

# Spontaneous Calcium Transients in Developing Cortical Neurons Regulate Axon Outgrowth

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Growth cones of cortical axons pause for many hours in preparation for axon branching. They become large and complex compared with small advancing growth cones. We wanted to investigate whether calcium transients regulate the advance of mammalian CNS growth cones. We found that spontaneous calcium transients in developing cortical neurons have characteristic patterns, frequencies, and amplitudes. Importantly, neurons with large paused growth cones exhibit high-frequency spontaneous calcium transients, which are rare in those with small advancing growth cones. The incidence, frequencies, and amplitudes of calcium transients are inversely related to rates of axon outgrowth. The transients are mediated primarily by L-type voltage-gated calcium channels, and silencing them with channel blockers promotes axon outgrowth. Thus calcium transients regulate growth cone advance by direct effects on the growth cone.

**Key words:** calcium transients; growth cone; cortical development; axon guidance; axon branching; L-type calcium channels

## Introduction

Axons are guided to their targets by changes in the behaviors of their terminal growth cones. In the mammalian CNS, however, interstitial axon branches rather than terminal growth cones often innervate target neurons (O'Leary et al., 1990). Previously we found that growth cones demarcate the locations of axon branch points. In the corpus callosum of living cortical slices, axonal growth cones develop large complex morphologies and pause for many hours in regions from which branches later emanate (Halloran and Kalil, 1994). In contrast, rapidly extending growth cones have small simple morphologies. Similarly, growth cones of dissociated cortical neurons become large and complex during stalling behaviors that can last for hours or days. When the growth cone re-extends, it leaves behind filopodial or lamellipodial remnants from which axon branches subsequently extend (Szebenyi et al., 1998). These findings link pausing behaviors of the growth cone to branching by the axon (Kalil et al., 2000). Large complex growth cones are found consistently at choice points in the nervous system where they make decisions about growth in new directions (Mason and Erskine, 2000) including branching. In the central regions of pausing growth cones, microtubules form prominent loops (Tsui et al., 1984; Sabry et al., 1991; Tanaka and Kirschner, 1991; Dent et al., 1999), but their role in regulating growth cone advance is unclear.

Calcium has been shown to be an important regulator of neurite extension (Gomez and Spitzer, 2000; Spitzer et al., 2000). Axon outgrowth occurs within optimal levels of intracellular cal-

cium but slows or ceases when calcium is above or below these levels (Kater and Mills, 1991). Recent studies have shown that transient as opposed to sustained levels of intracellular calcium are important in regulating growth cone advance. In growth cones of dissociated chick DRG and *Xenopus* spinal neurons (Gomez et al., 1995; Gu and Spitzer, 1995; Gomez and Spitzer, 1999), the frequencies of spontaneous Ca<sup>2+</sup> transients were shown to be inversely related to rates of axon outgrowth. In *in vivo* studies in *Xenopus* spinal cord (Gomez and Spitzer, 1999), imposition or suppression of Ca<sup>2+</sup> transients in specific growth cones was sufficient to slow or accelerate axon outgrowth, respectively.

Given the importance of growth cone pausing for axon guidance and development of axon branches, we wanted to investigate whether Ca<sup>2+</sup> transients might also regulate the advance of mammalian CNS growth cones. Spontaneous Ca<sup>2+</sup> transients have been documented in cortical neurons within living slices during development of cortical circuitry (Yuste et al., 1992; Garaschuk et al., 2000; Mao et al., 2001). Ca<sup>2+</sup> transients also play a role in dendritic development (Lohmann et al., 2002; Redmond et al., 2002). However, the nature of Ca<sup>2+</sup> transients in cortical neurons has not been extensively characterized, and their role in regulating axon outgrowth is not known. In the present study, we imaged Ca<sup>2+</sup> transients in developing cortical neurons. For the first time we report that Ca<sup>2+</sup> transients regulate the advance of mammalian CNS growth cones.

## Materials and Methods

**Dissociated cell cultures.** Cultures were prepared from cortical tissues obtained from embryonic day 14 (E14) and postnatal day 0 (P0)–P3 golden Syrian hamsters (*Mesocricetus auratus*) as described previously (Dent et al., 1999; Dent and Kalil, 2001; Szebenyi et al., 2001). Glass coverslips were coated with poly-D-lysine. We used etched grid glass coverslips (Bellco, Vineland, NJ) to provide landmarks for locating the positions of neuronal processes over time.

**Time-lapse Ca<sup>2+</sup> imaging of cortical neurons.** Twenty-four hours after plating, cortical neurons were loaded with 2–4 μM fluo-4 AM (Molecular Probes, Eugene, OR) predissolved in 0.01% pluronic acid (Molecular Probes) and 0.1% dimethylsulfoxide (DMSO) for 30 min. Excess dye was washed out with three to five rinses of serum-free medium. The cover-

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Movies of several figures can be viewed at <http://kalil.anatomy.wisc.edu>.

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slips containing the neurons were then enclosed in a chamber consisting of a 15 mm glass ring (Thomas Scientific, Swedesboro, NJ) and a 25 mm round coverslip (Fisher, Itasca, IL). The dishes were returned to the incubator for 30–60 min. Fluorescence imaging of intracellular  $\text{Ca}^{2+}$  dynamics for periods ranging from 10 min to 1 hr was performed with a Nikon (Tokyo, Japan) TE300 Quantum inverted epifluorescence microscope equipped with a Princeton Instruments (Trenton, NJ) MicroMax 512BFT cooled CCD camera containing a back-thinned, frame-transfer EEV CCD57-10 chip (Roper Scientific). The imaging system was controlled by Metamorph Software (Universal Imaging, West Chester, PA). Neurons were imaged in time lapse with a  $60\times$  magnification, 1.4 numerical aperture (NA) Plan Apo CF160 objective (Nikon). We were interested in the relationship between axon outgrowth and  $\text{Ca}^{2+}$  activities. Therefore we selected for study neurons with large pyramidal morphologies, because *in vivo* these neurons have long efferent axons. Images were captured every 1–15 sec, with 300–500 msec exposures, and under low-light level conditions. Images were collected at a slow transfer rate, which reduces background noise, and binned ( $2 \times 2$ ). In some experiments, differential interference contrast (DIC) images were taken in rapid succession with fluorescent  $\text{Ca}^{2+}$  images to monitor the behaviors of the growth cones.

**Pharmacological agents.** Stock solutions were prepared by solubilizing drugs in DMSO or methanol according to the manufacturer's recommendations. The following drugs in stock solutions were diluted in serum-free medium and bath applied to cultures: the general voltage-gated  $\text{Ca}^{2+}$  channel (VGCC) blocker  $\text{Ni}^{2+}$  (2 mM; Sigma, St. Louis, MO), the L-type VGCC antagonists nifedipine (Calbiochem, La Jolla, CA) and nimodipine (Calbiochem), the P/Q-type VGCC antagonist  $\omega$ -agatoxin IVA (Calbiochem), the N-type VGCC antagonist  $\omega$ -conotoxin GVIA (Calbiochem), the sodium channel blocker tetrodotoxin (TTX; Sigma), the endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase blocker thapsigargin (Alomone Labs, Jerusalem, Israel), and the ryanodine receptor antagonist dantrolene (Alomone Labs).

**Long-term drug treatment and immunocytochemistry.** For long-term treatments, 20  $\mu\text{M}$  nifedipine was added to the cultures 15 hr after plating. Cultures were fixed at 48 hr after plating. Fixation and immunocytochemistry were performed as described previously (Dent and Kalil, 2001). Primary antibodies to the  $\alpha$  1C and 1D subunits of the L-type  $\text{Ca}^{2+}$  channel (Calbiochem) were diluted 1:100 in blocking buffer and incubated with cortical neurons at 4°C overnight, followed by application of a Cy3-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:500 in blocking buffer for 1 hr. In some experiments quantifying and comparing the expression of L-type  $\text{Ca}^{2+}$  channels in embryonic and postnatal neurons, the E14 and P1 cultures were prepared on the same day. They were fixed nearly at the same time after plating and processed with first and secondary antibodies simultaneously. The images were taken on the same day and under the same conditions including exposure time, illumination, and scaling.

**Image processing and data analysis.** To evaluate changes in  $[\text{Ca}^{2+}]_i$ , average fluorescent pixel intensity of the entire growth cone in each image was digitally quantified with Metamorph software. This value was subtracted from background and normalized to baseline fluorescence intensity with Microsoft (Redmond, WA) Excel software. Fluorescence increases exceeding 150% of baseline were characterized as  $\text{Ca}^{2+}$  transients (Gu and Spitzer, 1994). This was reconfirmed by frame-by-frame examination of the time-lapse movie (available at <http://kalil.anatomy.wisc.edu>). Transients were readily distinguished from spurious fluctuations arising from environmental factors, because fluctuations had very low amplitudes, occurred randomly, had no particular pattern, and were not sustained. The kinetics of  $\text{Ca}^{2+}$  transients was estimated in the time-lapse images captured every second. The rise time of  $\text{Ca}^{2+}$  transients was measured as the interval between initiating baseline and peak signal, and the decay time of  $\text{Ca}^{2+}$  transients was defined as the time from peak signal to baseline. Fluorescence intensity of immunostained neurons was quantified with Metamorph software.

**Analysis of neurite length.** For measurements of axon and branch length, images of neurons were acquired at 48 hr with a  $20\times$  magnification, 0.7 NA Neofluor CF160 objective. The distance from the cell body

to the distal extent of the central region of the growth cone was measured as the axon length. To measure the rate of advance of growth cones, images of neurons were acquired at 24 hr, and their positions were recorded by etched markings on the coverslips. At 3 and 25 hr after drug application, the same population of neurons was again imaged. The outgrowth of the growth cone was determined by subtracting the axon length measured at 24 hr *in vitro* from the axon length measured at 27 or 49 hr. Statistical analysis was performed using Sigmaplot (Jandel Scientific, Corte Madera, CA). Graphs were created in Sigmaplot (SPSS, Chicago, IL). Images were processed with Metamorph 4.62 and Photoshop 6.0 (Adobe Systems, Mountain View, CA). Images shown in the figures were enhanced using the unsharp mask filter and brightness–contrast adjustment functions in Adobe Photoshop. Time lapse images were assembled into QuickTime movies (Premiere; Adobe Systems).

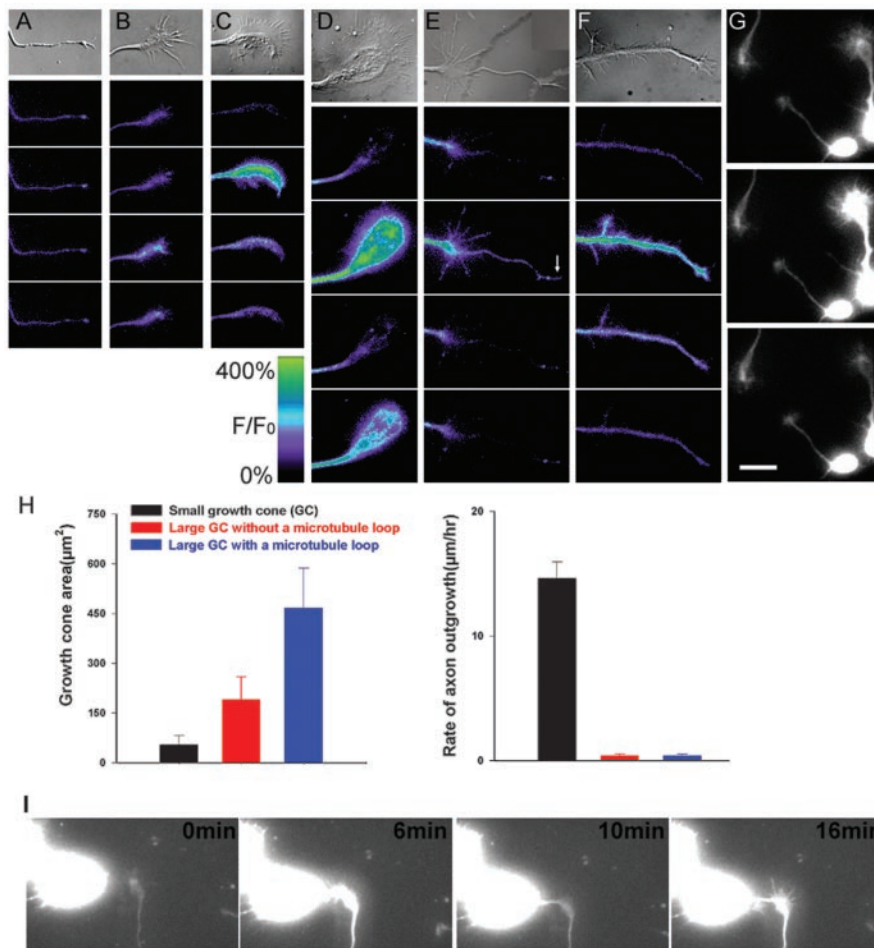
## Results

### Spontaneous $\text{Ca}^{2+}$ transients occur in developing cortical neurons

To determine whether spontaneous fluctuations in  $[\text{Ca}^{2+}]_i$  occur in dissociated cortical neurons, we loaded early postnatal (0–3 d) cortical neurons with the  $\text{Ca}^{2+}$  indicator dye fluo-4 and imaged the neurons for periods of 10–60 min at intervals ranging from every second to every 15 sec. These procedures did not cause detectable damage to the neurons, and their dendritic and axonal processes maintained motility during the entire imaging period. Imaging was performed on cultures 24 hr after plating at low density. At this stage of development, neurons were still extending processes, but the single long axon was already distinguishable from the uniformly short dendrites. Because most of the processes were not in contact with other cells, there was probably little synaptic activity among cortical neurons. A large percentage (57.4%) of the neurons in the cultures showed spontaneous fluctuations in levels of  $[\text{Ca}^{2+}]_i$ . These  $\text{Ca}^{2+}$  transients occurred rapidly and appeared instantaneously throughout the cell body, dendrites, the axon, and the entire growth cone, including filopodia. However, even in neurons with axons longer than 300  $\mu\text{m}$ , transients were propagated too rapidly to determine whether they originated in the cell body or the growth cone.

### $\text{Ca}^{2+}$ transients are most prevalent in cortical neurons with large paused growth cones

To determine whether transient elevations in  $[\text{Ca}^{2+}]_i$  are related to axon outgrowth, we focused on measurements of  $\text{Ca}^{2+}$  transients in axonal growth cones. In previous studies, we found that cortical growth cone morphologies are well correlated with their rates of extension (Halloran and Kalil, 1994; Szebenyi et al., 1998). Small, simple, bullet-shaped growth cones usually extend rapidly whereas, large complex growth cones with expanded lamellipodia and numerous filopodia pause for many hours. These growth cones develop prominent microtubule loops in their central region before re-extension of the axon and development of branches (Dent et al., 1999; Dent and Kalil, 2001). Therefore, we compared the prevalence of  $\text{Ca}^{2+}$  transients in growth cones with morphologies characteristic of each of these stages in outgrowth, pausing, and branching (Fig. 1 and Table 1). Small simple growth cones showed very few transient elevations in  $[\text{Ca}^{2+}]_i$  (Fig. 1A), whereas all of the growth cones with large complex morphologies showed frequent  $\text{Ca}^{2+}$  transients (Fig. 1B–F). As illustrated in Figure 1, during the 30 sec imaging periods that were part of longer (20–60 min) sequences, large complex growth cones showed at least one  $\text{Ca}^{2+}$  transient. Moreover, the amplitude relative to baseline of the  $\text{Ca}^{2+}$  transients appeared highest in the large paused growth cones that contained a prominent microtubule loop (Fig. 1D). This growth cone also showed two  $\text{Ca}^{2+}$



**Figure 1.**  $\text{Ca}^{2+}$  transients occur preferentially in growth cones with large complex morphologies. *A–F*,  $\text{Ca}^{2+}$  transients in cortical growth cones with morphologies representative of progressive stages in growth cone extension, pausing, and branching. *Top*, DIC images of each growth cone followed by pseudocolor fluorescence images of  $[\text{Ca}^{2+}]_i$  in the growth cone at 10 sec intervals. *A*, A small simple growth cone shows no detectable  $\text{Ca}^{2+}$  fluctuations. *B*, A pausing growth cone without a central microtubule loop exhibits a single  $\text{Ca}^{2+}$  transient of relatively low amplitude. *C*, A large paused growth cone with a partially formed microtubule loop (a large “transitional” growth cone) shows a single high-amplitude  $\text{Ca}^{2+}$  transient at 10 sec. *D*, A large paused growth cone with a prominent microtubule loop has the highest amplitude  $\text{Ca}^{2+}$  transients, which occur at 10 and 30 sec. *E*, A pausing growth cone from which an axon is re-extending exhibits a single transient of moderate amplitude at 10 sec. The growth cone at the tip of the new axon (*arrow*) shows a simultaneous  $\text{Ca}^{2+}$  transient. *F*, A branching axon exhibits a single high-amplitude  $\text{Ca}^{2+}$  transient at 10 sec. *G*, Sequence of fluorescence images at 10 sec intervals showing changes in  $\text{Ca}^{2+}$  levels in three different cortical neurons in close proximity (see supplemental movie 1; available at <http://kalil.anatomy.wisc.edu>). The smaller growth cones to the *left* and *center* show little fluctuation in  $\text{Ca}^{2+}$  levels, in contrast to the large paused growth cone, which shows a very large  $\text{Ca}^{2+}$  transient at 10 sec. The scale shows relative fluorescence intensity over baseline in pseudocolor images. *H*, *Left*, Average areas of growth cones of three different morphologies, exemplified by growth cones in *A*, *B*, and *D*. *Right*, Average growth rates of these growth cones. *I*, Sequence of fluorescence images of a growth cone showing increase in calcium levels as the growth cone changes from extending (0 min) to pausing (6, 10, and 16 min) in response to contact with another cell in the dish (see supplemental movie 2; available at <http://kalil.anatomy.wisc.edu>). Scale bar: (in *G*), 10  $\mu\text{m}$ .

transients in 30 sec, in contrast to the one transient exhibited by the other growth cones shown in Figure 1. We found several examples (Fig. 1*G*) in which a large paused growth cone exhibited frequent large  $\text{Ca}^{2+}$  fluctuations, whereas within the same microscopic field, two nearby small growth cones showed no  $\text{Ca}^{2+}$  transients (see the movie available at <http://kalil.anatomy.wisc.edu>). To confirm that growth cone morphologies reflected rates of extension in these experimental conditions, we measured the areas and rates of advance of 45 growth cones (15 growth cones in each group) included in the  $\text{Ca}^{2+}$  measurements in Table 1 (Fig. 1*H*). Growth cones with prominent microtubule loops were the largest, more than twice the area of those lacking microtubule

loops. Measurements of the rates of extension of growth cones of different morphologies showed that large complex growth cones maintained motility but failed to advance during the 10–60 min imaging period, whereas small simple growth cones extended at an average rate of 14.6  $\mu\text{m}/\text{hr}$ . Together, these results show that  $\text{Ca}^{2+}$  transients are most prevalent in large pausing growth cones but occur only rarely in small advancing growth cones.

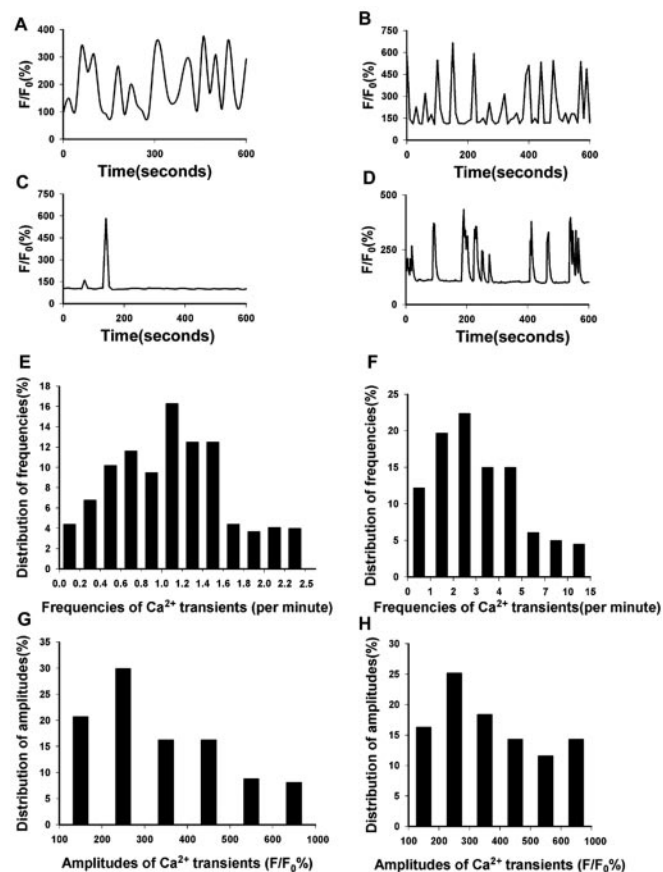
Cortical growth cones undergo transitions from paused to growth states over long time periods of up to several days (Szebenyi et al., 1998). Thus it was not possible to monitor fluctuations in  $\text{Ca}^{2+}$  levels in the same growth cone for many hours or several days as growth cones underwent transitions from pausing to extending. However, in one fortuitous case, we monitored  $\text{Ca}^{2+}$  activity in a growth cone that went from extending to pausing after contact with another cell (see the movie available at <http://kalil.anatomy.wisc.edu>). While the growth cone was advancing at 15  $\mu\text{m}/\text{hr}$ , it showed no  $\text{Ca}^{2+}$  activity (Fig. 1*I*). Within minutes after contact with a cell body, the growth cone stopped advancing and exhibited high  $\text{Ca}^{2+}$  activity (one transient per minute) that continued during the rest of the 1 hr imaging period. As shown in the image at 16 min, the growth cone also enlarged. This observation demonstrates that the transition from growth cone advancing to pausing is accompanied by a rapid development of persistent  $\text{Ca}^{2+}$  transients.

#### $\text{Ca}^{2+}$ transients in growth cones have characteristic patterns, frequencies, and amplitudes

Characterization of  $\text{Ca}^{2+}$  transients in pausing growth cones ( $n = 420$ ) revealed that patterns of  $\text{Ca}^{2+}$  oscillations occurred with varying temporal patterns in different neurons. As shown in Figure 2*A–D*, these repetitive patterns of  $\text{Ca}^{2+}$  transients were characteristic for individual growth cones and remained constant during the entire 20–60 min imaging period. Measurements of the frequencies and amplitudes of  $\text{Ca}^{2+}$  transients also showed wide variation among different growth cones. When imaging intervals of 10–15 sec were used, frequencies varied from 0.1 to 2.5 transients per minute. Analysis of frequency distributions in 295 neurons showed an average frequency of  $1.0 \pm 0.1$  transient per minute (Fig. 2*E*). However, imaging neurons every 1–5 sec ( $n = 147$ ) revealed a more accurate average frequency of  $3.2 \pm 0.1$  per minute (Fig. 2*F*). Amplitudes of the  $\text{Ca}^{2+}$  transients also varied widely, ranging from 50 to 1000% above baseline (Fig. 2*G,H*). To determine the kinetics of  $\text{Ca}^{2+}$  transients, we measured their rise and decay times in 35 large paused growth cones at intervals of 1 sec.  $\text{Ca}^{2+}$  transients

**Table 1. The occurrence of calcium transients in growth cones of different morphologies**

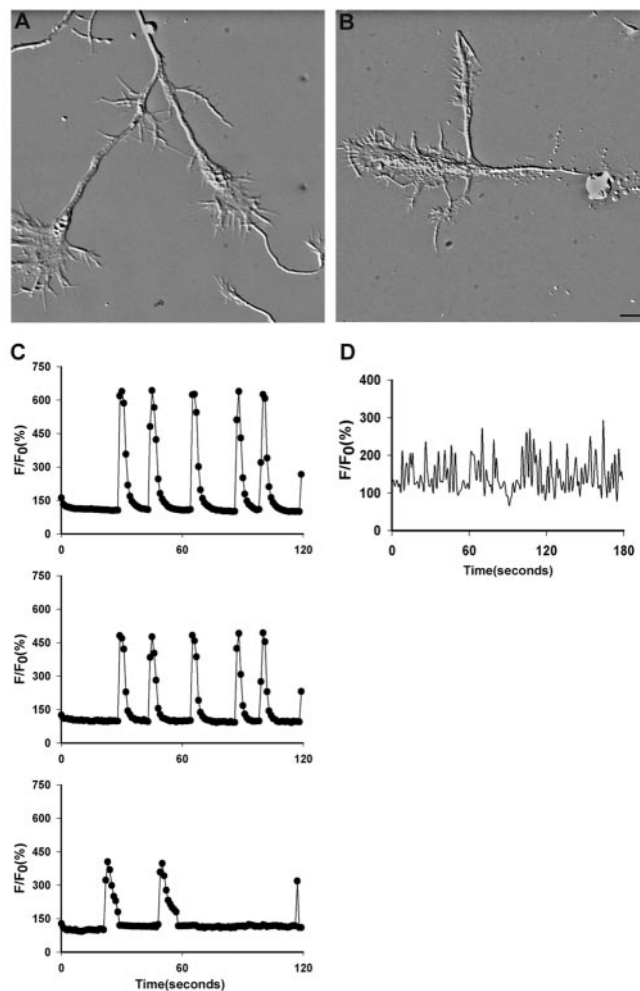
Growth cone (GC) type	Number of neurons with calcium transients	Number of neurons without calcium transients	% of neurons with calcium transients
Small GC	20	89	18%
Large GC without a microtubule loop	29	27	52%
Large transitional GC	124	50	71%
Large GC with a microtubule loop	89	18	83%
Large GC re-extending	28	12	70%
Branching axon (GC of primary axon)	35	8	81%



**Figure 2.**  $\text{Ca}^{2+}$  transients in growth cones have characteristic temporal patterns, frequencies, and amplitudes. *A–D*, Examples of characteristic temporal patterns of  $\text{Ca}^{2+}$  transients in four different large paused growth cones. Changes in fluorescence intensity are shown relative to baseline ( $F/F_0\%$ ). Note that, over the 10 min imaging period shown, the pattern of  $\text{Ca}^{2+}$  fluctuations is consistent and repetitive. These patterns persist for up to 1 hr (data not shown). *E, F*, Distribution of frequencies of  $\text{Ca}^{2+}$  transients per minute in the entire population of growth cones included in this study. *E*,  $\text{Ca}^{2+}$  imaging was performed at 10–15 sec intervals in growth cones of varying morphologies ( $n = 295$ ). *F*,  $\text{Ca}^{2+}$  imaging was performed at 1–5 sec intervals in growth cones of varying morphologies ( $n = 147$ ). *G, H*, Distribution of amplitudes of  $\text{Ca}^{2+}$  transients in percentages of growth cones with various morphologies. Amplitudes are expressed as a percentage of baseline fluorescence. The growth cone population in *G* and *H* corresponds to that in *E* and *F*, respectively. Values for amplitudes were obtained from the peak amplitudes in plots of transients for individual growth cones, as shown in *A–D*.

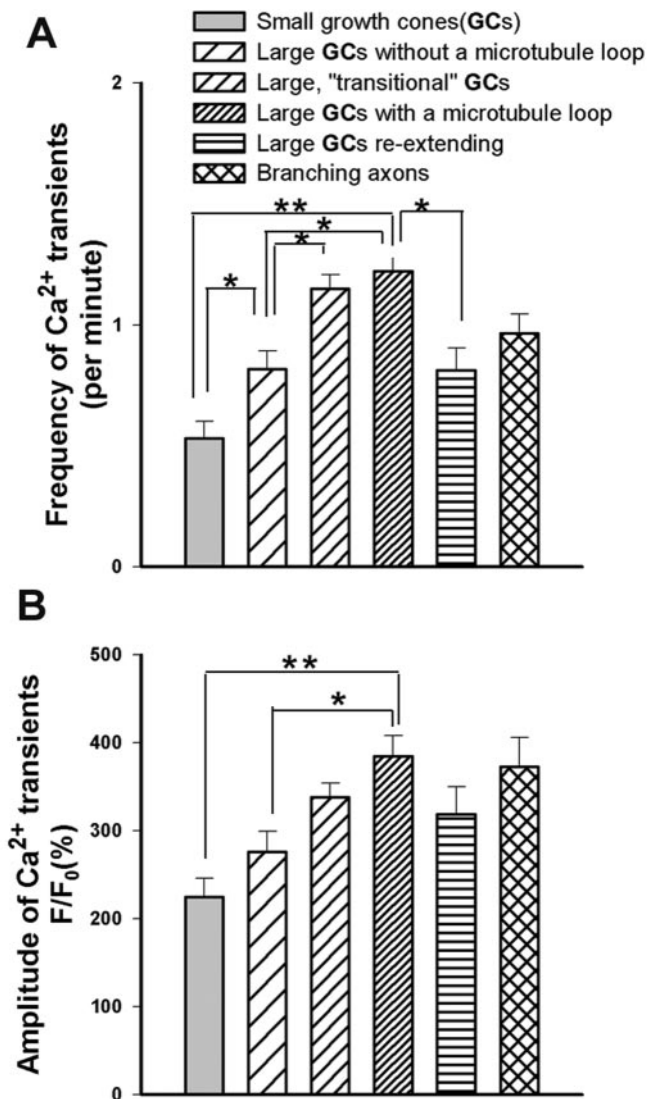
had a rapid rise time of  $2.14 \pm 0.2$  sec and a decay time of  $5.5 \pm 0.3$  sec.  $\text{Ca}^{2+}$  transients with a rapid rise and short duration are consistent with  $\text{Ca}^{2+}$  spikes observed in other systems (Gu et al., 1994).

Although  $\text{Ca}^{2+}$  transients observed in cortical growth cones generally reflected those in the cell body, we found several examples in which patterns of  $\text{Ca}^{2+}$  transients appeared to be endogenous to the growth cone. As shown in Figure 3*A*, two growth



**Figure 3.**  $\text{Ca}^{2+}$  transients can show endogenous patterns in growth cones. *A*, Two large complex growth cones branching from a single axon (*top*) and a small simple growth cone (*bottom*). *B*, A complex branching growth cone that has been severed from the cell body. Scale bar: (in *B*) *A, B*, 10  $\mu\text{m}$ . *C*, Patterns of  $\text{Ca}^{2+}$  transients at the *top*, *middle*, and *bottom* were measured in the growth cones in *A* at the *left* (branching growth cones), *right* (primary growth cone), and *bottom* (small advancing growth cone), respectively. Growth cones branching from the same axon show the same frequency but different amplitudes. The large complex growth cones show higher frequencies than the small growth cone. *D*, The pattern of  $\text{Ca}^{2+}$  transients imaged every second in the severed growth cone has a very high frequency and faster than normal kinetics.

cones branched from a single axon. Although the  $\text{Ca}^{2+}$  transients were initiated simultaneously and had similar frequencies in both growth cones, the characteristics of the rise and decay times and the peak amplitudes were different in each growth cone (Fig. 3*C*). In several other cases, we found growth cones that had been severed from the cell body. One such growth cone (Fig. 3*B*) had a large complex morphology and exhibited high-frequency  $\text{Ca}^{2+}$



**Figure 4.** Frequency and amplitude of Ca<sup>2+</sup> transients are inversely proportional to rates of axon outgrowth. *A, B*, Histograms plotting frequencies (*A*) and amplitudes (*B*) of growth cones (GCs) with different morphologies included in Table 1 and illustrated and described in Figure 1. Growth cones of progressively larger size and greater complexity (corresponding to slower rates of outgrowth) show increasingly higher frequencies and amplitudes of Ca<sup>2+</sup> transients. \**p* < 0.05 (*t* test); \*\**p* < 0.001 (*t* test).

transients, although their rise and decay times were faster than in normal attached growth cones (Fig. 3*D*). The growth cone appeared healthy and remained motile for several hours. These results suggest that growth cones can have endogenous Ca<sup>2+</sup> transients independent of the cell body.

#### Frequency and amplitude of Ca<sup>2+</sup> transients are inversely proportional to rates of axon outgrowth

To determine whether the frequency and amplitude of Ca<sup>2+</sup> transients are correlated with growth cone morphologies and rates of axon outgrowth, we plotted the average frequencies and amplitudes of Ca<sup>2+</sup> transients in growth cones (*n* = 249) of different morphologies (Fig. 4). The rates of outgrowth of a subset of these growth cones are shown in Figure 1*H*. Large paused growth cones with prominent microtubule loops (*n* = 77) had the highest average frequencies of Ca<sup>2+</sup> transients (1.2 ± 0.1 per minute) when images were acquired every 10 sec. Imaging an-

other group of large paused growth cones with similar morphologies at 1 sec intervals (*n* = 56) revealed even higher average frequencies (5.0 ± 0.5 per minute). In some large growth cones, frequencies could reach 14 transients per minute. For large growth cones with re-extending axons (*n* = 28), the average frequency of the transients (0.8 ± 0.1 per minute) was significantly less than in large paused growth cones. For smaller but complex growth cones without microtubule loops (*n* = 29) that extended slowly, the frequency of Ca<sup>2+</sup> transients averaged 0.8 ± 0.1 per minute. In contrast, small simple growth cones (*n* = 20) that were extending relatively rapidly had average frequencies of 0.5 ± 0.1 transients per minute. Amplitudes of the Ca<sup>2+</sup> transients were also well correlated with growth cone morphologies and rates of outgrowth. Plotting the maximum amplitudes of the same growth cones shown in the frequency plots demonstrated that amplitudes of Ca<sup>2+</sup> transient were highest in large paused growth cones and lowest in small advancing growth cones (Fig. 4*B*). Thus, measurements of Ca<sup>2+</sup> transients in large numbers of growth cones of different morphologies and rates of extension demonstrate that the frequency and amplitude of Ca<sup>2+</sup> transients are inversely correlated with rates of growth cone advance.

#### Ca<sup>2+</sup> transients are mediated primarily by L-type voltage-gated channels

To determine whether Ca<sup>2+</sup> transients are related to electrical activity, we tested the effects of the voltage-gated sodium channel blocker TTX. We chose large paused growth cones with microtubule loops for study, because these growth cones had the highest incidence of Ca<sup>2+</sup> transients with the highest frequencies and amplitudes. Application of 1 μM TTX to the cortical cultures completely abolished Ca<sup>2+</sup> transients within a few seconds (*n* = 20 growth cones; data not shown). To identify the channels mediating the Ca<sup>2+</sup> transients, we first applied 2 mM Ni<sup>2+</sup>, which at this concentration is a general calcium channel blocker. Ni<sup>2+</sup> immediately abolished all Ca<sup>2+</sup> transients (*n* = 6 growth cones; data not shown), suggesting a role for VGCCs in generating Ca<sup>2+</sup> transients. We subsequently applied blockers specific for L-type (nimodipine and nifedipine), N-type (ω-conotoxin), and P/Q-type (ω-agatoxin) VGCCs. Application of conotoxin or agatoxin did not have any effect on Ca<sup>2+</sup> transients (Fig. 5*D*). We tested a range of concentrations for conotoxin (1–5 μM) and agatoxin (0.1–0.5 μM) (*n* = 10), all of which gave the same results. In contrast, both nimodipine and nifedipine eliminated Ca<sup>2+</sup> transients in a dose-dependent manner.

As shown for the growth cone in Figure 5*A*, 5 μM nifedipine is capable of silencing Ca<sup>2+</sup> transients within seconds. However, in five additional growth cones tested, we found that 5 μM nifedipine completely silenced Ca<sup>2+</sup> transients in only one and partially decreased the amplitudes of Ca<sup>2+</sup> transients in the remaining growth cones. Higher concentrations of nifedipine more reliably silenced Ca<sup>2+</sup> transients. At 10 μM (Fig. 5*B*), calcium activity was completely silenced in one-half the growth cones tested (*n* = 6) and only partially attenuated in the others. At concentrations of 20 μM (*n* = 6), Ca<sup>2+</sup> activity was completely blocked in all six growth cones examined. For nimodipine (Fig. 5*C*), another L-type channel blocker, 10 μM nimodipine completely blocked all activity (*n* = 3). These results demonstrate that the L-type VGCCs are the major channels involved in mediating Ca<sup>2+</sup> transients in cortical neurons. However, the observation that concentrations of 20 μM were necessary to silence Ca<sup>2+</sup> transients completely in some neurons suggests that nifedipine at higher concentrations may also block other VGCCs.

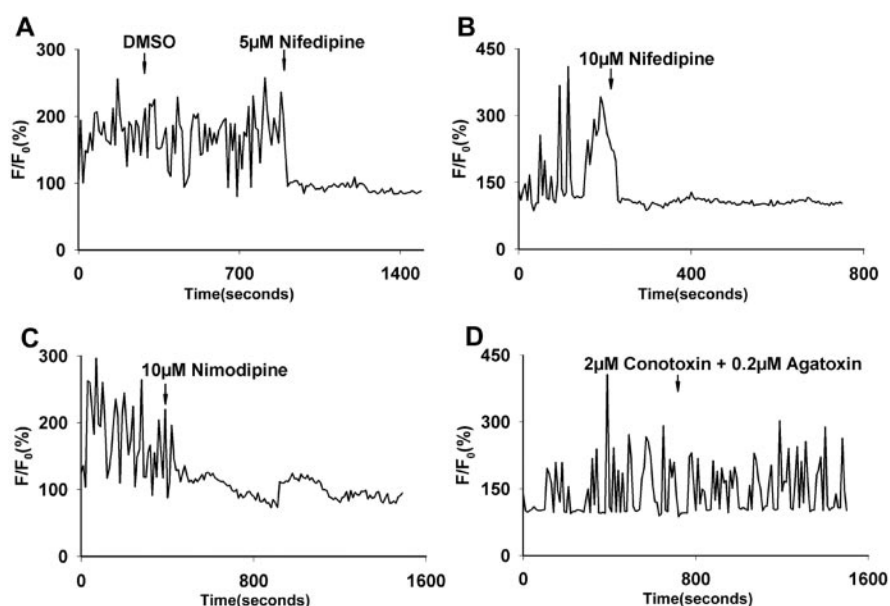
Previous studies have demonstrated the presence of L-type

VGCCs on developing cortical and hippocampal neurons (Hell et al., 1993; Dolmetsch et al., 2001; Timmermann et al., 2002). To determine the distribution of L-type channels on cortical neurons in our cultures, we stained late embryonic and early postnatal neurons with antibodies to the  $\alpha 1C$  and  $1D$  subunits of the L-type  $Ca^{2+}$  channel. As shown in Figure 6D, L-type  $Ca^{2+}$  channels were distributed on the entire postnatal neuron, including the dendritic and axonal processes. However, L-type  $Ca^{2+}$  channels were very sparse on embryonic neurons but did appear after several days in culture (Fig. 6B,C). Interestingly, in comparison with postnatal cortical neurons (0–3 d) E14 neurons showed very few  $Ca^{2+}$  transients (Fig. 6F). Postnatal neurons ( $n = 529$ ) had a much higher incidence of  $Ca^{2+}$  transients in their growth cones (61.4%) than did E14 neurons ( $n = 120$ ) (22.5%) (Fig. 6F), and amplitudes of  $Ca^{2+}$  transients were also greater in postnatal neurons. However, when embryonic neurons were allowed to mature for 5–7 d *in vitro* (DIV), they expressed L-type channels and concomitantly a large percentage (77.5%) of their axonal growth cones exhibited  $Ca^{2+}$  transients. These results demonstrate that the developmental expression of L-type channels is well correlated with the incidence of  $Ca^{2+}$  transients.

In addition to  $Ca^{2+}$  entry through L-type  $Ca^{2+}$  channels,  $Ca^{2+}$  is also known to be released from intracellular stores. To determine whether intracellular stores contributed to  $Ca^{2+}$  transients in cortical neurons, we applied thapsigargin and dantrolene, which block the endoplasmic reticulum  $Ca^{2+}$  ATPases and ryanodine receptors, respectively. Low concentrations of these blockers significantly reduced the amplitude ( $\sim 40\%$ ) but not the frequency of the  $Ca^{2+}$  transients (data not shown), demonstrating that at least some of the rise in  $[Ca^{2+}]_i$  is attributable to release from intracellular stores.

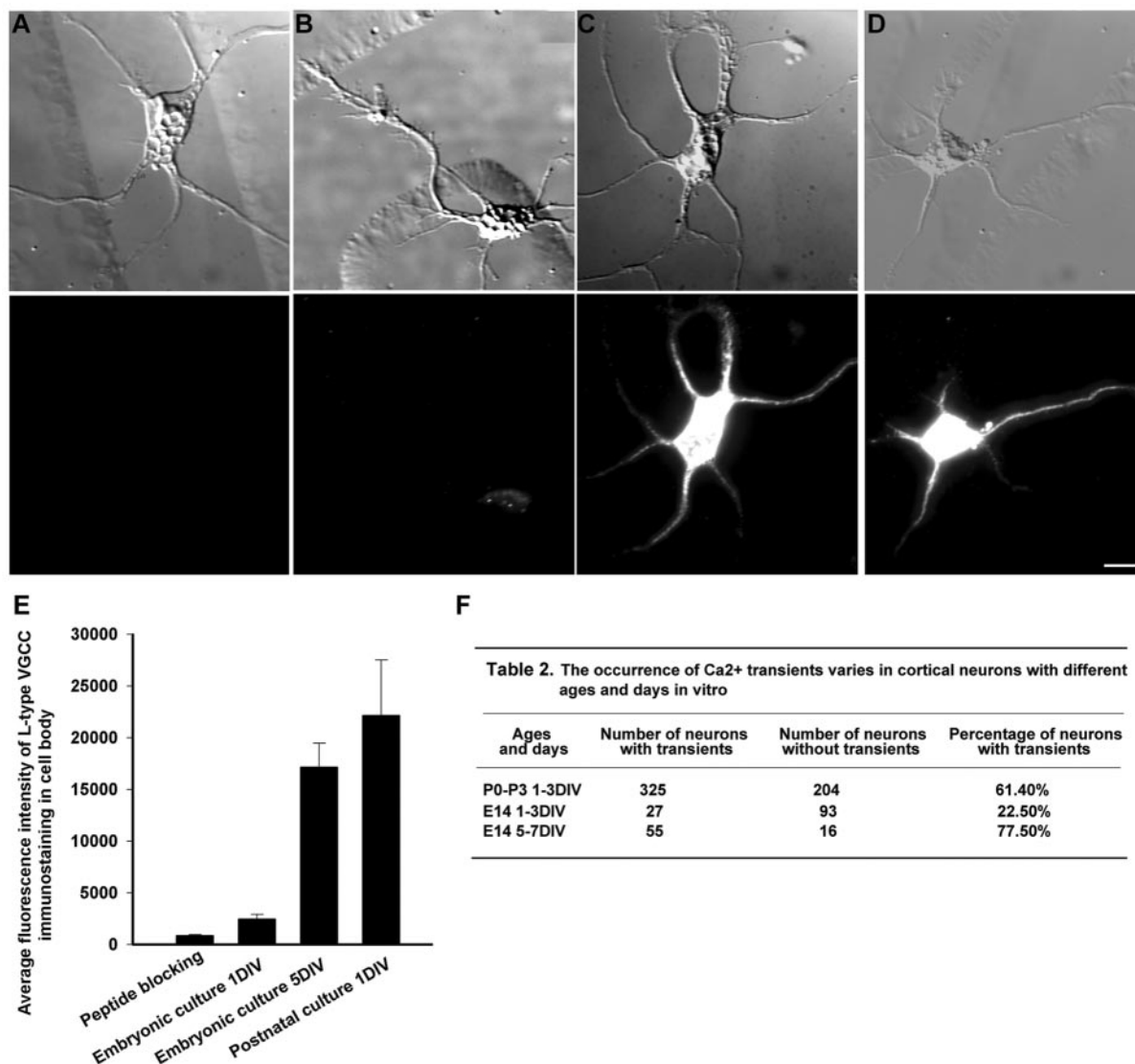
### Suppression of $Ca^{2+}$ transients by blocking L-type VGCCs promotes axon outgrowth

If high-frequency  $Ca^{2+}$  transients are associated with growth cone pausing, then silencing  $Ca^{2+}$  transients should induce axon outgrowth. We therefore applied nifedipine, which at concentrations of  $20 \mu M$  completely silences  $Ca^{2+}$  transients mediated by L-type VGCCs for as long as antagonist is present. In a few experiments, we used  $10 \mu M$  nimodipine. First we studied populations of cortical growth cones that were treated with nifedipine at 15 hr after plating. After exposure of the neurons to nifedipine (Fig. 7A,C,D) or nimodipine (Fig. 7B) for 33 hr, most of the cortical neurons had long axons tipped by small growth cones. This effect was dose dependent, and increasing concentrations of nifedipine resulted in longer axons. As shown in Figure 7C,  $5 \mu M$  nifedipine had only a small but statistically significant ( $p = 0.033$ ) effect on axon length, which is consistent with our finding that  $5 \mu M$  nifedipine only partially eliminated  $Ca^{2+}$  transients. Application of  $10 \mu M$  nifedipine or nimodipine more completely suppressed  $Ca^{2+}$  transients and had a greater effect on increasing axon outgrowth ( $p = 0.002$ ). At higher concentrations (Fig. 7C,D), nifedipine resulted in increasing axon lengths. Very few axons (Fig. 7E)



**Figure 5.**  $Ca^{2+}$  transients are mediated by L-type VGCCs. *A, B*, Effects of  $5 \mu M$  (*A*) and  $10 \mu M$  (*B*) nifedipine on silencing  $Ca^{2+}$  transients. *C*, A  $10 \mu M$  concentration of nimodipine also silenced  $Ca^{2+}$  transients in the growth cone. *D*, Conotoxin and agatoxin, which block N-type and P/Q-type channels, respectively, had no effect.

had large paused growth cones. In contrast, control cultures treated with DMSO had many large growth cones and fewer long axons. Next we compared rates of axon outgrowth in nifedipine-treated versus control (DMSO) cultures. After nifedipine application at 24 hr after plating, we monitored the outgrowth of 56 axons and their growth cones along with an equal number of controls. For both control and nifedipine conditions, we first imaged the neurons in phase microscopy before treatments and recorded their positions. We then imaged the entire neuron, including the cell body, the axon, and the growth cone at 3 hr and again at 25 hr after treatment. Rates of axon outgrowth were calculated by comparing positions of the axons and growth cones at these two time points. Application of nifedipine almost doubled the average rates of axon outgrowth compared with controls (Fig. 7F). This increase in rate of axon outgrowth was already apparent within the first 3 hr of nifedipine treatment, which suggests that suppression of  $Ca^{2+}$  transients has relatively acute effects on promotion of axon extension. In another set of experiments, we followed large pausing growth cones over time to determine changes in individual growth cones ( $n = 38$ ) induced by nifedipine. As shown in one example (Fig. 8B), at the beginning of the experiment the large growth cone had a prominent microtubule loop and was not advancing. After 7 hr, the axon had advanced  $85 \mu m$  and the remnants of the paused growth cone remained as an expanded region of the axon. By 19 hr, the axon had extended even further. In comparison, control cultures still had many large paused growth cones and much shorter axons (Fig. 8A). Analysis of all growth cones (Fig. 8C) showed that by 19 hr after nifedipine treatment, 68% of the large paused growth cones ( $n = 38$ ) had extended, in comparison with only 24% of controls ( $n = 42$ ). Axon outgrowth of nifedipine-treated neurons at this time point was  $181 \pm 17$  versus  $115 \pm 26 \mu m$  (Fig. 8D). Interestingly, in contrast to control cultures, nifedipine-treated cortical neurons failed to develop collateral branches from regions along the axon in which growth cones had paused. These results demonstrate that  $Ca^{2+}$  transients play a central role in regulating axon outgrowth.



**Figure 6.** The incidence of Ca<sup>2+</sup> transients correlates with developmental expression of L-type VGCCs. *A–D*, Images of cortical neurons (*top*) and fluorescence images (*bottom*) after staining with antibodies to the L-type VGCC. *A*, Control staining of a postnatal neuron with preabsorbed antibody. *B*, Staining of an embryonic neuron after 1 DIV showing very weak expression. *C*, Staining of an embryonic neuron after 5 DIV. *D*, Staining of a postnatal neuron after 1 DIV. Scale bar, 10  $\mu$ m. *E*, Fluorescence intensity of neurons at different ages and days *in vitro* after staining with antibodies to L-type VGCCs. Fluorescence intensity of immunostained neurons is expressed as grayscale values relative to the maximum levels that the camera can acquire. *F*, Table comparing the incidence of Ca<sup>2+</sup> transients at different ages and days *in vitro*. The ages at which Ca<sup>2+</sup> transients occur corresponds to development of L-type VGCCs.

We have interpreted the results of blocking L-type VGCCs as affecting Ca<sup>2+</sup> transients. However, it is possible that blocking L-type VGCCs can have other effects on Ca<sup>2+</sup>, such as lowering basal cytoplasmic levels, or on Ca<sup>2+</sup> release from intracellular stores. We have not examined these possibilities. However, even if these indirect effects contributed to our results, they would not obviate our conclusion that eliminating Ca<sup>2+</sup> transients promotes axon outgrowth.

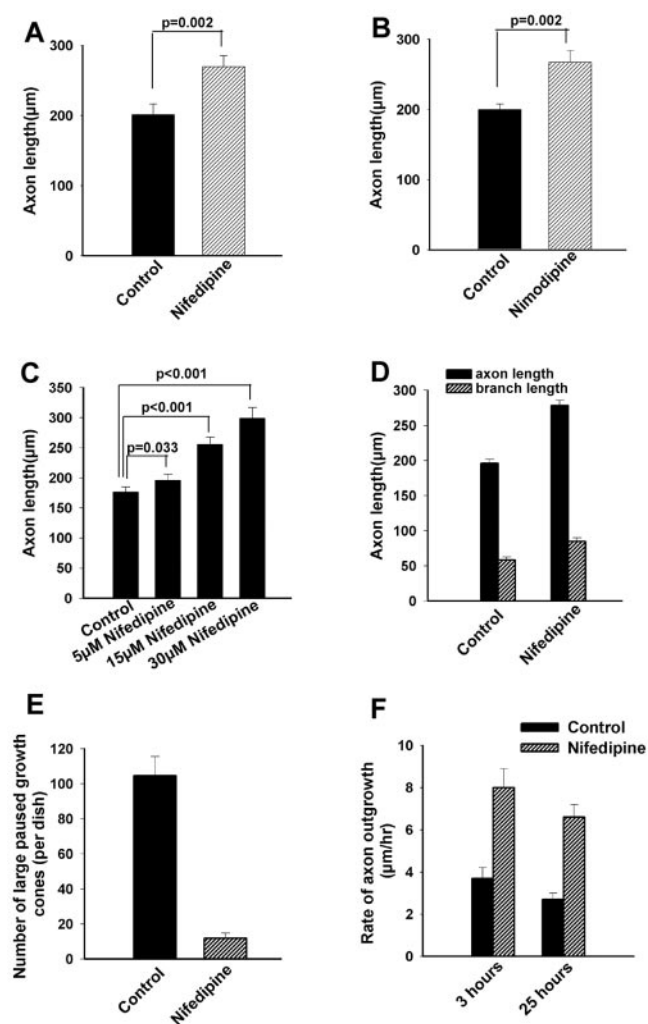
## Discussion

We demonstrate here that developing cortical neurons in dissociated cultures exhibited spontaneous Ca<sup>2+</sup> transients with varying frequencies, amplitudes, and temporal patterns. These transients were most prevalent in large paused growth cones but rare in small advancing growth cones. The frequency and amplitude of Ca<sup>2+</sup> transients were inversely correlated with rates of axon outgrowth. Ca<sup>2+</sup> entry occurred primarily through L-type VGCCs, although other VGCCs may also be involved. Remark-

ably, within a few hours, silencing Ca<sup>2+</sup> transients by blocking L-type Ca<sup>2+</sup> channels caused outgrowth of large paused growth cones that would otherwise remain stationary for many hours or several days. Together these results provide the first evidence that Ca<sup>2+</sup> transients in mammalian CNS neurons play a central role in regulating forward advance of the growth cone.

## Developing CNS neurons exhibit Ca<sup>2+</sup> transients related to electrical activity

A number of studies in slice preparations have reported spontaneous Ca<sup>2+</sup> transients in developing cortical neurons (Yuste et al., 1992; Owens and Kriegstein, 1998; Garaschuk et al., 2000; Mao et al., 2001). In developing postnatal cortical slices, neurons exhibited high-frequency spontaneous Ca<sup>2+</sup> transients that varied in their temporal patterns, but these transients were oscillatory (Mao et al., 2001). In embryonic cortical slices, precursor cells in the ventricular zone displayed intermittent spontaneous Ca<sup>2+</sup> transients in the cell bodies that occurred approximately



**Figure 7.** Suppressing  $\text{Ca}^{2+}$  transients in cortical growth cones promotes axon outgrowth. *A, B*, Histograms showing effects of  $10 \mu\text{M}$  nifedipine (*A*) and  $10 \mu\text{M}$  nimodipine on increasing axon length ( $n = 34$  for each experimental treatment). *C*, Histogram showing that application of nifedipine 15 hr after plating increased axon length in a dose-dependent manner ( $n = 32$ ). *D*, Histogram showing that application of  $20 \mu\text{M}$  nifedipine 15 hr after plating increases axon and branch length ( $n = 133$ ). *E*, Application of nifedipine virtually eliminates large paused growth cones after 33 hr. *F*, Histograms comparing rates of axon outgrowth in nifedipine-treated and control cultures at 3 and 25 hr after addition of  $20 \mu\text{M}$  nifedipine ( $n = 56$  for each condition).

once every 20 min and had slow onsets (Owens and Kriegstein, 1998). In contrast to our results, these transients were not activity dependent and did not involve VGCC activation but instead were mediated by calcium release from intracellular stores. These  $\text{Ca}^{2+}$  transients were thought to contribute to the regulation of neurogenesis. Other developing CNS neurons such as cerebellar Purkinje cells also exhibit spontaneous high-frequency  $\text{Ca}^{2+}$  transients that are mediated through L-type VGCCs and have been correlated with electrical activity (Liljelund et al., 2000). Although it was suggested that  $\text{Ca}^{2+}$  dynamics can influence downstream events such as gene expression, no correlations were made between  $\text{Ca}^{2+}$  transients and specific aspects of development.

#### $\text{Ca}^{2+}$ transients are mediated by VGCCs

Our results suggest that the L-type calcium channel is a major VGCC involved in the  $\text{Ca}^{2+}$  transients that we observed. Our

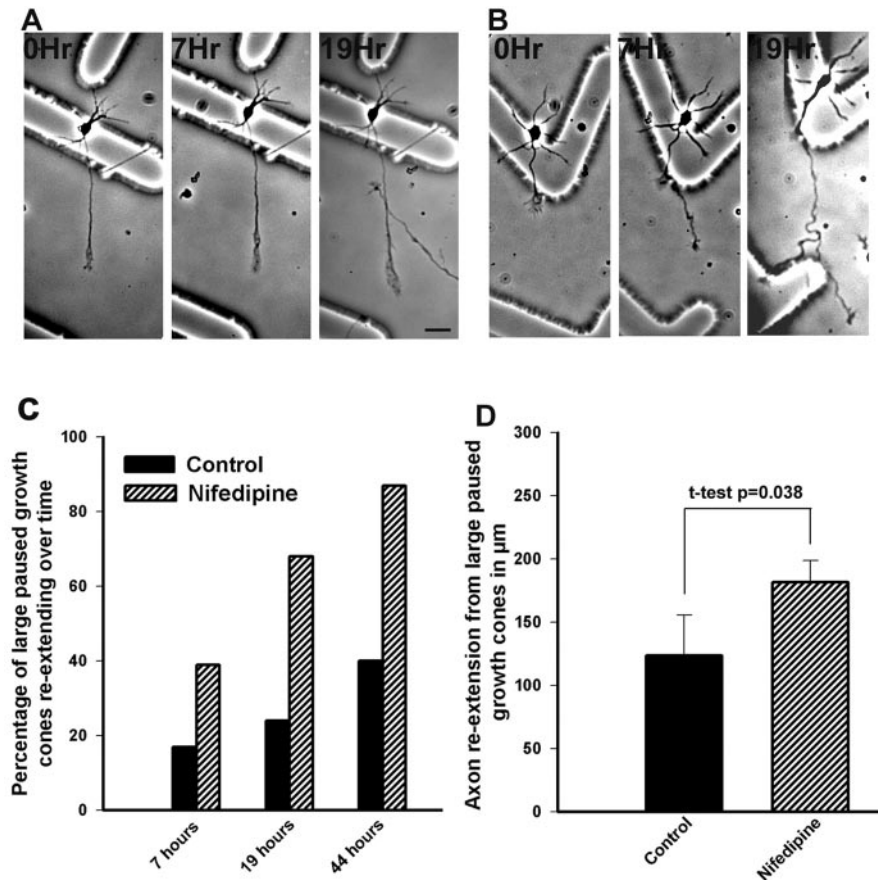
results and other studies (Dolmetsch et al., 2001) have shown that L-type channels are abundant in developing cortical neurons, which also contain other VGCCs such as N- and R-type channels (Timmermann et al., 2002). Thus it is possible that other VGCCs in addition to L-type channels are involved in  $\text{Ca}^{2+}$  transients in cortical neurons. Our results show that application of blockers to N- and P/Q-type channels at high concentrations failed to block calcium activity. However, we did not test for the involvement of R- and T-type channels. At a concentration of  $10 \mu\text{M}$ , the blocking action of nifedipine is generally regarded as specific to L-type calcium channels.  $\text{Ca}^{2+}$  transients in many of the cortical growth cones were completely silenced at this concentration. However, we also found that for some neurons, higher concentrations up to  $20 \mu\text{M}$  were required to completely silence  $\text{Ca}^{2+}$  transients, suggesting that at higher concentrations nifedipine may be blocking other VGCCs in addition to L-type channels. This interpretation is consistent with a recent study of calcium activity in developing cortical neurons (Redmond et al., 2002).

#### $\text{Ca}^{2+}$ transients play a role in regulating axon outgrowth

Previous studies have shown that levels of  $[\text{Ca}^{2+}]_i$  play an important role in regulating neurite outgrowth. Sustained elevation of intracellular  $\text{Ca}^{2+}$  concentrations inhibited growth cone advance (Kater et al., 1988), and maximal outgrowth occurred within an optimal range of calcium concentrations (Kater and Mills, 1991). Transient elevations of  $[\text{Ca}^{2+}]_i$  that were either spontaneous or induced by electrical stimulation also regulated neurite extension by slowing growth cone advance. Electrical stimulation of DRG neurons and elevation of  $[\text{Ca}^{2+}]_i$  (Fields et al., 1990) inhibited the growth rate of DRG neurites and induced collapse of the lamellipodia and filopodia. In contrast, our results show that high-frequency spontaneous  $\text{Ca}^{2+}$  transients did not cause growth cone collapse or retraction. Rather, cortical growth cones stalled in their advance but maintained motility while exhibiting large expanded lamellipodia and active filopodia. These differences may reflect differences in induced versus spontaneous  $\text{Ca}^{2+}$  transients. Highly variable temporal patterns of spontaneous  $\text{Ca}^{2+}$  transients may be intrinsic to specific types of cortical neurons. However, because large pyramidal neurons rather than small interneurons were selected for imaging, it is not likely that the incidence of  $\text{Ca}^{2+}$  transients reflects differences in neuronal cell types. The meaning encoded in these patterns of activity is unknown but may be difficult to mimic by imposed electrical stimulation.

Naturally occurring  $\text{Ca}^{2+}$  transients have been shown to regulate growth cone extension (Gomez et al., 1995; Goldberg and Grabham, 1999; Gomez and Spitzer, 1999). Two distinct types of  $\text{Ca}^{2+}$  transients were characterized in *Xenopus* spinal neurons (Gu and Spitzer, 1994; Gu et al., 1995). Spikes are generated by spontaneous calcium-dependent action potentials in an all-or-none bidirectional manner and act through VGCCs. In contrast, waves, which can arise in the growth cone as well as the cell body, are not elicited by depolarization, do not involve VGCCs, and are propagated at a rate consistent with diffusion of  $\text{Ca}^{2+}$ . Spikes and waves could be generated in the same cell, but waves rather than spikes were shown to regulate axon outgrowth (Gu and Spitzer, 1995). Gomez and Spitzer (1999) found that spontaneous  $\text{Ca}^{2+}$  transients regulate the rate of axon outgrowth *in vivo* in the embryonic *Xenopus* spinal cord, and that rates of axon outgrowth are inversely proportional to the frequency of  $\text{Ca}^{2+}$  transients. Like *Xenopus* spinal neurons, cortical neurons exhibited  $\text{Ca}^{2+}$  transients during growth cone pausing. Similarly, frequencies of  $\text{Ca}^{2+}$  transients were inversely related to rates of growth cone





**Figure 8.** Eliminating  $\text{Ca}^{2+}$  transients by blocking L-type VGCCs promotes axon outgrowth from large paused growth cones. *A*, A large pausing growth cone in control conditions imaged at three different time points. With reference to the markings on the coverslip, the growth cone has advanced only slightly during 19 hr. In contrast, at 19 hr a small growth cone has advanced into the field. *B*, A large pausing growth cone treated with nifedipine imaged at three different time points. At time 0, the growth cone contains a prominent microtubule loop visible even in phase microscopy. By 7 hr, a new axon tipped by a growth cone has extended from the pausing growth cone. By 19 hr, the axon has extended even further. *C*, Histograms comparing the percentage of large pausing growth cones that have re-extended in control versus nifedipine-treated cultures at successive times after addition of nifedipine or DMSO (control). *D*, Histograms comparing outgrowth of axons re-extending from large paused growth cones 19 hr after addition of nifedipine or DMSO (control). Scale bar: (in *A*) *A*, *B*, 20  $\mu\text{m}$ .

advance, and suppression of  $\text{Ca}^{2+}$  transients in both types of neurons increased rates of axon outgrowth. However, in cortical neurons  $\text{Ca}^{2+}$  transients that regulate axon outgrowth have the characteristics of spikes, act through VGCCs, and have frequencies that can reach 14 per minute, in contrast to waves, which have frequencies ranging from 3 to 16 per hour.  $\text{Ca}^{2+}$  transients in cortical neurons regulate not only axon outgrowth but also axon branching. Application of nifedipine, which blocks  $\text{Ca}^{2+}$  transients, interrupted growth cone pausing and promoted axon outgrowth. Consequently, development of axon branches from large paused growth cones failed to occur. Thus, at decision regions in the mammalian CNS, spontaneous  $\text{Ca}^{2+}$  transients, by slowing growth cone advance, could play an important role in the development of branches at appropriate regions along the axon.

#### Possible mechanisms for regulation of growth cone advance by $\text{Ca}^{2+}$ transients

The exact mechanisms whereby  $\text{Ca}^{2+}$  transients regulate growth cone advance have not been identified. It is likely that transient elevation of  $[\text{Ca}^{2+}]_i$  plays a role in the reorganization of the cytoskeleton that underlies changes in growth cone behaviors (Suter and Forscher, 2000). When growth cones slow their ad-

vance, they develop prominent microtubule loops (Sabry et al., 1991; Tanaka and Kirschner, 1991). Previously we found that during transitions from paused to growth states, the microtubule loop undergoes splaying and fragmentation (Dent et al., 1999), and initiation of new growth from the growth cone and the formation of axon branches require interaction between dynamic microtubules and actin filaments (Dent and Kalil, 2001). It is therefore intriguing that  $\text{Ca}^{2+}$  transients with the highest incidence, frequency, and amplitude were found in large paused growth cones with prominent microtubule loops. Preliminary results (our unpublished observations) show that application of nifedipine causes disruption of microtubule loops in large paused growth cones within 1 hr. This suggests that  $\text{Ca}^{2+}$  transients play a role in maintaining the stability of looped microtubules, thus slowing growth cone advance. Consistent with this finding, previous studies have suggested that neurite elongation may be regulated by calcium in part by influencing actin filament stability (Lankford and Letourneau, 1989) and consequently microtubule dynamics. There is recent evidence that  $\text{Ca}^{2+}$  transients acting via calcineurin may slow neurite extension by depolymerizing actin filaments (Lautermilch and Spitzer, 2000). Recent studies have shown that  $\text{Ca}^{2+}$  transients in localized regions of the growth cone can induce filopodial protrusion by actin polymerization (Lau et al., 1999), slow growth cone advance by reducing filopodial motility (Gomez et al., 2001), and induce growth cone turning behaviors (Hong et al., 2000; Zheng, 2000). In the future, it

will be important to examine how induction of  $\text{Ca}^{2+}$  transients in localized regions of the growth cone leads to local reorganization of the cytoskeleton to elicit changes in growth cone behaviors and the development of axon branches.

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