Development/Plasticity/Repair

Calcium/Calmodulin-Dependent Protein Kinase II Alters Structural Plasticity and Cytoskeletal Dynamics in Drosophila

Ryan Andersen, Yimei Li, Mary Resseguie, and Jay E. Brenman
Department of Cell and Developmental Biology and Neuroscience Center, University of North Carolina Chapel Hill, Chapel Hill, North Carolina 27599

Drosophila dendritic arborization (da) neurons contain subclasses of neurons with distinct dendritic morphologies. We investigated calcium/calmodulin-dependent protein kinase II (CaMKII) regulation of dendritic structure and dynamics in vivo using optically transparent Drosophila larvae. CaMKII increases the dynamic nature and formation of dendritic filopodia throughout larval development but only affects neurons that normally contain dendritic filopodia. In parallel, we examined the effects of Rac1 activity on dendritic structure to explore signaling specificity. In contrast to CaMKII activity, Rac1 does not alter filopodia stability but instead causes de novo filopodia formation on all da neurons. Although both mediators increase cytoskeletal turnover, measured by fluorescence recovery after photobleaching experiments, only CaMKII increases the dynamic nature of dendritic filopodia. CaMKII signaling thus appears to use mechanisms and machinery distinct from Rac1 signaling. This study illustrates a molecular means of uncoupling cytoskeletal regulation from morphological regulation. Our results suggest that Drosophila dendritic filopodia may share some cytoskeletal regulatory mechanisms with mammalian dendritic filopodia. Furthermore, general dendrite cytoskeletal compartmentalization is conserved in multipolar neurons.

Key words: actin; dendrites; Drosophila; filopodia; Rac1; CaMKII

Introduction

Neuronal dendrites process and receive information, either from other neurons or the external environment. Regulation of dendritic morphological diversity is a key factor in defining synaptic function and plasticity (for review, see Cline, 2001; Scott and Luo, 2001; Jan and Jan, 2003). Molecular/genetic signaling determines this morphology and changing function by modulating microtubule-based dendritic shafts and actin-rich dendritic filopodia or spines (Filkova and Delay, 1982; Matus et al., 1982; Micheva et al., 1998). Filopodia can serve multiple functions including acting as precursors to dendritic spines (Parnass et al., 2000) and pioneering the formation of dendritic arbors (Niell et al., 2004). Dendritic compartments undergo dynamic regulation and demonstrate both morphological and synaptic plasticity (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999; Yuste and Bonhoeffer, 2001). However, how these plasticity changes are regulated is poorly understood. We asked whether proteins thought to modulate the cytoskeleton could regulate the formation and stability of dendritic filopodia.

The Drosophila larval peripheral nervous system (PNS) contains external sensory, chordotonal, and multiple dendrite (md) neurons (Bodmer and Jan, 1987). PNS neurons are postulated to transduce and relay multiple sensory inputs (Dethier, 1963; Bodmer and Jan, 1987). Different md neurons are postulated to preferentially transduce different sensory modalities consistent with distinct morphologies of md neuron subtypes (Grueber et al., 2003). Studies have demonstrated that md neurons can function during thermosensation (Liu et al., 2003; Tracey et al., 2003), mechanosensation (Grueber et al., 2001), and locomotor behavior (Ainsley et al., 2003). A subset of md neurons, dendritic arborization (da) neurons, are structurally reminiscent of most multipolar highly branched vertebrate neurons and provide a genetically tractable assay for identifying dendritic molecular regulators (Gao et al., 1999).

Calcium/calmodulin-dependent protein kinase II (CaMKII) is implicated as a regulator of both morphological and electrophysiological plasticity and therefore provides a molecular link between neural activation and downstream changes in plasticity (Silva et al., 1992a; Pettit et al., 1994; Wu and Cline, 1998; Zou and Cline, 1999). CaMKII is an abundant brain serine/threonine kinase (~2% of total protein) (Erondu and Kennedy, 1985) represented by four distinct genes in mammals (α, β, γ, δ) (for review, see Lisman et al., 2002). Autophosphorylation of threonine 286 [or Drosophila CaMKII (dCaMKII) threonine 287] produces an activated enzyme that no longer requires bound calci-
primary antibodies overnight at 4°C and washed three times with PBST. Secondary antibodies, cyanine 2 (Cy2)-conjugated goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA) and Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch), were diluted 1:200 in 5% normal goat serum/PBST and incubated with samples for 4 h at room temperature. Images were from abdominal hemi-segment A6, acquired under similar settings using Zeiss (Oberkochen, Germany) LSM 510 confocal microscope with a 40× oil immersion lens. Briefly, system argon/helium-neon lasers were used to excite Cy2 and Cy3, respectively, and a 2 μm optical slice was taken. To ensure that all filopodia emanating from the dendritic shaft were imaged, approximately eight 0.2 μm optical slices were imaged as a Z-stack with a 4× digital zoom at 1024 × 1024 pixels. The Z-stack was then compiled into one image using the LSM 510 software. Images were sized and cropped with Adobe Photoshop and placed into Adobe Illustrator (Adobe Systems, San Jose, CA) for labels and arrangement.

Construction of Nod::GFP transgenic. The Nod::GFP fusion protein was made using a previously generated Nod::LacZ fusion construct (Clark et al., 1997). An EcoRI-KpnI fragment of enhanced GFP (Clontech, Mountain View, CA) was used to replace the corresponding fragment of LacZ in Nod::LacZ to create Nod::GFP. The Nod::GFP gene fragment was cloned into the pUAST vector (Brand and Perrimon, 1993). The transgenes were introduced into a w1118 stock by P-element-mediated transformation (Rubin and Spradling, 1982).

In vivo imaging. Homozygous recombinant second instar larvae were placed on an air-permeable membrane (cut-out 40 μm cell strainer, Corning, NY). Second-instar larvae mounted in halocarbon oil (27 Sigma, St. Louis, MO) and gently coverslipped (22 × 50 mm; Fisher Scientific, Pittsburgh, PA) to restrict movement but not cause bursting of the body wall. Confocal images of dendrite morphology were obtained with a Zeiss LSM 510 confocal microscope by exciting the 488 nm argon line to excite GFP. Abdominal hemi-segment A6 was imaged for all experiments. For filopodia counts (see Fig. 5A), the entire ddaA neuron dendritic arbor was imaged. Three micrometer Z-stacks (usually composed of 15 slices, each 0.2 μm thick) at 1024 × 1024 pixels with a 20× lens were compiled to ensure that all filopodia protruding from the dendritic structure were imaged. Using the Zeiss LSM software, the ddaA neuron was traced from seven animals of each genotype, and all dendritic filopodia were classified by length and counted. Similar laser settings and Z-stacks were used for time-lapse experiments (see Fig. 5B, C). Images were taken every 2–3 min for 15 min to ensure that filopodia did not emerge and retract during the 15 min and may have been missed. We measured the formation and disappearance of filopodia (see Fig. 5B) by tracing the entire 100 μm length of the first ventral branch of the ddaA neuron and classifying filopodia as either formed if they appeared after 0 and disappeared if they were no longer visible during the 15 min interval. Seven ddaA dendritic lengths for each genotype were counted and plotted with Excel software (Microsoft, Redmond, WA). A χ² test [calculated with SAS (Cary, NC) software] was used to determine differences in filopodia populations. To resolve the stability of a given filopodium over time (see Fig. 5C), 25 filopodia on the ddaA neuron (hemisegment A6) were observed for 15 min at 2–3 min intervals. The Zeiss LSM software overlay function allowed tracing and measurements of these filopodia, which were categorized as elongating, retracting, or stable (within 0.2 μm) at t = 0 and t = 15, and averaged across seven animals of each genotype. The coverslip was removed, ensuring no harm to the specimen, and the larvae were placed in yeast-cornmeal vials. Only data from larvae that eclosed as adults (>90%) were used.

Fluorescence recovery after photobleaching. Second instar larvae were mounted in halocarbon oil as described for in vivo imaging (above). Images were obtained using the confocal with a 488 nm argon line (Coherent, Santa Clara, CA) used to excite GFP. The argon line was set to 50% output, and the acousto-optical tunable filter (AOTF) slider was set to 0.5%, minimizing unwanted photobleaching. The pinhole was adjusted to 5 Airy disks to obtain an optical section of 4 μm. Once a baseline time point was captured, filopodia were bleached by increasing the zoom 4× and drawing a region of interest (ROI) (Zeiss software) around a ~2 μm filopodium excluding the dendrite (see Fig. 6A). At 50% output, the
Results
An assay system for cytoskeletal and dendritic analysis

We used the Gal4/UAS system (Brand and Perrimon, 1993) to express fluorescently tagged proteins, assessing the dendritic compartmentalization and structure of da neurons. The GAL4 109(2)80 enhancer line allows clear visualization of da neuron morphology, is highly specific to postmitotic md sensory neurons, and labels all six da neurons (ddaA–F), one bd neuron, and one tracheal dendrite neuron in the 12 neurons of the dorsal cluster (Gao et al., 1999). These neurons have been classified (I–IV) based on the complexity of the dendritic arbor (Grueber et al., 2003). Previously, we observed dendritic protrusions in larvae faintly visible with GFP but unlabeled by Tau::GFP (Gao et al., 1999). We hypothesized these protrusions would be actin-enriched, similar to mammalian dendritic filopodia and spines (Fifkova and Delay, 1982; Matus et al., 1982). Indeed, actin::GFP fusion protein (β-Actin5C) expressed in da neurons allows visualization of numerous finger-like dendritic filopodia (Fig. 1B), more intensely labeled than with GFP alone (Fig. 1A). Although GFP is not completely excluded from filopodia (Fig. 1A, inset), the small volume of dendritic filopodia results in decreased fluorescence compared with actin::GFP, which is specifically targeted to filopodia (Fig. 1B, inset). GMA (Dutta et al., 2002), a chimeric GFP construct containing 140 amino acids of the F-actin-binding protein Moesin, was expressed in transgenic animals to further characterize these filopodia (Fig. 1C–E). Both GMA (Fig. 1C–E) and rhodamine–phalloidin (data not shown) labeling gave qualitatively similar results with strong enrichment in filopodia. Furthermore, GMA has been demonstrated to give qualitatively similar staining as rhodamine–phalloidin in other cell types as well (Dutta et al., 2002). However, numerous in vivo microtubule reporters, including Tau::GFP, which binds microtubules in vivo (Brand, 1995), and tubulin::GFP (Grieder et al., 2000) failed to label dendritic filopodia (data not shown). Immunolabeling against Futsch, a microtubule–associated protein 1B-like protein, which binds microtubules (Hummel et al., 2000) (Fig. 1D), also failed to label these filopodia, suggesting these structures are devoid of microtubules.

Observing dendritic filopodia development in real time al-
allows the discrimination of subtle, but distinct, steps in filopodia development and F-actin regulation. Whole-animal time-lapse imaging demonstrates that F-actin-rich patches accumulate in the dendrite and premark the directed site of future dendritic filopodia emergence (Fig. 1E). In time-lapse analyses, every emerging filopodium is preceded by a GMA and their localizations is provided in Table 1 (available at www.jneurosci.org as supplemental material).

CaMKII can potentially regulate Drosophila dendritic filopodia

Structural similarities between Drosophila da dendrites and vertebrate dendrites prompted us to investigate whether functional regulation could be similar as well. CaMKII, present in da sensory neurons (see Fig. 5, inset) (Takamatsu et al., 2003), has been demonstrated to affect dendritic morphology (Cline, 2001) as well as filopodia and spine formation (Jourdain et al., 2003). We expressed CaMKII protein variants (Jin et al., 1998) to evaluate potential roles for CaMKII regulation of dendritic morphology. Wild-type CaMKII (UAS-CaMKII), or single point mutant versions in the autoregulatory domain, threonine 287 (UAS-CaMKII T287D, UAS-CaMKII T287A), were driven by the same promoter element, Gal4 109(2)80. To enable live-animal imaging, transgenic larvae bearing the Gal4 109(2)80 and UAS-actin::GFP element were used for the remaining experiments. The CaMKII T287D mutation is a phosphomimetic version of phospho-threonine that renders the enzyme constitutively active without requiring bound calcium/calmodulin. In contrast, the CaMKII T287A mutation allows the enzyme to become active in the presence of calcium/calmodulin but loses the ability to become persistently active when calcium levels fall (Fong et al., 1989; Waldmann et al., 1990; Wang et al., 1998).

There were no detectable differences of dendrite structure between wild-type (Fig. 3A, F,K) and wild-type UAS-CaMKII transgenic larvae (Fig. 3B, G,L). However, significant increases in dendritic filopodia density were observed in class III neurons expressing activated CaMKII T287D (Fig. 3D, N). The CaMKII T287D phenotype becomes most obvious when comparing equivalent dendrite branches from the same neurons in wild-type, UAS-CaMKII, UAS-CaMKII T287A, and UAS-CaMKII T287D larvae (Fig. 3K–N). We counted all dendritic filopodia on the entire ddaA dendritic arbor for second instar larvae bearing CaMKII transgenes. We further classified these filopodia based on length to detect any dramatic differences in morphology. The UAS-CaMKII T287D phenotype was confined to the dendritic compartment, because no formation of filopodia on axon shafts in CaMKII T287D animals occurred (Fig. 3N, yellow arrowhead). The CaMKII T287D phenotype demonstrates a more severe phenotype with increasing transgene number. Increasing CaMKII activity, by increasing CaMKII T287D transgene number, increased the number of filopodia formed without substantially changing the length/number distribution (Fig. 4A). In contrast, even two transgenes of CaMKII T287A or wild-type CaMKII had no effect on total numbers of filopodia formed (Fig. 4A). CaMKII
We examined a known modifier of the actin cytoskeleton and morphological phenotypes, Rac1 and CaMKII, which have distinct regulation of the actin cytoskeleton. Thus, CaMKII expression does not affect cell fate as Cut filopodia-bearing neurons (data not shown) (Grueber et al., 2003), class III neurons but simply converted other neurons into type II da neurons (Grueber et al., 2003), expression of this shown). Although Cut protein is known to alter cell fates of sub-variations of actin modulatory proteins such as Moesin (data not shown). Rho kinase (Winter et al., 2001), as well as active and inactive CaMKII T287D and was not observed with other kinases and CaMKII T287A did produce significantly longer filopodia (>10 μm) and fewer short filopodia (<2 μm) than wild-type CaMKII (χ² test; p < 0.0001) (Fig. 4A).

Increased filopodia formation appears highly specific to CaMKII T287D and was not observed with other kinases and actin modulatory proteins, including Gal4/UAS expression of Rho kinase (Winter et al., 2001), as well as active and inactive versions of actin modulatory proteins such as Moesin (data not shown). Although Cut protein is known to alter cell fates of sub-types of da neurons (Grueber et al., 2003), expression of this homeodomain protein did not increase filopodia formation on class III neurons but simply converted other neurons into filopodia-bearing neurons (data not shown) (Grueber et al., 2003). Thus, CaMKII expression does not affect cell fate as Cut expression does but uses signaling mechanisms that specifically regulate the actin cytoskeleton.

Rac1 and CaMKII expression have distinct morphological phenotypes. We examined a known modifier of the actin cytoskeleton and dendritic structure, Rac1, to further explore specificity of the CaMKII T287D phenotype. In Drosophila, two groups have demonstrated a genetic requirement for rac1 in da neurons. Loss of rac1 function results in decreased dendritic branching (Lee et al., 2003), whereas increased wild-type Rac1 expression results in increased branching (Lee et al., 2003; Emoto et al., 2004). Furthermore, dominant-negative Rac1 suppresses an increased branching phenotype produced by Furry kinase loss-of-function mutants (Emoto et al., 2004). When we expressed wild-type Rac1 using Gal4 109(2)80, we observed dramatic increases in dendritic branching (Fig. 3E, J), as reported previously (Lee et al., 2003; Emoto et al., 2004). We also observed actin::GFP-containing filopodia on da neuron classes that normally lack filopodia (Fig. 3J). In contrast, the CaMKII T287D phenotype was confined to class III neurons and did not create filopodia de novo in neurons that normally lack abundant filopodia (Fig. 3D, I). Densities of spines and filopodia also vary characteristically among different mammalian neuronal types, and not all vertebrate dendrites have filopodia or spines. Rac1 expression leads to more filopodia on transgenic larvae than wild-type or UAS-CaMKII larvae solely by increasing dendritic branching and forming filopodia on normally nonfilopodia bearing neurons (Fig. 3) without altering filopodia density (Fig. 3K, O). These observations suggest distinct mechanisms for CaMKII- and Rac1-mediated filopodia regulation.

Activated CaMKII, but not Rac1, increases the dynamic nature of dendritic filopodia. We compared the effects of CaMKII and Rac1 signaling on dendrite stability. The ability to repeatedly image equivalent neurons between different transgenic animals over time allows for direct comparisons of dendritic dynamics. In mammals, dendritic filopodia and dendritic spines are spontaneously dynamic, such that only a small percentage of spines persist after a few days (Trachtenberg et al., 2002). Drosophila dendritic filopodia are also intrinsically dynamic (Fig. 4B, C). Identical 100 μm dendrite segments of the class III neuron ddaA were imaged over a 15 min interval in wild-type, wild-type CaMKII-, CaMKII T287D-, CaMKII T287A-, and Rac1-expressing larvae (Fig. 4B, C). Each filopodium was monitored continuously for its stability over time and whether filopodia formed or disappeared on the dendritic segment. Time-lapse analysis revealed that CaMKII T287D dramatically increases the numbers of newly formed and disappearing filopodia during development (Fig. 4B). More filopodia...
variants would be associated with corresponding protein local-
phenotype appears relatively stable, with increased branching most prominent. Scale bars:
Rac1-expressing larvae (Fig. 4)
first instar larvae (Fig. 4)
branching phenotype is readily apparent early in newly hatched
stages supports the time-lapse experiments. Whereas the Rac1
mutants (Fig. 4)
are formed than disappear, explaining why CaMKII T287D ani-
imals have such a large increase in filopodia that becomes more
pronounced over time (Fig. 4).

The CaMKII and Rac1 increase actin turnover in dendrites
We further explored how CaMKII and Rac1 might modulate actin turnover within dendritic filopodia by examining actin dynamics in living larvae. A previous study using dissociated hippocampal ne-
urons expressing actin::GFP has demonstrated rapid turnover of F-actin in dendritic spines. Actin turnover in spines occurs on the seconds to minute time scale, although the spines themselves can be semistable (Star et al., 2002). We used a similar actin::GFP construct but con-
ducted fluorescent recovery after FRAP studies using intact second instar Drosophila larvae. da neurons are ideally suited for FRAP analysis. These neurons are attached to a single layer of optically transparent epidermal cells and allow localized photobleaching of a single filopodium (Fig. 6). Such FRAP studies are particularly powerful with Drosophila because pathways can be modulated genetically, instead of pharmacologically, and the location of these neurons allows noninvasive experimentation in intact animals. FRAP analysis was conducted with low laser settings and high detector gain for data acquisition and high-power focused pulses for pho-
tobleaching (Fig. 6A) (Star et al., 2002). Photobleaching itself did not cause retraction of filopodia (Fig. 6A). Furthermore, FRAP data were only used from larvae that could later eclose to adults (~90%). Similar to the mammalian studies, we observed rapid turnover of actin::GFP within dendritic filopodia but on a slightly slower time scale than mammals (data herein) (Fig. 6B) (Star et al., 2002). In addition, fluorescent recovery was usually observed at the distal filopodia tip first (data not shown), consistent with addition of new monomer at the plus end.

Dendrites of transgenic larvae expressing CaMKII T287D contain increasing filopodia in a gene-copy-
dependent manner, whereas CaMKII T287D activity, but not Rac1 activity, modulates dendritic filopodia stability. A. Second instar larvae expressing CaMKII T287D possess increasing numbers of filopodia on ddaA neurons with increasing transgene number. Expression of CaMKII T287A shifts the distribution toward slightly longer filopodia (p < 0.001; χ^2 test; n = 7 of wild-type CaMKII and CaMKII T287A) but does not increase total number (p < 0.06; ***p < 0.006; ***p < 0.0007; t test; n = 7 of each genotype; all filopodia from the entire ddaA dendritic arbor were counted). B. Time-lapse imaging (2–3 min intervals; total, 15 min) of the ventral dendrite segment (100 μm) of neuron ddaA demonstrates that CaMKII T287D, but not Rac1, activity increases formation and disappearance of filopodia (p < 0.02; ***p < 8 × 10^-10). C. CaMKII expression decreases the stable pool of filopodia when compared with WT, WT CaMKII, CaMKII T287A, and Rac1 (**p < 0.004; n = 7; 25 filopodia from a 100 μm ventral dendrite segment of neuron ddaA segment (similar to B) were imaged every 2–3 min for 15 min. The filopodia were then classified as stable, elongating/retracting, or disappeared. Seven animals for each genotype were quantified. Error bars represent SEM. D–F. Newly hatched first instar larvae and third instar larvae (6–1) reveal timing differences between CaMKII T287D and Rac1 mutant phenotypes. WT CaMKII (D) and CaMKII T287D (E) first instar larvae appear similar. In contrast, a Rac1 (F) expressing larva
shows an early branching and filopodia mutant phenotype. D–F. Third instar class III neuron ddaA demonstrates CaMKII T287D expression has a cumulative phenotype (H), consistent with continuous increased filopodia formation (B). The Rac1 phenotype appears relatively stable, with increased branching most prominent. Scale bars: D, 6, 10 μm. WT, Wild type.
CaMKII regulates dendritic structural plasticity
CaMKII, which is expressed in Drosophila da neurons (Fig. 5), is commonly suggested to link neural activation with functional modification of circuits. In mouse models, altered expression of CaMKII-α leads to abnormal behavior (Chen et al., 1994), altered LTP in the hippocampus (Silva et al., 1992a; Pettit et al., 1994), reduced experience-dependent plasticity in barrel cortex (Glazewski et al., 1996), and impaired spatial learning (Silva et al., 1992b). How CaMKII modulates the cytoskeleton to bring about these changes is primarily unknown. Two groups have shown that CaMKII activity can alter filopodia formation in mammals (Fink et al., 2003; Jourdain et al., 2003). We demonstrate, with intact animals in real time, that CaMKII signaling affects dendritic stability by altering cytoskeletal and structural dynamics.

Similar morphological effects of CaMKII signaling may reflect the highly conserved amino acid sequence and biochemical regulatory mechanisms shared between CaMKII in mammals and Drosophila (Wang et al., 1998). A version of CaMKII, T287A, which can still be activated but not autophosphorylated, appears to slightly shift the distribution of filopodia, such that neurons expressing T287A CaMKII have slightly longer (>10 μm), and crude aspects of Drosophila PNS and CNS dendrites are similar. Drosophila da neurons provide a unique opportunity to develop genetic assays for dendrite development entirely in vivo. Our studies exploit these benefits to identify novel phenotypes for CaMKII and Rac1 signaling in Drosophila.

Drosophila da neuron dendrites also share biochemical similarity with Drosophila CNS dendrites. da neurons display minus-end-out microtubule arrays (Fig. 2) found uniquely in dendrites. Developing neurons display a distal minus-end-out array in dendrites, a key event in dendrite versus axon differentiation (Yu et al., 2000). In contrast, axonal compartments have solely (−) to (+) microtubule arrays from cell body to axon terminal (Baas et al., 1988; Burton, 1988). Although da dendrites do not receive axonal contacts, Nod reporters demonstrate similar enrichment at the tips of dendrites in both da sensory and mushroom body (CNS) axonally innervated neurons (Lee et al., 2000). da sensory neurons also have distinct actin-rich dendritic compartments, similar to dendrites in the Drosophila brain (Scott et al., 2003).

Soluble GFP weakly labels filopodia when compared with actin::GFP or GMA, which target GFP specifically to filopodia. These labeling patterns are similar with mammalian neurites wherein GFP is a volumetric indicator in neurites that intensely labels dendrites, with weak labeling of spine necks of small diameter (~0.1 μm) (Harris and Kater, 1994; Sabatini et al., 2002).

FRAP analysis of actin-rich dendrite compartments in mammals demonstrates a fast (~2 s) (Star et al., 2002; Zito et al., 2004) diffusion-limited FRAP recovery stage followed by a slower, longer recovery stage. We obtained similar FRAP results whereby GFP recovery in dendrite shafts or filopodia occurs within seconds (data not shown), whereas actin::GFP FRAP recovery demonstrates a longer, slower time frame (Fig. 6). This postdiffusion, longer recovery stage is inferred to reflect dynamic and mobile pools of actin (Star et al., 2002; Zito et al., 2004). Actual measurements of globular actin and F-actin, however, require biochemical-based analysis. The actin::GFP used in our studies behaves similarly to actin::GFP in mammalian studies, except the small size of Drosophila larvae make them amenable to whole-animal unanesthetized studies. Furthermore, we demonstrate that the effects of genetic perturbations on cytoskeletal turnover can be analyzed in intact transgenic animals with FRAP, providing cytoskeletal information to link molecules to phenotypes.
Actin::GFP in dendritic spines of transfected hippocampal neurons (Star et al., 2002). Actin::GFP turnover in dendritic filopodia was demonstrated using fluorescence recovery after FRAP (arrows). A transgenic animal containing both UAS-actin::GFP and membrane-targeted UAS-myr::mRFP reveals that FRAP itself does not visibly affect visible filopodia structure (arrowhead). Pre, Prediffusion; Post, postdiffusion. B, Individual data points from seven animals of each genotype were fit to $F(t) = 1 - \left(1 - e^{-t/\tau}\right)^2$, an equation used previously to document FRAP for actin::GFP in dendritic spines of transfected hippocampal neurons (Star et al., 2002). Actin::GFP turnover in UAS-CaMKII T287D ($t_{1/2} = 132 \pm 10 \text{s}$; black diamond) and UAS-Rac1 ($t_{1/2} = 102 \pm 6 \text{s}$; red circle) animals dramatically increased compared with UAS-CaMKII T287A ($t_{1/2} = 347 \text{s}$; blue cross) or UAS-CaMKII animals (green triangles).

Figure 6. Intact CaMKII T287D and wild-type Rac1 transgenic larvae demonstrate significantly increased actin::GFP turnover in dendritic filopodia. A, Actin::GFP turnover in dendritic filopodia was demonstrated using fluorescence recovery after FRAP (arrows). A transgenic animal containing both UAS-actin::GFP and membrane-targeted UAS-myr::mRFP reveals that FRAP itself does not visibly affect visible filopodia structure (arrowhead). Pre, Prediffusion; Post, postdiffusion. B, Individual data points from seven animals of each genotype were fit to $F(t) = 1 - \left(1 - e^{-t/\tau}\right)^2$, an equation used previously to document FRAP for actin::GFP in dendritic spines of transfected hippocampal neurons (Star et al., 2002). Actin::GFP turnover in UAS-CaMKII T287D ($t_{1/2} = 132 \pm 10 \text{s}$; black diamond) and UAS-Rac1 ($t_{1/2} = 102 \pm 6 \text{s}$; red circle) animals dramatically increased compared with UAS-CaMKII T287A ($t_{1/2} = 347 \text{s}$; blue cross) or UAS-CaMKII animals (green triangles).

CaMKII and Rac1 function distinctly in Drosophila dendrites

Given the known roles for Rho subfamily signaling on the actin cytoskeleton, we analyzed the roles of Rac1 signaling on dendritic structure in relationship to CaMKII (Figs. 3, 4, 7). In our studies, CaMKII modulates both morphological stability and cytoskeletal dynamics. In contrast, Rac1 signaling alters cytoskeletal dynamics without changing morphological stability. Rac1 activity greatly increases F-actin turnover as well, but the effects on structural plasticity are indistinguishable from CaMKII T287A or wild type (Figs. 4, 6). Importantly, whereas CaMKII increases the number of dendritic filopodia on only da class III neurons, Rac1 increases filopodia on all da neurons, even those that are normally devoid of filopodia (Figs. 3, 7).

Although the morphology of filopodia on Rac1-expressing animals can be slightly unusual, unlike CaMKII T287D, the density on class III neurons is no greater than wild type (Fig. 3K, O). The combination of phenotypic analysis, time lapse, and FRAP suggest that Rac1 and CaMKII distinctly regulate dendritic filopodia. Furthermore, 50% reduction of all rac genes (Hakeda-Suzuki et al., 2002; Ng et al., 2002) in heterozygous rac mutants did not suppress the CaMKII phenotype (data not shown). Expression of a dominant-negative Rac1 construct (N17) (Luo et al., 1994), which should block Rac-mediated GEF signaling, also failed to block the CaMKII T287D phenotype (data not shown). We also expressed both transgenes simultaneously, which does not result in increased densities of filopodia on non-class III neurons (data not shown) as might be predicted. This suggests either CaMKII is downstream of Rac1 or they modulate filopodia by distinct mechanisms.

An alternative hypothesis is that CaMKII activity strictly modulates the dynamics of dendritic filopodia extension and retraction, whereas Rac1 signaling preferentially modulates initial formation. There is evidence that the same pathways are used differently to regulate morphology in distinct subpopulations of neurons in mammals. Transgenic mice expressing dominant-negative p21-activated kinase (PAK) have abnormal dendritic spines in some cortical regions but not all regions (Hayashi et al., 2004). Furthermore, it has been demonstrated in Drosophila that many developmental defects can be rescued in rac triple-null mutants by a version lacking the effector domain for PAK (Ng et al., 2002), supporting different roles for downstream effectors in different cellular contexts.
Implications for structural plasticity

In *Drosophila*, altering sensory neuron surface areas that function in thermosensation, mechanosensation, or regulating crawling behavior (Grueber et al., 2001; Ainsley et al., 2003; Liu et al., 2003; Tracey et al., 2003) would have profound consequences in transducing environmental cues. Changing the degree of coupling between morphology and the cytoskeleton could result in different elicited behaviors from the same environmental cue. In addition, preliminary observations suggest that larvae reared in different environments can result in filopodia with different morphologies (R. Andersen, P. Medina, Z. Blalock, and J. E. Brenman, unpublished observations). Changing environmental conditions could therefore regulate the dynamic nature or morphology of filopodia, thus controlling the animal’s behavioral response.

What are the benefits of independently regulating morphological stability and cytoskeletal stability? In other systems, mechanisms to distinctly regulate cytoskeletal dynamics and morphological plasticity could be important during synaptogenesis, modification of synaptic strength, or neurite outgrowth. Calcium activation of multiple pathways, including CaMKII, could help sculpt morphological shape during periods of specialized neural activity. Evidence suggests synaptic strengthening is associated with morphological changes of dendritic spines into mushroom-shaped or perforated spine structures (Harris et al., 1992). One could imagine simultaneous activation and deactivation of numerous signaling pathways could sculpt a filopodium into a spine or vice versa. In fact, spines and filopodia have been directly observed to interconvert (Parnass et al., 2000; Roelandse et al., 2003). Spine motility and filopodia motility (Korkotian and Segal, 2001; Yuste and Bonhoeffer, 2001) could be modulated by differential signaling in pathways, including CaMKII and Rac, to adjust the likelihood of contacting other cells during development. Delivery and internalization rates of postsynaptic scaffolding/stabilizing proteins and receptors could be tied to cytoskeletal turnover and thus alter the ability to process neural information or transduce molecular signals. Another possibility is that receptor function could depend on physical anchoring to the actin cytoskeleton, which would serve as an indirect means of regulating receptor signaling without necessarily altering protein amount. Comparisons of cytoskeletal turnover and structural stability of dendritic compartments using FRAP, fluorescence resonance energy transfer, two-photon microscopy, and other in vivo techniques may allow direct comparisons of specific molecules in mammalian and *Drosophila* dendrites in the future. Modulating different aspects of neural architecture, by regulating distinct pathways, could provide the neuron flexibility to respond to changing developmental or environmental cues. Although Rac has many documented roles for modulating cellular morphol-
ogy, less is known about Rac (and Rho GTPases) genetically in LTP. In contrast, CaMKII has many documented genetic roles for LTP but few known signaling pathways to regulate dendritic structure and the cytoskeleton. Genetically amenable systems asayed with in vivo approaches, as we demonstrate, should allow for such analysis.

References


