A dynamin-3 spliced variant modulates the actin/cortactin-dependent morphogenesis of dendritic spines

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Summary

Immature dendrites extend many actin-rich filopodial structures that can be replaced by synapse-containing dendritic spines as the neuron matures. The large GTPase dynamin-3 (Dyn3) is a component of the postsynapse in hippocampal neurons but its function is undefined. Here, we demonstrate that a specific Dyn3 variant (Dyn3baa) promotes the formation of immature dendritic filopodia in cultured neurons. This effect is dependent upon Dyn3 GTPase activity and a direct interaction with the F-actin-binding protein cortactin. Consistent with these findings, Dyn3baa binds to cortactin with a 200% higher affinity than Dyn3aaa, a near identical isoform that does not induce dendritic filopodia when expressed in cultured neurons. Finally, levels of Dyn3baa-encoding mRNA are tightly regulated during neuronal maturation and are markedly upregulated during synaptogenesis. Together, these findings provide the first evidence that an enhanced interaction between a specific Dyn3 splice variant and cortactin modulate actin-membrane dynamics in developing neurons to regulate the morphogenesis of dendritic spines.

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Introduction

Synaptogenesis is initiated by the dynamic activities of axons and growth cones, as well as by dendrites and dendritic protrusions (Jontes and Smith, 2000). During the development of cultured hippocampal neuron, synaptogenesis generally occurs between 6 and 14 days in vitro (DIV), with most dendritic protrusions maintaining a filopodial morphology (Dailey and Smith, 1996; Ziv and Smith, 1996). These filopodia are long, thin, actin-rich structures with or without an enlargement of the tip (Hering and Sheng, 2001). As these neurons and synapses mature, the immature filopodia are replaced by shorter, mature mushroom-shaped dendritic spines containing the postsynaptic density (Fiala et al., 1998; Papa et al., 1995).

The general morphological profile and dynamics of filopodia and dendritic spines have been previously described (Hering and Sheng, 2001), although little is known about the molecular mechanisms underlying the morphological conversion of filopodia to mature, mushroom-shaped spines. The dynamin family of large GTPases are potential candidates to participate in this important process, because they are believed to provide a dynamic link between cell membranes and the actin cytoskeleton via a pleckstrin homology (PH) domain and a proline/arginine-rich domain (PRD), respectively (Orth and McNiven, 2003; Schafer, 2002). Three conventional dynamin isoforms are expressed in neurons. Dyn1 is neuron specific (Nakata et al., 1991) and is found primarily in the presynapse (Gray et al., 2003), where it is required for synaptic-membrane endocytosis and recycling (Shupliakov et al., 1997; Takei et al., 1996). Dyn2 is expressed in all tissues (Cook et al., 1994; Sontag et al., 1994) and participates in a range of endocytic and secretory membrane-trafficking events. By contrast, little is known about the expression, cellular distribution and function of Dyn3. This poorly understood form exhibits some tissue-specific expression and is found primarily in the brain, lung and testis (Cook et al., 1996; Nakata et al., 1993).

In a previous study, we presented evidence suggesting that Dyn3 accumulated at the postsynapse and localized to dendritic spines and filopodia (Gray et al., 2003). There are two expressed spliceoforms of Dyn3, Dyn3aaa and Dyn3baa, which differ by only ten amino acids spliced into Dyn3baa immediately preceding the PH domain (Cao et al., 1998). Both spliceoforms were expressed in hippocampal neurons to test whether these proteins contributed to G-protein-coupled-receptor endocytosis at the synapse. Although Dyn3 did not appear to contribute to the endocytic recycling of the metabotropic glutamate receptor, we did observe a striking change in the morphology of mature neurons expressing a fusion between Dyn3baa and green fluorescent protein (GFP) (Gray et al., 2003), a phenotype that suggested a postsynaptic function. Specifically, these transfected neurons lacked mature dendritic spines and were covered instead by long, thin filopodia.

In the current study, we observe that early and constitutive
expression of Dyn3baa, but not Dyn3aaa, leads to an aberrant maturation of dendritic spines and a reduction in filopodia and spine synapses. This phenotype is dependent upon an intact actin cytoskeleton and the GTPase activity of Dyn3baa. Remarkably, filopodia can also be induced in epithelial cells that do not normally express Dyn3. To define how Dyn3baa might produce these changes, we tested for functional and physical interactions between Dyn3 and the actin binding protein cortactin, which is also a known structural component of the postsynapse (Hering and Sheng, 2003; Naisbitt et al., 1999). Cortactin and Dyn2 are known to bind directly (McNiven et al., 2000) and disruption of this interaction has been shown to have profound effects on actin-based structures within cells, such as stress fibers (McNiven et al., 2000), actin comets (Lee and De Camilli, 2002; Orth et al., 2002) and dorsal ruffles (Krueger et al., 2003). Importantly, reducing Dyn3-cortactin interactions in cultured neurons through the expression of C-terminally truncated proteins prevents the observed aberrant neuronal filopodial outgrowth. In support of this in vivo observation, we find that, although both Dyn3 spliceforms can bind to cortactin directly, the filopodium-inducing Dyn3baa exhibits a twofold greater affinity for cortactin than does Dyn3aaa, the form that allows the normal development of mature, mushroom-shaped dendritic spines. In support of these different effects, we also observe markedly distinct expression patterns of the two Dyn3 spliceforms during synaptogenesis and filopodium and spine maturation. This study provides the first functional insights for Dyn3 in any cell type or tissue and is consistent with a novel developmental role for this dynamin family member in spine synaptogenesis. We also discuss the contributions of Dyn3 to actin-based membrane dynamics during synaptic remodeling.

Materials and Methods

Plasmid construction and transfection
Dyn3aaa-GFP and Dyn3baa-GFP were produced as described (Gray et al., 2003), and Dyn3baa(K44A)-GFP was produced using the manufacturer’s protocol. For co-transfections, equal amounts of endotoxin-free DNA with Effectene (Qiagen) according to the manufacturer’s protocol. For co-transfections, equal amounts of Dyn3aaa-GFP and cortactin-RFP were used in the same transfection reaction. Neurons were used 5-12 days after transfection. In all cases, transfection-complex-containing medium was exchanged after a 4 hour incubation with the cells.

RNA isolation and RT-PCR
Three time-course sample collections were completed from three separate neuronal isolations. In addition, hippocampal tissues were collected from Sprague-Dawley rats (pooled from three different individuals) at various time points throughout development. For reverse-transcription PCR (RT-PCR), total RNA was isolated from either the neuronal cultures or the hippocampal tissue at each time point and used to make a cDNA library as previously described (Cao et al., 2003). Neurons were fed twice weekly by changing half of the medium. Neuronal cultures were transfected at 6DIV using Dyn3aaa-GFP and cortactin-RFP were used in the same transfection reaction. Neurons were used 5-12 days after transfection. In all cases, transfection-complex-containing medium was exchanged after a 4 hour incubation with the cells.

COS-7 cells were grown in DMEM + 10% fetal calf serum and antibiotics. Cells were transfected with Effectene as described above and used 48 hours later. All cell-culture reagents were from Invitrogen.

Quantitation of spine/filopodium phenotypes and immunofluorescence
Neurons were transfected with the following combinations: GFP and CFP empty vectors; Dyn3aaa-GFP and CFP; Dyn3baa-GFP and CFP; or Dyn3baa(K44A)-GFP and CFP. The neurons were then fixed and processed as previously described (Gray et al., 2003). Once a transfected cell was identified by epifluorescence, it was imaged using a Zeiss LSM 510 confocal microscope equipped with a Zeiss 63× objective (1.4 NA) and an argon/krypton laser set to a wavelength of 428 nm to excite only the CFP fluorophore. All measurements of dendritic length, filopodium/spine length and protrusion density were completed using IPLab software (Scanalytics). Spine heads were identified when the tip of a dendritic protrusion was 2 pixels larger than the shaft. For quantitation of synaptic contacts, Dyn3aaa-GFP- or Dyn3baa-GFP-expressing cells were immunolabeled with either PSD-95 (Affinity Bioreagents) or synapsin (gift of P. McPherson, McGill University) antibodies and imaged using a cooled CCD camera (Hamamatsu Photonics). Images were prepared for quantitation using Adobe Photoshop. Blinded observers completed all quantitation. For each transfection condition, spine length, density and spine head presence were determined from 15-20 cells, analysing 4-6 mm total measured dendritic length and 3000-5000 spines/filopodia in total. Quantitation of the proportion of spines containing PSD-95 or adjacent to synapsin was completed on 15-20 cells from each transfection condition in which 1000-1200 spines/filopodia in total were examined. A spine/filopodium was scored as possessing synaptic machinery if it either contained PSD-95 clusters or was directly adjacent to synapsin. For statistical analysis, analysis of variance (ANOVA) and Fisher’s protected-least-significant-difference (PLSD) tests were completed using StatView (SAS Institute). To test for actin sensitivity, neurons were treated with 1 µM latrunculin A (LatA) (Molecular Probes) or an equivalent volume of dimethylsulfoxide (DMSO) for 18 hours before fixation and staining with rhodamine-phalloidin (Sigma). To test for the localization of active synapses, uptake assays for the styryl dye N-(3-triethylammoniumpropyl)-4-[6-(4-diethylaminophenyl)hexatrienyl] pyridinium dibromide (FM4-64) were completed at 18DIV on transfected neurons. Neurons were pre-equilibrated in Tyrode’s solution [composed of (in mM) 145 NaCl, 5.4 KCl, 1.8 CaCl2, 0.8 MgCl2, 10 Hepes and 10 glucose (pH 7.4, osmolality 310 mosm)] followed by 30 seconds of depolarization in Tyrode’s solution adjusted to 90 mM KCl. Depolarization solution contained glutamate-receptor blockers [100 µM 2-amino-5-phosphono pentanoate (AP-5) and 20 µM 6,7-dinitroquinoxaline-2,3-dione (DNQX)] and 15 µM FM4-64. Cultures were washed with regular Tyrode’s solution containing glutamate-receptor blockers for 10 minutes, fixed and imaged immediately.
et al., 1998). Neurons were cultured as described above, except that cells were grown in poly-L-lysine-coated plastic dishes instead of on coverslips. Reactions were carried out as previously described (Cao et al., 1998). Primers were designed to flank the 30-nucleotide splicing region within the mid/PH domain of Dyn3; sequences were 5′-ACGTCGCAAGCTTTCGG3′ and 5′-ACTTTCCCAGACCTTGTG3′, producing products of 370 bp (Dyn3aaa) and 400 bp (Dyn3baa), small fragments of the entire transcript. Total levels of Dyn3 mRNA were determined using primers that were previously described (Cao et al., 1998). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with primers as previously described (Gray et al., 2003). In order to visualize multiple Dyn3 splice variants, samples were separated on a 3% agarose gel and post-stained with ethidium bromide.

Direct binding assays

100-200 nM GST, Dyn3aaa or Dyn3baa was combined with 200 nM His6-tagged recombinant cortactin and glutathione-Sepharose beads (Sigma) in immunoprecipitation (IP) buffer for 1 hour at 4°C. After several washes with IP buffer, complexes were boiled and eluted in 100-200 nM GST, Dyn3aaa or Dyn3baa was combined with 200 nM His6-tagged recombinant cortactin and glutathione-Sepharose beads (Sigma) in immunoprecipitation (IP) buffer for 1 hour at 4°C. After several washes with IP buffer, complexes were boiled and eluted in

Results

Dyn3baa induces filopodial outgrowth in mature neurons

We have previously observed that the expression of Dyn3baa-GFP induced a marked outgrowth of filopodia from transfected cells and a marked decrease in the normal distribution of mushroom-shaped dendritic spines (Gray et al., 2003) (Fig. 1B). This surprising finding provided a functional correlate to support our biochemical and by dividing densitometry measurements for Dyn3baa bands by measurements for Dyn3aaa bands and averaging the fold differences over three experiments. Errors were expressed as standard error of the mean.

Theoretical modeling

Molecular models of the Dyn3baa sequence were generated to produce a theoretical visual interpretation of the insert and its possible effects on protein structure. Sequences from the region surrounding the insert of Dyn3baa and Dyn3aaa proteins (residues 484-652 or 484-642, respectively) were aligned against the sequence for the pleckstrin homology (PH) domain of Rac. Alignment was achieved using the T-Coffee multiple sequence alignment server (http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee-cgi/index.cgi) (Notredame et al., 2000; Poirot et al., 2004). Alignment sequences were submitted to the Alignment Interface of the SWISS-MODEL Automated Protein Modeling Server (http://swissmodel.expasy.org//SWISS-MODEL.html) (Guex and Peitsch, 1997; Peitsch, 1995; Schwede et al., 2003) and modeled against the known crystal coordinates of the Rac PH domain.

Coordinate files results were received by email from SWISS-MODEL and visualized using RasMac (RasMac Molecular Graphics). Although the crystal structure of the PH domain of dynamin has been solved, the Dyn3baa insert extends beyond the known crystallized sequence and therefore could not be used for molecular modeling of the Dyn3baa insert region. Alignments generated against PH domains of Grp1 and Akt also produced results indicating the insert region of Dyn3baa folds into an extended turn region.
Table 1. Quantitation of spine/filopodium morphology from transfected neurons

<table>
<thead>
<tr>
<th>Age</th>
<th>GFP</th>
<th>Dyn3aaa</th>
<th>Dyn3baa</th>
<th>Dyn3baa KA</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 DIV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean number of spines per 10 µm of dendrite</td>
<td>6.4±0.9</td>
<td>5.5±1.0</td>
<td>5.6±1.2</td>
<td>5.5±1.0</td>
</tr>
<tr>
<td>Mean overall spine length (µm)</td>
<td>1.5±0.2</td>
<td>1.8±0.3</td>
<td>2.0±0.2</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>Number of spines ≥2 µm long per 100 µm of dendrite</td>
<td>13.5±4.3</td>
<td>16.9±4.7</td>
<td>20.3±5.0</td>
<td>6.1±2.6</td>
</tr>
<tr>
<td>Proportion of spines with heads (%)</td>
<td>14.9±4.2</td>
<td>21.4±8.5</td>
<td>13.0±4.2</td>
<td>55.3±8.7</td>
</tr>
<tr>
<td>18 DIV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean number of spines per 10 µm of dendrite</td>
<td>6.0±0.7</td>
<td>5.7±0.8</td>
<td>8.9±1.4</td>
<td>5.3±1.3</td>
</tr>
<tr>
<td>Mean overall spine length (µm)</td>
<td>0.9±0.1</td>
<td>1.1±0.1</td>
<td>1.4±0.1</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>Number of spines ≥2 µm long per 100 µm of dendrite</td>
<td>2.5±2.1</td>
<td>2.6±1.9</td>
<td>16.6±4.1</td>
<td>5.5±2.4</td>
</tr>
<tr>
<td>Proportion of spines with heads (%)</td>
<td>76.9±10.3</td>
<td>77.6±9.5</td>
<td>29.1±6.8</td>
<td>69.3±9.8</td>
</tr>
</tbody>
</table>

All values are expressed as the mean±s.d.

Bold numbers indicate a significant statistical difference (P<0.001) between that transfection condition and all other transfection conditions within each specific measurement. ANOVAs followed by Fisher’s PLSD test were performed for each measurement.
any transfection conditions (Fig. 3E,F). Conversely, at 18DIV, 75-80% of protrusions from cells transfected with GFP or Dyn3aaa-GFP were associated with synaptic markers (Fig. 3E,F). At 18DIV, Dyn3aaa-GFP was found in spine heads, where it was juxtaposed to the presynaptic sites (labeled with synapsin) of untransfected neurons (Fig. 3A, arrows) and localized with PSD-95 (Fig. 3C, arrows). These synaptic puncta were also determined to be active synapses by using the styryl dye FM4-64 to label sites of rapid endocytosis following high K+ depolarization, and most spines from these older cultures were juxtaposed to FM4-64-positive puncta (see Fig. S2 in supplementary material). At 18DIV, dendritic protrusions found on neurons transfected with Dyn3baa-GFP were rarely juxtaposed to synapsin-positive presynaptic terminals (Fig. 3B, arrows) and most did not contain PSD-95 clusters (Fig. 3D, arrows). Our quantitation confirmed these trends, with filopodia/spines from 18DIV Dyn3baa-GFP-expressing neurons exhibiting a similar association with synaptic markers as 11DIV neurons (Fig. 3E,F). Also, filopodia from Dyn3baa-GFP neurons did not associate with FM4-64-positive puncta, although some dye accumulation was observed along the dendritic shafts of Dyn3baa-expressing neurons (see Fig. S2 in supplementary material). Taken together, these morphological observations indicated that synaptogenesis onto dendritic protrusions is significantly reduced in neurons expressing Dyn3baa-GFP.

Fig. 2. Dyn3baa inhibits the development of normal neuronal morphology. Neuron morphology was quantified using two morphological criteria to distinguish between filopodia and spines. 18DIV Dyn3baa-expressing cells remained similar to 11DIV cultures with respect to protrusion length (A) and presence of a spine head (B), indicating that filopodia at 11DIV do not mature into spines by 18DIV in these neurons. By contrast, control neurons expressing Dyn3aaa-GFP or GFP exhibit this normal progression during development from filopodia to spines. Expression of the GTPase-deficient Dyn3baaKA-GFP did not affect the morphology of transfected neurons at 18DIV but did cause immature (11DIV) transfected neurons to contain morphologically mature spines. All error bars represent s.d.

Fig. 3. Synaptogenesis is impaired in Dyn3baa-expressing neurons. Neurons transfected with Dyn3aaa-GFP (A, C) or Dyn3baa-GFP (B, D) at 18DIV were labeled for the presynaptic marker synapsin (A, B) or the postsynaptic marker PSD-95 (C, D). Dyn3aaa-GFP was found to be directly juxtaposed to synapsin-positive puncta (arrows in A) and colocalized with PSD-95 (arrows in C). Dyn3baa-GFP and dendritic filopodia were neither directly juxtaposed to synapsin-positive puncta (arrows in B) nor colocalized with PSD-95 (arrows in D). (E, F) Quantitation of association between dendritic protrusions and synaptic markers revealed that control neurons exhibited little association with synaptic markers at 11DIV but extensive association at 18DIV. By contrast, neurons transfected with Dyn3baa-GFP exhibited impaired dendritic spine synaptogenesis, with 20% of filopodia containing PSD-95 (E) and less than 10% of filopodia juxtaposed to synapsin (F) at 18DIV. Neurons expressing a GTPase-deficient mutant Dyn3baa-GFP (KA mutant) did not exhibit this maturation defect and progressed normally through synaptogenesis with culture age. All error bars represent s.d. Scale bar, 5 µm.
Dyn3baa induces filopodia in a heterologous system

To test whether Dyn3baa-induced filopodium formation was a conserved function and not an expression artefact specifically in neurons, we expressed Dyn3baa-GFP in COS-7 cells, a cell type that is normally disoidal in shape, does not abundantly produce filopodia and does not endogenously express Dyn3. COS-7 cells expressing Dyn3aaa-GFP maintained a characteristically smooth plasma-membrane perimeter, lacking numerous filopodia (Fig. 4A,A'). By dramatic contrast, cells expressing Dyn3baa-GFP were uniquely shaped, with numerous small membranous protrusions and rows of filopodia (Fig. 4B,B'). Nearly all transfected cells lost their normal smooth, rounded appearance and adopted a more spindly and spiky shape. Based on these data in COS-7 cells, this filopodium-inducing function for Dyn3baa is not peculiar to neurons, with Dyn3baa seeming to perform an important role in the machinery that produces and maintains filopodia in cells.

Dyn3baa induces filopodia in non-neuronal cells

Fig. 4. Dyn3baa-GFP induces filopodial outgrowth in non-neuronal cells. COS-7 cells transfected with Dyn3aaa-GFP (A) maintained a normal morphology, with a smooth lamellar perimeter (arrows in A, close-up in A') and a rounded shape. By contrast, COS-7 cells transfected with Dyn3baa-GFP exhibited radical shape changes (B), with the smooth perimeter often replaced with numerous large membranous protrusions and long filopodia that were remarkably similar to the filopodia in neurons (arrows in B, close-up in B'). Transfection with Dyn3baa(KA)-GFP caused a phenotype resembling Dyn3aaa-GFP transfection, causing cells to maintain their normal smooth perimeter (C). Interestingly, mutant Dyn3baa remained at the membrane perimeter (arrows in C, close-up in C') but did not induce the shape changes observed with Dyn3baa wild-type transfection. Scale bar, 10 µm.

Dyn3baa GTPase activity is required for filopodium formation

To test whether the enzymatic activity of Dyn3baa was necessary for the induction and/or maintenance of dendritic filopodia, a GTPase-deficient Dyn3baa was constructed containing a mutation (K44A) in the highly conserved GTPase region of the protein (Damke et al., 1994). Surprisingly, Dyn3baa(K44A)-GFP overexpression produced a normal morphological phenotype in neurons at 18DIV, with these cells displaying short, mushroom-shaped spines (Fig. 2A,B, Table 1). Interestingly, based on our quantitation at 11DIV, neurons transfected with the GTPase-defective Dyn3baa(K44A)-GFP developed mature morphological characteristics at this earlier time, making these 11DIV neurons more similar to mature 18DIV neurons, the opposite effect to wild-type-Dyn3baa/GFP expression. Although this morphological difference at 11DIV was observed in cells expressing the Dyn3baa mutant, no effect on spine/filopodium synaptogenesis was observed (Fig. 3E,F).

 Taken together, these results suggest that GTPase-deficient Dyn3baa might actually enhance or facilitate the morphological development of dendritic spines but has no effect on spine synapse assembly.

In parallel studies with cultured epithelial cells, GTPase-dead Dyn3baa did not induce filopodia in this heterologous system (Fig. 4C,C'), consistent with the notion that the wild-type enzymatic activity is important during the reorganization of the cell periphery. Interestingly, although filopodia were not induced in these Dyn3baa-mutant-expressing cells, the exogenous protein was recruited to the membrane perimeter in putative lamellipodia (Fig. 4C, arrows), even incorporating into what appeared to be membrane ruffles. By contrast, Dyn3aaa-GFP seldom extended all the way out to the plasma membrane of the COS-7 cells (Fig. 4A'). This suggests that, in both epithelial and neuronal cell types, the targeting of Dyn3baa does not require enzymatic activity but the formation of discrete filopodia does.

Dyn3 spliced variants bind differently to cortactin

Previously, the PRD of Dyn2 was found to bind to the SH3 domain of the actin-binding protein cortactin (McNiven et al., 2000). Interestingly, the region of the Dyn2 PRD that mediates this interaction is nearly identical in Dyn3 (Fig. 5A). To test whether Dyn3 and cortactin also bind, we performed immunoprecipitation experiments using Dyn3-GST and His6-cortactin. Although GST alone did not pull down cortactin, both Dyn3 splicing forms isolated cortactin, confirming a direct interaction between these two proteins (Fig. 5B). This novel interaction between these two proteins (Fig. 5B). This novel link to the actin cytoskeleton for Dyn3 is interesting considering the actin dependence of the Dyn3baa-induced filopodia and the fact that cortactin is a prominent component of the post-synapse (Hering and Sheng, 2003; Naisbitt et al., 1999).

Because these initial cortactin-Dyn3 immunoprecipitation-based binding experiments were largely qualitative and we wanted to gain insight into how this direct interaction might contribute to spine formation, we next tested for different binding affinities for the two Dyn3 spliceoforms to cortactin using quantitative blot-overlay experiments. In each case, 3-500 ng of each Dyn3 spliced variant was spotted onto membranes followed by an overlay with an excess of recombinant cortactin. The cortactin was detected with antibodies, and binding was quantified using a densitometer (Fig. 5C). A control experiment using antibodies against Dyn3 was performed and quantified by densitometry to assure even spotting of dynamin protein (data not shown). Signals were normalized to the maximum intensity so that comparisons could be made across experiments. Binding curves were generated from the densitometry data (Fig. 5D) and the steeper slope and higher maximum intensity of the Dyn3baa curve
demonstrated its stronger affinity for cortactin than that of Dyn3aaa. Differences in binding were also calculated, showing at least a twofold increase in cortactin binding for Dyn3baa over Dyn3aaa at all tested concentrations (Fig. 5E). The larger binding variations at lower cortactin concentrations are most likely due to the spotting of small protein quantities and film inconsistencies. The difference in cortactin affinity between Dyn3aaa and Dyn3baa was significant ($P < 0.05$ for 300 ng and 500 ng) and reflected three separate experiments. Thus, although the PRDs of both Dyn3aaa and Dyn3baa are identical, the insert present in Dyn3baa is likely to impose certain structural modifications on the rest of the C-terminal region of the protein, which results in a markedly increased affinity for cortactin.

 Dyn3baa-cortactin interactions are needed to maintain dendritic filopodia

Cortactin has been previously shown to localize to dendritic spines (Hering and Sheng, 2003; Naisbitt et al., 1999), a finding we have confirmed using antibodies and exogenous cortactin-RFP expression (see Fig. S3 in supplementary material). In addition, we have previously demonstrated that disrupting the Dyn2-cortactin interaction through the expression of C-terminal deletion constructs results in the alteration of actin organization (McNiven et al., 2000). To test the physiological relevance of the Dyn3-cortactin interaction with respect to spine morphology, C-terminal truncation mutant constructs of the Dyn3 spliced forms (Dyn3 $\Delta$PRD) were engineered and expressed in cultured neurons (Fig. 6A). Neurons expressing Dyn3aaa $\Delta$PRD developed normally, with an extensive distribution of mushroom-shaped dendritic spines by 18DIV (Fig. 6B). These spines appeared to assemble synapses normally, based on the clustering of synapsin and PSD-95 (data not shown). Overall, the Dyn3aaa $\Delta$PRD signal was more diffuse, with fewer Dyn3aaa-GFP puncta than were observed for the wild-type GFP-tagged protein. By contrast, expression of the mutant Dyn3baa $\Delta$PRD did not induce filopodia but rather allowed the normal maturation program to proceed, with spines becoming the predominant dendritic protrusion by 18DIV (Fig. 6C). These spines assembled synapses normally, because both presynaptic and postsynaptic markers were observed at the tips of spines in Dyn3baa $\Delta$PRD-transfected neurons (data not shown). This was in marked contrast to mature neurons expressing wild-type Dyn3baa, which failed to exhibit clusters of PSD-95 at dendritic protrusions, indicating a reduction in neuronal synaptogenesis (Fig. 3B,D). These data suggest that the PRD of Dyn3baa is necessary for targeting this spliced variant to the appropriate machinery to modulate spinogenesis, potentially by binding to cortactin.

In reciprocal experiments designed to disrupt the Dyn3-cortactin interaction, neurons were co-transfected with a mutant cortactin missing the C-terminal SH3 domain (Cort $\Delta$SH3) (Fig. 6D) and either wild-type Dyn3aaa or Dyn3baa. Initially, we tested whether Cort $\Delta$SH3 affected neuronal development independent of Dyn3 expression. Synaptogenesis appeared to be unaffected by this mutant protein and it also continued to localize to dendritic spines, suggesting that the SH3 domain is not necessary for cortactin targeting (data not shown), a finding previously described by others (Hering and Sheng, 2003). There were no observed morphological changes in neurons expressing Dyn3aaa-GFP and Cort $\Delta$SH3, with neurons exhibiting normal dendritic spines at 18DIV (Fig. 6E). By contrast, neurons expressing...
both Dyn3baa-GFP and CortΔSH3 no longer displayed numerous filopodia at 18DIV (Fig. 6F), suggesting that the Dyn3baa-induced increase in filopodia is dependent upon binding to cortactin. Interestingly, although the immature filopodia normally induced by Dyn3baa were lost, Dyn3baa-GFP still trafficked to dendritic spines in the presence of CortΔSH3. This suggests that Dyn3baa can target to dendritic protrusions independent of cortactin, but, in order to function during filopodial induction, it must be recruited to actin filaments by cortactin.

Dyn3baa transcription is enhanced selectively during neuronal spinogenesis in vitro and in situ

The data described above suggest that increased levels of Dyn3baa might stimulate the production of filopodia on neurons (an event that normally occurs during development) but also maintains these filopodia in older neurons when filopodia are usually replaced by dendritic spines. Therefore, in transfected neurons, a sustained increase in Dyn3baa expression during maturation could cause a filopodium-dominated immature stage to persist, blocking the maturation of filopodia into spines. To explore the possibility that the neuron regulates the relative levels of endogenous Dyn3 spliceoforms, RNA was collected from cultured neurons at various stages of development for use in RT-PCR experiments.

Dyn3aaa mRNA levels were relatively stable between 1DIV and 21DIV, with a slight decrease in transcript copy number during synaptogenesis (7-14DIV) that was recovered by 21DIV (Fig. 7A). By contrast, Dyn3baa was barely detectable at the earlier stages of neuronal development, when neurites were forming and general polarity is established, but was substantially upregulated between 7DIV and 14DIV (Fig. 7A), the general timeframe for filopodium generation, synaptogenesis and a filopodium-to-spine transition (Jontes and Smith, 2000). By 14-21DIV, the ratio between the Dyn3aaa and Dyn3baa messages was roughly 1:1 but, in adult rat brains, there was a severalfold increase in the levels of Dyn3aaa over Dyn3baa. This suggested that, in the adult, either Dyn3aaa is upregulated with respect to Dyn3baa or the production of Dyn3baa is decreased with respect to Dyn3aaa after the critical time of synaptogenesis and filopodium/spine formation, possibly attenuating filopodium production in the adult neuron through a dilution of the Dyn3baa spliceoforms by the Dyn3aaa spliceoforms. To demonstrate equal transcript-copy-number loading in each PCR reaction, primers recognizing
GAPDH were also used as a control (Fig. 7A). As an additional control, we also used primers to amplify a fragment of Dyn3 that is present in all known spliced variants (Cao et al., 1998). In this reaction, there was little difference in overall dynamin levels after 1DIV (Fig. 7C), suggesting that the tight regulation observed for Dyn3baa might be unique to this spliceoform and not a global phenomenon for other spliced variants.

A third band was observed in most of our reactions using these primers. In order to assess whether or not this band represented an additional Dyn3 spliced variant, we attempted to clone this fragment using a range of strategies or to amplify additional spliced variants using this band as template. In all cases, a unique fragment was not isolated nor amplified but, rather, Dyn3aaa and Dyn3baa fragments were consistently retrieved (data not shown). This led us to believe that the higher band at 430 bp is an artefact of the RT-PCR, with a heteroduplex forming between one strand from each of Dyn3aaa and Dyn3baa. This technical artefact has been well documented in other studies using RT-PCR to examine spliced variants (Zacharias et al., 1994).

To test whether the altered expression of the Dyn3baa form occurs in neurons in situ (in addition to in vitro), we isolated the hippocampus from rats at various stages of development and completed identical experiments to those described above. Interestingly, similar patterns of Dyn3 spliceoform expression were observed from whole hippocampus, with Dyn3aaa levels appearing to be unchanged throughout development, whereas Dyn3baa was dramatically upregulated at P4, the equivalent time as was observed in culture (Fig. 7B). Again, GAPDH levels were assessed at each time point, demonstrating an equal loading of transcript copy numbers for each reaction (Fig. 7B). Dyn3baa levels were maintained at an elevated level through P30 compared with Dyn3aaa but, again, in the adult, Dyn3aaa was the predominant spliceoform. As with the cultures, we also amplified all Dyn3 spliced variants and again saw little difference in overall Dyn3 levels after embryonic day 15, suggesting that the tight regulation of Dyn3baa might be unique to this spliceoform (Fig. 7D). In conclusion, there is a striking correlation between levels of endogenous Dyn3baa expression and filopodial growth in both cultured neurons and intact hippocampi that is consistent with a specific role for the Dyn3baa spliceoform in dendritic spine morphogenesis.

Discussion

In this study, we demonstrate a new and important function for the dynamin family of proteins, specifically a role for a distinct Dyn3 spliceoform (Dyn3baa) in the regulation of dendritic spine development and filopodium induction. When expressed in developing hippocampal neurons in culture, Dyn3baa localizes to and induces the formation of long filopodium-like immature spines. Exogenous expression of this Dyn3 form in epithelial cells also leads to the formation of numerous filopodia. Interestingly, a mutant form of this dynamin variant Dyn3baa(K44A) not only prevents filopodium formation in mature neurons and epithelial cells but also inhibits the normal formation of these structures in immature neurons. Instead, 11DIV neurons expressing Dyn3baa(K44A) produce morphologically mature spines, a characteristic of older (18-21DIV) neurons. Importantly, Dyn3baa binds with a 200% greater affinity to cortactin than does Dyn3aaa, an interaction mediated by the tails of these proteins. When the tail-to-tail interaction between Dyn3baa and cortactin is perturbed through the expression of truncation mutants, Dyn3baa can no longer mediate and sustain filopodial outgrowth in mature neurons. Last, although Dyn3aaa is constitutively expressed in neurons, a marked upregulation of Dyn3baa mRNA was observed in both cultured cells and whole hippocampi during the period of rapid synaptogenesis and spino genesis, suggesting that the temporal expression of Dyn3 spliceoforms plays an important role during development. These results represent the first demonstration of a cooperative role for Dyn3 and cortactin in neuronal morphological remodeling, as well as additional evidence implicating the dynamin family of large GTPases in actin-dependent processes.

Dyn3, filopodia and actin

It has become clear that actin dynamics are important during the development and function of dendritic spines (Matus, 2000). Actin filaments are organized into a meshwork within both the head and spine neck, often with parallel actin bundles in the spine neck extending the barbed ends toward the spine head, with an extensive branching of those filaments within the head (Hirokawa, 1989; Landis and Reese, 1983). In addition, the actin cytoskeleton within the spines intimately associates with lipid microdomains, modulating the stability of the associating filaments (Itoh et al., 2002). This actin-membrane interplay was shown to be significant, because depletion of lipid rafts in cultured hippocampal neurons led to an overall decrease in spine density, implicating the membrane-cytoskeleton interaction as an integral modulator of spine development (Hering et al., 2003).

Despite these advances, few studies have identified protein links between actin and membranes during neuronal development. The dynamin family of GTPases is an attractive candidate as this link, because the association between dynamin and the membrane is well established and the number of studies implicating an interplay between dynamin and the actin cytoskeleton is steadily expanding (Orth and McNiven, 2003). Several groups have demonstrated a role for dynamin in the propulsion of vesicle comets (Lee and De Camilli, 2002; Orth et al., 2002) and in actin polymerization directly (Schafer et al., 2002). In previous work, enhanced expression of numerous actin-associated proteins, including debrin (Hayashi and Shirao, 1999), spinophilin/neurabin (Feng et al., 2000) and SPAR (Pak et al., 2001), have led to morphological changes in spines and filopodia. These studies, in conjunction with our own, underline the importance of the actin cytoskeleton and the proteins that modify it during normal filopodium and spine development.

An interesting finding of this study is the fact that two closely related Dyn3 forms mediate distinct spine morphologies when expressed in neurons. Alternative splicing in the dynamin family of mechanoenzymes is extremely important in determining the functional or localization behavior of each particular dynamin isoform. Dyn2 currently has four known spliced variants, with one particular spliceoform enriching at the Golgi and clathrin-coated pits, whereas another maintains a more cortical localization, associating with many endocytic structures (Cao et al., 1998). These two forms differ by only four amino acids, which reside...
Fig. 8. Dyn3 interacts with PSD proteins to regulate dendritic spine maturation. (A) During synaptogenesis and early development, enhancing the expression of one of two PSD binding partners can lead to opposite dendritic spine phenotypes. Expression of Homer causes a supermaturation of spines, with these protrusions becoming larger and fully functional at an immature stage [11DIV (Sala et al., 2001)]. Conversely, expression of Dyn3baa inhibits the neuron from maturing and assembling spine synapses, leaving the cell in an immature state into the later stages of development (17DIV). An overabundance of another Dyn3 spliced variant (Dyn3aaa) or the GTPase-deficient Dyn3baa (Dyn3baaKA) has little effect on the normal spine developmental program; filopodia are eventually replaced by mushroom-shaped spines. (B,C) Theoretical modeling of Dyn3aaa (B) and Dyn3baa (C) of 100 amino acids around the PH domain based on the solved structure of Rac. Proline and charged residues in the insert [red in Dyn3aaa (C)] might give rise to a protruding turned region extending away from the compact structure of the PH domain, as seen for Dyn3aaa in (B).

at precisely the same location in the molecule as the ten-amino-acid insert that differentiates Dyn3aaa and Dyn3baa. In addition, only particular spliceoforms of Dyn2 are involved in the modulation and maintenance of the centrosome (Thompson et al., 2004), another example of splicing leading to functional differences. Thus, important information for targeting and function are contained in these spliced areas, regardless of the identity between other domains between different spliceoforms.

In these studies, we provide additional evidence demonstrating a biochemical difference in binding affinity between Dyn3aaa and Dyn3baa (Fig. 5C), possibly owing to a conformational change in the two proteins induced by the ten-amino-acid insert, as our modeling suggests, despite the identity between the PRDs of Dyn3aaa and Dyn3baa. This slight modification in primary structure could induce a variation in the Dyn3baa three-dimensional conformation, optimally exposing surface epitopes and protein-protein interaction sites within the PRD. Because cortactin binds dynamin through an SH3-PRD interaction, folding differences that alter accessibility to the PRD might alter binding affinity. Molecular modeling using SWISS-MODEL of 100 amino acids around the PH domain of Dyn3, including the ten-amino-acid insert of Dyn3baa, was performed based on the solved crystal structure of the Rac PH domain (Hirschberg et al., 1997). The RasMac program allowed visualization of the theoretical structural differences caused by the ten-amino-acid insert in the Dyn3baa spliceoform (Fig. 8B,C). The modeling suggests that the combination of prolines and charged residues might promote the formation of a pronounced turn region extended away from the compact structure adjacent to the PH domain.

Based on three-dimensional cryoelectron micrograph reconstructions of dynamin crystals (Zhang and Hinshaw, 2001), it is possible that any structural changes to the region around the PH domain could alter the interactions between multiple molecules of the ‘stalk’ region of dynamin oligomers and affect the way in which the PRD tail protrudes from the polymer. This, in turn, might affect binding properties with cortactin. We intend to explore this possibility using structural studies.

Dyn3, cortactin and spine formation

The pronounced interaction between the specific Dyn3 spliceoform (Dyn3baa) and cortactin defined above by in vitro binding experiments are consistent with the in vivo studies of Fig. 6, demonstrating that an immature neuronal phenotype in mature neurons is induced upon the disruption of the complex through expression of either Dyn3baaΔPRD or CortΔSH3.

Cortactin was originally identified as a cytoskeleton-interacting protein and substrate of Src (Wu and Parsons, 1993) and was recently linked to endocytosis (Cao et al., 2003). The cortactin protein bundles actin when it is dephosphorylated but, upon phosphorylation by Src, it exhibits reduced actin-cross-linking abilities (Huang et al., 1997). More recently, studies have demonstrated that cortactin might also function in cell migration owing to its dynamic and responsive relocalization to the cortex and actin-rich ruffles upon stimulation with growth factors (Krueger et al., 2003; McNiven et al., 2000). These examples emphasize the importance of cortactin-modulated actin dynamics, a concept that has since been applied to the neuronal actin cytoskeleton. Indeed, cortactin has been observed in the tips of ruffling growth cones, along with both CortBP1 (Du et al., 1998) and dynamin (N.W.G. and M.A.M., unpublished). In addition, cortactin localizes to dendritic spines, where it can interact with the scaffolding molecule Shank (Hering and Sheng, 2003; Naisbitt et al., 1999). This was a significant discovery, because it provides a link between the complex PSD network and the actin cytoskeleton, presumably the anchor for this receptor-containing complex. Our data demonstrating a role for Dyn3baa and cortactin in the morphological regulation of dendritic spines are consistent with this prediction, because the overexpression of Dyn3baa could change the properties of either the actin within the dendritic protrusion or the cortactin bundles themselves, and diminish the ability of these cytoskeletal structures to bind to the PSD complex.

Interestingly, both the GTPase activity of Dyn3baa and the PRD domain are necessary to initiate the production of filopodia that eventually develop into mature spines. Overexpression of Dyn3baa results in filopodia being abundantly present at 18DIV when spines are usually the...
predominant dendritic structure (Fig. 1). When mutant Dyn3baa lacking the PRD is overexpressed, there is no phenotype, suggesting that this protein is rendered nonfunctional and irrelevant from the standpoint of spine homeostasis (owing to a lack of proper targeting) and that the neuron can thus develop normally. Improper targeting induced by the removal of the dynamin PRD has been exploited in a number of studies to demonstrate a loss-of-function for dynamin (Orth et al., 2002). When Dyn3baa(K44A) (lacking GTPase activity) is expressed, there is no ‘filopodial phenotype’ (i.e. normal spines develop). Interestingly, with the expression of Dyn3baa(K44A), there is in fact a reduction in overall filopodial density, with more mature protrusions present instead. This suggests that Dyn3baa does indeed perform a direct role in dendritic filopodial growth because, when the GTPase-dead mutant is expressed, it acts as a dominant-negative mutant, incorporating into Dyn3baa-containing tetramers and disrupting the functionality of the complex. Without functional Dyn3baa tetramers, the modulation of dendritic protrusion growth is shifted away from actin-rich filopodia, thus causing the appearance of mature-looking protrusions at an earlier age. With dynamin classified as a mechanoenzyme, it follows that GTPase activity can cause a conformational shift in the structure of the dynamin oligomeric complex, a necessary task to sever vesicles during endocytosis (Danino et al., 2004). This same conformational shift could change the way that dynamin interacts with other proteins or performs tasks related to actin regulation through these interacting proteins (like cortactin). Precedence for this comes from studies demonstrating a reduction of actin-based vescle motility in cells expressing mutant Dyn2 (Lee and De Camilli, 2002; Orth et al., 2002), a reduction of in vivo actin dynamics at the periphery of motile cells (Schafer et al., 2002) and a reduction of actin turnover at podosomes (Ochoa et al., 2000). Thus, links between the GTPase activity of dynamin isoforms, the consequences of GTPase-deficient mutants on actin-based structures (such as dendritic filopodia and spines) and actin binding proteins (like cortactin) are well established in the literature, leading to the conclusions in this report.

Transcriptional regulation of Dyn3 spliceoforms

To explore the mechanisms mediating the filopodial induction observed by the overexpression of Dyn3baa, we first examined the expression of Dyn3 spliceoform mRNA to determine whether there was a difference in expression between Dyn3aaa and Dyn3baa as neurons matured. A hypothesis to explain our original results assumed that the constitutive increase in Dyn3aaa transcript production in adulthood, when Dyn3baa(K44A) (lacking GTPase activity) is expressed, could reduce filopodium formation. The persistent overexpression of exogenous Dyn3baa might disrupt this delicate balance of Dyn3 spliceoform control, producing the observed phenotypes in this study. Because these changes in spliceoform transcript levels are observed not only in cultured neurons, but also from whole hippocampus, it is likely that a regulation of Dyn3 splicing plays an important role during in vivo synaptogenesis.

This study provides novel evidence indicating a functional relevance for alternative splicing within the Dyn3 transcript and subsequent implications during synaptogenesis. With 13 putative Dyn3 spliceoforms (Cao et al., 1998), many undiscovered functional possibilities remain for Dyn3 in the neuron.

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