N- and C-Terminal Domains of β -Catenin, Respectively, Are Required to Initiate and Shape Axon Arbors of Retinal Ganglion Cells *In Vivo*

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We used deletion mutants to study β -catenin function in axon arborization of retinal ganglion cells (RGCs) in live *Xenopus laevis* tadpoles. A deletion mutant β cat Δ ARM consists of the N- and C-terminal domains of wild-type β -catenin that contain, respectively, α -catenin and postsynaptic density-95 (PSD-95)/discs large (Dlg)/zona occludens-1 (ZO-1) (PDZ) binding sites but lacks the central armadillo repeat region that binds cadherins and other proteins. Expression of Δ ARM in RGCs of live tadpoles perturbed axon arborization in two distinct ways: some RGC axons did not form arbors, whereas the remaining RGC axons formed arbors with abnormally long and tangled branches. Expression of the N- and C-terminal domains of β -catenin separately in RGCs resulted in segregation of these two phenotypes. The axons of RGCs overexpressing the N-terminal domain of β -catenin developed no or very few branches, whereas axons of RGCs overexpressing the C-terminal domain of β -catenin formed arbors with long, tangled branches. Additional analysis revealed that the axons of RGCs that did not form arbors after overexpression of Δ ARM or the N-terminal domain of β -catenin were frequently mistargeted within the tectum. These results suggest that interactions of the N-terminal domain of β -catenin with α -catenin and of the C-terminal domain with PDZ domain-containing proteins are required, respectively, to initiate and shape axon arbors of RGCs *in vivo*.

Key words: β -catenin; α -catenin; PDZ proteins; lipofection; axon arborization; axon branching; retinal ganglion cells; *Xenopus laevis*

Introduction

In many types of neurons, axons branch or arborize when they reach their target tissue. Axon arborization is spatiotemporally correlated with neuron–target interactions (Sakaguchi and Murphy, 1985; Yates et al., 2001) and with synapse formation and function (Cantalupo et al., 2000; Alsins et al., 2001), but in contrast to neuron–target interactions and synaptogenesis, we know very little about the molecules that control axon arborization, with a few notable exceptions (Cohen-Cory and Fraser, 1995; Wang et al., 1999; Zou and Cline, 1999).

β -catenin participates in several molecular interactions that could regulate axon arborization. First, β -catenin links the cadherin family of cell adhesion molecules to α -catenin and the cytoskeleton, thereby strengthening adhesion (for review, see Ivanov et al., 2001; Schneider et al., 2003). N-cadherin, β -catenin, and α -catenin are expressed in axons (Murphy-Erdosh, 1994; Riehl et al., 1996; Benson and Tanaka, 1998) and at synapses (Uchida et al., 1996; Benson and Tanaka, 1998; Miskevich et al., 1998). N-cadherin is required for normal axonal targeting and arborization (Inoue and Sanes, 1997; Lee et al., 2001), as well as for normal synapse formation and function (Tang et al., 1998;

Togashi et al., 2002). We do not know currently whether β -catenin is also required for these processes. At the synapse, β -catenin interacts with at least two postsynaptic density-95 (PSD-95)/discs large (Dlg)/zona occludens-1 (ZO-1) (PDZ) proteins, Lin-7/Velis and synaptic scaffolding molecule (S-SCAM) (Perego et al., 2001; Nishimura et al., 2002). Together with cadherins, these proteins recruit and localize proteins at synaptic junctions (Perego et al., 2001; Nishimura et al., 2002). Here we use deletion mutants to examine how disrupting the interactions mediated by the N- and C-terminal domains of β -catenin, which interact, respectively, with α -catenin and synaptic PDZ proteins, specifically affects stages in axonal arborization.

We have studied β -catenin function in axonal arborization of RGCs in tecta of live *Xenopus* tadpoles. An advantage of this system is that the effects of β -catenin on arborization of single axons *in vivo* can be examined over several days (O'Rourke and Fraser, 1990; Cohen-Cory and Fraser, 1995; Zou and Cline, 1999). We first show using *Xenopus* brain sections that β -catenin is expressed in RGC axon arbors. We then express deletion mutants of β -catenin in RGCs and examine their effects on the formation of axonal arbors during development *in vivo*. These experiments suggest that interactions mediated by the N- and C-terminal domains of β -catenin, which contain α -catenin and PDZ interaction sites, respectively, initiate and shape the axonal arbors of RGCs *in vivo*.

Materials and Methods

Xenopus laevis tadpoles. *Xenopus laevis* embryos were generated by *in vitro* fertilization of eggs obtained from females primed with human

Received April 14, 2003; revised May 23, 2003; accepted May 23, 2003.

This work was supported by National Research Service Award F32MH 12613 to T.M.E. and by the Howard Hughes Medical Institute (HHMI). L.F.R. is an investigator of the HHMI. We thank members of our laboratory, especially S. Bamji, L. Elia, and B. Rico, for comments on this manuscript.

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chorionic gonadotrophin. Embryos were cultured in a 10% modified Ringer's solution (MMR) and staged according to Nieuwkoop and Faber (1956); 0.001% phenylthiocarbamide was added to the Ringer's solution to reduce pigmentation (Cohen-Cory and Fraser, 1995).

Antibody staining. Brains with and without green fluorescent protein (GFP)-expressing retinal ganglion cell (RGC) axon arbors were dissected from stage 45/46 tadpoles. Tadpoles were anesthetized in a 0.02% benzocaine solution before dissection. The dissected brains were fixed overnight at 4°C in a freshly made 4% paraformaldehyde solution and sunk in sucrose over 2 nights (15 and 30% sucrose in phosphate buffer). The brains were then embedded in an optimal cutting temperature compound mold, cryostat sectioned into 30- μ m-thick horizontal sections, and mounted onto glass slides. Antibody staining was performed as follows. Sections were rinsed in PBS for 5 min, blocked with normal goat serum [5% in PBS plus 0.1% Triton X-100 (PBST)] for 30 min at room temperature, and incubated overnight at 4°C with a polyclonal rabbit anti- β -catenin antibody generated against *Xenopus* β -catenin (1:500 in PBST) (Kypta et al., 1996). Sections were then rinsed in PBS three times for 8 min and incubated with a Texas Red-tagged goat anti-rabbit secondary antibody (1:200 in PBST) (Molecular Probes, OR) for 1 hr at room temperature. Control sections underwent the same procedure but were incubated with only the Texas Red secondary antibody.

To determine whether β -catenin staining colocalized with GFP axon arbors, we followed a procedure similar to the one described previously by Pinches and Cline (1998). Antibody-stained tectal sections (30 μ m) containing a single GFP arbor were imaged with a confocal microscope (Bio-Rad 600) using a 100 $\times$ objective [Zeiss Plan Apochromat; numerical aperture (NA) = 1.40] and a z-series protocol (z-interval 0.5–1.0 μ m). We then opened each optic slice individually in NIH image (Version 1.62) and traced the portion of the GFP arbor in that optic slice. We noted all yellow β -catenin puncta that colocalized with the GFP arbor in that particular optic slice. Two criteria were used to define a yellow spot as a countable puncta. First, in the image containing the green (GFP)/red (anti- β -catenin) overlay, we counted only yellow spots of >1 μ m diameter. Second, we counted only those spots with intensity in the red channel that was twofold greater than that of the average intensity in the neuropil region of the corresponding control section (stained only with a secondary antibody). All of the individual tracings for a single tectal section were combined into one image, and thus the arbor and corresponding β -catenin staining were reconstructed. In all cases, this constituted a partial reconstruction of the total arbor, comprising only that portion of arbor present in the 15 μ m of the tectal section that had optimal antibody penetration. In each of these reconstructed GFP arbors, we counted the total number of branches, measured the length of each of the branches, and counted the number of β -catenin puncta in each of the branches. From these data, we calculated the average number of β -catenin puncta per 10 μ m of branch length. To confirm the specificity of overlap of red β -catenin puncta with the green GFP arbor branches, we mismatched the red and green images along the y-axis and then calculated the number of yellow puncta in the mismatched images. On average, mismatched images contained 33% of the number of yellow puncta as did the correctly matched images.

Lipofection. To transfect RGCs with DNA constructs, we used the lipofection protocol first developed for studying axon outgrowth by *Xenopus* RGCs (Holt et al., 1990; Riehl et al., 1996). Briefly, 24 hr after fertilization, at stages 20–23, we manually removed vitelline envelopes from embryos and transferred the embryos to a high salt solution (1 $\times$ MMR). We back filled micropipettes with a mixture of DNA and DOTAP lipofection reagent (at a ratio of 1:3) and pressure injected 50–200 nl of the DNA–DOTAP solution into eye bud primordia. We then transferred embryos to low-salt 0.1 $\times$ MMR containing 0.001% phenylthiocarbamide to inhibit pigmentation. Embryos were raised in the dark at 23°C for 4 d (until stage 45/46). Expression of proteins has been shown to peak 4 d after lipofection (Holt et al., 1990). This corresponds to stage 45/46, by which time RGC axons have reached the tectum and are forming arbors.

Arbor imaging. At stage 45/46, the heads of tadpoles are transparent, so that GFP-expressing axon arbors can be imaged in live animals using fluorescent illumination. For screening and confocal imaging of arbors, we anesthetized tadpoles in a 0.02% benzocaine solution and then

mounted tadpoles in a chamber made of silicone and sealed with a coverslip. To screen tadpoles for GFP arbors, we used a 10 $\times$ objective (Nikon Plan Apo; NA = 0.45) on an upright microscope (Nikon Microphot-FXA). For each tadpole that contained a GFP arbor we drew a rough sketch of the morphology of the arbor and its location in the tectum. We confirmed that GFP and GFP Δ ARM-expressing arbors originated from cells located in the contralateral retina by following several brighter axons back to the contralateral eye. Animals containing fluorescent arbors were taken to an inverted confocal microscope (Bio-Rad 600) and imaged using a 40 $\times$ objective (Zeiss Plan Neofluor; NA = 0.75). Some of the arbors were confocal imaged at 24 hr intervals over a 2 or 3 d period. All of the arbors that we imaged [the thicker, unbranched axons expressing GFP Δ ARM and GFP tagged to the N-terminal domain of β -catenin (GFPNTERM), as well as the thinner, branched arbors expressing GFP, GFP Δ ARM and GFP tagged to the C-terminal domain of β -catenin (GFPCTERM)] were located at a relatively superficial depth within the tectum.

Arbor analysis. Confocal images were projected using the NIH Image (version 1.62) projection algorithm. From this projected image we counted the number of branches in the arbors and measured the total length of arbors. By using the projection algorithm in NIH image, we underestimate both the number of branch tips and the length of arbors, relative to other studies that manually reconstructed arbors by tracing each individual slice (Zou and Cline, 1996).

DNA plasmid construction. The β -catenin deletion mutant Δ ARM contains amino acid residues 1–151 of β -catenin fused to residues 648–781. NTERM contains residues 1–151 of β -catenin, and CTERM contains residues 648–781 of β -catenin.

All constructs, including GFP, were subcloned into the *Xenopus* expression vector pCS2 [originally constructed by D. Turner (University of Michigan) and R. Rupp (Max-Planck-Institute, Tuebingen, Germany)]. Δ ARM and CTERM were constructed previously in our laboratory from a full-length *Xenopus* β -catenin that had a myc-epitope tag fused to its C terminus and were subcloned into the expression vector pEGFP-C1 (Clontech) (Kypta et al., 1996). We subcloned Δ ARM and CTERM (with GFP at their N termini and myc tags at their C termini) from pEGFP-C1 into pCS2 using *NheI* and *XbaI*. To make pCS2 Δ ARM without GFP we subcloned Δ ARM (with the myc tag) from pEGFP-C1 into pCS2 using *EcoRI*. To make CTERM without GFP or a myc tag, we performed PCR on a full-length PBS *Xenopus* β -catenin (without a myc tag) and subcloned the resulting PCR fragment into the pCR-Blunt II-TOPO vector. We then subcloned CTERM (no myc) from pCR Blunt II-TOPO into pCS2 using *XbaI*.

To make pCS2-GFPNTERM we digested pEGFP-C1- β -catenin (full length) with *XhoI* and then subcloned the *Xho* fragment (containing the N-terminal domain of β -catenin) into pEGFP-C1. We then subcloned GFPNTERM out of pEGFP-C1 into pCS2 with *NheI* and *XbaI*.

Results

β -catenin is expressed in RGC axon arbors in the tectum

To determine whether β -catenin was expressed in RGC axon arbors in their target tissue, the tectal midbrain, sections from brains of stage 45/46 tadpoles were stained with anti- β -catenin. Results showed β -catenin expression throughout the neuropil region of the tectum (Fig. 1A) (tectal sections from six different brains showed similar staining patterns). Within the neuropil, β -catenin was expressed in a nonuniform pattern, with the strongest, most dense expression in the lateral neuropil (Fig. 1A). This corresponds to the outermost region of the tectum that is first invaded by RGC axons. In the more medial neuropil, the region of the tectum in which RGC axons form arbors, the expression of β -catenin appeared more sparse and punctate (Fig. 1A,C). N-cadherin is also expressed in a similar pattern in the neuropil region of the *Xenopus* tectum (Riehl et al., 1996).

To examine directly whether RGC axon arbors in the tectum contained β -catenin puncta, single RGC axon arbors were labeled with GFP (Fig. 1B,D). Tectal sections containing a single

Figure 1. β -catenin is expressed in RGC axon arbors in the tectum. Confocal image (single optic section) of a tectal section taken from a stage 45/46 tadpole stained with anti β -catenin antibody (red) shows expression in the neurophil area, the target region for RGC axons (A). Confocal image of a GFP-expressing RGC axon arbor (green) in the same optic section shows that the axon arbor is in the neurophil region of the tectal section (B). Higher magnification zoom of boxed regions in A and B shows that β -catenin staining is punctate in the neurophil (C) and that the GFP-expressing RGC axon arbor contains β -catenin puncta (E, arrows, double arrow, arrowhead). β -catenin puncta are located at branch points (D, arrows), in growth cones (D, double arrow), and along branch shafts (D, arrowhead). np, Neurophil region of the tectum; cb, cell body region of the tectum. Scale bars: A, B, 10 μ m; C–E, 5 μ m.

GFP RGC axon arbor were then stained for β -catenin and imaged on a confocal microscope. Examination of these images revealed specific (yellow) puncta of overlap between the green GFP-labeled RGC arbors and red-stained β -catenin puncta (Figs. 1C–E). The density of β -catenin puncta in RGC axon arbors was 3.4 ± 0.85 β -catenin puncta per 10 μ m of branch length (440 μ m of arbor length analyzed, 100 μ m or more per arbor). Within the RGC axon arbors, β -catenin puncta were found at branch points (Fig. 1E, arrows), along shafts of branches (Fig. 1E, arrowhead), and in branch tips (Fig. 1E, double arrow). To confirm the specificity of overlap of red β -catenin puncta with the green GFP arbor branches, we mismatched the red and green images along the y-axis and then compared the number of yellow puncta in the mismatched images with that in correctly matched red and green images. On average, mismatched images contained 33% of the number of yellow puncta as observed in the correctly matched images (see Materials and Methods). In summary, these data demonstrate that β -catenin is expressed in RGC axon arbors in the tectum. Thus, β -catenin may play a role in RGC axon arborization.

Perturbation of β -catenin function does not inhibit RGC axon outgrowth to the tectum

To perturb β -catenin function in RGCs, we used a deletion mutant of β -catenin named Δ ARM that is a fusion of the N-terminal and C-terminal domains of β -catenin (Fig. 2). The N-terminal domain of β -catenin contains a highly conserved subdomain that mediates interactions with α -catenin that in turn associates with the F-actin cytoskeleton (Ivanov et al., 2001; Schneider et al.,

2003). The interactions between β -catenin and α -catenin are required for strong cadherin-based cell–cell adhesion (Ivanov et al., 2001). The C-terminal domain contains a PDZ binding motif that has been shown to interact with two synaptic PDZ proteins (Perego et al., 2001; Nishimura et al., 2002). Δ ARM lacks the central armadillo repeat region of β -catenin that contains interaction sites for cadherins and several proteins that mediate Wnt signaling, including APC (adenomatous polyposis coli) and TCF/LEF (T-cell factor/lymphoid enhancing factor) (Hulskens et al., 1994; Gottardi and Gumbiner, 2001; Pokutta and Weis, 2002). Previous studies have shown that Δ ARM does not activate (or interfere with) Wnt signaling when expressed in cleavage stage *Xenopus* embryos (Funayama et al., 1995; Sehgal et al., 1997). Because Δ ARM is expected to compete with endogenous, full-length β -catenin for binding to α -catenin and PDZ-containing proteins, we hypothesized that overexpression of these proteins to cadherins, thereby perturbing cadherin-based adhesion and recruitment of PDZ-containing synaptic proteins (see Discussion).

We first asked whether Δ ARM inhibited RGC axon outgrowth from the retina to the tectum. To address this issue we examined whether GFP Δ ARM-expressing RGC axons were present in the tecta of

stage 45/46 tadpoles. In wild-type animals, a significant number of RGC axons have reached the tectum by this stage (Holt, 1984). RGCs lipofected with GFP Δ ARM extended green fluorescent axons to the tectum at stage 45/46. To determine whether GFP Δ ARM- and GFP-expressing axons reached the tectum at the same frequency, we lipofected these two plasmids into two groups of 30 tadpoles. By stage 45/46, one-third of the tadpoles in each of the two groups showed green fluorescent RGC axons in the tectum (data not shown). In addition, embryos injected with Δ ARM in the eyebud of a single, determined hemisphere extended axons only to the contralateral tectum, similar to embryos expressing GFP alone (eight embryos analyzed; data not shown). Thus, overexpression of Δ ARM does not inhibit the extension of RGC axons from the retina to the tectum. These data are consistent with previous findings showing that β -catenin function is not essential for axon outgrowth (Loureiro and Peifer, 1998; Loureiro et al., 2001) and that overexpression of the region of the cytoplasmic tail of cadherin that interacts with β -catenin is not sufficient to inhibit growth of *Xenopus* RGC axons (Riehl et al., 1996).

β cat Δ ARM expression perturbs axon arborization of RGCs *in vivo* in two different ways

Is β -catenin function required for axon arborization of RGCs? Before examining the arbors of RGCs expressing Δ ARM, we imaged and measured GFP-expressing RGC axon arbors in live tadpoles. At stage 45/46, arbors containing GFP were moderately branched, with an average branch number of ~ 11 and total arbor length of 300 μ m (Figs. 3A, 4A, 5A, B; Table 1). In a 24 hr period,

Figure 2. Schematic diagram of mutant β -catenin constructs. Wild-type β -catenin consists of three distinct functional and structural domains: an N-terminal domain, a central core consisting of 12 armadillo repeats, and a C-terminal domain. Within these three domains, β -catenin contains binding sites for α -catenin (N-terminal domain and armadillo repeats R1–R2; amino acid residues 120–150), cadherins (binding spans throughout armadillo repeats R3–R12), and PDZ-domain-containing proteins (C-terminal domain; amino acid residues 777–781). The deletion mutant Δ ARM lacks almost the entire armadillo repeat region found in wild-type β -catenin; it consists of the N-terminal residues 1–151 of wild-type β -catenin fused to the C-terminal residues 648–781. Thus, Δ ARM retains binding to α -catenin and PDZ-binding proteins but not to cadherin. The deletion mutants NTERM and CTERM are simply the N- and C-terminal domains of β -catenin that together constitute Δ ARM. Each protein had GFP fused to its N terminus and was expressed using the *Xenopus* expression vector pCS2. Δ ARM and CTERM also contained a myc-epitope tag at their C termini. We also confirmed the effects of Δ ARM without an attached GFP tag and of CTERM without attached GFP or myc tags (see Results).

these arbors increased their average branch number to 16 and their average total arbor branch length to 350 μ m (Figs. 4A, 5B, C, Table 1). These axonal arborization parameters agree with values for wild-type RGC axon arbors at stage 45/46 reported in a previous study (O'Rourke and Fraser, 1990).

Many axons of RGCs that express Δ ARM do not form arbors

In contrast to RGC axons expressing GFP alone, approximately half of RGC axons expressing Δ ARM failed to form arbors in the tectum (Figs. 3B, 4B, 5A). At stage 45/46, these axons had no or very few (at most three) terminal branches (Figs. 3B, 4B, 5A, B). Moreover, when imaged over several days, we observed that these axons did not form additional branches with time (Figs. 4B, 5B). The first example of an axon shown in Figure 4B shows a representative Δ ARM-expressing RGC axon that initially had two branches; during the following 2 d period, this axon did not add additional branches. Thus, in a significant fraction of Δ ARM-expressing RGCs, axon branching is severely inhibited.

The axons of RGCs expressing Δ ARM that formed severely reduced arbors also exhibited other morphological features distinct from those of control axons of RGCs expressing GFP. First, the axons (and a few secondary branches) formed by this subpopulation of Δ ARM-expressing RGCs were thicker than the axons and branches formed by control GFP-expressing RGCs (Fig. 3, compare A, B). Second, the tips of the axons and branches formed by these Δ ARM-expressing RGCs with reduced arborization often terminated in bulbs or club-like endings (Figs. 3B, arrow, 4B). Both of these features of Δ ARM-expressing RGC axons became more pronounced over time. On the second (and third) day of observation, axons of RGCs expressing Δ ARM became thicker, and the bulbs at the tips of their branches became larger (Fig. 4B).

Figure 3. Axonal arborization phenotypes of RGCs expressing β -catenin deletion mutants. Projected confocal z-series images taken of axon arbors in live tadpoles show arbor morphologies for RGCs expressing GFP (A) or GFP-tagged- β -catenin deletion mutants (B–E). RGC axons that express the β -catenin deletion mutant Δ ARM have two distinct phenotypes. They either do not arborize (B) or they arborize but contain longer, tangled branches (C). RGC axons expressing the β -catenin deletion mutant NTERM form severely reduced arbors (D) that are similar to the subpopulation of Δ ARM arbors with no or few branches (B). RGC axons expressing CTERM form arbors with long, tangled branches (E) that are similar to the subpopulation of Δ ARM arbors with effusive branching (C). RGC axons that express Δ ARM and NTERM and form severely reduced arbors (B, D) also have bulbous endings at their axon/branch tips (B, D, arrows) and appear thicker than RGC arbors that express GFP, Δ ARM, and CTERM (compare axon/branch thicknesses in B, D with those in A, C, E). Note that at the level of magnification of these projected confocal z-series (40 $\times$), all of the GFP-tagged- β -catenin deletion mutant constructs appear to be uniformly distributed within the axon terminals and arbors. Scale bars: A–E, 20 μ m.

Figure 4. Tracings of projected confocal z-series of RGC axon arbors expressing β -catenin deletion mutants confirm morphological phenotypes. Compared with controls, RGC axons that express Δ ARM have two distinct phenotypes. They either do not arborize (*B*) or they arborize but have abnormally long and tangled branches (*C*). NTERM-expressing arbors (*D*) are similar to the subpopulation of Δ ARM arbors with no or few branches (*B*). CTERM-expressing arbors (*E*) are similar to the subpopulation of Δ ARM arbors with abnormally long, tangled branches (*C*). For each condition (*A–E*), two examples of arbors are shown. In each example of an arbor, two (or three) (*B*, left) tracings are shown that correspond to confocal images taken ~ 24 hr apart. The first tracing of each example corresponds to a confocal image taken at stage 45/46. The leftmost arbor tracing in *A–E* corresponds, respectively, to the actual confocal z-series projection image shown in Figure 3*A–E*. Stippling over a single arbor branch in *C* and *E* illustrates a particularly tangled branch. Rostral is to the top and lateral is to the right in all images. Scale bars, 20 μ m (*A–E*).

Figure 5. Quantification of morphological parameters for arbors from RGCs expressing β -catenin deletion mutants confirm phenotypes shown in Figures 3 and 4. The distribution of branch numbers in arbors at stage 45 shows that GFP axon arbors are distributed about a mean of ~ 11 branches (*A*). In contrast, Δ ARM axons are distributed about two distinct means: Δ ARM arbors either have very few (1–2) branches or they have approximately the same number of branches as control GFP arbors (*A*). In addition, NTERM arbors have no or very few (1–6) branches, whereas CTERM arbors have numbers of branches comparable with GFP arbors (*A*). Plot of mean numbers of branches in arbors over 2 d shows that GFP arbors acquire more branches over time (*B*). Δ ARM arbors with branches and CTERM arbors behave similarly to GFP arbors (*B*). In contrast, Δ ARM axons without arbors and NTERM axons form few or no additional branches over time (*B*). Branched Δ ARM arbors (with 9 or more branches) and CTERM arbors also grow faster than GFP arbors (*C*) ($p < 0.05$ for both, Student's *t* test). We did not measure the growth rate for unbranched Δ ARM and NTERM arbors because many RGCs in these two classes did not form arbors (*C*). The number of axons analyzed is as follows: *A*, GFP (21), Δ ARM (24), NTERM (7), CTERM (10); *B*, GFP (10), Δ ARM-branched (6), Δ ARM-unbranched (6), NTERM (7), CTERM (10); *C*, GFP (9), Δ ARM-branched (6), CTERM (10).

Table 1. Total arbor branch length (TABL) in RGCs expressing β -catenin deletion mutants

Construct	TABL (μ m) $\pm$ SE (n)	
	Day 1	Day 2
GFP	319 $\pm$ 27 (15)	385 $\pm$ 35 (9)
Δ ARM (with branches)	470 $\pm$ 34** (13)	708 $\pm$ 70* (6)
CTERM	434 $\pm$ 46* (10)	615 $\pm$ 72* (10)

RGC axon arbors expressing β -catenin deletion mutants Δ ARM and CTERM are longer than controls. Measurements of total arbor length for both branched Δ ARM arbors and CTERM arbors over 2 d (day 1 corresponds to stage 45/46) show that they are significantly longer than arbors of control RGCs expressing GFP. Student's *t* test was used to test for statistical significance of differences. **p* < 0.05; ***p* < 0.005.

Other RGCs overexpressing Δ ARM form misshapen arbors

The remaining Δ ARM-expressing RGC axons that we imaged in tecta of live tadpoles did form arbors (Figs. 3C, 4C). At stage 45/46, these arbors had comparable numbers of branches to control arbors of RGCs expressing GFP alone (Figs. 3C, 4C, 5A,B). The mean number of branches for Δ ARM arbors with branches was $\sim$ 12, compared with a mean number of branches of 11 for arbors of controls expressing GFP alone (Fig. 5A,B). Over a 2 d period, these Δ ARM arbors increased their total branch number, with an increase comparable with that observed in GFP arbors (Fig. 5B). Thus in this group of arbors, branching per se was not inhibited by expression of Δ ARM.

The arbors of axons expressing Δ ARM, however, formed branches that were shaped differently than control arbors. First, the total length of axonal branches of arbors expressing Δ ARM was greater than the length of control arbors expressing GFP (Figs. 3C, 4C, Table 1). When examined over several days, arbors of Δ ARM-expressing RGCs also grew faster than GFP arbors (Figs. 4C, 5C). Second, the branches of these arbors were more tangled than branches of control arbors. In the arbors of RGCs expressing Δ ARM, secondary branches crossed back and forth several times across the main axis of the arbor (Fig. 4C, stippling). In contrast, in control arbors, secondary branches extended straight from the main axis of arborization and rarely recrossed this axis (Fig. 4A). Thus, in this group of RGCs, expression of Δ ARM resulted in accelerated elongation and more tangled morphology of arbor branches.

The two arborization phenotypes that we observed in GFP/ Δ ARM-expressing axons were not caused by the inadvertent expression of GFP/ Δ ARM in neurons that were not RGCs. For both phenotypes we followed several bright fluorescent axons to their points of origin in the retinal ganglion cell layer of the contralateral eye. The GFP/ Δ ARM arborization phenotypes were also not caused by localization of the fusion protein GFP/ Δ ARM to only a portion of the arbor of RGCs. RGCs co-lipofected with GFP and Δ ARM in separate plasmids showed the same arborization phenotypes as did GFP/ Δ ARM-expressing RGCs [*n* = 10 RGCs co-lipofected; 4 did not arborize, and 6 arborized but had long, tangled arbors (data not shown)]. Thus, these data implicate β -catenin in axon arborization of RGCs *in vivo*. They show that β -catenin is required for both the initiation and shaping of RGC arbors.

Overexpression of the N- and C-terminal domain fragments of β -catenin, respectively, inhibit and misshape axon arbors *in vivo*

Δ ARM is a fusion of the N-terminal and C-terminal domains of β -catenin. To determine whether the two distinct arborization phenotypes observed in Δ ARM RGCs reflect distinct activities of these two domains, we expressed each domain alone in RGCs (Fig. 2).

RGC axons expressing NTERM had no or severely reduced arborization (Figs. 3D, 4D). At stage 45/46, NTERM RGC axons had very few branches (one to six branches) (Figs. 3D, 4D, 5A,B). Similar to Δ ARM axons without branches, NTERM axons did not acquire more branches over time (Figs. 4D, 5B). Instead, the average number of branches formed by these neurons was slightly reduced with time (Fig. 5B). Moreover, NTERM axons were also thicker than control axons and contained bulbs at their terminal ends (Figs. 3D, arrow, 4D). Thus, NTERM axons appeared and behaved very similar to the subpopulation of Δ ARM axons without arbors (Fig. 3B,D, compare with Fig. 4B, D).

In contrast, RGCs expressing CTERM formed severely misshapen arbors (Figs. 3E, 4E). At stage 45, CTERM arbors had numbers of branches comparable with those arbors formed by RGCs expressing GFP (Fig. 5A,B). Over 2 d, the mean number of branches formed by RGCs expressing CTERM increased significantly (Fig. 5B). However, similar to the arbors of Δ ARM-expressing RGCs with extensive branching, the arbors formed by RGCs expressing CTERM were significantly longer and grew faster than arbors formed by control RGCs expressing GFP alone (Figs. 3E, 4E, 5C, Table 1). CTERM arbors were also very tangled with long branches that crossed and overlapped other branches of the arbor (Figs. 3E, stippling, 4E). Thus, expression of CTERM in RGCs generates arbors that resemble those formed by the second class of RGCs expressing Δ ARM (Figs. 3, 4, compare C, E).

We confirmed that the presence of overextended, misshapen arbors in RGCs expressing CTERM was not caused by interference of protein interactions by the GFP and myc tags attached to the CTERM construct. Co-lipofection of a modified CTERM without the attached GFP and myc tags (see Materials and Methods) and GFP into RGCs also generated misshapen axon arbors with long, tangled branches (in three of three RGC axons). Consistent with this finding, recent work in our laboratory also indicates that attachment of a myc tag at the C-terminal end of β -catenin does not abolish (although it may weaken) PDZ protein binding to this domain (S. Bamji, N. E. Kimes, and L. F. Reichardt, personal communication).

These results demonstrate that the N- and C-terminal domains of β -catenin have distinct functions in axon arborization. They suggest that interactions mediated through the N-terminal domain of β -catenin are required for formation of arbors by RGC axons in the tectum, whereas interactions mediated by the C-terminal domain are required for normal extension and shaping of arbors.

RGC axons that express the β -catenin deletion mutants Δ ARM and NTERM are frequently mistargeted within the tectum

Previous studies have shown that in *Xenopus* tadpoles RGC axons do not branch until they reach their topographically appropriate locations in the tectal neuropil (Sakaguchi and Murphey, 1985). This suggests that specific topographic cues are required to promote formation of axonal arbors by RGCs. Thus, one possibility is that Δ ARM- and NTERM-expressing RGC axons do not form arbors because they are mistargeted within the tectum. Examination of the tectal projections of the subpopulation of Δ ARM-expressing axons without arbors and of NTERM-expressing axons demonstrated that they did not always terminate in a topographically appropriate location (Table 2). Two types of targeting errors were observed. The poorly branched arbors of RGCs expressing Δ ARM or NTERM frequently formed abnormally short projections into the tectum, or they had unusually long projections that mean-

Table 2. Mistargeting of RGC axons that express β -catenin deletion mutants

Construct	Types of targeting errors		Total axons mistargeted within the tectum	No. of axons analyzed
	Very short axonal projection into tectum (<40 μ m along the mediolateral axis)	Long axonal projection extending beyond tectal midline		
GFP	0	0	0	10
Δ ARM (unbranched)	4	2	6	10
Δ ARM (branched)	0	0	0	9
NTERM	3	1	4	7
CTERM	0	0	0	10

dered over the tectum and extended beyond the midline of the tectum, terminating in the ipsilateral tectum (Table 2). In contrast, the well branched arbors formed by RGC neurons expressing GFP, Δ ARM, or CTERM appeared to project consistently into and terminate within a relatively central region of the appropriate tectal hemisphere and to thus be correctly targeted (Table 2). In conclusion, some of the poorly branched axons of RGCs expressing Δ ARM or NTERM were clearly mistargeted within the tectum.

Discussion

N- and C-terminal domains of β -catenin, respectively, initiate and shape axon arbors in RGCs in vivo

Results presented above show that overexpression of the N- and C-terminal domains of β -catenin, respectively, inhibit and misshape axon arbors in RGCs in the tecta of live *Xenopus laevis* tadpoles. These results suggest that interactions of the N- and C-terminal domains of β -catenin are required, respectively, to form and shape axon arbors. This evidence implicates two distinct domains of β -catenin in two different processes of axon arborization.

Cadherin-based adhesion and formation of axon arbors in RGCs

Overexpression of β -cat Δ ARM or β -catNTERM in RGCs is expected to interfere with functions mediated by the N-terminal domain of endogenous β -catenin by sequestering proteins that normally bind to this domain. Results in this paper show that interference with interactions mediated by this domain prevents formation of axon arbors by RGCs in the optic tectum. The N-terminal domain of β -catenin has been shown to interact with α -catenin, which in turn links the cadherin- β -catenin complex to the F-actin cytoskeleton, thereby strengthening cadherin-based cell–cell adhesion (Ivanov et al., 2001; Schneider et al., 2003). Thus, the N-terminal domain of endogenous β -catenin may promote axon branching in RGCs by strengthening cadherin-based cell adhesion. How could strong cadherin-mediated cell–cell adhesion promote axon arborization in RGCs? We observed that NTERM- and Δ ARM-expressing RGC axons that did not form arbors were also frequently mistargeted within the tectum. Other work has suggested that in *Xenopus laevis* specific topographic signals in the tectal target promote branching (Sakaguchi and Murphey, 1985). Thus, one possibility is that cadherin-based cell–cell adhesion is required for cell–cell interactions that target RGC axons to their correct location within the tectum. Consistent with this possibility, in two different species, cadherin function has been shown to be required for targeting optic axons within their target tissue (Inoue and Sanes, 1997; Lee et al., 2001).

The N-terminal domain of β -catenin also contains a glycogen synthase kinase-3 β (GSK-3 β) binding site, and several phosphorylation sites that regulate ubiquitination and degradation of

the protein (Schneider et al., 2003). Thus another possibility for the mechanism of action of the overexpressed N-terminal domain of β -catenin is that it sequesters GSK-3 β and the ubiquitination machinery away from endogenous, full-length β -catenin. This could conceivably reduce the normal level of phosphorylation and destruction of β -catenin, thereby mimicking the action of activated Wnt-based signaling. However, in a different system, activation of Wnt-based signaling has been shown to promote, not inhibit, axon branching (Hall et al., 2000), the opposite phenotype of what we observe in *Xenopus* RGCs overexpressing the N-terminal domain of β -catenin.

β -catenin–PDZ protein interactions and shaping of RGC axon arbors

Our results also demonstrate that interactions mediated by the C-terminal domain of β -catenin are required to shape RGC axon arbors. When this domain was overexpressed in RGCs, longer and more tangled axon branches were formed by RGCs. These overgrown, tangled axon arbors of RGCs overexpressing the C-terminal domain of β -catenin may correlate with and perhaps be caused by an inhibition of synaptogenesis. Cadherin function has been shown to be required for synapse formation in hippocampal neurons (Togashi et al., 2002). Introduction of dominant-negative cadherin molecules into cultured hippocampal cells removed endogenous cadherin and β -catenin from synapses, which in turn dispersed synaptic proteins, disrupted synaptic function, and caused morphological changes in dendritic spines. Moreover, the last three amino acids of the C-terminal domain of β -catenin comprise a PDZ-binding motif through which β -catenin interacts with, and thereby links cadherin to, at least two synaptic PDZ proteins (Perego et al., 2001; Nishimura et al., 2002). Indeed, overexpression of the C-terminal domain of β -catenin in hippocampal neurons has been shown to inhibit recruitment of the synaptic PDZ protein S-SCAM (Nishimura et al., 2002). Consistent with the possibility that the tangled arbors are caused by an inhibition of synaptogenesis, perturbation of other proteins involved in synaptogenesis has been shown to also cause excessive growth of arbors. For example, in the *Xenopus* retinotectal system, inhibition of CaMKII (Ca²⁺/calmodulin-dependent protein kinase II) function inhibits maturation of synapses and also promotes excessive growth of axonal arbors (Zou and Cline, 1999). In addition, in mice lacking either agrin or MuSK (muscle-specific kinase), the neuromuscular junction fails to differentiate. In these mice, the axons that innervate the neuromuscular junction also form extremely abnormal arbors that extend over the entire surface of the muscle instead of being restricted to a small region, as in normal mice (Lin et al., 2001).

Several transcriptional coactivators have also been shown to bind to the C-terminal region of β -catenin from armadillo repeat 10 through the C-terminal end of the protein (Hecht et al., 1999; Takemaru and Moon, 2000). Thus, another possibility is that the overexpressed C-terminal domain of β -catenin competes with en-

ogenous full-length β -catenin for binding to these transcriptional activators and perturbs the normal pattern of transcription.

β -cat Δ ARM generates two distinct arborization phenotypes in RGCs that appear to be regulated by distinct signaling pathways

The deletion mutant β -cat Δ ARM is a fusion of the N- and C-terminal domains of β -catenin. Expression of Δ ARM in RGCs generated two distinct arborization phenotypes that mimicked the separate activities of the N- and C-terminal domains of β -catenin. These results suggest that in Δ ARM-expressing RGCs that do not form arbors, binding of proteins to the N-terminal domain of β -catenin is perturbed, whereas in Δ ARM-expressing RGCs that form extended, misshapen arbors, perturbation of C-terminal domain interactions is dominant. How does β -cat Δ ARM result in two very distinctive phenotypes that appear to be caused by perturbation of two distinct signaling pathways? A likely possibility is that in the two classes of RGCs, different levels of Δ ARM are present that selectively perturb distinct protein–protein interactions. In RGCs with lower levels of Δ ARM, binding of proteins to the C-terminal domain could be perturbed, resulting in misshapen arbors. In RGCs with higher levels of Δ ARM, N-terminal (and C-terminal) domain interactions could be perturbed, thereby inhibiting formation of arbors. Consistent with this hypothesis, we observed that GFP Δ ARM-expressing RGCs that did not form arbors tended to exhibit brighter GFP fluorescence than GFP Δ ARM-expressing RGCs that formed overextended, misshapen arbors (data not shown). This suggests that those RGCs that did not form arbors may have contained higher levels of GFP Δ ARM.

Regulation of β -catenin–cadherin interactions during axon outgrowth and arborization in RGCs

Results in this study show that RGC axons expressing β -catenin deletion mutants grow normally from the retina to the tectum (Loureiro and Peifer, 1998; Loureiro et al., 2001). In particular, our data indicate that interactions of the N-terminal domain of β -catenin with proteins such as α -catenin are not required for growth of RGC axons from the retina to the tectum. A previous study showed that N-cadherin function is required for RGC axon outgrowth to the tectum (Riehl et al., 1996). However, this study showed that overexpression of the juxtamembrane domain of N-cadherin (required for p120 catenin binding) and not the more C-terminal β -catenin binding domain interfered with RGC axon outgrowth (Riehl et al., 1996). Consistent with this, our data also indicate that normal RGC axon growth does not require cadherin interaction with β -catenin (and α -catenin).

We also show here that interactions of the N-terminal domain of β -catenin with proteins such as α -catenin are required within the tectum for targeting and arbor formation by RGC axons (see Discussion above). Cadherin function is also required for targeting and formation of arbors by RGC axons in the tectum (Inoue and Sanes, 1997). Thus, the cadherin– β -catenin– α -catenin adhesion complex may function together at a later stage to promote targeting and formation of arbors by RGC axons in the tectum. One mechanism to regulate the associations and function of the cadherin– β -catenin– α -catenin adhesion complex is through tyrosine phosphorylation of β -catenin (Fujita et al., 2002; Lilien et al., 2002). Previous work has suggested that tyrosine phosphorylation of β -catenin may mediate axon outgrowth by RGCs, likely by dissociation of the cadherin– β -catenin– α -catenin adhesion complex (Kypta et al., 1996; Lilien et al., 2002). It will be interesting to test whether dephosphorylation of tyrosine residues on

β -catenin is subsequently required during the later developmental events of targeting and formation of arbors by RGC axons in the tectum.

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