell and Developmental Biology, and Neuroscience Center, University of North Carolina, Chapel Hill, North Carolina 27599-7090

Changes in the morphology of a dendritic spine require remodeling of its actin-based cytoskeleton. Biochemical mechanisms underlying actin remodeling have been studied extensively, but little is known about the physical organization of the actin-binding proteins that mediate remodeling in spines. Long-term potentiation-inducing stimuli trigger expansion of the spine head, suggesting local extension and branching of actin filaments. Because filament branching requires the Arp2/3 complex, we used quantitative immunoelectron microscopy to elucidate the organization of ARPC-2 (Arp2/3 complex subunit 2), an essential component of the complex. Our data from CA1 hippocampus indicate that Arp2/3 concentrates within spines in a previously unrecognized torroidal domain, apparently specialized to mediate actin filament branching.

**Key words:** actin; cytoskeleton; hippocampus; LTP; pyramidal cell; synaptic plasticity

## Introduction

Spines, actin-rich protrusions that emerge from the dendritic shaft of excitatory neurons in the mammalian forebrain, are the primary locus of synaptic contacts. Spines can change their size and shape in response to synaptic activity (Lippman and Dunae-vsky, 2005). Recent *in vitro* evidence shows that long-term potentiation (LTP) induction protocols lead to spine enlargement (Kopec et al., 2006; Park et al., 2006; Harvey and Svoboda, 2007); moreover, the synapses on these enlarged spines express more AMPA-type glutamate receptors (Matsuzaki et al., 2001; Kasai et al., 2003). That similar conditions obtain *in vivo* is suggested by ultrastructural evidence that larger spines have larger postsynaptic densities (PSDs) (Harris and Stevens, 1989), and that larger PSDs host more AMPA receptors (Kharazia and Weinberg, 1999; Takumi et al., 1999; Nusser, 2000). Therefore, spine size is tightly linked to synaptic strength, implying machinery within the spine that coordinates modifications in synaptic efficacy and spine size. Changes in spine morphology require remodeling of the actin filaments comprising the “spinoskeleton” (Chen et al., 2007; Lynch et al., 2007). That actin remodeling itself may play a causal role in LTP (Fifkova and Delay, 1982) is supported by evidence that spines enlarge before AMPA receptors accumulate at the spine surface (Kopec et al., 2006), and more directly, by evidence that blocking actin remodeling pharmacologically interferes with LTP (Fukazawa et al., 2003).

The actin cytoskeleton is reorganized dynamically via an elaborate network of biochemical cascades. Filament branching re-

quires the actin-related protein 2/3 (Arp2/3) complex (Goley and Welch, 2006; Pollard, 2007). After activation, this evolutionarily conserved supramolecular assembly initiates a new “daughter” actin filament, branching from the side of an existing “mother” filament. The Arp2/3 complex plays a central role in a wide range of basic processes throughout the eukaryotes, including cell migration, phagocytosis, exocytosis, and endocytosis (Bretschneider et al., 2004; Hurst et al., 2004; Toshima et al., 2005; Zuo et al., 2006; Cai et al., 2007; Galletta et al., 2008). Recently, Arp2/3 was shown also to play a key role in regulating the morphology of dendritic spines (Kim et al., 2006). Recent work suggests considerable organization within hippocampal spines (Blanpied et al., 2002; Cooney et al., 2002; Rácz and Weinberg, 2004, 2006; Rácz et al., 2004; Park et al., 2006), leading us to wonder whether compartmentalization of Arp2/3 within the spine might help to maintain this internal organization. Using immunoelectron microscopy, we show here that Arp2/3 complex subunit 2 (ARPC-2; an essential component of the Arp2/3 complex) (Mullins et al., 1997; Gournier et al., 2001) in the spine head concentrates in a restricted domain likely to play a special role in actin remodeling.

## Materials and Methods

Experiments were performed on adult Sprague Dawley rats; housing and experimental procedures complied with Institutional Animal Care and Use Committee guidelines. Animals anesthetized with pentobarbital (60 mg/kg, i.p.) were perfused intracardially with saline, followed by a mixture of depolymerized paraformaldehyde (PFA) (4%), glutaraldehyde (0.2–0.5%), and picric acid (1%) in 0.1 M phosphate buffer, pH 7.4 (for EM), or 4% PFA (for LM). Fifty micrometer coronal Vibratome sections were processed for immunohistochemistry (Rácz et al., 2004; Rácz and Weinberg, 2004, 2006).

The primary antibody was a polyclonal rabbit anti-ARPC-2 (also called p34 in mammals, and arc35 in yeast) generated against a peptide corresponding to amino acids 285–298 of human ARPC-2 (Upstate Biotechnology #07-227). This widely used antibody has been well characterized (Di Nardo et al., 2005; Kempniak et al., 2005; Hotulainen and Lappalainen, 2006). Western blot analysis confirmed that the antibody recognizes a single band in rat brain homogenate, migrating at ~34 kDa

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Correspondence should be addressed to Bence Rácz, Szent Istvan University, István u. 2, 1078 Budapest, Hungary. E-mail: racz.bence@aotk.szie.hu.

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(supplemental Fig. 1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material).

Electron micrographs of 125 randomly selected immunolabeled spine profiles, including synapses, were taken from proximal and middle regions of CA1 stratum radiatum from three animals. Membrane perimeters, profile areas, and distances were measured using NIH ImageJ (v1.38; see <http://rsb.info.nih.gov/ij/>). To determine “axo-dendritic” position of immunogold particles, we defined the lateral edges of the PSD for a random sample of clearly defined synapses and measured the distance from each gold particle lying within this synaptic region to the postsynaptic membrane (supplemental Fig. 2, inset, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material) (Valtschanoff and Weinberg, 2001; Rácz and Weinberg, 2004).

Using the same micrographs, we measured distances of gold particles from the closest point on the spine plasma membrane and from the geometric center of the spine profile (defined as the bisection of a line from the PSD center to the furthest point on the spine profile) to determine “radial position” (see Fig. 3A, inset).

Normalized radial position,  $r_N$  (the fraction of the distance from the center to the plasma membrane), was computed according to the following equation:

$$r_N = 1 - \left( \frac{r_1}{r_1 + r_2} \right),$$

where  $r_1$  is the radial distance from the membrane to the particle, and  $r_2$  is the radial distance of the particle from the geometric center of the spine. Therefore, 1.0 corresponds to a particle at the plasma membrane, and 0 corresponds to a particle at the spine center. Only particles lying within the spine were considered.

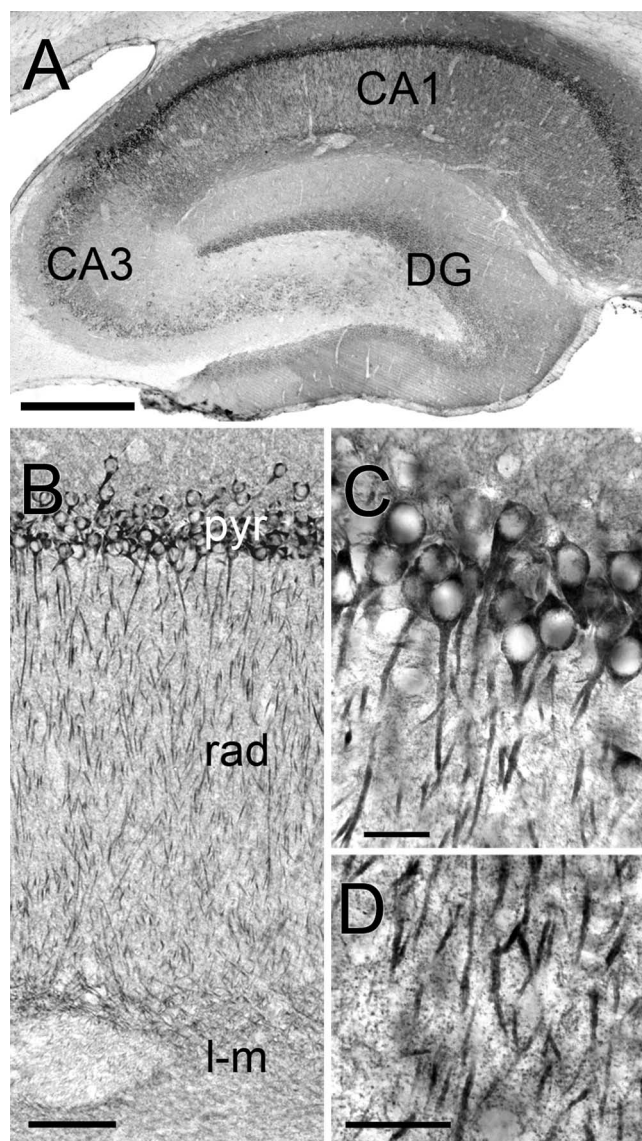
To investigate “tangential” distribution of ARPC-2 within the spinoplasm, we identified the point on the plasma membrane closest to each particle and determined the distance from there to the PSD edge, measured along the membrane (see Fig. 4A, inset). To compensate for differences in spine sizes, we normalized particle positions, such that 0 corresponded to the PSD edge and 1.0 to a point equidistant from both edges of the PSD (Fig. 4B, inset).

Data were collected in Microsoft Excel for additional analysis; Kaleidagraph (Synergy Software) and CricketGraph (Cricket Software) were used to compute statistics and generate graphs. We detected no significant differences in data from different animals ( $p > 0.2$ ; two-sided  $t$  tests) and therefore pooled the data. Line graphs were smoothed digitally, using a three-point weighted running average. For all but the end points,  $y_{j(\text{smoothed})} = [y_{(j-1)} + 2y_j + y_{(j+1)}]/4$ . For the end points,  $y_{1(\text{smoothed})} = (2y_1 + y_2)/3$ , and  $y_{n(\text{smoothed})} = [y_{(n-1)} + 2y_n]/3$ .

## Results

Immunostaining for the Arp2/3 complex was widespread throughout the gray matter of the brain. Staining was prominent in areas with numerous spiny neurons. In the hippocampus, staining was remarkably strong in the CA1 subregion (Fig. 1A). ARPC-2 immunostaining in CA1 concentrated in the cell bodies and dendrites of pyramidal neurons, sparing the nucleus (Fig. 1B,C). Numerous intensely stained puncta were visible at high magnification (Fig. 1D), raising the suspicion that ARPC-2 might be associated with spines.

To obtain a clearer understanding of its subcellular organization, we performed immunogold labeling for ARPC-2, using pre-embedding methods to optimize sensitivity. After labeling with silver-enhanced 1.4 nm gold, numerous electron-dense particles were seen in dendrites and spines, and occasionally in axon terminals. Particles were seen throughout the spine, seemingly more common away from the plasma membrane (Fig. 2A–D). Particles in spines and dendritic shafts were often associated with filamentous structures resembling F-actin, especially at branch points (Fig. 2C,D). Particles were also associated with vesicular struc-

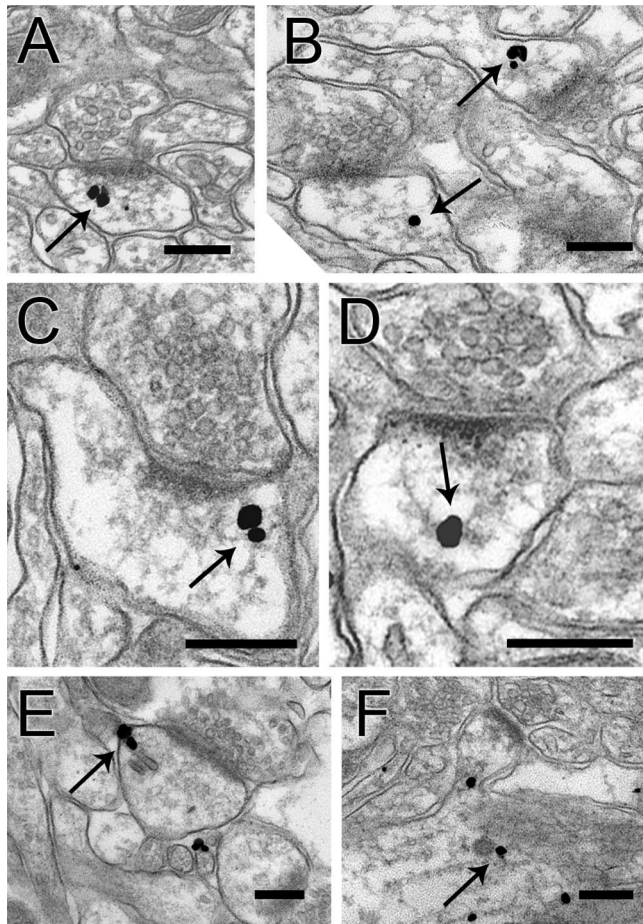


**Figure 1.** Immunoperoxidase staining for ARPC-2 in hippocampus. **A**, Low-magnification view; staining is strongest in CA1. **B**, Higher-magnification view shows laminar staining. pyr, Pyramidal layer; rad, stratum radiatum; l-m, stratum lacunosum-moleculare. **C**, Pyramidal cell layer of CA1. Staining is prominent in perikarya and apical dendrites but spares nuclei. **D**, High magnification reveals immunopositive puncta in the neuropil of stratum radiatum, likely corresponding to dendritic spines. Scale bars: **A**, 1 mm; **B**, 250  $\mu\text{m}$ ; **C**, **D**, 50  $\mu\text{m}$ .

tures occasionally visible within spines or in shafts near the spine neck (Fig. 2E,F).

We performed quantitative analysis to determine whether the Arp2/3 complex is restricted to specific spine domains. Because the domain defined by the synapse may exhibit distinct properties, we compared gold particles lying within the wedge defined by the lateral edges of the PSD and the geometric center of the spine with particles lying outside of this area (Fig. 3A). Finding no obvious differences, we pooled the two samples for subsequent analysis. ARPC-2 concentrated away from the plasma membrane but also lay away from the geometric center of the spine, peaking between 20 and 100 nm from the plasma membrane (Fig. 3A). Data were generally consistent among animals (means of  $66.7 \pm 3.2$ ,  $62.6 \pm 3.7$ , and  $59.9 \pm 4.4$  nm). Pooling data from the three animals, the label was an average of  $63.3 \pm 2.1$  nm from the plasma membrane ( $n = 263$  particles). These data suggest that





**Figure 2.** Preembedding immunogold labeling in dendritic spines. *A, B*, Immunogold particles (arrows) within the spine cytoplasm, typically lying away from the membrane. *C, D*, Particles coding for ARPC-2 were often associated with thin filaments, in the vicinity of branch points (arrows). *E*, Labeling associated with a likely endosome in a spine profile. *F*, Gold particles associated with filamentous structures and a coated vesicle (arrow) within a dendritic shaft. Another gold particle is associated with a vesicle lying in the neck of a spine from this shaft. Scale bars, 250 nm.

the Arp2/3 complex concentrates in a shell between the plasma membrane and the center of the spine.

Gold particles were uncommon >100 nm from the membrane (Fig. 3*A*). However, these data come from spine profiles of different radii (range, 80–470 nm; median, 177 nm). To pool the data more effectively, we normalized radial position (see Materials and Methods). Labeling again concentrated in a radial zone close to the plasma membrane (Fig. 3*B*). Further adjustment to correct for unequal bin areas did not change our conclusion that Arp2/3 concentrates in an intermediate zone between the center and the plasma membrane (supplemental Fig. 3*A*, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material).

We examined the distance of particles from the membrane, and from the spine center, as a function of spine radius (Fig. 3*C*). Unexpectedly, we found no relationship between spine size and distance to the membrane ( $r^2 < 0.01$ ) but a very strong relationship between spine size and distance to the center ( $r^2 > 0.76$ ;  $p < 0.001$ ). We conclude that the antigen tends to lie at a fixed distance from the plasma membrane, independent of spine size.

We then asked whether the Arp2/3 complex has a preferred “angular” location with respect to the axis defined by the PSD. We found that ARPC-2 was sparse in the wedge defined by the synaptic specialization, instead concentrating in a region 200–

400 nm from the PSD edge, measured along the membrane (Fig. 4*A*). Because this tangential distance was correlated with the total perimeter of the spine (data not shown), we normalized particle positions such that 0 corresponded to the PSD edge and 1.0 to the point equidistant along the perimeter of the spine from both edges of the PSD (Fig. 4*B*, inset). We considered only particles lying away from the spine center (normalized radial distance > 0.4) (Fig. 3*B*, supplemental Fig. 3*B*, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material), because the true tangential position of the few particles lying in the center is inherently poorly defined. Pooling these data, we found that ARPC-2 concentrated in a region 0.4–0.6 normalized units from the PSD (Fig. 4*B*) (mean,  $0.44 \pm 0.02$ ;  $n = 189$  particles). We conclude that in three dimensions, ARPC-2 concentrates within a donut-shaped zone lying halfway between the plasma membrane and the center and halfway between the PSD and the spine neck (Fig. 4*C*).

## Discussion

### Methodological constraints

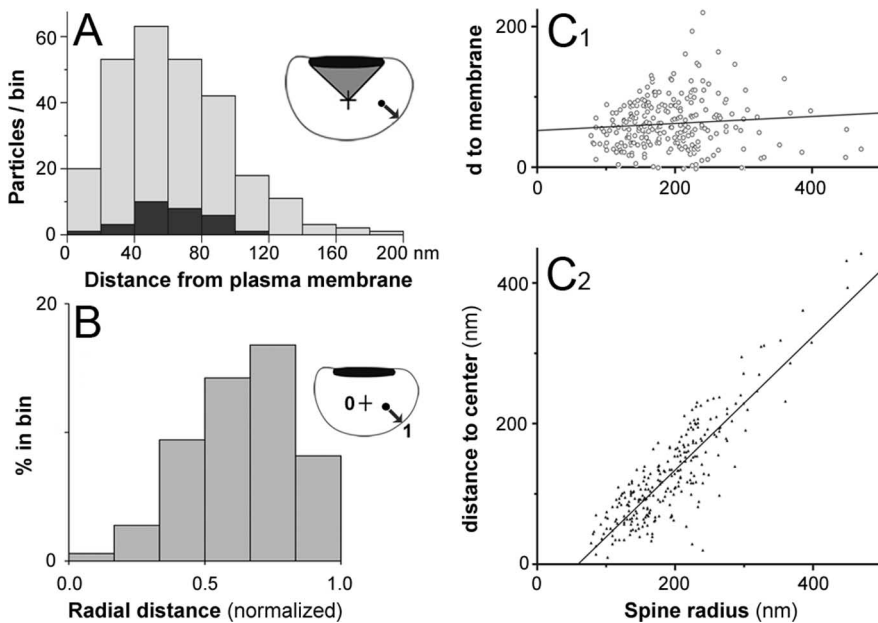
The Arp2/3 complex contains ARPC-2 and six other proteins assembled into a tightly linked biochemical package (Robinson et al., 2001), suggesting that ARPC-2 is a reliable probe for this complex, although this has not been demonstrated directly in brain. Proteomic studies have identified Arp2/3 subunits within the PSD (Li et al., 2004; Peng et al., 2004). Our results also suggest the Arp2/3 complex is present (although sparse) within the PSD. However, because pre-embedding techniques may fail to detect antigens within the PSD, because of limited access of antibodies (Lorincz et al., 2002; Rácz and Weinberg, 2004), pre-embedding may underestimate the fraction of antigen lying within the PSD.

The intrinsic noise of the immunogold technique can be reduced by averaging data from a large unbiased sample. This approach revealed unexpected features of the spatial organization of the Arp2/3 complex within the spine head. By focusing on average values, we may have failed to detect heterogeneity among spines. However, because the averaging process should attenuate any nonrandom organization detected, the true distribution of Arp2/3 *in vivo* likely peaks even more sharply within the domain we identified. An error is introduced by examining random single sections to estimate antigen distribution, rather than serial sections. That this error is relatively modest is suggested by the strong correlation between spine size and PSD length ( $r = 0.68$ ) (supplemental Fig. 4, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material), similar to, although less impressive than, the  $r = 0.88$  from fully reconstructed CA1 spines (Harris and Stevens, 1989). Therefore, our method should provide a good qualitative estimate of protein organization but is likely to underestimate the true extent of ARPC-2 compartmentalization.

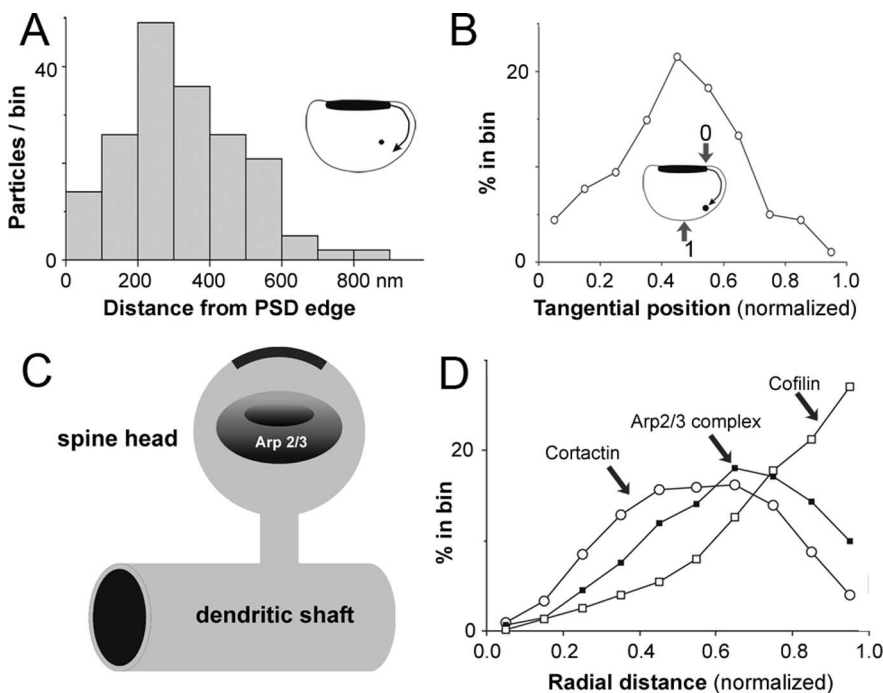
The techniques used here provide an averaged “snapshot” of a dynamic process, which reflects the most common distribution of the Arp2/3 complex in spines, but misses transients. However, even in very dynamic systems like the leading edges of migrating cells, the spatial location of Arp2/3 must be highly restricted to maintain typical actin treadmilling (Svitkina and Borisy, 1999; Atilgan et al., 2005; Shao et al., 2006). Therefore, the restricted spatial organization within the spinoplasm seen here likely represents the focus of activity of the Arp2/3 complex.

### Actin dynamics and the spinoskeleton

Activity-dependent changes in spine morphology are tightly linked to long-term synaptic plasticity (Fifkova and Delay, 1982; Matus, 2000; Knott et al., 2006; Chen et al., 2007; Harvey and



**Figure 3.** Radial organization of labeling. **A**, Stacked histogram shows labeling in relationship to the spine membrane; data from the PSD wedge (inset) are shown in black. Immunogold particles concentrated 20–100 nm from the membrane. **B**, Histogram shows distribution of normalized positions (0 corresponds to a particle at the spine center; 1.0 to a particle at the plasma membrane) (for details, see Materials and Methods). Particles concentrated between 0.4 and 0.8 normalized radial units within the spine head (see inset; black dot represents gold particle, and the arrow represents distance from the particle to the membrane). **C<sub>1</sub>**, Scatterplot: radius of the spine head is unrelated to distance to the plasma membrane; the line shows least-squares linear regression fit ( $r = 0.09$ ). **C<sub>2</sub>**, In contrast, note the striking relationship between spine radius and distance to spine center ( $r = 0.87$ ).



**Figure 4.** Organization of ABPs in spines. **A**, Tangential distribution of ARPC-2 (see inset and Materials and Methods). ARPC-2 was sparse near the synapse, peaking 200–400 nm from the PSD. **B**, Normalized particle positions: 0 corresponds to PSD edge, and 1.0 corresponds to the point equidistant from both edges of the PSD (inset). Particles concentrate  $\sim 0.4$ – $0.6$  U away from the PSD. **C**, Schematic representation of the Arp2/3 complex-enriched domain. **D**, Graph showing spatial distribution of cofilin (white squares), the Arp2/3 complex (black squares), and cortactin (white circles) within spines. Cofilin concentrates closest to the plasma membrane [postembedding immunogold data from Rácz and Weinberg (2006)], Arp2/3 at an intermediate position, and cortactin peaks farthest from the membrane [preembedding (Rácz and Weinberg, 2004)].

Svoboda, 2007). Underlying these shape changes is a complex interplay of molecular cascades triggered by synaptic input, leading ultimately to reorganization of the actin cytoskeleton (for review, see Carlisle and Kennedy, 2005; Calabrese et al., 2006; Schubert and Dotti, 2007; Honkura et al., 2008). Recent studies suggest that Arp2/3 plays a crucial role in synaptic plasticity: disruption of WAVE-1 (Wiskott-Aldrich syndrome protein family member 1), an upstream activator of the Arp2/3 complex, impairs learning and memory (Soderling et al., 2003, 2007). Moreover, loss of WAVE-1 also leads to abnormal spine morphology (Kim et al., 2006).

During morphogenesis, filopodia are thought to transform into mature, mushroom-shaped spines (Ethell and Pasquale, 2005) as a result of the protrusive force created by actin polymerization (Tada and Sheng, 2006). A linear bundle of actin reported in the spine, densest in the neck, may represent the primordial axis of the original filopodial extension (Fifkova and Delay, 1982; Smart and Halpain, 2000). Honkura et al. (2008) found that a dynamic pool of actin treadmills at the periphery, whereas a stable actin pool resides at the base of the spine. In this context, evidence that Arp2/3 plays a key role in the expansion of the head associated with mature dendritic spines might be predicted (Kim et al., 2006). The data reported here show that the Arp2/3 complex is restricted primarily to a toroidal domain within the spinoplasm, presumably representing a zone specialized for branch-point creation (Kiehart and Franke, 2002; Goley and Welch, 2006). Growth of filaments from this domain may generate the forces necessary to cause protrusion of the spine membrane during activity-dependent spine morphing, as observed in model systems (Pollard, 2007).

#### Endocytosis and the Arp2/3 complex in spines

Specialized endosomal compartments have been reported in hippocampal spines (Blanpied et al., 2002; Cooney et al., 2002). We reported previously that three key endocytic proteins localize in a lateral domain of dendritic spines (tangential distance,  $\sim 0.4$ – $0.6$  normalized units), corresponding to the functionally defined “endocytic zone,” a region that plays a special role in the activity-dependent endocytosis necessary for synaptic trafficking of receptors (Perez-Otano and Ehlers, 2004; Rácz et al., 2004; Lu et al., 2007). Recent evidence from non-neuronal systems implicates the actin cytoskeleton in regulated



endocytosis (for review, see Kaksonen et al., 2006; Smythe and Ayscough, 2006; Galletta et al., 2008); moreover, Arp2/3 complex-mediated actin polymerization is a prerequisite for clathrin-coated vesicle formation and movement (Merrifield et al., 2004; Martin et al., 2006). Recent *in vitro* experiments show that proper regulation of Arp2/3 is important for NMDA receptor-mediated endocytosis of AMPA receptors (Rocca et al., 2008). The present *in vivo* results support this finding, raising the intriguing possibility that this Arp2/3 complex-rich domain may contribute to efficient regulation of postsynaptic endocytosis.

### Organization of actin-binding proteins within the spine

Actin-binding proteins (ABPs) within the spine exhibit remarkable spatial segregation. Previous work found that cofilin concentrates at the plasma membrane (Rácz and Weinberg, 2006; Zhou et al., 2007), where filament growth is likely restricted by its depolymerizing activity. By activating cofilin, long-term depression-evoking stimuli lead to spine shrinkage (Zhou et al., 2004, 2007); conversely, stimuli that trigger LTP also inactivate cofilin, permitting stable spine enlargement (Meng et al., 2003; Lang et al., 2004). In contrast, cortactin concentrates in the spine core, and its loss decreases spine density (Hering and Sheng, 2003), consistent with the suggestion that the core is stabilized by cortactin (Rácz and Weinberg, 2004). The present data indicate that the Arp2/3 complex concentrates in an annulus within the spinoplasm, presumably representing a zone specialized for actin branching. Together, these data (summarized in Fig. 4D) suggest a previously unrecognized compartmentalization of actin regulation in dendritic spines.

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