BDNF stabilizes synapses and maintains the structural complexity of optic axons in vivo

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Summary

Brain-derived neurotrophic factor (BDNF) modulates synaptic connectivity by increasing synapse number and by promoting activity-dependent axon arbor growth. Patterned neuronal activity is also thought to influence the morphological maturation of axonal arbors by directly influencing the stability of developing synapses. Here, we used in vivo time-lapse imaging to examine the relationship between synapse stabilization and axon branch stabilization, and to better understand the participation of BDNF in synaptogenesis. Green fluorescent protein (GFP)-tagged synaptobrevin II was used to visualize presynaptic specializations in individual DsRed2-labeled *Xenopus* retinal axons arborizing in the optic tectum. Neutralizing endogenous tectal BDNF with function-blocking antibodies significantly enhanced GFP-synaptobrevin cluster elimination, a response that was paralleled by enhanced branch elimination. Thus, synapse dismantling was associated with axon branch pruning when endogenous BDNF levels were reduced. To obtain a second measure of the role of BDNF during synapse stabilization, we injected recombinant BDNF in tadpoles with altered glutamate receptor transmission in the optic tectum. Tectal injection of the NMDA receptor antagonists APV or MK801 transiently induced GFP-synaptobrevin cluster dismantling, but did not significantly influence axon branch addition or elimination. BDNF treatment rescued synapses affected by NMDA receptor blockade: BDNF maintained GFP-synaptobrevin cluster density by maintaining their addition rate and rapidly inducing their stabilization. Consequently, BDNF influences synaptic connectivity in multiple ways, promoting not only the morphological maturation of axonal arbors, but also their stabilization, by a mechanism that influences both synapses and axon branches.

Key words: *Xenopus laevis*, Retinal ganglion cell, Axon branching, In vivo imaging

Introduction

During development, synapses form, mature and stabilize, and are also eliminated by mechanisms that require intimate communication between pre- and postsynaptic neurons. Embryological and in vivo imaging techniques have recently demonstrated that rapid changes in axon and dendritic arbor structures are necessary to initiate central nervous system (CNS) synaptogenesis (Alsina et al., 2001; Cline, 2001; Cohen-Cory, 2002; Jontes et al., 2000; Jontes and Smith, 2000; Niell et al., 2004; Trachtenberg et al., 2002). Structural changes in developing axon and dendritic arbors can therefore reflect the formation, stabilization and elimination of synapses. In the CNS, as in the neuromuscular junction (NMJ), activity-dependent structural development and remodeling of neuronal connectivity requires not only the addition but also the selective stabilization of synapses (Cohen-Cory, 2002; Goda and Davis, 2003; Sanes and Lichtman, 2001; Walsh and Lichtman, 2003). Thus, synapse stabilization and the associated elimination of excess synaptic inputs are crucial steps in the maturation of synaptic circuits. Our previous studies have provided a direct correlation between structural changes in axon arbor complexity and synapse formation (Alsina et al., 2001). Dual-color imaging of GFP-synaptobrevin tagged presynaptic sites in DsRed-labeled *Xenopus* axons in vivo revealed that while most synapses remain stable, synapses are also formed and eliminated as axons branch and increase their complexity. Moreover, our studies demonstrated a role for brain-derived neurotrophic factor (BDNF) in this process, enhancing synapse formation.

Neurotrophins, originally identified for their ability to promote neuronal survival and differentiation, are potent modulators of synaptic connectivity in the CNS, influencing synaptic structure and function (Poo, 2001; Vicario-Abejon et al., 2002). Specifically, BDNF influences the morphological complexity of axons and dendrites (Cohen-Cory and Fraser, 1995; Lom and Cohen-Cory, 1999; McAllister et al., 1995), increases synapse number in the developing brain (Aguado et al., 2003; Alsina et al., 2001; Causing et al., 1997; Luikart et al., 2005; Rico et al., 2002), modulates synapse maturation (Collin et al., 2001; Huang et al., 1999), controls the ultrastructural composition of synapses (Carter et al., 2002; Collin et al., 2001; Tyler and Pozzo-Miller, 2001; Wang et al., 2003) and may regulate the incorporation of synaptic proteins into synaptic vesicle membranes (Pozzo-Miller et al., 1999). Thus, BDNF is involved in multiple aspects of synaptogenesis, from the formation to the functional maturation of synapses. Our previous work specifically demonstrated both permissive

and instructive roles for BDNF during synaptogenesis (Alsina et al., 2001). In vivo time-lapse imaging of *Xenopus* retinal ganglion cell (RGC) axon arbors showed that BDNF increases arbor complexity, thereby increasing the number of presynaptic sites in the more elaborate axons, while also influencing synapses directly, increasing synapse density per axon branch. Although these observations suggested that BDNF influences the formation and therefore the stabilization of newly formed synapses, our studies did not directly differentiate between these two dynamic events. Here, we examined the relationship between axon branch and synapse stabilization to obtain a better understanding of the participation of BDNF in this important aspect of synaptogenesis. Manipulations that decrease endogenous tectal BDNF show that the stability of axon branches and of GFP-synaptobrevin identified synapses depends on BDNF, but that the rate of synapse turnover, a component of normal axon remodeling, is unaffected by alterations in BDNF. Moreover, by manipulating NMDAR transmission directly in the optic tectum, we demonstrate that BDNF can rescue synaptic sites that would normally be affected when NMDAR activity is altered.

Materials and methods

Xenopus laevis tadpoles were obtained by in vitro fertilization of oocytes from adult females primed with human chorionic gonadotropin. Tadpoles were raised in rearing solution [60 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 10 mM HEPES pH 7.4, 40 mg/l gentamycin] plus 0.001% phenylthiocarbamide to prevent melanocyte pigmentation. Tadpoles were anesthetized during experimental manipulations with 0.05% tricane methanesulfonate (Finquel, Argent Laboratories, Redmond, WA). Staging was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). Animal procedures were approved by the University of California, Irvine.

GFP-Synaptobrevin in vivo expression and axon labeling

The method used for the simultaneous visualization of axon arbor morphology and presynaptic sites in individual RGC axons in vivo was as described previously (Alsina et al., 2001), with minor modifications. In brief, a chimeric gene coding for wild-type GFP and the complete sequence of *Xenopus* synaptobrevin II was used to target GFP expression to synaptic vesicles in live tadpoles. Retinal progenitor cells of stage 20-24 tadpoles were co-transfected with equimolar amounts of GFP-synaptobrevin and pDsRed2 (Clontech, Palo Alto, CA) expression plasmids by lipofection (Holt et al., 1990). Tadpoles were reared under filtered illumination, in 12-hour dark/light cycles, until stage 45 when used for experimentation and imaging. Only a few neurons per retina were transfected, with 80-90% of transfected neurons expressing both plasmids (Alsina et al., 2001).

Electron microscopy

Stage 45 tadpoles with only a few RGCs expressing GFP-synaptobrevin in their axon terminals were selected and processed for pre-embedding immunoelectron microscopy. Tadpoles were anesthetized and fixed in 2% paraformaldehyde, 3.75% acrolein in 0.1 M phosphate buffer (pH 7.4). Brains were removed, post-fixed and embedded in 1% agarose. Vibratome sections (50 μm) were collected, incubated in 1% sodium borohydride in phosphate buffer, cryoprotected, quickly permeabilized in liquid nitrogen and blocked in 0.5% bovine serum albumin (BSA), 0.1 M Tris buffer saline (TBS) (pH 7.5). Sections were incubated overnight in a primary mouse monoclonal antibody against GFP (1:10 dilution in 0.1% BSA in TBS; Molecular Probes, Eugene, OR) followed by 2 hours in a secondary goat anti-mouse IgG coupled to 1 nm gold particles [1:50 dilution in 0.5% fish gelatin, 0.8% BSA in 0.01 M PBS (pH 7.4); Aurion-EMS, Hatfield, PA]. Sections were incubated in 2% glutaraldehyde and gold particles were enlarged using a British BioCell silver intensification kit (Ted Pella, Redding, CA). Sections were post-fixed in 2% osmium tetroxide, dehydrated and flat embedded in 100% Epon between Aclar sheets. Sections (70 nm) were obtained using a Reichert ultramicrotome with a diamond knife (Diatome) and counterstained with 2% uranyl acetate and Reynolds lead citrate. Ultrastructural analysis was performed using a Philips CM20 transmission electron microscope.

Drug treatment and time-lapse imaging

The behavior of individual, fluorescently labeled RGC axons was followed with confocal microscopy in stage 45 tadpoles expressing GFP-synaptobrevin. Only tadpoles with individual RGC axons labeled with DsRed2 showing specific, punctate GFP labeling in their terminals were selected. Tadpoles containing one or two clearly distinguishable double-labeled axons, with at least six branches were imaged every 2 hours for 8 hours, then again at 24 hours. Immediately after the first observation, 0.2-1.0 nl of anti-BDNF (330 μg/ml of purified IgG; R&D systems, Minneapolis, MN), APV (50 μM solution; Tocris Cookson, Ellisville, MO), MK801 (20 μM solution; Tocris Cookson), recombinant BDNF (200 ng/μl; Amgen, Thousand Oaks, CA) or vehicle solution (50% Niu Twitty) was pressure injected into the ventricle and subpial space overlying the optic tectum. The specificity of the BDNF antibody versus non-immune IgG, and its ability to influence RGC differentiation were determined in control experiments as previously described (Lom and Cohen-Cory, 1999). Axon arbors in tadpoles injected with control, non-immune IgG exhibited branch and GFP-synaptobrevin cluster dynamics comparable with those of vehicle-treated tadpoles (data not shown). Microinjection of APV and MK801 into the optic tectum of developing tadpoles has been shown to eliminate NMDAR-mediated synaptic currents completely (Zhou et al., 2003), and was effective in blocking neuronal activity up to 8 hours after treatment (B.H., unpublished). To correlate GFP-synaptobrevin distribution with axon morphology, thin optical sections (1.0 μm) through the entire extent of the arbor were collected at 60× magnification (1.00 NA water-immersion objective) with a Nikon PCM2000 laser-scanning confocal microscope (Melville, NY) equipped with Argon (488 nm excitation; 10% neutral density filter) and HeNe (543 nm excitation) lasers. A 515/30 nm (barrier) and a 605/32 nm (band-pass) emission filters were used for GFP-synaptobrevin and DsRed2 visualization, respectively. GFP-synaptobrevin and DsRed2 confocal images were obtained simultaneously, below saturation levels, with minimal gain and contrast enhancements.

Data analysis

All analysis was performed from raw confocal images without any post acquisition manipulation or thresholding. Analysis was performed blind to the treatment group. Digital three-dimensional reconstructions of DsRed2-labeled arbors (red only) were obtained from individual optical sections through the entire extent of the arbor with the aid of the MetaMorph software (Universal Imaging, West Chester, PA). To characterize the distribution of GFP-synaptobrevin puncta to particular axonal regions, pixel-by-pixel overlaps from individual optical sections obtained at the two wavelengths were analyzed. Yellow regions of complete red and green overlap were identified, counted and related to arbor morphology. GFP-synaptobrevin labeled puncta of 0.5-1.0 μm^2 in size (size of smallest puncta), and hue and pixel intensity values between 16-67 and 150-255, respectively, were considered to be single synaptic clusters. Discrete GFP-synaptobrevin puncta classified in this manner exhibited median pixel values 2.0 to 3.0 times greater than the median pixel values of background non-punctate GFP within the same axon arbor. During data analysis, we ensured that similar ratios were maintained for every axon arbor analyzed throughout the 24 hour

observation period (see Fig. 2). Synaptic cluster values were obtained by manual counting of yellow puncta and similar values were obtained by digital counting. To obtain a detailed analysis of synaptic cluster dynamics at each observation interval, several parameters were measured: the number of clusters per branch or per unit arbor length, the number of clusters added or eliminated, the number of clusters maintained from one observation interval to the next, and the location of each synaptic cluster along the axon arbor. For the quantitative analysis of axon branching, the following morphological parameters were measured: total arbor branch length (length of total branches), total branch number, the number of individual branches gained or lost, and the number of branches remaining from one observation interval to the next. Extensions from the main axon of more than 5 μm were classified as branches. Total arbor length was measured from binarized images of the digitally reconstructed axons. A relative measure of cumulative length of all branches per axon terminal was obtained by counting total pixel number from the first branch point. A total of 10-14 axon arbors per condition were analyzed, with one axon analyzed per tadpole. Axons analyzed had between 6-41 branches and 13-229 clusters. Data are presented as percent increase from the initial observation interval to each subsequent interval, or as percent increase for each 2 hour observation interval. Two-sample unpaired *t*-tests, one-way ANOVA Tukey's multiple comparison tests, and Fisher's exact and chi-square tests (Systat, SPSS) were used for the statistical analysis of data. Results were classed as significant as follows: $*P \leq 0.05$, $**P \leq 0.005$, $***P \leq 0.0005$.

Results

GFP-synaptobrevin serves as a suitable marker to investigate cellular and molecular mechanisms of synaptogenesis at the single cell level as it preferentially concentrates at presynaptic contact sites (Ahmari et al., 2000; Alsina et al., 2001; Nonet, 1999). The punctate distribution of GFP-synaptobrevin labeling along the axon arbor (Fig. 1E), and its co-localization with endogenous pre- and postsynaptic proteins (Alsina et al., 2001), demonstrate that GFP-synaptobrevin is targeted to presynaptic specializations in RGC axon terminals of live, developing tadpoles. To further validate GFP-synaptobrevin as an in vivo synaptic marker, we examined the distribution of GFP immunoreactivity in GFP-synaptobrevin-labeled RGC axon arbors by electron microscopy. Plasmid lipofection was used for the selective expression of GFP-synaptobrevin by retinal neurons and to target GFP-synaptobrevin exclusively to RGC axons within the brain (see Materials and methods). GFP-synaptobrevin is localized to ultrastructurally identified synapses in the tectal neuropil of stage 45 tadpoles (Fig. 1A-D). GFP immunoreactivity was associated to synaptic vesicles and preferentially localized to presynaptic terminals in morphologically mature retinotectal synapses.

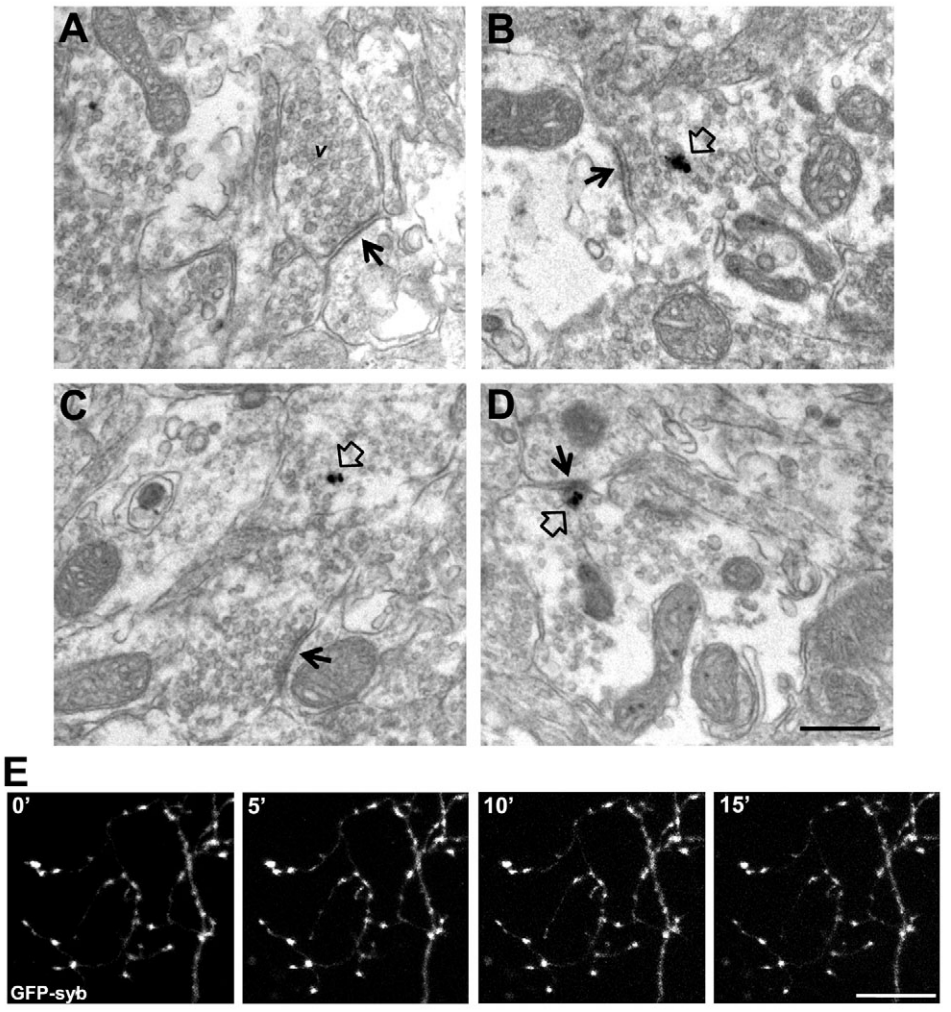

Fig. 1. GFP-synaptobrevin specifically localizes to presynaptic sites in RGC axon terminals. (A-D) The localization of GFP-synaptobrevin was determined by examining the distribution of GFP immunoreactivity by electron microscopy. Morphologically mature synapses (black arrows), containing presynaptic terminals with numerous synaptic vesicles (v) and clearly defined pre- and postsynaptic specializations, are present in the tectal neuropil of stage 45 tadpoles. (B-D) Electron photomicrographs of tadpole brains immunostained with an antibody to GFP show the localization of gold particles (open arrows) to presynaptic terminals in the tectal neuropil. Silver enhancement of the secondary antibody coupled to 1 nm gold particles shows that the GFP immunolabel is preferentially associated to synaptic vesicles in morphologically mature retinotectal synapses (B,D), as well as in presynaptic terminals near contact sites (C). Scale bar: 0.2 μm. (E) Regions of an individual RGC axon arbor imaged at 5 minute intervals illustrate the distribution of GFP-synaptobrevin puncta. The majority of the GFP-synaptobrevin puncta remain constant throughout time. This is in contrast to motile GFP-synaptobrevin puncta present in small transport packets, prevalent in axon terminals of neurons grown in culture (Ahmari et al., 2000). Scale bar: 20 μm.

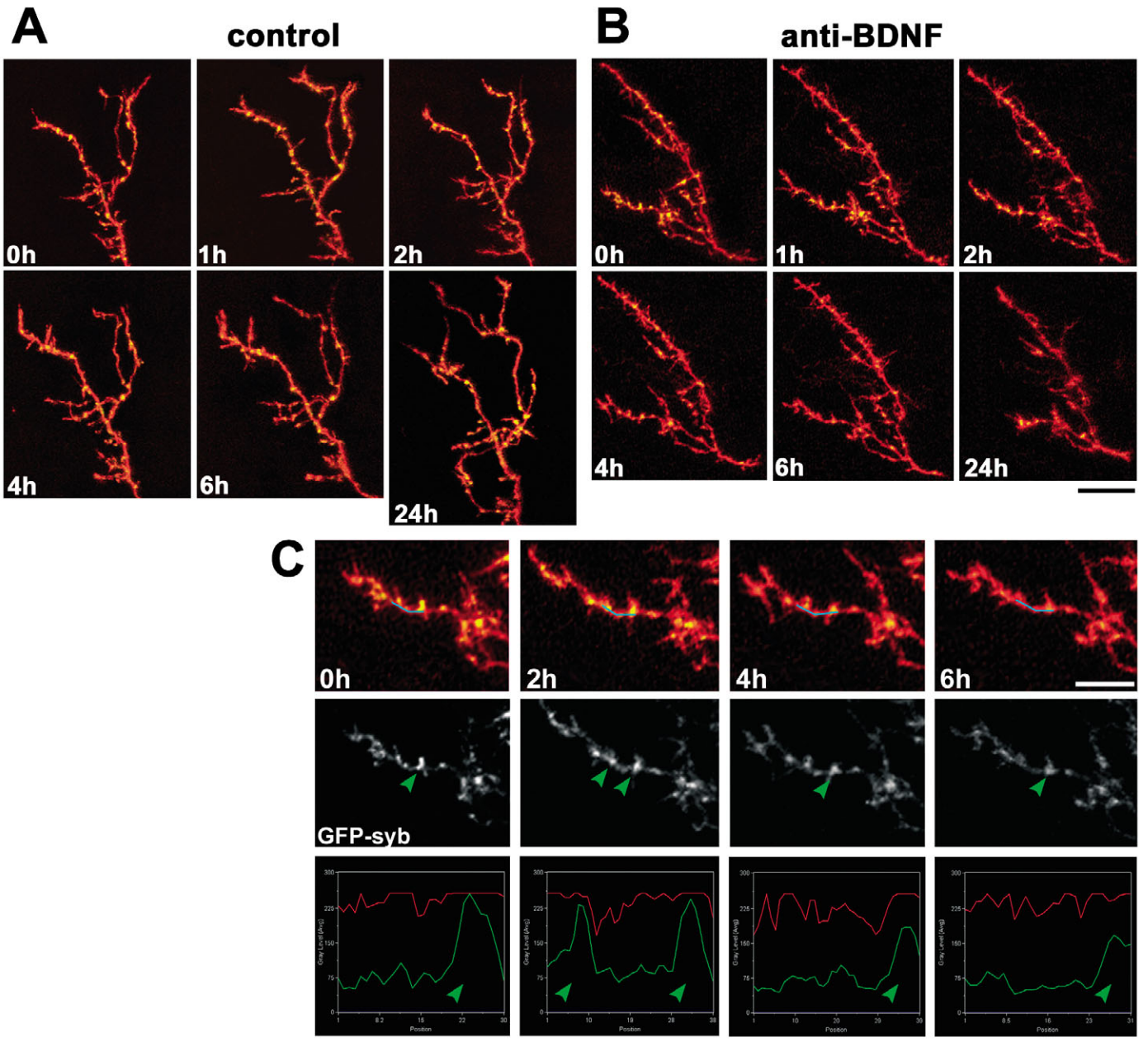

Fig. 2. Neutralizing endogenous BDNF influences presynaptic sites in individual RGC arbors. Time-lapse confocal imaging of individual DsRed2-labeled RGC axons expressing GFP-synaptobrevin illustrates the effects of neutralizing endogenous tectal BDNF on synapse number and axon arbor morphology. (A) Image reconstructions of a RGC axon in a vehicle-treated (control) tadpole show the localization of GFP-synaptobrevin clusters (yellow) within specific regions of the arborizing, DsRed2-labeled axons (red). (B) The number and distribution of GFP-synaptobrevin clusters was significantly altered in RGC arbors in tadpoles that received a single injection of anti-BDNF following the first imaging session (0h). Anti-BDNF not only influences axon arbor complexity but also decreases the number and density of GFP-synaptobrevin clusters per axon arbor. (C) Magnified region of the arbor shown in B illustrates the localization of GFP-synaptobrevin clusters to branch points and branch termini, and their disappearance after anti-BDNF treatment. By separating the green component (middle panel, GFP fluorescence) from the red component (overlay DsRed2 and GFP fluorescence; top panel) one can clearly distinguish specific GFP-synaptobrevin puncta from the background fluorescence signal. The line scans (bottom panels) obtained from raw confocal data show the intensity of the DsRed2 and GFP-synaptobrevin signals at the level of the axon branch demarcated by the light-blue hairlines (top panels). The green arrowheads (middle panels) indicate sites containing GFP-synaptobrevin clusters that are crossed by the line scan. In the 0 and 4 hour images, the proximal part of the line scan (1 pixel width) travels near GFP-synaptobrevin puncta but only crosses the arbor area where background fluorescent signal is observed. Background fluorescence intensity values remain similar after repeated imaging and that fluorescence intensity values of specific GFP-synaptobrevin puncta are at least twice as great as those of background signals. Scale bar: 20 μm in A,B; 10 μm in C. Posterior is upwards, anterior is downwards.

Blocking endogenous BDNF induces GFP-synaptobrevin cluster dismantling and axon branch elimination

Our previous studies show that BDNF influences the morphological maturation of RGC axon arbors primarily by promoting axon arbor growth. Specifically, increasing BDNF tectal levels induces axon branch addition without affecting the degree of stabilization (Cohen-Cory and Fraser, 1995; Cohen-Cory, 1999). Exogenous BDNF, however, can influence axon branch stability under conditions where stability is experimentally altered. For example, BDNF prevents the destabilizing effects that blocking retinal activity exerts on axon branches by maintaining the normal rates of branch addition and elimination (Cohen-Cory, 1999). Thus, to determine directly whether endogenous BDNF participates in presynaptic site stabilization, we imaged RGC axon terminals double labeled with GFP-synaptobrevin and DsRed2 in tadpoles treated with function-blocking antibodies to BDNF. Tectal injection of anti-BDNF induced a rapid decrease in GFP-synaptobrevin labeled synapses in RGC axon arbors examined at 2, 4, 6, 8 and 24 hour time points following treatment (Fig. 2). GFP-synaptobrevin cluster number was significantly decreased 4 hours after anti-BDNF treatment (55.8±11.0% versus 110.3±7.9% in control; $P<0.0005$; Fig. 3A), an effect that was paralleled by a significant decrease in total branch number (84.3±5.8% versus 101.6±5.0% in control; $P<0.03$; Fig. 3B). The decrease in GFP-synaptobrevin cluster and branch number was maintained throughout the observation period (4, 6, 8 and 24 hours; Fig. 2 and Fig. 3A,B). Anti-BDNF not only decreased total GFP-synaptobrevin cluster and branch number, but also significantly decreased GFP-synaptobrevin clusters per axon branch and per unit arbor length (Fig. 3C,D), indicating that endogenous BDNF significantly influences synapse density per axon arbor. Therefore, the effects of increased tectal BDNF on synapse number, branch number and synapse density we have previously reported (Alsina et al., 2001) mirror the actions of endogenous BDNF.

A detailed analysis of GFP-synaptobrevin cluster dynamics was used to further characterize the actions of endogenous BDNF during synapse stabilization (Fig. 4A). A single

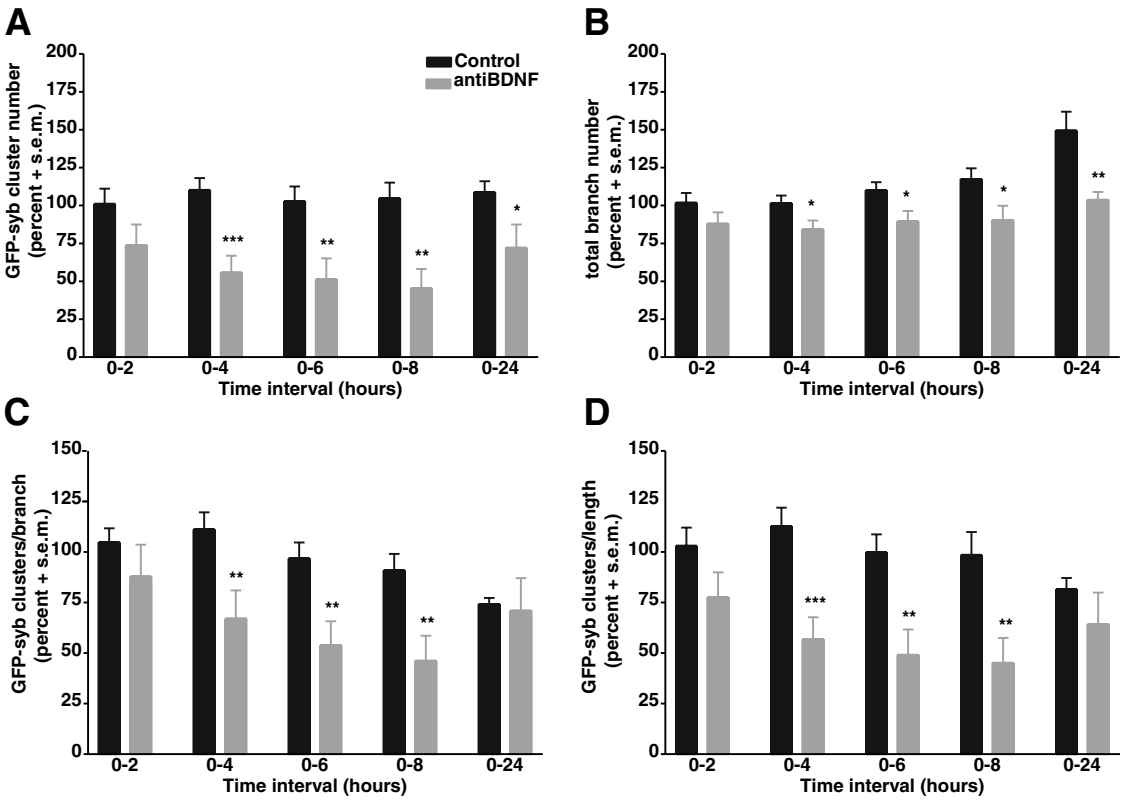

Fig. 3. Anti-BDNF significantly decreases GFP-synaptobrevin cluster number and influences axon arbor complexity. Several morphological parameters illustrate the dynamic changes in GFP-synaptobrevin labeled presynaptic sites and axon arborization in control and anti-BDNF treated tadpoles followed every 2 hours for 8 hours and again at 24 hours. All parameters are expressed as percent change from their initial value at the time of treatment. (A) Total GFP-synaptobrevin cluster number per axon terminal in control- and anti-BDNF-treated tadpoles. Anti-BDNF significantly decreases GFP-synaptobrevin cluster number versus control 4 hours after treatment. (B) The complexity of both control and anti-BDNF-treated arbors is illustrated by the net increase in total branch number per axon terminal. A significant decrease in branch number by 4 hours in anti-BDNF treated tadpoles versus control parallels the decrease in synaptic cluster number. (C,D) A measure of synapse density in both control- and anti-BDNF-treated tadpoles is provided by comparing the increase in GFP-synaptobrevin cluster number with the increase in (C) branch number or (D) total arbor length (expressed as a ratio). In controls, there is a one-to-one relationship in the increase in GFP-synaptobrevin cluster number to arbor length, while in anti-BDNF-treated tadpoles GFP-synaptobrevin cluster density is decreased to 50-60%. This difference is significant from 4 to 8 hours after treatment. Bars indicate mean±s.e.m. $n=14$ axon arbors in control and $n=10$ arbors in anti-BDNF; $*P\leqslant0.05$; $**P\leqslant0.005$; $***P\leqslant0.0005$.

treatment with anti-BDNF significantly reduced the number of GFP-synaptobrevin clusters maintained from one observation interval to the next (stable clusters) throughout the 24 hour imaging period when compared with control, with the number of stable clusters reduced already during the first 2 hours after treatment (Fig. 4B). The number of GFP-synaptobrevin clusters added per RGC axon arbor, however, was not significantly affected at any observation interval (the average number of GFP-synaptobrevin clusters added every 2 hours was 38.12±7.3% in anti-BDNF versus 36.7±3.42% in controls; in tadpoles treated with recombinant BDNF, 57.9±7.5% new clusters were added every 2 hours, data not shown). Thus, these in vivo imaging studies revealed that presynaptic sites are rapidly destabilized and eliminated in the absence of BDNF. Our in vivo imaging studies also revealed that a decrease in the number of stable branches parallels the decrease in stable GFP-synaptobrevin clusters during the first 6 hours after anti-BDNF treatment (0-2, 2-4 and 4-6 hour intervals only; Fig. 4C). This resulted in a cumulative branch elimination effect: more branches were eliminated and less stabilized over time. Together, these results indicate that endogenous BDNF simultaneously modulates presynaptic site and axon branch stabilization.

Analysis of GFP-synaptobrevin cluster and branch elimination also revealed that most of the branches eliminated did not contain GFP-synaptobrevin clusters prior to their

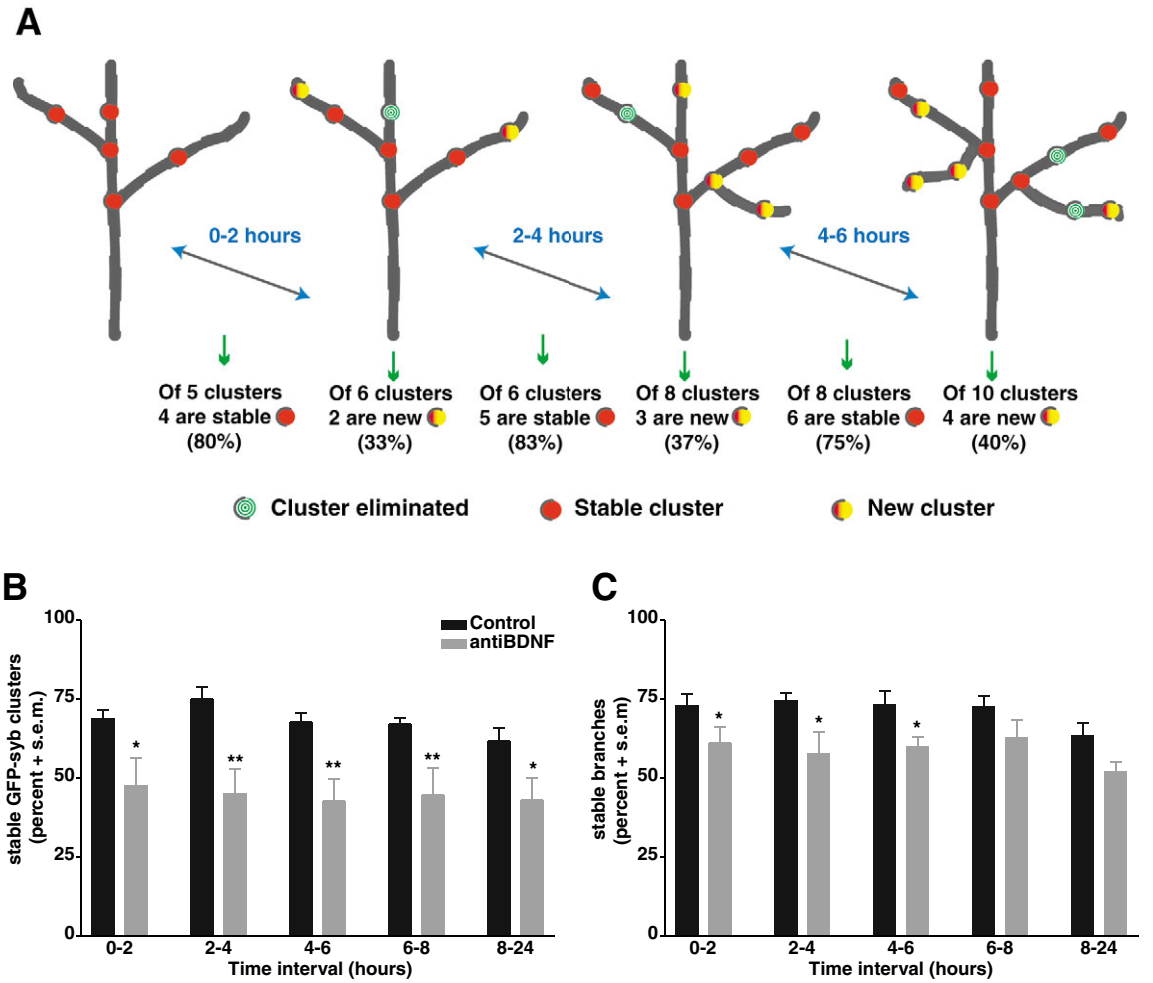

Fig. 4. Anti-BDNF rapidly influences presynaptic site and axon branch stability. (A) Diagrammatic representation of GFP-synaptobrevin cluster dynamics and arbor growth. The number of GFP-synaptobrevin clusters stabilized and eliminated, and the number of new GFP-synaptobrevin clusters added between observation intervals was calculated and normalized for each time interval to obtain a dynamic measure of synapse addition and stabilization over time. As new GFP-synaptobrevin clusters are added, the absolute number of clusters that are stabilized increases, but as a proportion it remains relatively constant. The hypothetical axon depicted here exhibits rates of synapse stabilization that are slightly higher than those observed for RGC axon arbors in vehicle-treated tadpoles (control). (B) Detailed analysis of the number and distribution of GFP-synaptobrevin clusters per axon branch, and of the lifetimes of individual GFP-synaptobrevin clusters for every observation period reveal the effects of neutralizing endogenous BDNF on synapse stabilization. Anti-BDNF significantly reduced the stability of GFP-synaptobrevin clusters by 2 hours (0-2 hours), an effect that was maintained through every observation period. (C) Analysis of the number of axon branches that are retained or eliminated from one observation interval to the next provides a measure of the effects of anti-BDNF on axon branch stability. Axon branches are significantly destabilized and eliminated 0-2 hours after treatment and this effect is maintained for the first 6 hours following treatment. On average, 60.2±2.6% of branches are stable every 2 hours in anti-BDNF treated arbors versus 73.3±1.6% in controls. $*P \leq 0.05$; $**P \leq 0.005$.

Table 1. Average number of branches eliminated that did or did not exhibit GFP-synaptobrevin clusters prior to their retraction

	Branches eliminated with clusters	Branches eliminated without clusters	Branches eliminated of total branches
Control	13.78±1.06%	13.36±1.59%	27.14±1.68% (n=52)
Anti-BDNF	15.16±1.67%	26.02±2.29%*	41.17±2.49%* (n=37)

*Significantly different from control by Fisher's exact and chi-square tests ($P \leqslant 0.05$).

retraction (Table 1), suggesting that presynaptic site stabilization and axon branch stabilization may be related. Our previous observations support a link between presynaptic site stabilization and axon branch formation (Alsina et al., 2001) (see also Fig. 5A). Time-lapse imaging demonstrates that new branch extension occurs preferentially at RGC arbor sites where GFP-synaptobrevin clusters localize (Fig. 5A). By contrast, in anti-BDNF treated tadpoles, GFP-synaptobrevin cluster dismantling preceded axon branch retraction in a significant fraction of the eliminated branches (31.5%, n=11 axons; Fig. 5B,C). For the rest of the eliminated branches, GFP-synaptobrevin cluster dismantling and branch retraction occurred simultaneously or sequentially within the 2 hour period between observations. The observation that some branches retracted as far back as the site where a GFP-synaptobrevin cluster localized suggests that axon branch pruning may also be associated to presynaptic site elimination, at least when endogenous BDNF levels are altered. In a few instances, however, axon branch retraction did not immediately follow the disappearance of a GFP-synaptobrevin cluster, at least during the period of observation (see APV data below). Thus, although our observations suggest that presynaptic site

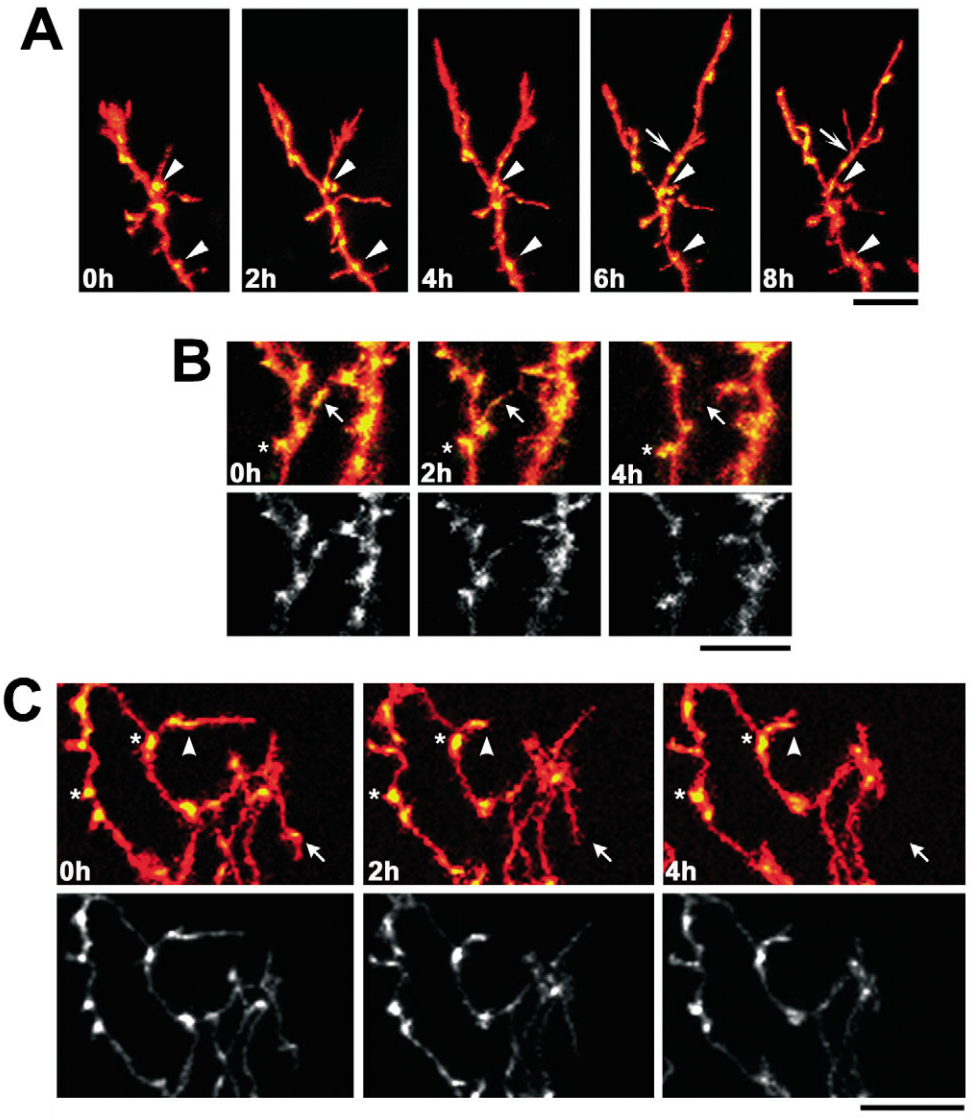

Fig. 5. Distribution and dynamics of GFP-synaptobrevin labeled presynaptic sites along RGC axon terminals. (A) Time-lapse sequence of a region of a control arbor illustrates the dynamic relationship between presynaptic site location and axon branch formation. New axonal branches originate from sites rich in GFP-synaptobrevin puncta (arrowheads), while new GFP-synaptobrevin clusters appear along an axon branch (Alsina et al., 2001). (B) Magnified region of an arbor illustrates the localization of GFP-synaptobrevin puncta to a nascent branch (arrows) in a DsRed2 labeled axon and its disappearance after anti-BDNF treatment (overlay, top panel; GFP-synaptobrevin fluorescence only, bottom panel). In some branches, GFP-synaptobrevin cluster dismantling precedes axon branch elimination (arrow), as indicated by the significant decrease in GFP fluorescence at the 2 hour time point. (C) Time-lapse sequence of a region of an anti-BDNF treated axon arbor shows the disappearance of GFP-synaptobrevin clusters and the retraction of an axon branch (arrow). The arrowhead indicates a site where a decrease in punctuate GFP-synaptobrevin fluorescence correlates with the shortening of the distal region of the axon branch. Asterisks indicate arbor sites with stable GFP-synaptobrevin clusters. Scale bars: 20 μm in A; 10 μm in B,C. Posterior is upwards, anterior is downwards.

elimination influences axon branch elimination, a causal relationship could not be inferred.

BDNF rescues presynaptic specializations that are destabilized by altering tectal NMDA receptor transmission

In the developing retinotectal system, N-methyl-D-aspartate receptor (NMDAR)-mediated synaptic transmission has been hypothesized to influence axon arbor morphology and the stability of synapses (Debski and Cline, 2002; Yen et al., 1995). Thus, to test whether exogenous BDNF can also stabilize presynaptic sites in tadpoles deprived of normal patterns of synaptic activity, we experimentally increased the probability of axon branch destabilization by altering NMDAR transmission in the tadpole optic tectum. Tectal microinjection of APV (50 μM) resulted in rapid (less than 2 hours) and significant GFP-synaptobrevin cluster dismantling in the individual arbors (Fig. 6). A single injection of APV significantly reduced total GFP-synaptobrevin cluster number (40.5±7.0% of initial value after 2 hours of treatment versus 101.1±9.9% in control, $P<0.0002$; Fig. 7A). APV, however, did not influence total axon branch number at 2, 4, 6 or 8 hours (89.2±7.6% versus 101.9±6.4% in control at 2 hours, $P>0.05$; Fig. 7B), but had a moderate effect by 24 hours (Fig. 7B). Synaptic cluster density, expressed as the number of GFP-synaptobrevin clusters per unit arbor length, was therefore significantly decreased throughout the 24-hour observation period (Fig. 7C). Similarly, MK801 (an open-state NMDAR channel blocker) decreased GFP-synaptobrevin cluster number and density in RGC axon arbor terminals without influencing branch number or length (Fig. 7A-C). Thus, blocking NMDAR transmission in the optic tectum significantly affected presynaptic specializations on RGC axon arbors without altering their morphology.

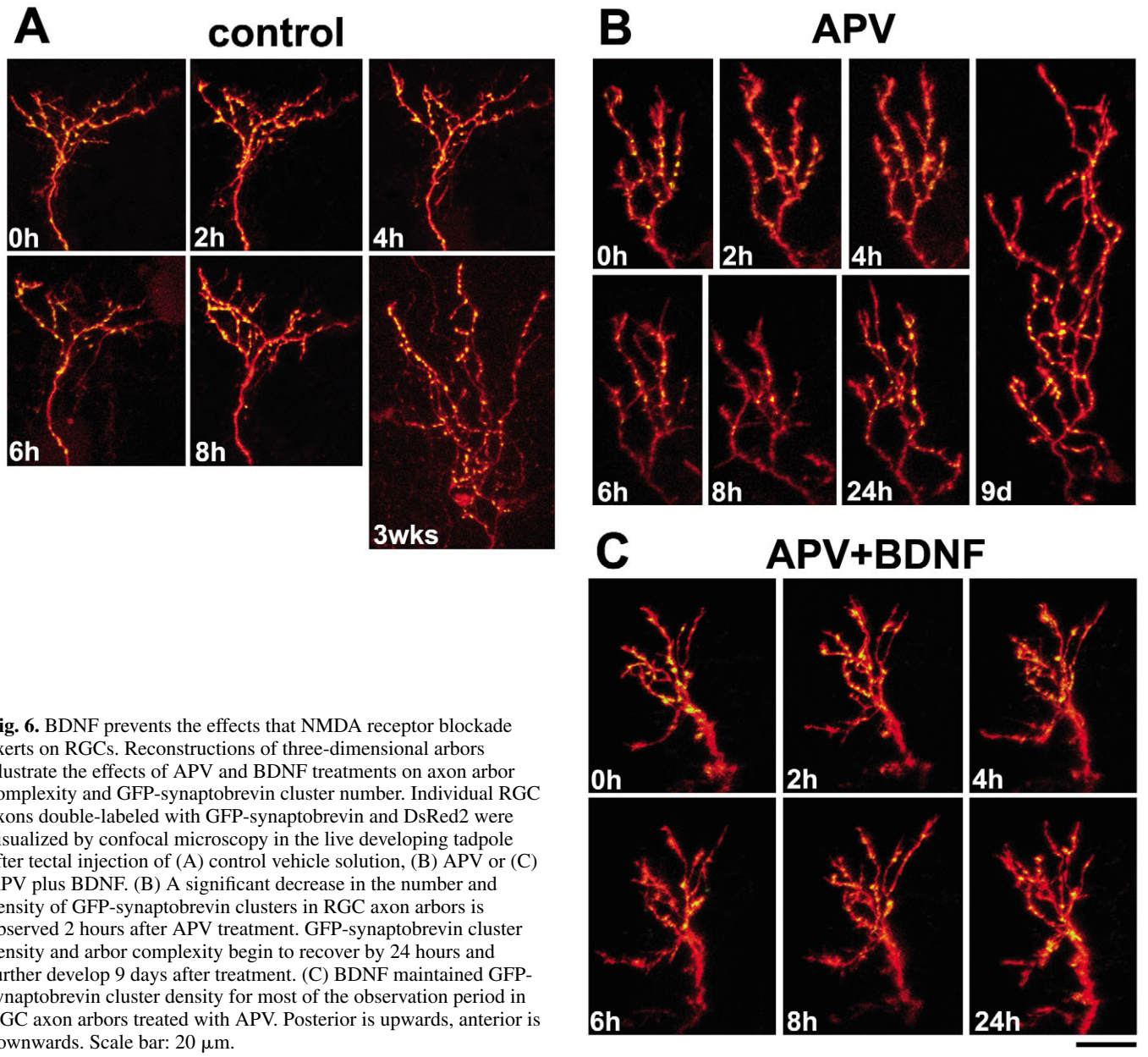

Fig. 6. BDNF prevents the effects that NMDA receptor blockade exerts on RGCs. Reconstructions of three-dimensional arbors illustrate the effects of APV and BDNF treatments on axon arbor complexity and GFP-synaptobrevin cluster number. Individual RGC axons double-labeled with GFP-synaptobrevin and DsRed2 were visualized by confocal microscopy in the live developing tadpole after tectal injection of (A) control vehicle solution, (B) APV or (C) APV plus BDNF. (B) A significant decrease in the number and density of GFP-synaptobrevin clusters in RGC axon arbors is observed 2 hours after APV treatment. GFP-synaptobrevin cluster density and arbor complexity begin to recover by 24 hours and further develop 9 days after treatment. (C) BDNF maintained GFP-synaptobrevin cluster density for most of the observation period in RGC axon arbors treated with APV. Posterior is upwards, anterior is downwards. Scale bar: 20 μm.

Simultaneous treatment with BDNF and APV significantly attenuated the effects of blocking NMDAR activity (Fig. 6C and Fig. 8A). Total GFP-synaptobrevin cluster number in tadpoles treated with APV+BDNF was significantly different from that in tadpoles receiving a single injection of APV (86.7±12.7% in APV+BDNF versus 40.5±7.0% in APV alone 2 hours after treatment, $P<0.006$; Fig. 8A). Similarly, GFP-synaptobrevin cluster density per axon arbor was higher in tadpoles treated with APV+BDNF versus those treated with APV alone (80.2±9.0% GFP-synaptobrevin clusters/unit arbor length in APV+BDNF versus 43.2±7.1% in APV alone 2 hours after treatment, $P<0.005$; Fig. 8C). Total axon branch number was not affected by the APV or APV+BDNF treatments (Fig. 8B), suggesting that synapse elaboration (Alsina et al., 2001) and/or normal patterns of neuronal activity (Cohen-Cory, 1999) are needed for BDNF to significantly influence axon branch extension. Together, these results demonstrate that BDNF can prevent the effects of blocking NMDAR on RGC axon arbors.

Detailed analysis of GFP-synaptobrevin cluster dynamics revealed that APV treatment induced a rapid but transient (lasting from 0 to 4 hours) decrease in stable GFP-synaptobrevin clusters that was accompanied by a significant increase in cluster elimination (Fig. 9A). BDNF rescued GFP-synaptobrevin clusters by stabilizing them, preventing their elimination. More than 50% (51±5.9%) of the GFP-synaptobrevin clusters were stable in axons of APV+BDNF-treated tadpoles, while only 32.8±5.3% of clusters were stable in tadpoles treated with APV alone (in controls 68.8±2.7% of clusters are stable Fig. 9A). In addition, BDNF maintained the rate of GFP-synaptobrevin cluster addition at control levels, while APV induced a rapid and significant decrease in cluster addition during the first 2 hours (35.26±9.4% in APV+BDNF versus 12.23±3.01% in APV alone, $P<0.05$; Fig. 9B). Thus, our analysis of GFP-synaptobrevin cluster dynamics reveals that BDNF significantly reduced the effects of APV when APV was most active. The acute, APV-elicited decrease in GFP-synaptobrevin cluster addition was followed by a sharp

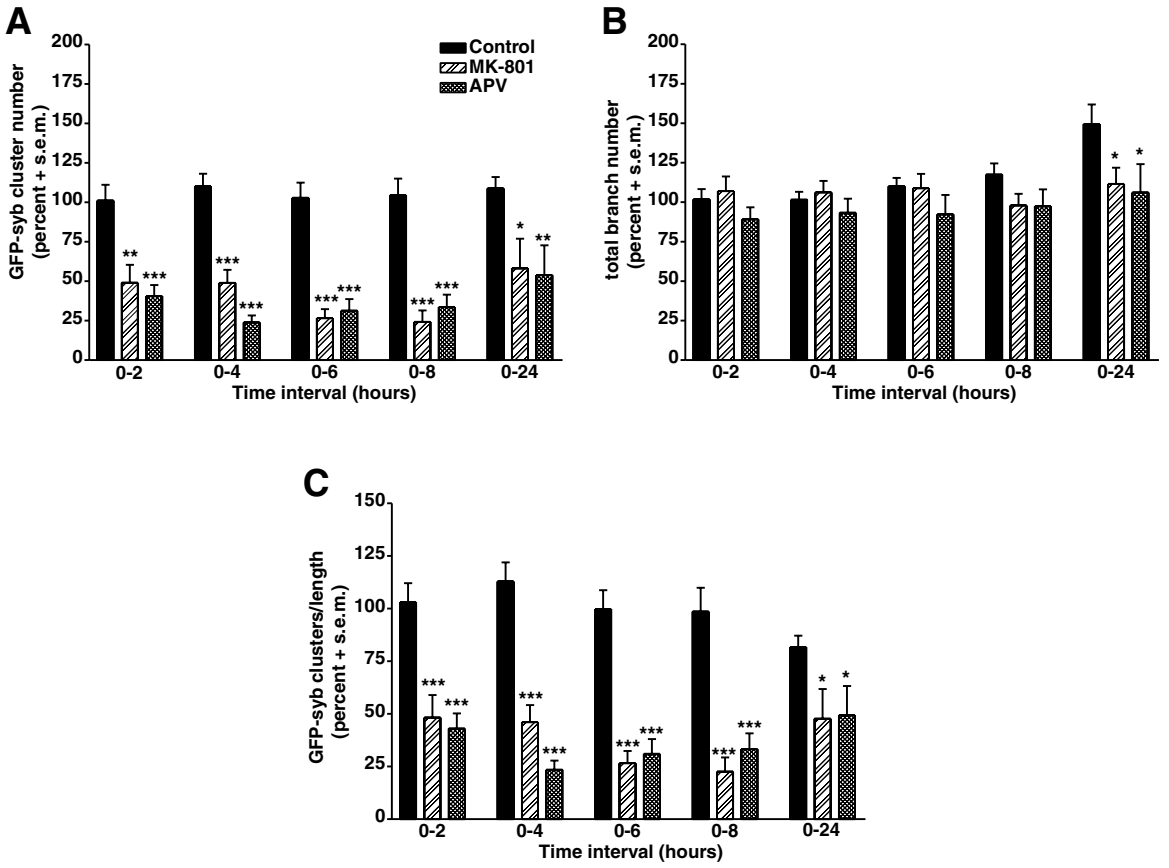

Fig. 7. NMDA receptor blockade specifically influences presynaptic sites without altering RGC axon arbor complexity. The effects of altering NMDAR transmission in the optic tectum on GFP-synaptobrevin cluster number and axon branching in tadpoles that received single tectal injections of APV or MK801 is shown as the percent change from their initial value at the time of treatment. (A) Both APV and MK801 significantly decreased GFP-synaptobrevin cluster number versus control 2 hours after treatment. The peak cumulative effects of APV on GFP-synaptobrevin cluster number occur 4 hours after treatment (0-4 hours), while the MK801 cumulative effects peak 6 hours after treatment (0-6 hours). (B) RGC axon arbor complexity, expressed as the increase in total branch number per axon terminal, is affected by the APV and MK801 treatments by 24 hours only. (C) A measure of synapse density is provided by comparing the change in GFP-synaptobrevin cluster number with the change in total arbor length from the initial observation. In controls, there is a one-to-one relationship in the increase in GFP-synaptobrevin cluster number to arbor length, while in APV and MK801-treated tadpoles GFP-synaptobrevin cluster density is significantly decreased to 50% or below. This difference is significant for all observation time points. $n=14$ axon arbors in control, $n=10$ in APV and $n=12$ in MK801. *$P\leqslant0.05$; **$P\leqslant0.005$; ***$P\leqslant0.0005$.

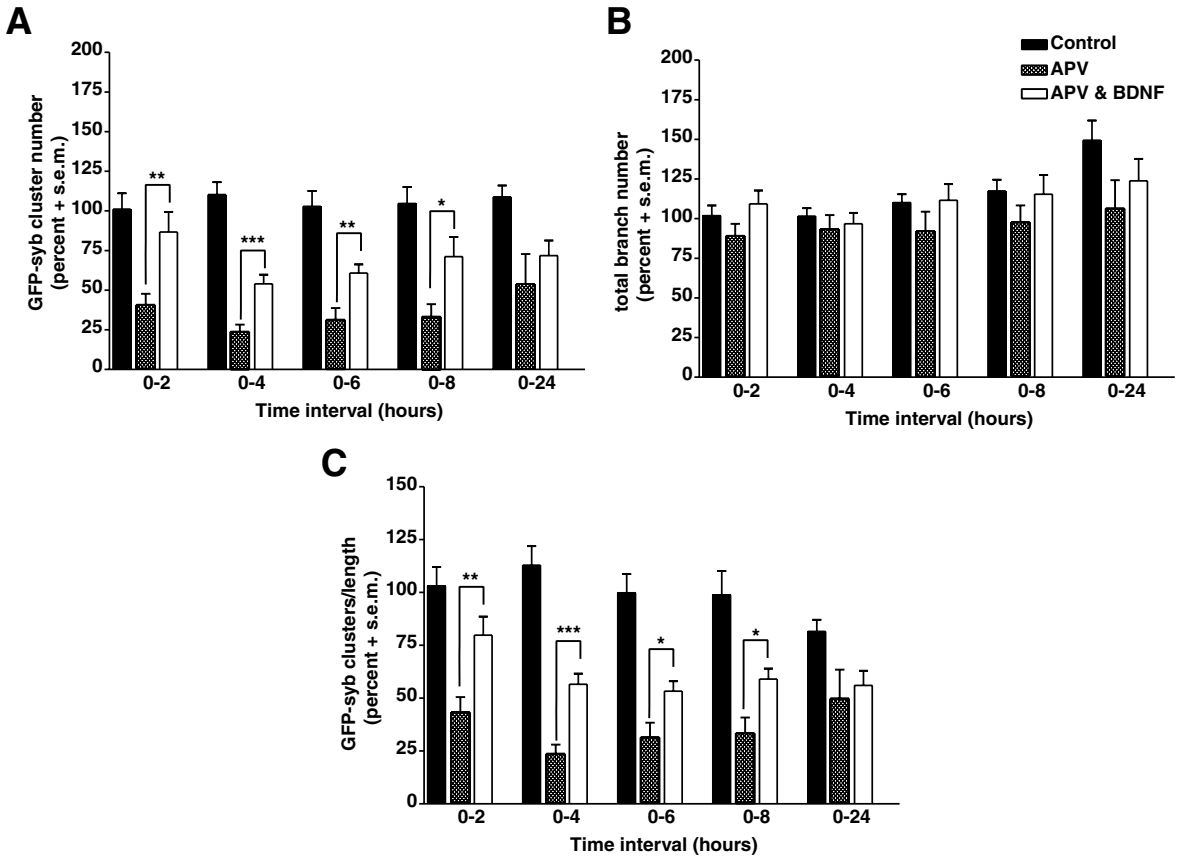

Fig. 8. BDNF maintains the number and density of presynaptic specializations in RGC axon terminals affected by NMDA receptor blockade. (A) APV significantly decreased GFP-synaptobrevin cluster number versus control 2 hours after treatment. Co-injection of APV and BDNF rescued GFP-synaptobrevin labeled presynaptic sites, significantly reducing the effects of APV on GFP-synaptobrevin cluster number for a period of 8 hours. (B) Total branch number was not affected by APV+ BDNF. (C) GFP-synaptobrevin cluster density was significantly decreased in the APV-treated tadpoles. BDNF maintained GFP-synaptobrevin cluster density in RGC axons co-treated with APV. The asterisks indicate significance between APV alone and APV+BDNF. (A,C) APV+BDNF is significantly different from control at the 0-4, 0-6, 0-8, 0-24 time intervals; *$P \leqslant 0.05$; **$P \leqslant 0.005$; ***$P \leqslant 0.0005$. n=14 axon arbors in control, n=10 in APV, n=12 in MK801 and n=11 in APV+BDNF.

increase in the addition of new clusters between 4 and 6 hours after treatment, indicating that axons swiftly recover from the acute APV-induced synapse loss. Indeed, the number and density of GFP-synaptobrevin clusters and the complexity of axon arbors that received a single APV treatment were comparable with that of controls when imaged more than 1 week after treatment (Fig. 6). Morphologically, RGC axons in APV or APV+BDNF-treated tadpoles were indistinguishable from controls after treatment (total branch number and arbor length; Fig. 8B); only a small and non-significant effect on the elimination of filopodial-like, short branches was observed during the first 2 hours after APV treatment (data not shown). Thus, blocking NMDAR neurotransmission in the optic tectum induces a rapid decrease in the number of GFP-labeled presynaptic sites without equivalently influencing axon branch dynamics. Taken together, our findings demonstrate significant synapse destabilization following NMDAR blockade, and further demonstrate that BDNF can influence the synaptic complexity of axon arbors not only by enhancing synapse formation but also by stabilizing synapses, even in the absence of NMDAR activity.

Discussion

Numerous studies implicate BDNF in the modulation of synapse structure and function. Observations that *Trkb* mutant mice have fewer synapses and simpler axon arbors have supported a role for BDNF during synaptogenesis (Causing et al., 1997; Martinez et al., 1998; Rico et al., 2002). Evidence that TrkB signaling is necessary for neurotransmitter receptor maintenance suggest that BDNF may stabilize synapses or synaptic components (Gonzalez et al., 1999). That BDNF is required not only for the formation (Horch and Katz, 2002; McAllister et al., 1995; Wirth et al., 2003) but also for the maintenance of dendritic structure (Gorski et al., 2003) further supports a role for BDNF in synapse stabilization. However, studies that examine the long-term effects of manipulating BDNF or TrkB expression in mammalian embryos or in slice cultures cannot directly differentiate between synapse formation and synapse stabilization. In vitro studies have provided more direct proof that neurotrophins are needed for synapse maintenance, as synaptic efficacy and the number of FM4-64-identified synapses are concurrently reduced by alterations in TrkB signaling (Klau et al., 2001). Our study distinguishes between long-term and acute effects of BDNF on

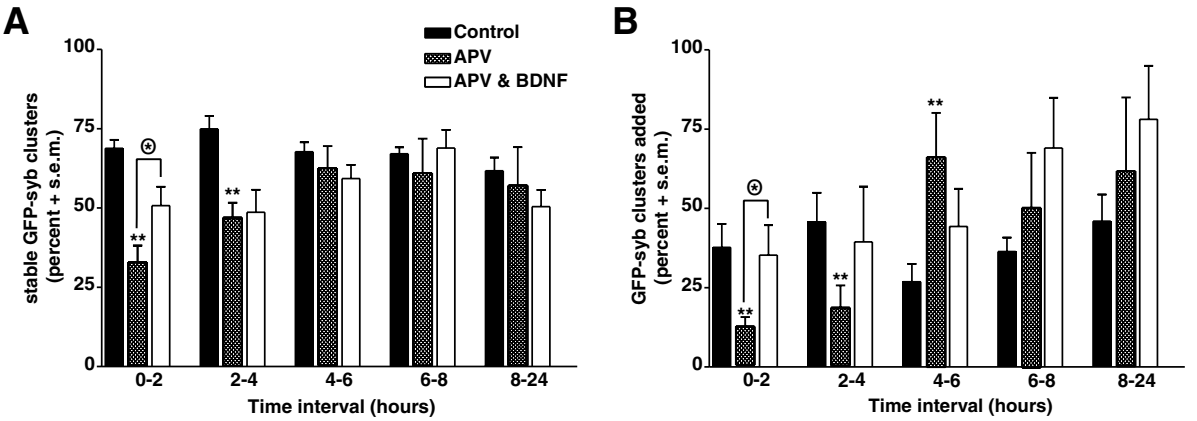

Fig. 9. BDNF prevents the acute effects of NMDAR blockade by maintaining GFP-synaptobrevin cluster addition and stabilization rates. (A,B) Detailed analyses of the number and distribution of GFP-synaptobrevin clusters per axon branch and of the lifetimes of individual presynaptic clusters for every observation period reveal the effects of BDNF on synapse dynamics following NMDAR blockade. APV significantly decreased the stability (A) of GFP-synaptobrevin clusters during the first 4 hours after treatment (0-2 and 2-4 hour observation periods). The proportion of GFP-synaptobrevin clusters that are stabilized in the presence of APV was significantly influenced by BDNF (APV+BDNF) during the first 2 hours of treatment. (B) A significant decrease in GFP-synaptobrevin cluster addition (0-2 and 2-4 hours) preceded an increase in cluster addition following APV treatment. BDNF rescued GFP-synaptobrevin cluster number by maintaining the rate of GFP-synaptobrevin cluster addition. GFP-synaptobrevin cluster dynamics is presented for APV-treated tadpoles, and was similar for axons in MK-801-treated tadpoles. The circled asterisks indicate significant differences between APV alone and APV+BDNF ($P \leqslant 0.05$). Double asterisks indicate significant differences between control and APV ($P \leqslant 0.005$). (A) APV+BDNF was significantly different from control at 0-2 and 2-4 hours only.

synapses in vivo, allowing the visualization of these events in the environment normally experienced by a developing neuron. The ability to visualize presynaptic sites within individual RGC axons in vivo has allowed us to follow dynamic changes in localization of candidate synapses within the arbor structure, and to differentiate between synapse formation and stabilization. Neutralization of endogenous BDNF produced very rapid effects, reducing GFP-synaptobrevin cluster stabilization within 2 hours. By manipulating synaptic activity in the optic tectum we have further demonstrated that BDNF can rapidly rescue synapses. A single injection of BDNF reversed the acute effects of NMDAR blockade, either with APV or MK801, on GFP-synaptobrevin-labeled presynaptic sites.

An important observation obtained from our previous studies is that a high proportion of branches destined to be eliminated during the active remodeling of axonal arbors lacked GFP-synaptobrevin clusters (Alsina et al., 2001). Microinjection of specific BDNF blocking antibodies into the tectum allowed us to directly examine the relationship between branch elimination and synapse elimination and to test whether endogenous BDNF stabilizes candidate synapses. When endogenous BDNF was neutralized in vivo, a high proportion of the GFP-synaptobrevin labeled presynaptic specializations were dismantled, an effect that was associated with significant axon branch elimination. Further, anti-BDNF reduced GFP-synaptobrevin cluster density to less than 50% 8 hours after treatment. The effects of neutralizing endogenous tectal BDNF on synapse density described here complement the effects of increasing tectal BDNF levels on RGC axon arbors: recombinant BDNF increases GFP-synaptobrevin cluster density by 50%, with a similar time course (Alsina et al., 2001). Our analysis of GFP-synaptobrevin cluster dynamics indicates, however, that BDNF may preferentially influence a subset of synapses, as the two types of alterations in BDNF levels do not exert reciprocal effects. Increasing tectal BDNF with recombinant BDNF induces new candidate synapses to be formed and new branches to be extended, thus promoting arbor growth (Alsina et al., 2001). BDNF stabilized the newly formed synapses but did not influence the subset of synapses that are normally remodeled because the fraction of GFP-synaptobrevin labeled presynaptic sites and branches that are normally eliminated remained the same (B. Alsina and S.C.C., unpublished) (see Cohen-Cory and Fraser, 1995; Cohen-Cory, 1999). However, blocking endogenous tectal BDNF affected a fraction of already stable GFP-synaptobrevin clusters, resulting in significant synapse and axon branch elimination. The number of GFP-synaptobrevin clusters that are normally added as part of the remodeling process remained the same when endogenous BDNF levels were lowered. Therefore, our observations confirm a role for BDNF in modulating synaptogenesis and further demonstrate a dual function for BDNF during the stabilization of both synapses and axon branches.

Synapse elimination is a necessary step during the remodeling of neuronal connectivity (Goda and Davis, 2003). In most instances, the disassembly of previously functional synapses can be correlated with presynaptic input elimination, as demonstrated for the NMJ (Eaton et al., 2002; Sanes and Lichtman, 2001; Walsh and Lichtman, 2003). Synapse disassembly can also occur as a mechanism that modulates the strength of connectivity between two neurons without influencing arbor morphology (Goda and Davis, 2003). An NMDAR mechanism that mediates synapse strengthening has been hypothesized to influence axon branch and synapse dynamics in the developing retinotectal system (Debski and Cline, 2002). Evidence that RGC axon branch stabilization relates to the stabilization of structural synapses, however, was

missing. The present study supports the idea that synapse disassembly can occur without input elimination (Buffelli et al., 2003; Hata et al., 1999; Hopf et al., 2002; Goda and Davis, 2003). Our findings demonstrate that synaptic changes induced by acute widespread alterations in NMDAR-mediated synaptic transmission in the optic tectum influence presynaptic site stability, but are not sufficient to destabilize and eliminate RGC axonal branches. The disappearance of GFP-synaptobrevin clusters in response to NMDAR blockers may reflect an increase in synaptic vesicle dispersion or recycling (Bacci et al., 2001), rather than complete removal of the synapse. It is noteworthy that endogenous BDNF simultaneously and independently influences the stability of both synapses and axon branches, and that BDNF can contribute to synapse and branch stability even when neuronal activity is altered (Cohen-Cory, 1999).

Neuronal activity and neurotrophins interact to modulate neuronal structure and function (Vicario-Abejon et al., 2002). Neuronal activity regulates gene transcription, transport and secretion of BDNF protein (Righi et al., 2000; Chytrova and Johnson, 2004; Lessmann et al., 2003). TrkB receptor trafficking to the membrane (Du et al., 2000; Meyer-Franke et al., 1998), and BDNF-TrkB receptor complex internalization (Lu, 2003) also depend on neuronal activity. Even though BDNF signaling is tightly modulated by neuronal activity and it is believed that neurotrophins preferentially modulate active synapses (Lu, 2003), BDNF can also influence synapses and neuronal connectivity independently of whether neurons are synaptically active (Cohen-Cory, 1999; Collin et al., 2001). The observation that BDNF can act rapidly to maintain candidate synapses for a significant time period following NMDAR blockade supports a role for BDNF independent of target NMDAR activation and has important implications for understanding the function of BDNF and potential therapeutic properties. BDNF exerted robust and rapid effects on synapses following APV treatment, although a single dose of BDNF was not sufficient to maintain GFP-synaptobrevin cluster number at control levels for a prolonged period of time. The rapid effects of BDNF observed are consistent with the localized, rapid changes in Ca^{2+} signaling that BDNF elicits on axon terminals (Zhang and Poo, 2002) (B.H. and S.C.C., unpublished), and with rapid, depolarizing effects of BDNF on cultured neurons (Kafitz et al., 1999). The inability of BDNF to rescue synapses back to control levels for a prolonged period of time, however, may be due to differences in potencies or pharmacokinetics between APV and BDNF, or to rapid TrkB receptor downregulation following NMDAR blockade (Kingsbury et al., 2003).

Correlated synaptic activity is thought to modulate retinotectal map refinement by regulating presynaptic axon branch dynamics (Debski and Cline, 2002). Pharmacological manipulations that alter neuronal activity demonstrate that the stability of RGC axon arbors depends on activity. For example, presynaptic activity blockade by intraocular injection of TTX influences RGC axon branch stabilization by increasing the rates of branch addition and elimination, influencing arbor structure by 24 hours (Cohen-Cory, 1999). Chronic NMDAR blockade in whole tadpoles decreases RGC axon branch lifetimes but only transiently (Rajan et al., 1999; Ruthazer et al., 2003). Our observations that acute tectal administration of APV and MK801 does not significantly influence RGC axon branching suggest that differences in acute versus chronic effects of the inhibitors (and/or that relative contributions of pre- and postsynaptic activity to axon branch stabilization) may be responsible for the differential influences of activity blockade on synapse and axon branch stabilization. In tadpoles with doubly innervated tecta, axon branches with synchronized activity are selectively stabilized through a NMDAR-dependent process (Ruthazer et al., 2003). Because BDNF modulates RGC responses to altered activity levels by stabilizing synapses, it is possible that BDNF may actively participate in selective synapse and axon branch stabilization in territories where input activity is correlated.

An important question that remains is whether the structural, GFP-synaptobrevin identified synapses that are stabilized by BDNF are physiologically active (Ahmari and Smith, 2002). BDNF can potentiate developing synapses in spatially localized (Zhang and Poo, 2002) and temporally restricted (Kafitz et al., 1999) manners. Structural modifications at synapses, moreover, correlate with activation of synaptic responses by neurotrophins (Vicario-Abejon et al., 2002). For example, the number of docked synaptic vesicles and synaptic vesicle distribution are altered in BDNF-deficient mice, an ultrastructural defect that correlates with altered presynaptic function (Carter et al., 2002). Conversely, an increase in the number of docked synaptic vesicles correlates with the activation of synaptic responses elicited by neurotrophins in young cultured hippocampal neurons (Collin et al., 2001). In this regard, loss of presynaptic function has been correlated with the removal of synaptic vesicles and synaptic vesicle components from individual synaptic sites (Hopf et al., 2002). Although we cannot rule out the possibility that the effects that we observed relate to the redistribution of GFP-labeled synaptic vesicles or synaptic vesicle components (as BDNF can regulate the mobilization of vesicles from a reserve pool to a docked synaptic pool) (Carter et al., 2002; Collin et al., 2001; Pozzo-Miller et al., 1999; Vicario-Abejon et al., 2002), the structural modifications of synapses that we observed may represent, or eventually lead to, alterations in synaptic function (Du and Poo, 2004). Our experiments demonstrating that a significant portion of GFP-synaptobrevin clusters is eliminated following MK801 treatment suggest that active synapses are involved in a BDNF response, as MK801 selectively blocks open NMDAR channels. The localization of GFP-synaptobrevin to mature ultrastructurally identified RGC synapses and the activity-dependent recycling of GFP-labeled presynaptic sites, as determined by FM4-64 co-staining of GFP-synaptobrevin puncta (Alsina et al., 2001), also suggests that GFP-synaptobrevin localizes to functional synapses.

How does BDNF influence axon arbor complexity and synapse number? While the direct signaling mechanisms that modulate these two processes remain to be elucidated, it is likely that BDNF signaling promotes changes in actin polymerization and the reorganization of the actin cytoskeleton at synapses. Actin polymerization and microtubule dynamics are necessary for growth cone steering and axon branching (Dent et al., 2004; Kornack and Giger, 2005). BDNF regulates growth cone motility and filopodial dynamics by modulating F-actin stabilization and polymerization through a Rho GTPase-dependent pathway (Gehler et al., 2004; Yuan et al., 2003). F-actin is enriched at synapses and the integrity of the actin cytoskeleton at pre- or postsynaptic terminals can also

directly influence the stability of developing synapses (Dillon and Goda, 2005; Zhang and Benson, 2001). It is therefore possible that common signaling pathways that influence cytoskeletal dynamics at both synapses and axon branches may be used by BDNF. The identification and characterization of BDNF signaling events that coordinate synapse formation and axon branching remain.

In conclusion, our imaging studies provide a direct link between the cellular and molecular mechanisms underlying synaptogenesis in vivo and reveal BDNF as a modulator of multiple aspects of synaptogenesis, from synapse formation to stabilization. The selective disassembly of presynaptic specializations in RGC axon arbors correlates with axon branch pruning when BDNF is withdrawn, but not when overall synaptic activity is decreased. Thus, structural rearrangements in RGC synaptic connectivity are modulated by BDNF, where BDNF influences the morphological maturation of axonal arbors and their stabilization, by a mechanism that influences both synapses and axon branches.

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