Light-Induced Calcium Influx Into Retinal Axons Is Regulated by Presynaptic Nicotinic Acetylcholine Receptor Activity In Vivo

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Edwards, James A. and Hollis T. Cline. Light-induced calcium influx into retinal axons is regulated by presynaptic nicotinic acetylcholine receptor activity in vivo. J. Neurophysiol. 81: 895–907, 1999. Visual activity is thought to be a critical factor in controlling the development of central retinal projections. Neuronal activity increases cytosolic calcium, which was hypothesized to regulate process outgrowth in neurons. We performed an in vivo imaging study in the retinotectal system of albino Xenopus laevis tadpoles with the fluorescent calcium indicator calcium green 1 dextran (CaGD) to test the role of calcium in regulating axon arbor development. We find that visual stimuli to the retina increased CaGD fluorescence intensity in retinal ganglion cell (RGC) axon arbors within the optic tectum and that branch additions to retinotectal axon arbors correlated with a local rise in calcium in the parent branch. We find three types of responses to visual stimulus, which roughly correlate with the ON, OFF, and SUSTAINED response types of RGC reported by physiological criteria. Imaging in bandscan mode indicated that patterns of calcium transients were nonuniform throughout the axons. We tested whether the increase in calcium in the retinotectal axons required synaptic activity in the retina; intraocular application of tetrodotoxin (10 μM) or nifedipine (1 and 10 μM) blocked the stimulus-induced increase in RGC axonal fluorescence. A second series of pharmacological investigations was designed to determine the mechanism of the calcium elevation in the axon terminals within the optic tectum. Injection of bis-(o-aminophenoxy)-N,N,N′,N′-tetraacetic acid-AM (BAPTA-AM) (20 mM) into the tectal ventricle reduced axonal calcium levels, supporting the idea that visual stimulation increases axonal calcium. Injection of BAPTA (20 mM) into the tectal ventricle to chelate extracellular calcium also attenuated the calcium response to visual stimulation, indicating that calcium enters the axon from the extracellular medium. Caffeine (10 mM) caused a large increase in axonal calcium, indicating that intracellular stores contribute to the calcium signal. Presynaptic nicotinic acetylcholine receptors (nAChRs) may play a role in axon arbor development and the formation of the topographic retinotectal projection. Injection of nicotine (10 μM) into the tectal ventricle significantly elevated RGC axonal calcium levels, whereas application of the nAChR antagonist αBTX (100 nM) reduced the stimulus-evoked rise in RGC calcium fluorescence. These data suggest that light stimulus to the retina increases calcium in the axon terminal arbors through a mechanism that includes influx through nAChRs and amplification by calcium-induced calcium release from intracellular calcium stores. Such a mechanism may contribute to developmental plasticity of the retinotectal system by influencing both axon arbor elaboration and the strength of synaptic transmission.

INTRODUCTION

Retinal ganglion cells axons form complex arbors in the optic tectum. Visual activity is thought to play a role in the growth of the retinal axon arbor; however, the mechanisms by which activity might influence axon arbor elaboration are poorly understood. One possibility is that visually induced synaptic activity in the retina increases calcium concentrations in the axon arbors, which could have a rapid impact on growth-controlling machinery in the axon branches. We performed an in vivo calcium imaging study to test whether visual stimulation of the retina increases calcium in axon terminals and the mechanisms underlying such changes in calcium. We further determined whether local changes in intraaxonal calcium levels correlated with changes in axon arbor morphology.

Retinal axons first innervate the optic tectum at stage 39 of development (Holt 1989) and form synapses with target tectal neurons soon thereafter (Holt and Harris 1983; Zhang et al. 1998). A crude topography is present in the retinotectal projection at stage 40, according to anatomic and electrophysiological criteria (Holt and Harris 1983; Zhang et al. 1998). Over the next week, the map becomes more refined, likely based on activity-dependent rules as the visual system becomes functional. During this time, the retinal axon arbors in the optic tectum continue to elaborate even after they formed synapses (Easter and Stuermer 1984; Reh and Constantine-Paton 1984; Sakaguchi and Murphy 1985). In vivo imaging of Di-I-labeled retinotectal axon arbors during these stages of development indicates that they constantly add and retract branches (O’Rourke et al. 1994; Witte et al. 1996). The axon arbor enlarges because of the selective maintenance of a small fraction of the newly added branches.

Increases in calcium can promote neurite extension and growth cone motility in vitro (Gomez et al. 1995) and in vivo (Bentley et al. 1991); however, the role of calcium in the elaboration of complex axon arbors in vivo was not explored. Calcium transients in retinal axon terminals in the optic tectum were recorded in isolated frog brains in response to optic nerve stimulation (Feller et al. 1996). These calcium signals were identified by a number of criteria to originate in the retinal terminals and to correspond to presynaptic calcium signals likely associated with synaptic transmission and presynaptic plasticity. Notably, calcium transients associated with synaptic transmission occur on a timescale of milliseconds (Augustine et al. 1985; Feller et al. 1996; Linas et al. 1982; Sabatini and Regehr 1996). More prolonged calcium signals in afferents, lasting ≤ 2 s, are associated with presynaptic plasticity of transmitter release in the adult frog RGCs (Feller et al. 1996) and with process outgrowth in developing systems (Bentley et al. 1991).

Retinotectal axon terminals in frogs and fish have nicotinic acetylcholine receptors (nAChRs) located at extrasynaptic sites (Sargent et al. 1989). In fish, nAChR on retinal axons have
been shown to contain the α7 subunit (Henley et al. 1986). The α7 subunit confers sensitivity to αBTX and renders the channel highly permeable to calcium (Lindstrom 1996; Patrick et al. 1993; Role and Berg 1996; Sargent 1993). Presynaptic nAChRs modulate synaptic transmission of glutamatergic synapses in the CNS (Gil et al. 1997; McGeehee et al. 1995; Role and Berg 1996; Wonacott 1997), including retinotectal synapses (King 1990; Titmus et al. 1998). Blocking nAChRs with αBTX in the optic tectum of fish prevented the reestablishment of topography in the regenerated retinotectal projection (Schmidt 1985) caused by the apparent retraction of retinal arbors from the site of drug infusion. Similarly, eliminating cholinergic inputs with the neurotoxin AF64A disrupted the topography of the regenerated retinotectal projection in fish (Schmidt 1985). ACh was reported to both enhance and decrease neurite outgrowth in different neuronal cell types (Lipton et al. 1988; Pugh and Berg 1994; Zheng et al. 1994). These studies suggest that extrasynaptic nAChRs located on retinal axons and activated by cholinergic inputs from the midbrain nucleus isthmi (Desan et al. 1987; Gruberg and Udin 1978; Udin and Fisher 1985) might influence retinotectal arbor development in a calcium-dependent manner.

METHODS

Dye labeling

Calcium green 1 dextran (CAGD) was chosen for this study because of its relatively large change in fluorescence emission on binding calcium and because it has a significant emission at basal levels of calcium (O’Donovan et al. 1993), which would permit us to determine axonal morphology even for those axons with resting calcium levels. CAGD or Texas Red dextran [10 kDa dextran, 5% in phosphate-buffered saline (PBS), Molecular Probes] was iontophoresed into the retina of stage 45/46 albino Xenopus laevis tadpoles with 1- to 10-nA positive current for about 1 s or until dye could be seen in the eyeball. Animals were anesthetized with 0.02% MS222 in Steinbergs solution for all injection, screening, and imaging procedures. Animals were maintained overnight in a 23°C incubator under Steinbergs solution for all injection, screening, and imaging procedures. Animals were not used for imaging if dye spilled out of the ventricle into the body cavity. All reagents are from Sigma, unless stated otherwise.

In vivo confocal imaging

Confocal images of labeled axons were collected in 2-μm steps in the Z dimension at 3-min intervals with a Noran laser scanning confocal attachment mounted on an upright Nikon microscope with a ×40 lens (0.8 NA). Images consisted of an average of eight individual optical sections, except for images collected in bandscan and linescan modes, which were not averaged. Depending on the depth of the labeled axons in the Z dimension, image collection for one complete Z series took 1–2 min. Animals were maintained under anesthesia throughout the imaging protocol. Blood flow was verified at the end of the imaging session. Data were not analyzed if the animal did not recover from anesthesia.

Imaging was performed on anesthetized animals held in a Silgard chamber under a coverslip. The Silgard was prepared with india ink to minimize light scattering. The Silgard was sculpted so that the animal was positioned exactly horizontally with the dorsal optic lobes in contact with the coverslip, but the animal was not compressed by the coverslip. This customized chamber allows us to repeatedly position the animal in the same orientation (Wu and Cline 1998) before and after drug injections. Two types of coverslips were used. One was a standard transparent coverslip. This permitted exposure of the scattered laser light to the retina and is referred to as light OFF. The second coverslip was painted with an opaque black paint, except for an opening directly above the optic tectum. This coverslip minimized laser illumination of the retina and is referred to as light ON. By carefully sliding the coverslip over the top of the chamber, clear and black coverslips could be exchanged without disturbing the orientation of the animal in the recording chamber.

After placing the specimen on the stage of the microscope, visual stimulation and confocal imaging were initiated simultaneously when the laser was turned on. Light stimulus applied to the eye takes ~100–150 ms to produce an action potential in RGC axons in the optic tectum of stage 47 Xenopus tadpoles (O’Rourke et al. 1994; Witte 1995). It took ~250 ms to acquire each image in the Z series. Therefore acquisition of data for the dorsal most image plane in the Z series occurred simultaneously with the visual stimulation.

Image processing

Three-dimensional (3D) reconstructions of the image stacks were rendered for each time point in the series with Noran Intervention software. To quantify the changes in CagD fluorescence in retinal axons within the tectum, 3D reconstructions of image stacks at each time point were aligned by fiduciary points in NIH image. The mean intensity value of the image for each time point was determined. Intensity values for the optimal response were expressed relative to the minimal response.

To identify sites of branch additions, the 3D reconstructions for each time point were imported into NIH image, aligned by fiduciary points and viewed as a movie. Regions of interest (ROIs) were marked on the major axon branch from which the new branch emerged. Fluorescence intensity values in the ROI were determined and plotted for all of the time points in the series, before and after the branch additions.

RESULTS

Retinal stimulation changes axonal calcium dynamics

The elaboration of retinal axon arbors can be influenced by visual activity (Constantine-Paton et al. 1990). To test whether visual stimulus alters axonal calcium levels, CagD fluorescence...
Fluorescence in retinotectal axons was imaged in animals whose eyes were alternately exposed to the illuminating laser light or shielded from the illumination by an opaque black coverslip with a small window over the optic tectum for imaging the axons (see METHODS). Images were collected in a Z series every 3 min, and fluorescence intensity was determined from the reconstructed images at each time point.

CaGD fluorescence intensity increased and decreased in correlation with direct or shielded illumination of the retina (Fig. 1). Calcium fluorescence was greater when the animal was imaged through a transparent coverslip and less when the opaque coverslip was placed over the eyes of the animal. Fluorescence intensity values increased and decreased repeatedly in this example as the retinotectal axons were imaged every 3 min for a total of 45 min. Figure 1, inset, shows a plot of the relative fluorescence for each observation. CaGD fluorescence intensity changes continued at a comparable magnitude throughout the observation period.

Our ability to stimulate the retinal axons reproducibly at 3-min intervals for ≤45 min indicates that we are not damaging the retina with the stimulus or other aspects of the experimental protocol. There is no apparent change in the magnitude of the overall calcium signal with repeated visual stimulation. This further indicates that the experimental protocol does not damage the system and that bleaching of the dye is not a significant factor.

We observed three types of behaviors of retinal axons with respect to their calcium dynamics in response to relative increases and decreases in retinal illumination (Fig. 2). In one response type, CaGD fluorescence intensity increased and decreased in correlation with direct or shielded illumination of the retina (Fig. 2A). In the second response type, CaGD fluorescence intensity changes exhibited the opposite pattern. Fluorescence intensity was relatively low when the animal was imaged through a transparent coverslip and greater when the eyes were shielded by the opaque coverslip (Fig. 2C). In the third response type, fluorescence intensity was relatively high, independent of the imaging conditions (Fig. 2B). In this last

**FIG. 1.** CaGD fluorescence intensity in retinotectal axon arbors changes with increased and decreased retinal illumination. Calcium signals repeatedly increase and decrease in retinal axon arbors over a period of 45 min as the retina is repeatedly illuminated and shielded from light. Inset: calcium green 1 dextran (CaGD) fluorescence intensity values in retinotectal axon arbors at each observation. Scale bar = 25 μm.
case, TTX injection into the retina significantly reduced the calcium signal seen with either the clear or opaque coverslip, demonstrating that the calcium signal was in response to visual stimulation.

Removing and replacing the same coverslip does not result in fluorescence intensity changes (Fig. 2D; n = 4 animals). Texas Red dextran did not show any changes in fluorescence intensity when the animal was imaged with the transparent or opaque coverslips (n = 4 animals; data not shown). These data indicate that the intensity changes were not an artifact of the imaging conditions, such as changing the coverslip for each 3-min time point or positioning the window of the opaque coverslip over the optic tectum with the labeled axons. The fluorescence signal likely represents changes in calcium in retinal axons, rather than glia, because the dextrans do not have access to glia in the optic tract and because tadpoles of these early stages do not yet have myelinated axons (Steen et al. 1989).

These data suggest that CaGD fluorescence intensity in retinal axon arbors changes in response to retinal stimulation. The CaGD fluorescence appeared to remain elevated during the ~1–2 min required to collect the Z series, suggesting that retinal stimulation produced prolonged increases in axonal calcium levels.

**Time course of calcium changes in retinal axon arbors**

To determine the time course of calcium changes in axon terminals, we recorded CaGD fluorescence in linescan and bandscan modes. In linescan mode images that were a single pixel in the y-dimension were collected continuously with a sweep rate of 68 μs. In bandscan mode, the imaged field was set to a band of several pixels in the y-dimension. Under this imaging protocol, images shown in Figs. 3 and 4 were collected approximately every 400 μs.

We first screened for animals that showed a maximal increase in CaGD fluorescence under LIGHT ON conditions. This allowed us to image the calcium signal under continuous stimulation (LIGHT ON) conditions. We selected a region of the arbor in which LIGHT ON triggered a large rise in calcium fluorescence during the initial screening and collected images continuously in bandscan mode for 8 s (Fig. 3). Changes in CaGD fluorescence intensity were analyzed in six ROIs: a portion of the major axon branch, a branch point, two small branch tips, a lamellar growth cone, and one of its filopodia. All ROIs, except the growth cone filopodium, showed an increased fluorescence signal with LIGHT ON stimulus compared with LIGHT OFF conditions (Fig. 3B). CaGD fluorescence intensity increased to different magnitudes in the different ROIs. We noticed three general features of the change in CaGD fluorescence. 1) Fluorescence intensity reached maximal values at the first time point after LIGHT ON stimulus. Data acquisition in linescan mode showed comparable kinetics of the initial calcium response to visual stimulation. 2) Calcium signals remained elevated for several seconds after the onset of light stimulation of the retina. 3) The dynamics of the prolonged calcium signal varied at the different sites analyzed.
In four of the six ROI (ROI 2–5), the CaGD fluorescence intensity was highest when LIGHT ON stimulus started and decreased gradually over the first 3 s of imaging, after which calcium levels either leveled off (ROI 2,4) or continued to fall (ROI 3,5) depending on the site analyzed. Even within this general pattern of calcium changes over the 8 s of data acquisition, all ROIs showed rapid fluctuations in CaGD fluorescence intensity (Fig. 3D), suggesting significant increases and decreases in local calcium levels.

Previous studies have shown that complex lamellar growth cones tend not to be the sites of branch extension in these tadpole retinotectal arbors, whereas simpler branch tips do elongate (Witte et al. 1996). We compared calcium dynamics in lamellar growth cones and simple branch tips. As shown in the example in Fig. 3, simple branch tips have lower CaGD fluorescence intensity values than lamellar growth cones, likely reflecting the small volume of these structures; however, both branch tips in this example have rapid calcium dynamics. (Fig. 3D and Fig. 4). CaGD signals are also dynamic within the palm of the growth cone, showing rapid changes in intensity within the millisecond timeframe of image collection. Growth cones labeled with Texas Red dextran do not show changes in fluorescence intensity, indicating that the changes in CaGD fluorescence intensity likely do not reflect volume changes. Filopodial fluorescence intensity values are lower than in the lamellar portion of the growth cone, again because of their small volume. It is interesting to note that, although the CaGD fluorescence intensity in the growth cone filopodium did not increase compared with LIGHT OFF conditions, it did show large fluctuations in calcium levels (Fig. 3D, ROI 6). Movies generated from the bandscans show waves of high calcium traveling from the base of the filopodium to the tip. All sites along the axon arbor, including simpler branch tips, lamellar growth cones, and filopodia, show considerable local changes in calcium dynamics.

To test whether individual sites within the axonal arbor show reproducible changes in calcium with repeated visual stimulation, we acquired images in bandscan mode from the same axon with four additional LIGHT ON stimulations. The LIGHT ON stimulus was maintained for 30 s with an interval of 4 min between stimuli. Each ROI showed comparable increases in CaGD fluorescence intensity with repeated stimulation (Fig. 4). Furthermore, the general pattern of changes in CaGD fluorescence intensity seen in the first stimulus (Fig. 3) was repeated.
for each of the ROI in the subsequent 4 stimulations. For instance, ROI 1 showed large fluctuations in CaGD fluorescence intensity at the first time point. Comparable fluctuations were seen in all the following time points. Similarly, ROI 4 decreased CaGD fluorescence intensity over the data acquisition period with the same time course for each of the four time points. In contrast, ROI 2, the branchpoint, showed different calcium responses with the different time points.

These data indicate that different sites within the arbor have different responses to visual stimuli in terms of the magnitude of overall calcium changes and in terms of the kinetics and magnitude of rapid fluctuations in calcium at individual sites. The time course of these calcium signals indicated that the rise in calcium we were detecting in the retinal arbors would likely outlast the burst of action potentials that RGCs fire in response to light stimulation. The dynamics of calcium changes at each ROI indicate that calcium levels can be controlled locally within the arbor and may correlate with the growth potential of different sites within the arbor. The data further indicate that different sites in the arbor show reproducible calcium responses to repeated visual stimuli.

**Retinal activity blockade prevents axonal calcium dynamics**

If retinotectal axon arbor calcium dynamics respond to retinal stimulation, blocking retinal activity would be predicted to decrease the calcium dynamics. To test this hypothesis, we imaged sequential changes in CaGD fluorescence intensity in retinal axon arbors in response to stimulation through a transparent or opaque coverslip, as described previously, to deter-

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**FIG. 4.** Reproducible changes in calcium with repeated visual stimulations. A: drawing of the part of the axon imaged repeatedly with **LIGHT ON** stimulus. ROIs are marked. The time course of visual stimulation and data acquisition is shown next to the axon. The retina was stimulated with **LIGHT ON** for 30 s every 4 min. Images were collected continuously for 30 s while the visual stimulus was maintained on. B: changes in CaGD fluorescence intensity for each ROI (in rows) for each time point (in columns).
mine the optimal stimulus conditions for the labeled axons. The retina was then injected with either TTX (100 nM, *n* = 10 animals or 10 μM, *n* = 23 animals) or the L-type calcium channel blocker nifedipine (1 μM, *n* = 8 animals or 10 μM, *n* = 4 animals). TTX blocks sodium-dependent action potentials in RGCs and should therefore prevent transmission of visual stimulated activity from the retina. Nifedipine blocks synaptic transmission within the retina of *Xenopus* and fish (Heidelberger and Matthews 1992; Schmitz and Witkovsky 1997; Tachibana et al. 1993; Witkovsky et al. 1997). Thirty minutes after drug injection into the retina, CaGD fluorescence intensity was imaged again under conditions (either transparent or opaque coverslip) that gave the maximal intensity values.

TTX injections (10 μM TTX) into the retina reduced the stimulus-induced calcium dynamics in the retinotectal axons (Figs. 5 and 6). We observed no effect on calcium dynamics after injections of 100 nM TTX into the retina (Fig. 6). Nifedipine injection into the retina also reduced the calcium dynamics (Figs. 5 and 6). The higher concentration of nifedipine was slightly less effective at blocking the calcium dynamics than 1 μM nifedipine, but the difference was not significant (*P* = 0.15). The difference between the effect of TTX and nifedipine may be due to incomplete block of all retinal activity by nifedipine (Heidelberger and Matthews 1992; Schmitz and Witkovsky 1997; Tachibana et al. 1993; Witkovsky et al. 1997). Injection of a comparable volume of PBS into the retina did not alter calcium dynamics in retinotectal axons (*n* = 5 animals, Fig. 6). Puncture of the back of the retina by the injection pipette or leak of the drugs out of the retina by another means killed the animals. This indicates that, for the animals analyzed here, the site of action of TTX and nifedipine was confined to the retina. These data support the conclusion that visual stimulation of the retina causes changes in calcium levels within the axon arbors in the optic tectum.

Because the visual stimulation itself increases calcium levels, either with LIGHT ON or LIGHT OFF conditions, the very act of making the calcium measurements in the intact animal makes it difficult to estimate basal calcium levels. TTX injections into the retina reduce the CAGD fluorescence intensity in the arbors to 31 ± 4% of the control value in response to the nonpreferred stimulus. The remaining fluorescence intensity likely reflects emission of the dye at basal calcium levels in the axons, which is unaffected by TTX (O’Donovan et al. 1993). Because TTX is irreversible and could not be combined with the other drug treatments, basal calcium levels could not be estimated for all experiments. Therefore we expressed the changes in calcium levels in response to different stimulus conditions as a value relative to the CaGD fluorescence seen with the nonpreferred stimulus, set at 100%. When only the animals with preferential LIGHT ON or LIGHT OFF responses are considered, the increase in CaGD fluorescence intensity for the preferred stimulus is 165 ± 3% of the CaGD fluorescence intensity levels for the control nonpreferred stimulus (Fig. 6). The observations that TTX, BAPTA, and αBTX reduce calcium levels even
below that seen with the nonpreferred stimulus indicate that the control nonpreferred stimulus does indeed cause a rise in axonal calcium above basal levels.

Control of axonal calcium dynamics by nAChR

To further test whether light stimulus increases calcium in RGC axons above basal calcium levels, animals were injected in the tectal ventricle with BAPTA-AM (20 mM). BAPTA-AM enters cells and is trapped there after deesterification of the AM moiety. The intracellular BAPTA chelates cytosolic calcium after a calcium rise but does not reduce calcium below basal levels (Adler et al. 1991; Swandulla et al. 1991). BAPTA-AM severely attenuated the visually evoked calcium dynamics recorded from retinal axons to 80 ± 6% of control nonpreferred response (n = 15 animals, Fig. 7). As in the TTX experiments, the remaining fluorescence likely represents CaGD emission at basal calcium levels. This suggested that visual activity caused a rise in cytosolic calcium in the axon arbors.

To determine whether the visual response requires calcium influx into the retinal axon arbors, BAPTA (20 mM) was injected into the brain ventricle. Lowering extracellular calcium with BAPTA decreased the visually evoked calcium response (n = 7 animals, data not shown).

Considerable data have shown that retinal axons have extrasynaptically located nicotinic acetylcholine receptors, containing the α7 subunit, which renders them permeable to calcium and sensitive to αBTX (Lindstrom 1996; Patrick et al. 1993; Role and Berg 1996; Sargent 1993). The α7 containing nAChRs are more permeable to calcium than nAChRs at the neuromuscular junction and even more permeable to calcium than N-methyl-D-aspartate receptors (Castro and Albuquerque 1995; Rogers and Dani 1995). Cholinergic inputs to the optic tectum project from the nucleus isthmi, which has reciprocal topographic connections with the ipsilateral optic tectum (Grobstein and Comer 1983; Gruberg and Udin 1978). Cholinergic afferents terminate in the superficial neuropil of the tectum in close association with retinal axons (Desan et al. 1987; Schmidt 1995; Udin and Fisher 1985). To test whether nAChR might be a means of calcium entry into retinal axon arbors, we imaged calcium dynamics in response to retinal illumination, as described previously, and again after injection of either nicotine or αBTX into the tectal ventricle.

Nicotine (10 μM, n = 10 animals or 50 μM, n = 9 animals) causes a huge increase in CaGD fluorescence intensity (260 ± 20% control nonpreferred response) with retinal stimulation (Figs. 7 and 8). αBTX (100 nM) blocked the calcium dynamics irreversibly (65 ± 7% control nonpreferred response) for >1 h after injection. Furthermore, the αBTX block of calcium dynamics could not be overcome by subsequent injection of nicotine (n = 6 animals, BTX + Nic in Fig. 8). Tectal injection of a comparable

![Fig. 7. Nicotinic acetylcholine receptors and caffeine mediate increases in calcium in retinal axon arbors. Images of CaGD fluorescence intensity in retinal axons. The light-induced increase in CaGD fluorescence is reduced by αBTX (100 nM) (A) and enhanced by nicotine (10 μM) (B) and caffeine (C).](http://jn.physiology.org/Download)
The visually induced increase in CaGD fluorescence intensity was significantly increased by caffeine (10 mM) (230 ± 19 control nonpreferred response), supporting a contribution from calcium-induced calcium release (CICR) from intracellular stores to the calcium dynamics recorded from retinal axons. This could account for the prolonged time course of the light-induced calcium signal seen in the retinal axons. These data indicate that calcium influx into retinal axons can occur through nAChR and that the calcium can then trigger a further rise in calcium through release from caffeine-sensitive stores.

**Branch additions correlate with local increases in calcium**

Short interval observations of retinal ganglion cell axons labeled with DiI indicate that an average of 20% of the initial branchtip number is added to axon arbors over each 3-min observation interval. The majority of branches are rapidly retracted so that the average lifetime of short branches is ~10 min (Witte et al. 1996). To test whether local increases in calcium levels occurred at sites of axon branch additions, retinal axon arbors were labeled with CaGD, and confocal Z series of images of the axons were collected in vivo at 3-min intervals. CaGD was used because its emission at relatively low intracellular calcium levels permitted us to reconstruct the axon arbor structure, whereas the increased fluorescence intensity reported changes in calcium within the arbor. 3D reconstructions of the axon arbor morphology at each time point were generated and imported as a single image for each time point into NIH image. The reconstructions were aligned by fiducial points, and the time series was viewed as a movie to identify sites of branch additions. Complete or partial reconstruction of seven axon arbors from six animals was used. Sites of branch additions along the parent axon branch were designated as ROIs. CaGD emission intensity in the ROI was determined for several observations before and after the new branch emerged (Fig. 9). A rise in CaGD fluorescence in the ROI correlated with a subsequent branch addition at that site in 10 of 12 cases. The remaining two branch additions were not preceded by a detectable local increase in calcium. The majority (7/12) of newly added branches were subsequently retracted within the next one to three observations, as shown in the example in Fig. 9. Retractions were not associated with changes in CaGD fluorescence intensity in the ROI. The brief lifetime of short branches (range for this sample: <6 min – <12 min) is comparable with that seen in DiI-labeled axons (Witte et al. 1996).

**DISCUSSION**

We used in vivo calcium imaging to address the question of whether visual stimulation can cause changes in calcium levels within retinotectal axon arbors, which in turn might regulate axon arbor elaboration. Visual stimulation causes a prolonged rise in calcium within axon arbors, which outlasts the period of release of synaptic transmitter. The rise in axonal calcium blocked by αBTX suggests that α7-containing nAChR participates in the calcium influx. The calcium influx triggered by activation of nAChR is large, although it contributes relatively little to the total current through the channel (Mulle et al. 1992; Seguela et al. 1993). In retinal axons the calcium influx through nAChR appears to be augmented by CICR because caffeine increases the visually stimulated calcium rise. CICR may partially account for the prolonged elevation in calcium we observe in the axons. In addition, we have shown that local calcium transients within axon branches correlate spatially and temporally with sites of branch additions. The data suggest a role for presynaptic nAChR in mediating visually induced calcium entry into retinal axon arbors, which can then influence axon arbor growth as well as retinotectal synaptic transmission (King 1990; Titmus et al. 1998).

**Visual stimulation increases axonal calcium**

We observe three types of responses in the axonal calcium dynamics after visual stimulation, which correlate with the electrophysiological response properties of the three classes of RGCs that project to the optic tectum in frog (Gruss and Grusser-Cornelis 1976; Hartline 1938). As demonstrated by extracellular recordings from RGC somata and their axon terminals within the optic tectal neuropil, ON-OFF ganglion cells respond with a brief burst of action potentials to increases and decreases in illumination or a stationary stimulus within the receptive field. ON ganglion cells fire a burst of action potentials when illumination is increased. ON ganglion cells fire a burst of action potentials when the center of the receptive field is illuminated. A common feature of RGC responses is that continuous exposure to the optimal stimulus does not result in continuous action potential activity. We find an increase in CaGD fluorescence intensity in retinotectal axons of some animals when the retina is directly illuminated. In others, the maximal calcium response occurs when the retinas are shielded from illumination, and in a third group CaGD fluorescence intensity is elevated to a comparable extent under both stimulus conditions. Since the similarities of these calcium response types to ON, OFF, and ON/OFF ganglion cell responses, there are differences between electrophysiological response properties and the calcium responses we recorded. For instance, calcium signals in RGC axons remain elevated during continuous visual stimulation, in contrast to the brief burst of action potentials recorded electrophysiologically. In addition, the ages of the animals tested and the stimulation and recording conditions
differ between the experiments. Therefore the extent to which the different response types we observe with calcium imaging correspond to the electrophysiologically defined RGC classes awaits further experimentation.

Calcium transients and branch additions

Most information on calcium dynamics and structural rearrangements is from studies of neurite outgrowth and growth cone motility in vitro (Letourneau et al. 1994). In vitro, elevated calcium within a certain concentration range may promote neurite extension, whereas calcium concentrations above or below the optimal level slows extension (Kater and Mills 1991). More recent work extended these studies to determine the role of calcium in growth cone behaviors in vivo, where high calcium levels are correlated with slower migration rates in growth cones (T. Gomez, unpublished data) and greater filopodial restructuring (Bentley et al. 1991). The high CaGD fluorescence that we observe in lamellar growth cones together with our previous observations that growth cones within complex axonal arbors are not the principle sites of branch extension (Cline 1996; Witte et al. 1996) are consistent with the idea that high calcium levels in growth cones are associated with local exploratory behaviors and slow extension rates in vivo.

In contrast, calcium-dependent mechanisms regulating branch additions within complex axon arbors appear to differ from those regulating growth cone extension. We find a strong temporal correlation between local calcium increases in the parent axon branch and subsequent branch addition at the site of the calcium transient. Our images collected at 3-min intervals cannot resolve whether the rise in calcium precedes the extension of the new branch or is coincident with branch

![FIG. 9. Branch additions correlate with local increases in calcium. Images of a portion of a retinal axon arbor imaged in vivo at 3-min intervals. At the third observation a new branch emerged from a parent branch and was retracted before the following observation. The ROI in which CaGD fluorescence intensity was measured is marked by the box. Numbers in the upper left-hand corners refer to the time point for image collection. Graph: Change in CaGD fluorescence intensity from that in the previous image. CaGD fluorescence intensity in the ROI increases when the new branch emerges. Scale bar in A = 20 μm.](http://jn.physiology.org/)

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calcium transients were not seen in retinotectal axons of adult Xenopus tadpoles by promoting the addition of side branches. Such prolonged axon arbor elaboration may contribute to the growth of axon arbors that may reinnervate that site after optic nerve crush (Schmidt 1985).

Localization of calcium transients within arbors

Several lines of evidence support the idea that the calcium signals we observe in growing retinal axons of Xenopus tadpoles are not related to retinotectal synaptic transmission and may contribute to axon arbor elaboration. First, calcium imaging at the spatial and temporal resolution used here is unlikely to reveal calcium transients associated with synaptic transmission (Smith et al. 1993). Second, elevations in CaGD fluorescence are widespread throughout the axon arbor. This distribution of elevated calcium is in contrast to the punctate calcium transients detected in retinal axons in isolated brains from adult Rana pipiens (Feller et al. 1996) and in contrast to our observations of the punctate distribution of presynaptic sites within retinal axons of Xenopus tadpoles of the same stages as used in this study (Pinches and Cline 1998). Third, calcium levels in retinal axon arbors remain elevated for a prolonged period, longer than the burst of retinal action potentials corresponding to the stimulus (Grusser and Grusser-Cornelis 1976; Hartline 1938), longer than light-evoked synaptic responses in optic tectal neurons in Xenopus tadpoles (Zhang, Tao, and Poo, unpublished observations), and longer than calcium transients shown to correspond to synaptic transmission and presynaptic plasticity in retinal axons from adult Rana (Feller et al. 1996). Fourth, local calcium transients correlate with branch additions. Finally, local introduction of αBTX into the optic tectum of fish results in a failure to reinnervate the site after optic nerve crush (Schmidt 1985). Therefore our in vivo imaging demonstrated that calcium signals in retinal axon arbors have a time course and subcellular distribution that suggest a role for the calcium in cellular processes other than synaptic transmission. One possibility is that the visually induced calcium dynamics in developing retinal axon arbors may contribute to the growth of axon arbors by promoting the addition of side branches. Such prolonged calcium transients were not seen in retinotectal axons of adult frogs (Feller et al. 1996), suggesting that the kinetics of the calcium dynamics may be developmentally regulated.

Presynaptic nAChR regulate calcium influx

Our data indicate that calcium enters the axons from the extracellular solution through Ca2+ containing, αBTX-sensitive nAChR after visual stimulation. Calcium may also enter the axons through voltage-dependent calcium channels (VDCCs), which would be blocked indirectly by αBTX, because αBTX blocks ACh-dependent depolarization. Participation of VDCCs cannot be tested directly because injection of drugs that block VDCCs into the brain ventricle killed the animals. The calcium signal is further augmented and prolonged by CICR based on our observation of caffeine-sensitive calcium stores within the axon arbors.

The most likely source of the cholinergic input to the retinal axon terminals in the optic tectum is the reciprocal topographic connection with the midbrain n. isthmi, which is present but relatively sparse during development (Udin and Fisher 1985). Because cholinergic isthmi inputs do not synapse directly on retinal afferents (Gruberg et al. 1984), ACh may act in a paracrine fashion (Sargent et al. 1989). The reciprocal loop from the optic tectum to the n. isthmi and back to the topographically same site in the optic tectum provides cholinergic input within 10 ms of retinal stimulation (Scherer and Udin 1991). Therefore isthmo tectal cholinergic feedback could occur within the timeframe of the calcium signal detected in our studies. One potential function of the cholinergic feedback loop may be to promote branch additions within topographically correct sites (Fig. 10).

In vivo imaging demonstrated that retinal axon arbor elaboration is regulated by a retrograde signal reflecting the strength of postsynaptic glutamate receptor activity (O’Rourke et al. 1994; Rajan et al. 1999) and postsynaptic CaMKII activity (Zou and Cline 1996). Tectal cells with strong synaptic inputs have more stable dendritic arbors (Wu and Cline 1998; Wu et al. 1996), and their presynaptic retinal axon arbors are also more stable (Zou and Cline 1996). Presynaptic nAChR may also play a role in stabilization of the retinal axon arbor by augmenting retinotectal synaptic transmission. Presynaptic nAChRs gate retinotectal synaptic transmission in goldfish and Xenopus (King 1990; Titmus et al. 1998). This suggests that
the more efficacious synapses gated by nAChR may also be more stable than other sites that do not benefit from positive feedback through the topographic cholinergic input from n. isthmi.

nAChR and neuronal plasticity

Cholinergic inputs were implicated in sensory system plasticity (Bear and Singer 1986; Gu and Singer 1993; Liu et al. 1994; Schmidt 1985, 1995); however, their mechanism(s) of action is not yet clear. nAChRs are located presynaptically in sensory projections (Bina et al. 1995; Broide et al. 1995; Prusky and Cyander 1988; Prusky et al. 1987; Sargent et al. 1989), where they can enhance synaptic transmission (Gil et al. 1997; King 1990; McGehee et al. 1995; Titmus et al. 1998). In the retinotectal system, these receptors also influence the reestablishment of the regenerating retinotopic projection (Schmidt 1985, 1995). We used in vivo imaging of CaGd-labeled retinotectal axon arbors in albino Xenopus to demonstrate that visual stimulation causes a prolonged increase in cytosolic calcium within retinotectal axon arbors, which is blocked by the nAChR antagonist BTX. The data support a model in which visual activity can promote the elaboration of the retinal arbor by a calcium-dependent mechanism triggered by local calcium entry through extrasynaptic nAChRs. These results suggest that nAChR activity may contribute to neuronal plasticity by promoting axon sprouting and thereby providing more release sites. Such an effect onafferent morphology could cooperate with an nAChR-mediated increase in synaptic transmission to enhance synaptic efficacy in the short term and the organization of circuits in the longer term.

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