Development/Plasticity/Repair

Netrin-1 Induces Axon Branching in Developing Cortical Neurons by Frequency-Dependent Calcium Signaling Pathways

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A single axon can innervate multiple targets by collateral branching. Axon branching is thus essential for establishing CNS connectivity. However, surprisingly little is known about the mechanisms by which branching is regulated. Axons often stop elongating before branches develop and anatomical and molecular data suggest that axon branching occurs independent of axon outgrowth. We found that netrin-1 dramatically increases cortical axon branching. Here, we sought to identify intracellular signaling components involved in netrin-1-induced axon branching. Using live cell imaging of dissociated developing cortical neurons, we show that netrin-1 rapidly increases the frequency of repetitive calcium transients. These transients are often restricted to small regions of the axon. Simultaneous imaging of calcium activity and development of axon branches revealed that Ca^{2+} transients coincide spatially and temporally with protrusion of branches from the axon. Remarkably, fully formed branches with motile growth cones could develop *de novo* within 20 min. Netrin-1-induced Ca^{2+} transients involve release from intracellular stores and Ca^{2+} signaling is essential for netrin-1-induced axon branching. Using techniques to overexpress or suppress kinase activity, we find that calcium/calmodulin-dependent protein kinase II (CaMKII) and mitogen-activated protein kinase (MAPK) are major downstream targets of the netrin-1 calcium signaling pathway and are required for axon branching. CaMKII, but not MAPK, is also involved in axon outgrowth. The role of CaMKII and MAPKs in axon branching is consistent with the sensitivity of these kinases to changes in the frequency Ca^{2+} transients. Together, these novel findings define calcium signaling mechanisms required for development of new axon branches promoted by a guidance cue.

Key words: **cortical development; axon guidance; netrin-1; axon branching; calcium transients; CaMKII**

Introduction

Axons are guided toward targets by responses of their motile growth cones to environmental cues (Dickson, 2002). However, in the complex environment of the mammalian CNS, growth cones guide axons along pathways but do not typically extend into targets. Rather, axons extend collateral branches, and, in this way, a single axon can innervate multiple targets. Thus, axon branching is a fundamental mechanism for establishing connectivity in the developing CNS. Axon branching is also important in regenerative sprouting after injury (Schwab, 2002). During development, axons are known to stop elongating before branches begin to extend (O'Leary et al., 1990), and anatomical and molecular data suggest that axon branching may occur independent of axon outgrowth (Halloran and Kalil, 1994; Ng et al., 2002; Colavita and Tessier-Lavigne, 2003). Thus, for example, we found that netrin-1 increases cortical axon branching without affecting outgrowth of the primary axon (Dent et al., 2004). However, mechanisms regulating axon branching independent of axon outgrowth are poorly understood.

Axon branching is essential for establishing CNS connectivity, but surprisingly little is known about the mechanisms by which branching is regulated (Dent et al., 2003; Kornack and Giger, 2005). Calcium signaling is an important regulator of axon outgrowth and guidance (Gomez and Spitzer, 2000; Henley and Poo, 2004). Previously, we found that, in cortical neurons, spontaneous global Ca^{2+} transients regulate axon outgrowth in a frequency-dependent manner such that high-frequency repetitive transients were correlated with slowing of axon outgrowth, whereas low-frequency transients were associated with more rapid outgrowth (Tang et al., 2003). Recently, we found that application of netrin-1 induces extensive axon branching on cortical neurons (Dent et al., 2004). How does netrin-1 promote axon branching? Because netrin-1 has been shown to elevate Ca^{2+} levels in growth cones during steering events (Hong et al., 2000; Ming et al., 2002; Nishiyama et al., 2003), we hypothesized that netrin-1 may promote axon branching through Ca^{2+} changes in the axon. Moreover, because Ca^{2+} frequency-dependent mechanisms are important in regulating cortical axon outgrowth (Tang et al., 2003), we hypothesized that repetitive

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Ca^{2+} transients may also play a role in netrin-1-induced axon branching. Activation of specific signaling components are known to be related to frequencies of Ca^{2+} transients. Calcium/calmodulin-dependent kinase II (CaMKII) is known to function as a Ca^{2+} spike-frequency detector (Hudmon and Schulman, 2002), and mitogen-activated protein kinases (MAPKs) are also sensitive to intracellular Ca^{2+} changes (Cullen and Lockyer, 2002). Therefore, if repetitive Ca^{2+} transients are indeed involved in axon branching, CaMKII and MAPKs could be likely candidates as downstream targets of Ca^{2+} signaling. In this study, we sought to identify the intracellular signaling components involved in netrin-1-induced axon branching.

Here, we show for the first time that netrin-1 promotes rapid and extensive cortical axon branching by evoking repetitive Ca^{2+} transients in regions of the axon. Netrin-1 was also able to modulate endogenous Ca^{2+} activity by increasing the frequency of Ca^{2+} transients. We show that CaMKII and MAPK are major components of the netrin-1 calcium signaling pathway essential for axon branching and that CaMKII, but not MAPK, also plays a role in axon outgrowth. Together, these novel findings define mechanisms by which netrin-1 can promote a dramatic increase in axon branching in developing cortical neurons.

Materials and Methods

Cell culture. Dissociated cultures were prepared from sensorimotor cortices obtained from postnatal day 0 (P0)–P3 golden Syrian hamster (*Mesocricetus auratus*), as described previously (Dent and Kalil, 2003). Neurons were cultured in dishes coated with 1.0 mg/ml poly-D-lysine at a density of 4–8 k/cm^2 for calcium imaging and immunocytochemistry, 0.8 k/cm^2 for netrin-1-induced branching experiments, and 15 k/cm^2 for immunoblotting assay. Neurons in our cultures have already developed dendrites and a long axon with a growth cone at 15 h after plating (Szebenyi et al., 1998). At this stage, axons and dendrites can be clearly identified. Thus, we performed our calcium imaging experiments as early as 15 h after plating. In some experiments, when a Ca^{2+}-free medium was required to test the necessity of extracellular Ca^{2+} for axon branching, Neurobasal medium was replaced by calcium-free DMEM medium in which cortical neurons survived well.

Experimental reagents. Recombinant chicken protein netrin-1 was purchased from R & D Systems (Minneapolis, MN). Stock solutions were prepared by solubilizing drugs in water, dimethylsulfoxide (DMSO), or methanol according to the recommendations of the manufacturer. The following drugs in stock solutions were diluted in serum-free medium and bath applied to cultures: the L-type voltage-gated Ca^{2+} channel (VGCC) antagonist nifedipine (15 μm; Calbiochem, La Jolla, CA), the ryanodine receptor antagonist dantrolene (20 μm; Alomone Labs, Jerusalem, Israel), the inositol 1,4,5 trisphosphate (IP_3) receptor antagonist 2-aminoethoxydiphenyl borate (100 μm; Calbiochem), the general inhibitor to CaM kinases (5 μm KN62; Calbiochem), the specific inhibitor to CaMKII (200 nM cell-permeable AIPII; Calbiochem), the MAPK inhibitors 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto) butadiene (U0126; 10 μM; Cell Signaling Technology, Beverly, MA) and 2-(2-amino-3-methyoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059; 20 μM; Promega, Madison, WI). The antibody to αCaMKII were obtained from Zymed Laboratories (South San Francisco, CA). The antibody against CaMKII, when phosphorylated at threonine 286, was purchased from Cell Signaling Technology and Promega. Phospho-MAP kinase kinase 1/2 (MEK1/2) and extracellular signal-regulated kinase 1/2 (Erk1/2) were all acquired from Cell Signaling.

DNA constructs, cell transfection, and image acquisition. DNA constructs were amplified by QIAfilter Plasmid Maxi kits (QIAGEN Sciences, Germantown, MD). Neurons at 1 d *in vitro* (DIV) were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Dishes were treated with 1 μg of DNA and 1 μl of Lipofectamine 2000 reagent in OptiMEM buffer (Invitrogen). Enhanced green fluorescent protein (EGFP) was cotransfected with non-GFP or myc-tagged plasmids. The cotransfection efficiency was monitored by immunostaining of EGFP-transfected cells with antibodies to proteins expressed by the plasmids and was found to reach >90%. Neurons with moderate levels of EGFP fluorescence developed typical dendritic and axonal process similar to nontransfected cells and remained healthy. At 3 DIV, cells were fixed and images of fluorescent neurons were acquired with a 40×/1.0 numerical aperture (NA) Plan Apo CF160 objective (Nikon, Tokyo, Japan). Neurons transfected with myc-tagged plasmids were subjected to immunostaining by anti-myc antibody (Oncogene Research, San Diego, CA) before image acquisition. Montages of representative neurons were constructed using Adobe Photoshop CS (Adobe Systems, Mountain View, CA). The length of axons and branches and number of branches was measured with MetaMorph software (Universal Imaging, Downingtown, PA).

Local application of netrin-1. Gradients of netrin-1 applied to localized regions of axons and growth cones were established by locally puffing netrin-1 from a 0.5–1.0 μm pipette connected to an Eppendorf (Hamburg, Germany) microinjection system. The pipette was loaded with a stock solution of netrin-1 (25 μg/ml) and positioned 10–20 μm from a growth cone or axon. Netrin-1 was ejected from the pipette tip by a pressure of 20–30 kPa every 1 s for 15–60 min during differential interference contrast (DIC) and fluorescence time-lapse imaging. The pipette loaded with a fluorescent dextran was used to demonstrate local gradients.

Time-lapse calcium and DIC imaging. Cortical neurons were loaded with membrane-permeable 2–4 μm Fluo-4 AM (Molecular Probes, Eugene, OR) predissolved in 0.01% pluronic acid (Molecular Probes) and 0.1% DMSO for 30 min. Excess dye was washed out with three to five rinses of serum-free medium. In some experiments, when netrin-1 was bath applied, coverslips on which neurons were plated were enclosed in a chamber consisting of a 15 mm glass ring (Thomas Scientific, Swedesboro, NJ). In a few experiments, TTX was bath applied to neurons to eliminate spontaneous electrical activity. This allowed us to determine whether Ca^{2+} activity could be evoked by netrin-1 in neurons without ongoing Ca^{2+} transients. The dishes were returned to the incubator for 30–60 min. Fluorescence imaging of intracellular Ca^{2+} dynamics in time-lapse for periods ranging from 10 min to 1 h was performed with a Nikon 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, Tucson, AZ). The imaging system was controlled by MetaMorph Software (Universal Imaging). Neurons were imaged in time lapse with either a 60× or a 100× 1.4 NA Plan Apo CF160 objective (Nikon). Images were captured every 5–15 s, with 150–500 ms exposures, and under low light-level conditions achieved by illumination-attenuating neutral-density filters (Chroma Technology, Brattleboro, VT). Images were collected at a slow transfer rate, which reduces background noise, and binned (2 × 2). In some experiments, DIC images were taken in rapid succession with fluorescence Ca^{2+} imaging to determine the relationship between development of new branches and intracellular Ca^{2+} changes. Alternate acquisition of both DIC and fluorescence images were automatically controlled by a Lambda 10–2 dual-filter wheel (Sutter Instruments, Novato, CA).

Measurement and analysis of Ca^{2+} transients. Measurements of transients in growth cones and axons were obtained as described previously (Tang et al., 2003). Briefly, average fluorescent pixel intensity of the region of interest in a growth cone or an axon was digitally quantified with MetaMorph software, subtracted from background, and normalized to baseline fluorescence intensity. Fluorescence increases >150% of baseline were generally characterized as Ca^{2+} transients, which were further confirmed by frame-by-frame examination of the time-lapse movie.

Quantification of axon branches and image processing. In experiments with long-term treatments, 100 ng/ml netrin-1 and other reagents were bath applied to cultures 7–10 h after plating. At 3 DIV, cultures were fixed, and images were acquired with a 10×/0.5 NA Neofluar phase-contrast objective on a Zeiss (Thornwood, NY) 35M inverted microscope. Processes extending >20 μm at orthogonal angles to the axon were characterized as branches. We counted only the number and length of branches extending from primary axons. Each individual experiment was repeated at least three times. Images shown in the figures were enhanced using the unsharp mask filter, high pass, and brightness-contrast

adjustment functions in Adobe Photoshop. Time-lapse images were assembled into QuickTime movies (Premiere; Adobe Systems). Graphs were created in SigmaPlot (SPSS, Chicago, IL). Images were processed with MetaMorph 4.62 and all figures were constructed in Photoshop CS (Adobe Systems).

Statistical analysis. Statistical analyses of control and experimental groups were performed using Sigmastat (Jandel Scientific, Corte Madera, CA). Significant difference between control neurons and those in a single experimental condition was determined by Student's *t* test. Multiple experimental groups were compared with controls with the Krustal–Wallis one-way ANOVA on ranks with Dunn's *post hoc* test. In pharmacological experiments in which cortical neurons were treated with various drugs for 3 d, at least 60 neurons in each experimental condition were chosen for measurements of branch number and length of axons and branches. In experiments in which overexpression of different constructs of CaMKII and MAPK were used, at least 30 transfected neurons in each experimental condition were used for measurements of branch number and length of axons and branches. Each experiment was repeated at least three different times. In experiments using time-lapse microscopic imaging, we tested the effects of local repetitive application of netrin-1 on 30 axons, of which 15 formed new branches within 1 h in response to netrin-1. In contrast, in control experiments ($n > 10$) in which 0.5% BSA in PBS buffer was locally applied to cortical axons, localized branch formation on axons rarely occurred. In four separate experiments, gradients of netrin-1 locally applied to cortical axons induced transient filopodial activity, which disappeared after withdrawal of netrin.

Immunocytochemistry. At 15–60 min after addition of netrin-1 to cortical neurons at 2 or 3 DIV, neurons were fixed and immunostained as described previously (Dent and Kalil, 2001). Primary antibodies (phospho-CaMKII and phospho-MAPK) from both Cell Signaling Technology and Promega and secondary antibodies from Jackson ImmunoResearch (West Grove, PA) were used to detect phosphorylation of CaMKII and MAPK. In some experiments, to quantify and compare phosphorylation of CaMKII or MAPK at different times after stimulation of netrin-1, the same cultures were fixed and processed with first and secondary antibodies at the same time. Images were taken on the same day and under the same conditions, including exposure time, illumination, and digital scaling. In a few experiments, to determine the relationship between netrin-induced calcium activity and phosphorylation of CaMKII, we first performed time-lapse calcium imaging for 10–15 min and rapidly fixed the cultures on the microscope stage, followed by immunocytochemistry. In these experiments, we used dishes attached with etched grid glass coverslips to provide landmarks for relocating the cells (Bellco Glass, Vineland, NJ).

Western blotting. Neurons at 3 DIV were treated with 100 ng/ml netrin-1 or 50 mM KCl, solubilized in 60 μl of ice-cold extraction buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 0.5% sodium deoxycholate, 1% IGEPAL CA-630, 10 mg/ml PMSF, 100 mM sodium vanadate (all from Sigma, St. Louis, MO), and complete protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN)], and centrifuged at 14,000 rpm for 5 min. Supernatants were absorbed for immunoblotting with rabbit antibodies to phosphorylated forms of CaMKII (Promega) and protein kinase C (PKC) (Cell Signaling Technology). Blots were developed in ECL+ (Amersham Biosciences, Arlington Heights, IL).

Results

Netrin-1 induces rapid axon branching

Previously (Dent et al., 2004), we found that bath application of netrin-1 to cortical neurons over several days promotes extensive axon branching, but not axon elongation, and that local application induces rapid localized axon branching. Here, to investigate the time course of branching, we bath applied netrin-1 (100 ng/ml) to cortical cultures for 3 d, which increased axon branching by more than threefold, consistent with our previous findings. Untreated axons typically have only a few branches, whereas netrin-1-treated axons had elaborate multibranched arbors ($n = 176$) (Fig. 1*A*) with ~40% increase in branch length. Within 1 h of netrin-1 treatment, axons had developed branches up to 22 μm in length ($n = 8$) (Fig. 1*B*,*C*), whereas controls did not develop significant branching until several days in culture. Branches on netrin-1-treated neurons could develop from growth cone filopodia that thickened, elongated, and elaborated a growth cone (Fig. 1*B*). Branches also developed from axonal filopodial processes that elongated and branched again into arbors (Fig. 1*C*). Local application of netrin-1 pulsed through a glass pipette positioned 25 μm from the axon elicited new branches within a few minutes in ~50% of axons tested ($n = 30$). In close proximity to the pipette tip, branches developed from preexisting filopodia (Fig. 1*D*), *de novo* from smooth regions of the axon (Fig. 1*E*), or from increased lammellipodia along the axon shaft (Fig. 1*F*). In several cases ($n = 4$), netrin-1 elicited transient filopodia (Fig. 1*G*) for 16 min that disappeared within 16 min after the pipette was withdrawn. Thereafter, filopodial activity did not change in the absence of netrin-1. These results suggest that netrin-1 can induce rapid branching on responsive neurons within 5 min by either additional growth of existing filopodia or *de novo* protrusions from the axon shaft. Moreover, netrin-1 is required for the maintenance of developing branches.

Netrin-1 induces repetitive calcium transients in cortical axons

To determine the effects of netrin-1 on Ca^{2+} activity in cortical neurons, we bath or locally applied netrin-1 and measured changes in intracellular Ca^{2+} with the calcium indicator Fluo-4. Application of netrin-1 evoked increases in Ca^{2+} activity within minutes in 59.4% of neurons tested ($n = 128$). In 32% of neurons, there were no detectable changes in activity, and, in 8.6% of neurons, calcium activity decreased. Within a few minutes, netrin-1 elicited repetitive Ca^{2+} transients in neurons showing no previous activity (Fig. 2*A*) or increased the frequency and amplitude of endogenous Ca^{2+} transients (Fig. 2*B*). In a few experiments, we used TTX to silence endogenous Ca^{2+} transients. We found that netrin-1 could evoke Ca^{2+} transients in neurons showing no endogenous activity (data not shown). BSA (15 control experiments) had no effect on calcium transients (Fig. 2*C*). Increased frequency was the most common type of change in Ca^{2+} transients after netrin-1 application. Measurements of calcium changes in 32 neurons responsive to netrin-1 showed that the average frequency of Ca^{2+} transients was increased fourfold (*t* test $p < 001$) from 0.2 to 0.8 per minute (Fig. 2*D*). Netrin-1 application could elicit localized Ca^{2+} changes that spread globally along the entire axon (Fig. 2*E1–E7*), or such changes could remain restricted to small regions of the axon shaft averaging 15.3 μm in length (Fig. 2*F1–F4*). Netrin-1-evoked Ca^{2+} transients in an axon and its branches often occurred in patterns, frequencies, and amplitudes that were different and distinct in the axon and its branches (Fig. 2*G1–G7*) (supplemental movie, available at www.jneurosci.org as supplemental material). Changes in Ca^{2+} transients were maintained for the entire observation period (5–30 min), but when netrin-1 was withdrawn, Ca^{2+} activity gradually declined within minutes to baseline levels (data not shown).

Calcium activity is required for axon branching

Subsequently, we asked whether netrin-1-induced branching requires Ca^{2+} signaling. First, we sought to determine the source of netrin-1-induced Ca^{2+} activity. Cortical axon elongation is regulated by calcium entry through voltage gated L-type channels (Tang et al., 2003). To determine whether this mode of calcium entry is also involved in netrin-1-induced activity, we applied the L-type

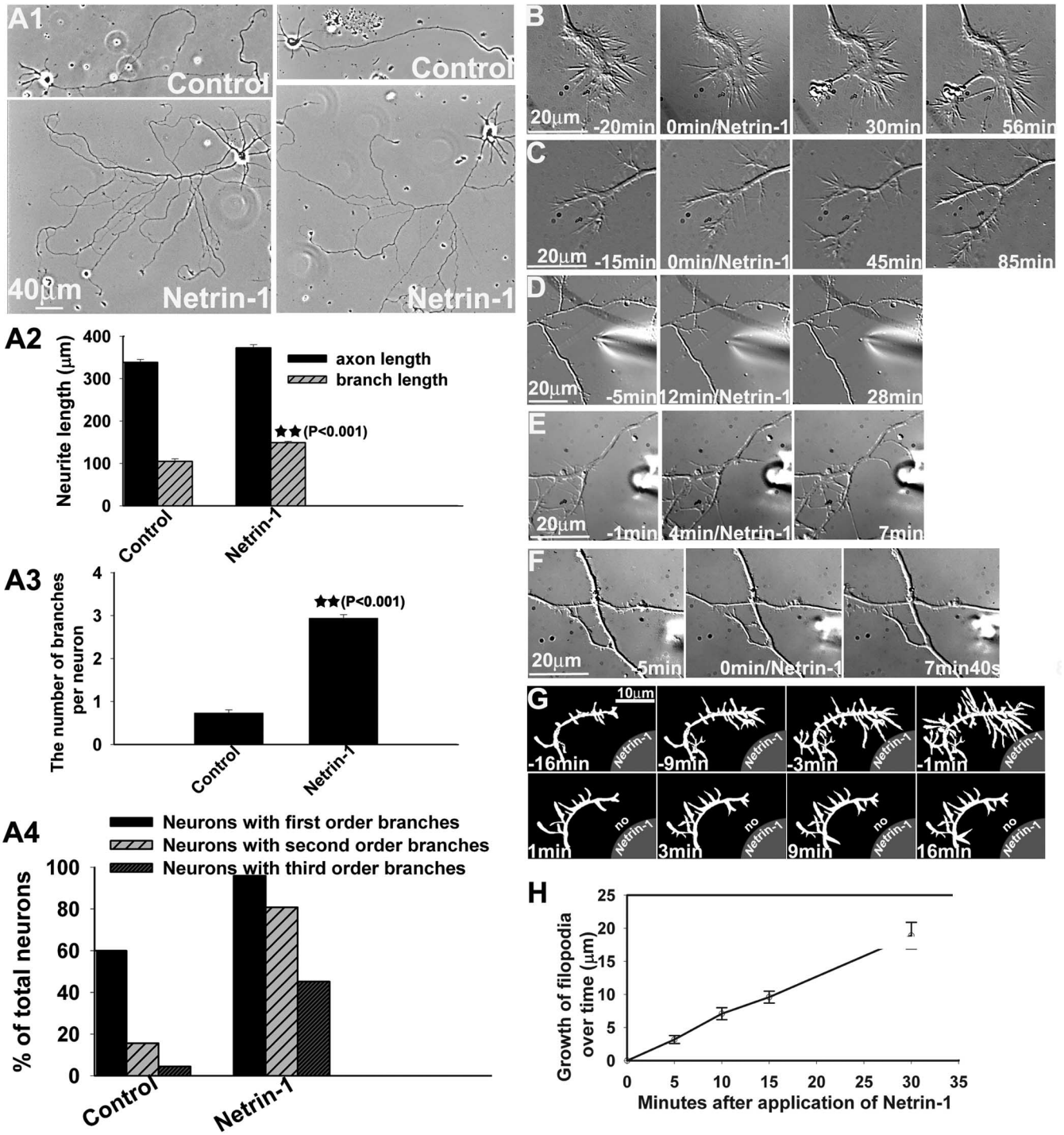

Figure 1. Bath-applied netrin-1 increases axon branching without affecting axon length, and locally applied netrin-1 induces filopodial protrusions on the axon shaft. ***A1***, Examples of cortical neurons treated for 3 d with BSA (controls) and for 3 d with bath-applied netrin-1 (100 ng/ml) showing dramatic increase in axon branching with netrin-1. ***A2–A4***, Bar graphs showing no increase in axon length but a significant increase in branch length (***A2***), a threefold increase in numbers of branches (***A3***), and a large increase in neurons with higher-order axon branches (***A4***) after netrin-1 treatment for 3 d. ***B***, ***C***, Sequences of time-lapse DIC images showing rapid development of axon branches after bath-applied netrin-1. ***B***, ***C***, An example of a branch extending from a growth cone and developing its own growth cone (***B***) and increased axon branching by filopodial protrusions (***C***). ***D–F***, Locally applied netrin-1 induces rapid protrusion of filopodia (***D***, ***E***) and lammelipodia (***F***) on the axon in the region of the pipette tip. ***G***, Series of tracings of DIC images showing extension of transient filopodia during 15 min time period when netrin-1 is locally pulsed every 15 s (at top). Transient filopodia are illustrated as cumulative over time. When netrin-1 is withdrawn (bottom), filopodial protrusions cease. ***H***, Graph plotting average growth of 12 axonal filopodia induced *de novo* from 10 neurons by netrin-1. Filopodia extend to 20 μm over 30 min.

channel blocker nifedipine and found in all cases ($n = 7$) that this did not interfere with the ability of netrin-1 to induce robust Ca^{2+} transients (Fig. 3*A*). To determine the involvement of Ca^{2+} release from intracellular stores, we applied blockers to IP_3 ($n = 6$) and ryanodine receptors ($n = 6$) and found that, in all cases, Ca^{2+} transients were gradually attenuated (Fig. 3*B*,*C*). Thus, netrin-1-induced Ca^{2+} transients involve release from intracellular stores rather than Ca^{2+} entry through L-type channels.

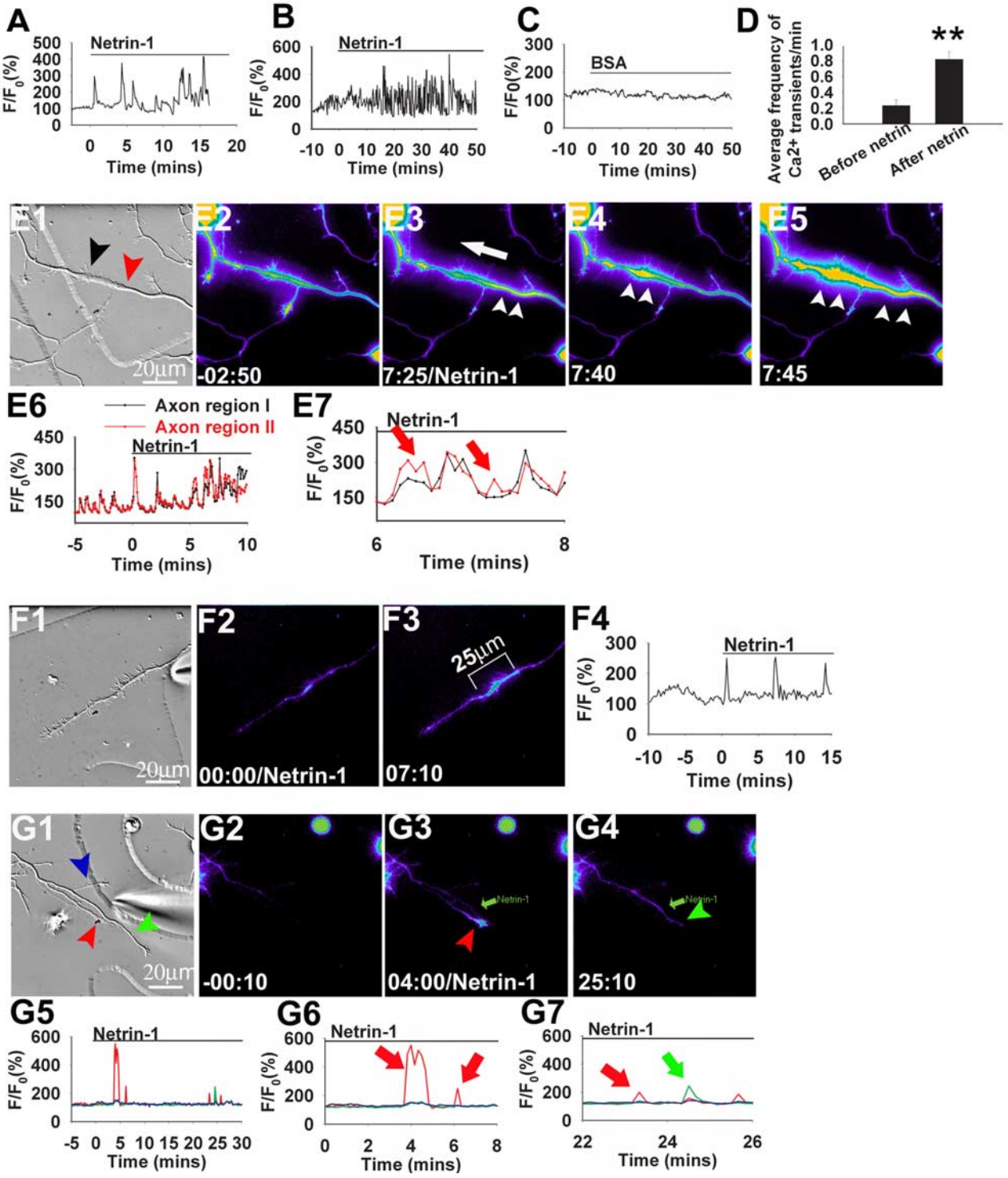

Figure 2. Repetitive calcium transients in cortical axons are evoked by netrin-1 application within minutes. ***A***, Netrin-1 evokes calcium activity in a previously silent axon. ***B***, Netrin-1 increases the frequency and amplitude of calcium transients. ***C***, BSA (control) elicits no calcium changes. Changes in fluorescence intensity are shown relative to baseline (F/F_0%), and measurements of calcium changes in all examples were obtained from axons and their growth cones. ***D***, Graph comparing average frequency of calcium transients in 32 neurons before and after netrin-1 application. Frequency of calcium transients increases fourfold after netrin-1 application. ***E1–E5***, DIC image (***E1***) and time-lapse images in pseudocolor (***E2–E5***), showing changes in calcium activity in a region of an axon after application of netrin-1. Calcium images were acquired every 5 s. ***E3***, A calcium transient begins in the region of the arrowheads and is propagated along the axon in the direction indicated by the arrow. ***E2–E5***, Regions of high calcium activity correspond to the region of the red and black arrowheads shown in the DIC image in ***E1***. Measurements of frequencies and amplitudes of calcium transients in the two axon segments corresponding to the red (region I) and black (region II) arrowheads are shown in ***E6*** and magnified in ***E7*** for the time period of 6 – 8 min. Red arrows indicate time points when peak amplitudes occur only in axon region I. ***F1–F4***, Localized pulsed application of netrin-1 can evoke calcium repetitive calcium transients in small localized regions of the axon. ***F4***, Changes in frequency of calcium transients in the region of the axon bracketed in ***F3***. ***G1–G4***, DIC image (***G1***) and time-lapse images in pseudocolor (***G2–G4***) showing changes in calcium activity in an axon (green arrowhead) and its two branches (blue and red arrowheads) after local application of netrin-1 indicated by the green arrow. Localized calcium transients occur in one branch at 4 min (***G3***) and in the primary axon at 25 min (***G4***). Measurements of calcium transients in the axon (green) and in the branch (red) are shown during different time periods (***G5–G7***). Local transients in the branch are indicated by red arrows and are indicated in the primary axon by green arrow (see supplemental movie 1, available at www.jneurosci.org as supplemental material). As shown in ***G5–G7***, calcium transients induced by netrin-1 can occur at different frequencies and asynchronously in an axon and its branches.

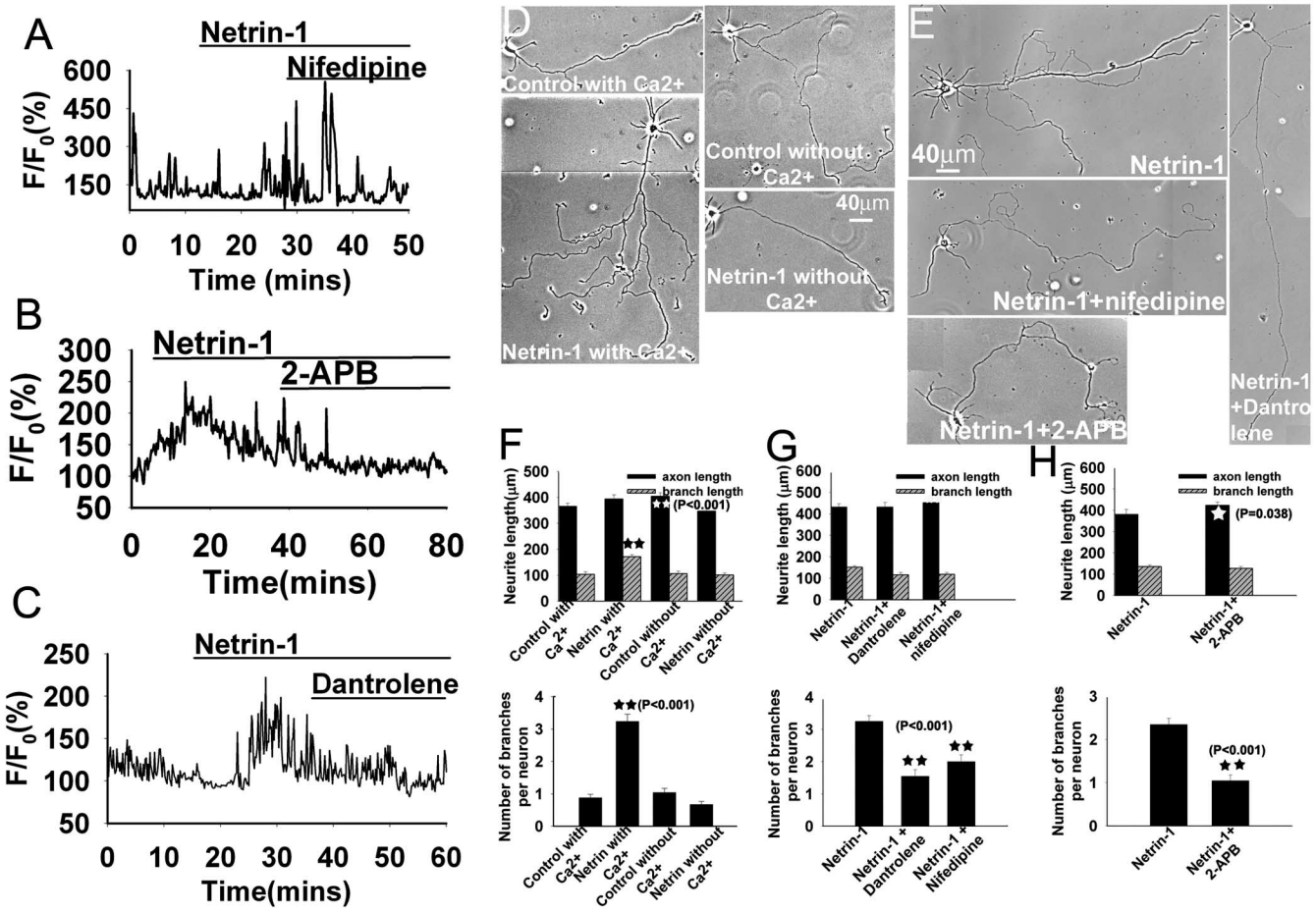

Figure 3. Calcium activity evoked by netrin-1 involves release from intracellular stores and is required for axon branching. ***A–C***, Examples of neurons in the presence of netrin-1 treated with nifedipine to block L-type voltage-gated channels (***A***), 2-aminoethoxydiphenyl borate (2-APB) to block IP_3 receptors (***B***), and dantrolene to block ryanodine receptors (***C***). Neurons treated with 2-APB and dantrolene, but not nifedipine, show attenuation of calcium activity over time. ***D***, Examples of neurons cultured with and without calcium in the medium in the presence of 0.5% BSA (control) or 100 ng/ml netrin-1. In the absence of extracellular calcium, netrin-1 does not promote axon branching, but axons are slightly longer. ***E***, Examples of neurons treated with netrin-1 or with netrin-1 and the addition of nifedipine, 2-APB, or dantrolene, demonstrating that blocking calcium release from intracellular stores diminishes axon branching. ***F–H***, Bar graphs comparing axon length, branch length, and numbers of branches per neuron in all experimental conditions shown in the examples in ***D*** and ***E***.

We then measured axon branching under various conditions in which calcium activity was experimentally reduced. When cortical neurons were cultured in Ca^{2+} free medium (Fig. 3*D*,*F*), axon branching was similar to control levels without netrin-1, although axons were significantly longer. Importantly, netrin-1 applied to neurons in the absence of extracellular Ca^{2+} failed to elicit axon branching above control levels. In the subsequent set of experiments, in which calcium was present in the medium, we examined the source of calcium involved in netrin-1-induced axon branching. We found that application of the L-type channel blocker nifedipine partially suppressed netrin-1-induced axon branching, which is surprising in view of the result that nifedipine has no effect on netrin-1-induced Ca^{2+} transients (Fig. 3*E*,*G*). One possibility to explain this result is that netrin-induced calcium transients normally activate L-type channels that act on downstream kinases (Dolmetsch et al., 2001) to promote branching. When L-type channels are blocked, these signaling pathways are inactivated, and branching is reduced in the presence of calcium transients that could arise from entry through other types of channels. When Ca^{2+} release from intracellular stores was prevented by blockers to IP_3 and ryanodine receptors, we found that axon branching was severely inhibited (Fig. 3*G*,*H*). These results show that Ca^{2+} signaling is required for netrin-1-induced axon branching and that release from intracellular stores is a major source of this Ca^{2+} activity. Nevertheless, we cannot rule out the possibility that calcium entry through L-type channels could contribute to netrin-1-induced axon branching.

To further demonstrate the importance of Ca^{2+} signaling in axon branching, we imaged netrin-1-induced branching events simultaneously with live cell Ca^{2+} imaging (Fig. 4). In four sequences lasting up to 30 min, we were able to capture axon branching events that precisely coincided spatially and temporally with induction of Ca^{2+} transients. In one sequence, for example, application of netrin-1 to a localized region of the axon almost immediately elicited large Ca^{2+} changes along a 30 μm length of the axon from which a 20 μm branch subsequently developed (Fig. 4). Ca^{2+} transients in this region of the axon shaft greatly increased in frequency and amplitude over the subsequent 20 min during continuous application of netrin-1 (Fig. 4*C*). Remarkably, by 12 min a branch began to protrude *de novo* from the region of high calcium activity. By 20 min, a motile growth cone had elaborated a dynamic arbor on which filopodia extended and retracted (supplemental movie, available at www.jneurosci.org as supplemental material). Thus, the axonal processes that developed in regions of high Ca^{2+} activity were not transient filopodia but stable arborizing branches with motile growth cones that

persisted throughout the observation period. Several other branches also developed *de novo* from this region of the axon within 20 min. Interestingly, the single long branch already present on the axon also showed localized high Ca^{2+} activity in a region from which numerous transient lamellar and filopodial processes protruded. These observations show that netrin-1 stimulation can induce high levels of Ca^{2+} transients on restricted regions of an axon coincident with rapid development of branches.

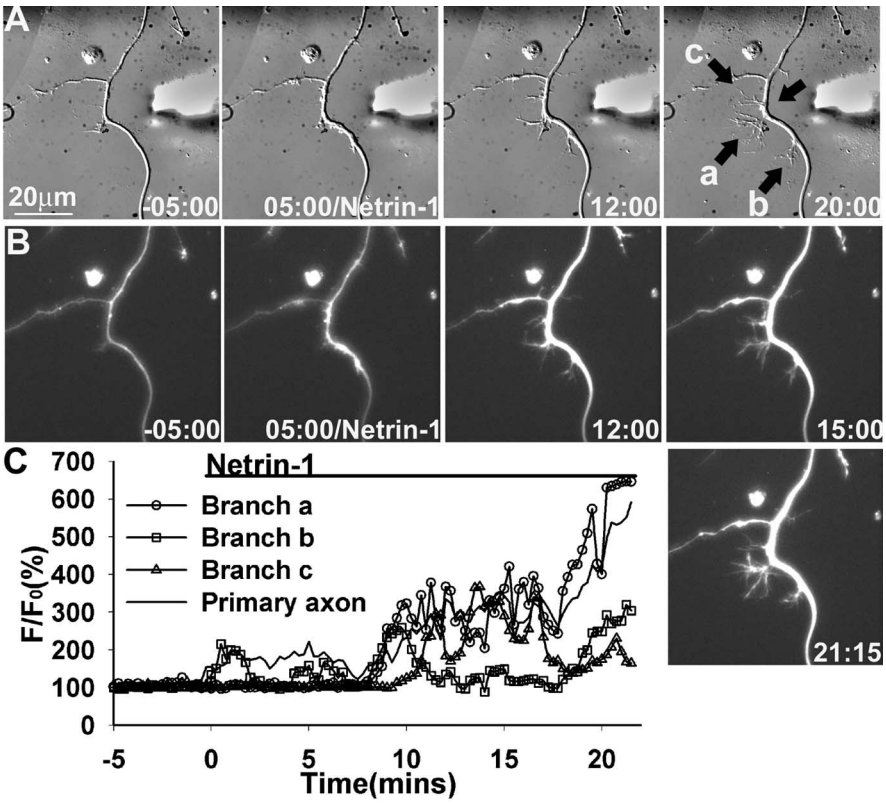

Figure 4. Localized netrin-1 application evokes repetitive calcium transients that are simultaneous with development of axon branches in the same region of the axon. ***A***, DIC time-lapse images showing protrusion of filopodia in response to local application of netrin-1. Arrows indicate three different branches. The branch at the arrow begins as a short filopodium but by 20 min has elongated and developed a complex growth cone. ***B***, The corresponding time-lapse fluorescence images show high levels of repetitive calcium transients (see supplemental movie, available at www.jneurosci.org as supplemental material) that coincide with newly developing branches. As can be seen in the movie, calcium activity begins before filopodial protrusion and continues during development of branches over 20 min. ***C***, Measurements of calcium transients in the primary axon and the three branches corresponding to lines of the arrows. The growth cone developing from the region of arrow a shows the largest changes in calcium activity.

CaMKII is involved in calcium signaling, regulating axon outgrowth and branching

We were interested in the downstream targets of netrin-1-induced Ca^{2+} signaling involved in axon branching. Because we found that the major effect of netrin-1 on Ca^{2+} transients was to increase their frequency, one likely downstream target is CaMKII, which is known to function as a detector of Ca^{2+} spike frequency (Hudmon and Schulman, 2002; Soderling et al., 2001). To determine whether netrin-1 activates CaMKII, we stained neurons with antibodies to phosphorylated CaMKII after treatment with either KCl or netrin-1, both of which increase intracellular Ca^{2+}. Activation of CaMKII occurred within 15 min and persisted up to 1 h (Fig. 5*A*,*B*). Importantly, we performed live cell imaging of Ca^{2+} transients in neurons before and after netrin-1 treatment. We then rapidly fixed these cells and stained them with phospho-CaMKII antibodies. These experiments (Fig. 5*C*) revealed that only cells in which repetitive Ca^{2+} transients were induced by netrin-1 had strong phospho-CaMKII staining, demonstrating a strong correlation between increased frequency of Ca^{2+} transients and activation of CaMKII. In the subsequent set of experiments, we applied the CaMKII inhibitor KN62, which can inhibit several CaM-kinases, and AIPII which has been shown to be a specific inhibitor for CaMKII (Jourdain et al., 2003). KN62 completely abolished axon branching in the presence of netrin-1 and significantly reduced axon outgrowth (Fig. 6 *A–C*). Although a previous study showed that CaMKII is necessary for KCl-induced survival of sensory neurons (Vaillant et al., 1999), we found no adverse effects on neuron survival after treatment with CaMKII inhibitors. As shown in Figure 6, axon and dendritic outgrowth are robust even after 3 d of exposure to these inhibitors which selectively affect axon length and branching. AIPII treatment dramatically reduced numbers of axon branches, which were significantly shorter and lacking in higher-order branches (Fig. 6*D–G*) compared with netrin-1-stimulated neurons. Similar to KN62, AIPII also reduced axon outgrowth. These pharmacological experiments were performed by adding the CaMKII inhibitors to the cultures along with netrin-1 before branches had developed. To determine whether CaMKII is important for initiation or maintenance of axon branches we added the inhibitors 2 d after netrin-1 treatment when most branches had already formed. Branching was only slightly reduced, suggesting that CaMKII is important for the initiation and growth of branches rather than their maintenance (data not shown). Together, these results suggest that CaMKII is involved in the netrin-1 signaling pathway underlying axon branching and also plays a role in axon outgrowth.

To provide additional evidence for the importance of CaMKII in axon branching, we transfected cortical neurons with several wild-type and mutant CaMKII constructs fused to EGFP. CaMKII is normally expressed in cortical neurons by 1 d in culture and increases over the next several days, during which axon branches are also developing (data not shown). Netrin-1 application increased CaMKII expression over this 3 d period (data not shown). We transfected neurons after 1 d in culture with either EGFP wild-type αCaMKII or βCaMKII. Overexpression of αCaMKII but not βCaMKII promoted a more than threefold increase in axon branching (Fig. 7*A–D*), and the morphologies of axon arbors were similar to those after netrin-1 treatment. Axon length was also significantly increased. Transfection with an autophosphorylation mutant of CaMKII (Fig. 7*D*) resulted in neurons with branching above control levels but still significantly less than in neurons transfected with wild-type CaMKII. This could mean that autophosphorylation is not responsible for 100% of the activity of CaMKII during netrin-1-induced axon branching. Transfection of neurons with constitutively active αCaMKII sim-

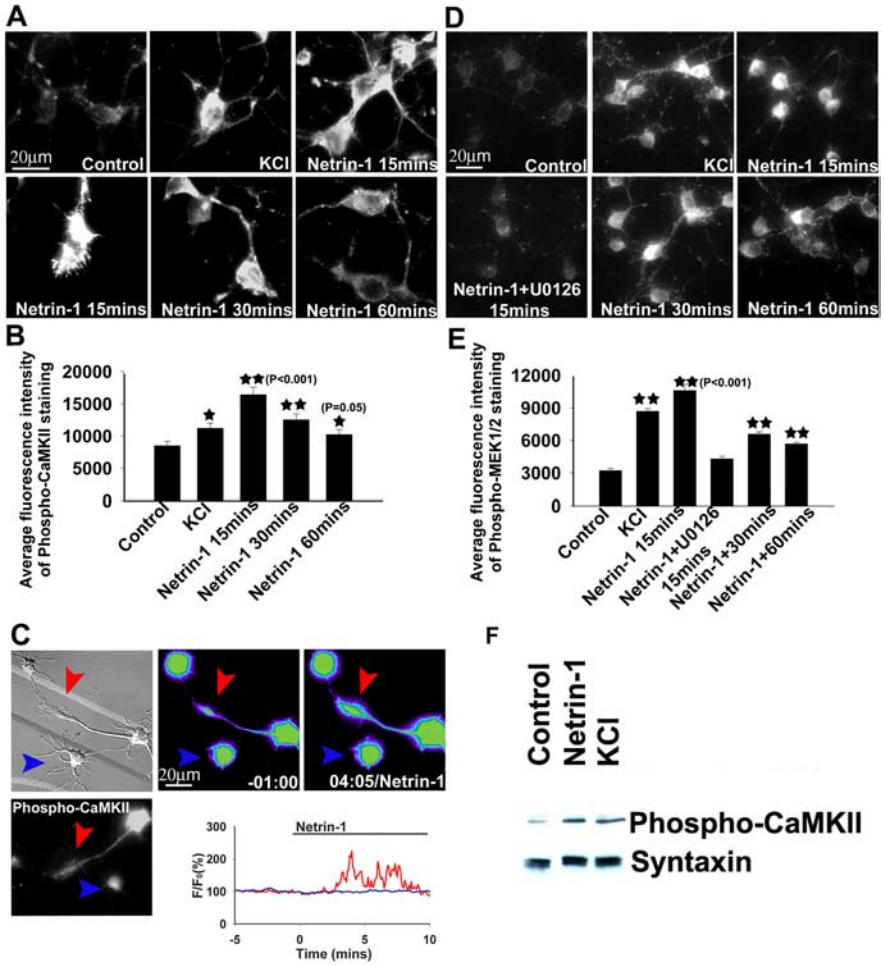

Figure 5. Netrin-1 activates both CaMKII and MAPK. ***A***, Examples of neurons treated with 0.5% BSA (control) for 15 min, KCl for 5 min, or netrin-1 for 15, 30, or 60 min and then immunostained with an antibody to CaMKII phosphorylated at the site of threonine 286. ***B***, Bar graphs comparing average fluorescence intensity of immunostained neurons subjected to treatments described in ***A***. ***C***, Examples of neurons (shown in DIC) imaged first for 15 min to measure calcium activity after application of netrin-1, followed by rapid fixation and immunostaining of phospho-CaMKII. Intense staining only occurred in neurons showing repetitive netrin-1-induced calcium transients (red arrowhead points to growth cone of neuron with high calcium activity) but not in neurons that failed to show calcium activity in response to netrin-1 (blue arrow). Measurements of calcium activity were obtained from neurons indicated by red and blue arrowheads. ***D***, Examples of neurons treated with 0.5% BSA (control) for 15 min, KCl for 5 min, netrin-1 for 15 min with or without the MAPK inhibitor U0126, or netrin-1 for 30 or 60 min followed by immunostaining with an antibody to the phosphorylated MAPK, MEK1/2. ***E***, Bar graphs comparing average fluorescence intensity of immunostained neurons subjected to treatments described in ***B***. ***F***, Western blot showing increases in phosphorylated CaMKII after application of netrin-1 for 15 min or KCl for 5 min. Syntaxin was used to control for loading of the same amount of protein sample.

ilarly increased axon length and branching (Fig. 7*F*). We also overexpressed a naturally occurring CaMKII inhibitory protein CaMKIIN. This protein has been well characterized with respect to its specificity in inhibiting CaMKII (Chang et al., 1998). CaMKIIN reduced axon branching to below control levels (Fig. 7*G–I*) and also resulted in shorter axons. In additional experiments, we examined effects of CaMKII overexpression in the presence of pharmacological inhibitors and found that branching was almost completely abolished (Fig. 7*J–P*). In a final set of experiments, we used several approaches to test the effects of inhibiting CaMKII on netrin-1-induced axon branching. First, we transfected neurons with the inhibitory protein CaMKIIN. Overexpression of CaMKIIN prevented netrin-1-induced axon branching but did not reduce axon length (Fig. 8*A–E*). In a second approach, we used an αCaMKII DNA vector-based RNA interference (RNAi) knockdown strategy (Sui et al., 2002; Gaudilliere et al., 2004) to reduce expression of αCaMKII (Fig. 8*F–G*). Neurons transfected with the U6/αCaMKII construct showed ~50% reduction in αCaMKII fluorescence immunostaining (Fig. 8*G*,*H*), and neurons with reduced levels of αCaMKII showed no netrin-1-induced branching. In comparison, neurons transfected with a control vector U6 developed normal robust branching in the presence of netrin-1 (Fig. 8*F3*). These results demonstrate that when αCaMKII is suppressed, netrin-1 fails to induce axon branching. Together, these results provide compelling evidence that αCaMKII plays a central role in promoting axon branching through netrin-1-evoked calcium signaling.

MAPK is involved in calcium signaling underlying axon branching

MAPK signaling cascades (including Ras, MEK1/2, and Erk1/2) are also known to be sensitive to intracellular Ca^{2+} changes. We therefore investigated the possible role of MAPK in regulation of axon branching. As with CaMKII, netrin-1 led to rapid phosphorylation of MAPK (Fig. 5*D*,*E*). Application of the pharmacological inhibitors U0126 and PD98059 specific to MAPK resulted in a dramatic reduction in axon branching compared with netrin-1-induced branching (Fig. 9*A–C*). Surprisingly, axon outgrowth was not affected, and axons were actually slightly longer than those treated with netrin-1. However, simultaneous application of CaMKII and MAPK inhibitors abolished axon branching and reduced axon outgrowth. Overexpression of constitutively active constructs of the MAPKs elicited extensive axon branching compared with EGFP-transfected controls (Fig. 9*D–M*). Conversely, inhibition of MAPK by a dominantly negative construct of Ras prevented netrin-1-induced axon branching (Fig. 9*I*). Interestingly, branches induced by MAPK appeared to have fewer secondary and tertiary branches than those elicited by netrin-1 and by CaMKII overexpression. These results suggest that the MAPK pathway activated by netrin-1 plays an important role in axon branching but not in axon outgrowth.

Discussion

Despite the essential role of axon branching in establishing CNS connectivity, surprisingly little is known about the mechanisms by which branching is regulated (Dent et al., 2003; Kornack and Giger, 2005). In the present study, we show that netrin-1 induces rapid and extensive cortical axon branching through intracellular Ca^{2+} signaling pathways. Application of netrin-1-induced repetitive Ca^{2+} transients in the axon or increased the frequency of endogenous Ca^{2+} transients. Ca^{2+} transients could be evoked in the entire axon or could begin in localized axon regions and

spread throughout the axon. Experimental reduction of Ca^{2+} levels revealed that Ca^{2+} signaling is essential for netrin-1-induced axon branching, and the use of specific receptor blockers revealed that release from intracellular stores is a major source of this Ca^{2+} activity. We imaged netrin-1-induced Ca^{2+} transients in localized regions of the axon, and remarkably, we found that within minutes of netrin-1 application, new branches protruded from those regions of the axon showing high-frequency Ca^{2+} transients. Thus, for the first time, we show that induction of high-frequency calcium transients along the axon can promote rapid development of branches. In additional experiments, we explored the downstream targets of Ca^{2+} signaling involved in axon branching. Using a variety of approaches involving pharmacological inhibition, overexpression, transfection with mutagenized constructs, and RNAi knockdown, we found that CaMKII and MAPKs play a major role in axon branching, which is consistent with the sensitivity of these kinases to changes in the frequency Ca^{2+} transients. Interestingly, CaMKII promotes axon outgrowth as well as axon branching, whereas MAPK affects only axon branching but not axon outgrowth. Together, this study reveals novel mechanisms, whereby netrin-1 Ca^{2+} signaling promotes cortical axon branching independent of axon outgrowth. Netrin-1 has been shown to attract cortical axons *in vivo* (Serafini et al., 1996) and directed growth and branching of cortical axons by netrin-1 has also been demonstrated in explant cocultures (Richards et al., 1997). Although the role of netrin-1 in cortical axon branching *in vivo* is not known, it is possible that similar calcium signaling mechanisms elicited by target-derived cues such as netrin-1 may promote branching to appropriate targets at specific locations along the axon.

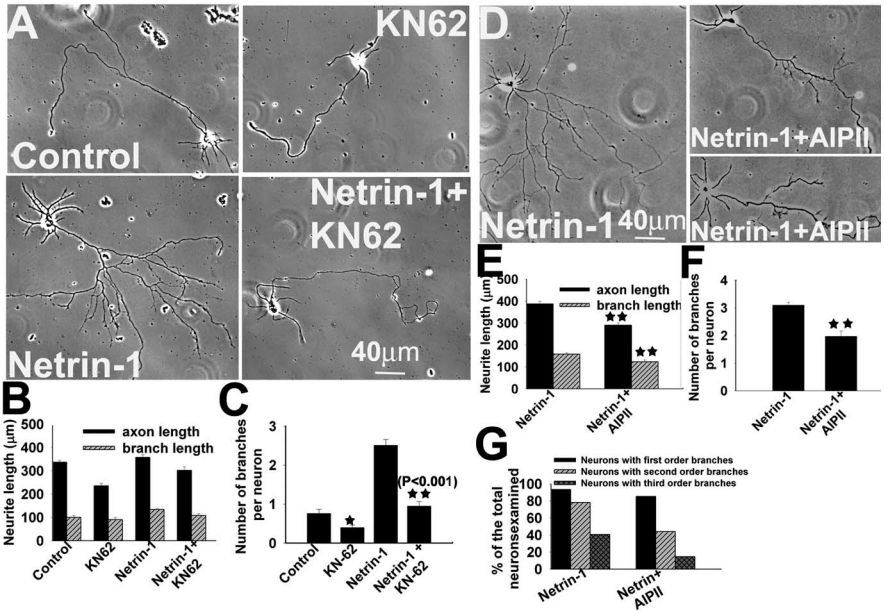

Figure 6. Pharmacological inhibition of CaMKII reduces axon outgrowth and branching. ***A***, Examples of neurons treated with BSA (control) or netrin-1 with or without KN62, a general inhibitor to CaM kinases. ***B***, ***C***, Bar graphs comparing axon length and branch length (***B***) and number of branches per neuron (***C***) in all conditions. KN62 reduces both spontaneous and netrin-1-induced branching as well as axon length. ***D***, Examples of neurons treated with netrin-1 with or without AIPII, a specific inhibitor to CaMKII. ***E–G***, Bar graphs comparing axon length and branch length (***E***), number of branches (***F***), and percentages of neurons with first-, second-, and third-order branches in all conditions. AIPII in the presence of netrin-1 reduces axon and branch length as well as primary and higher-order branching.

Calcium signaling in axon outgrowth and branching

Studies of axonal development have revealed diverse roles for calcium signaling (Kater and Mills, 1991; Letourneau et al., 1994; Gomez and Spitzer, 2000; Berridge et al., 2003; Henley and Poo, 2004). In the *Xenopus* spinal cord, axon outgrowth is regulated by frequencies of Ca^{2+} transients in the growth cone (Gu and Spitzer, 1995; Gomez and Spitzer, 1999). Suppression of Ca^{2+} transients accelerates axon outgrowth, whereas imposing Ca^{2+} transients slows growth cone advance (Gomez and Spitzer, 1999). Previously, we found that, in dissociated cortical neurons, spontaneous global Ca^{2+} transients regulate axon outgrowth in a frequency-dependent manner (Tang et al., 2003). When Ca^{2+} activity was silenced by L-type channel blockers, axons accelerated their advance. Thus, global Ca^{2+} transients appear to regulate cortical axon outgrowth in a frequency-dependent manner.

In contrast, local Ca^{2+} transients appear to be involved in axon guidance. Spatially restricted elevation of intracellular Ca^{2+} concentration in the growth cone induced by guidance cues has been shown to initiate turning behaviors in the direction of higher calcium (Zheng et al., 1994; Hong et al., 2000; Zheng, 2000). In response to gradients of netrin-1, intracellular Ca^{2+} concentrations are elevated in the growth cone to regulate changes in guidance behaviors (Hong et al., 2000; Ming et al., 2002; Nishiyama et al., 2003). Attractive turning was preceded by protrusion of lammelipodia and filopodia (Zheng, 2000). These effects involve relative Ca^{2+} concentrations that form a gradient across the growth cone (Zheng et al., 1994; Hong et al., 2000; Henley and Poo, 2004). Induction of localized elevations in Ca^{2+} concentration also promotes protrusion of filopodia from growth cones (Silver et al., 1990; Davenport and Kater, 1992; Gomez et al., 2001) and from axons (Lau et al., 1999). Our results show that, within minutes, axon branches develop from regions of the axon expressing repetitive high-frequency Ca^{2+} transients that persist up to 1 h in response to continuous netrin-1 application. In contrast to artificially imposed Ca^{2+} elevation, we used a relevant guidance cue to induce Ca^{2+} transients that reflect the physiological responses of the cortical neuron in branch formation. For this reason, we chose not to elevate calcium by localized application of nonphysiological reagents such as KCl, which transiently elevates levels of intracellular calcium (Gu and Spitzer, 1995) rather than evoking prolonged calcium transients or increasing frequencies of calcium transients, as does netrin-1. It might also be difficult to use localized electrical stimulation to impose calcium transients of frequencies optimal for the promotion of axon branching. Although previous studies of axon guidance have emphasized the role of local elevations in levels of intracellular Ca^{2+} during growth cone turning (Henley and Poo, 2004; Wen et al., 2004), we demonstrate here how axon branching is dependent on increases in the frequency of localized Ca^{2+} transients.

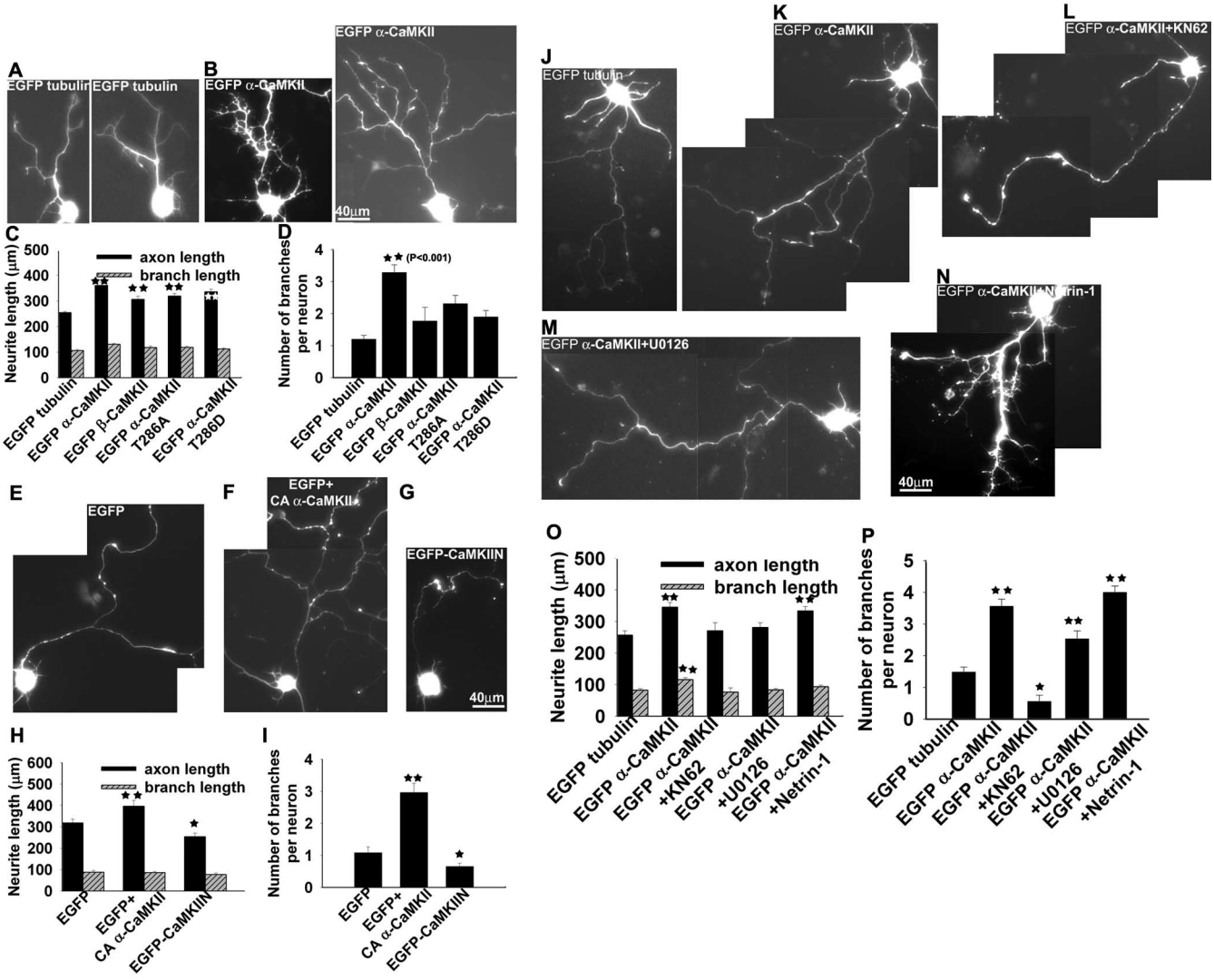

Figure 7. Overexpression of αCaMKII promotes axon branching and increases axon length. ***A***, ***B***, Examples of neurons transfected with EGFP–tubulin (***A***) and EGFP–αCaMKII (***B***). Neurons overexpressing αCaMKII develop highly branched arbors similar to those after netrin-1 treatment. ***C***, ***D***, Bar graphs comparing axon length and branch length (***C***) and number of branches per neuron (***D***) in neurons overexpressing tubulin, αCaMKII, βCAMKII, and two mutant forms of αCaMKII (T286A and T286D) mutated at the threonine 286 autophosphorylation site. Overexpression of wild-type αCaMKII alone promotes axon branching. ***E–G***, Examples of neurons transfected with EGFP alone (***E***), EGFP and a constitutively active (CA) mutant αCaMKII (***F***), and EGFP-CaMKIIKIIN, a CaMKII inhibitory protein (***G***). ***H***, ***I***, Bar graphs comparing axon length and branch length (***H***) and number of branches per neuron (***I***) in all conditions. ***J–N***, Examples of neurons transfected with EGFP–tubulin (***J***), EGFP–αCaMKII (***K***), and EGFP–αCaMKII with addition of KN 62 (***L***), U0126 (***M***), or netrin-1 (***N***). ***O***, ***P***, Bar graphs comparing axon length and branch length (***O***) and number of branches per neuron in all conditions. KN62 completely abolishes the branch-promoting effects of αCaMKII in contrast to partial reduction with U0126, and netrin-1 slightly enhances the branching effects of αCaMKII.

Downstream targets of calcium signaling during axon branching

What features of Ca^{2+} signaling activate specific downstream targets? Changes in the frequency of Ca^{2+} transients are known to activate genes for different neurotransmitters (Gomez and Spitzer, 2000; Borodinsky et al., 2004). Different frequencies as well as patterns of Ca^{2+} transients may also target different kinases and phosphatases in the cytoplasm to elicit different cellular responses (Tomida at al., 2003), and specific cellular events may be regulated by calcium transients at optimal frequencies (Eshete and Fields, 2001). Ca^{2+} oscillations are self renewing, repetitive, and persistent. Moreover, the mechanism of frequency modulation to decode information from calcium signals has advantages over mechanisms based on changes in amplitude of Ca^{2+} concentration alone (Cullen and Lockyer, 2002), because discrete Ca^{2+} oscillations can be more easily distinguished by calcium decoders such as CaMKII (De Koninck and Schulman, 1998) and PKC (Oancea and Meyer, 1998). Furthermore, as shown here, discrete Ca^{2+} oscillations can be shown to temporally precede and spatially coincide with discrete axon branching events.

Because calcium is a ubiquitous intracellular signal that participates in many diverse cellular processes, it has been difficult to identify specific downstream targets activated by calcium in axon growth and guidance (Lautermilch and Spitzer, 2000; Henley and Poo, 2004). Calcium may activate different targets to elicit opposing effects on the axon, such as inhibiting axon outgrowth but promoting the development of new branches. We identified αCaMKII as a major target of calcium signaling, which is essential for netrin-1-induced axon branching. Previous reports have shown that CaMKII plays an important role in neurite outgrowth and growth cone motility (Goshima et al., 1993) as well as growth

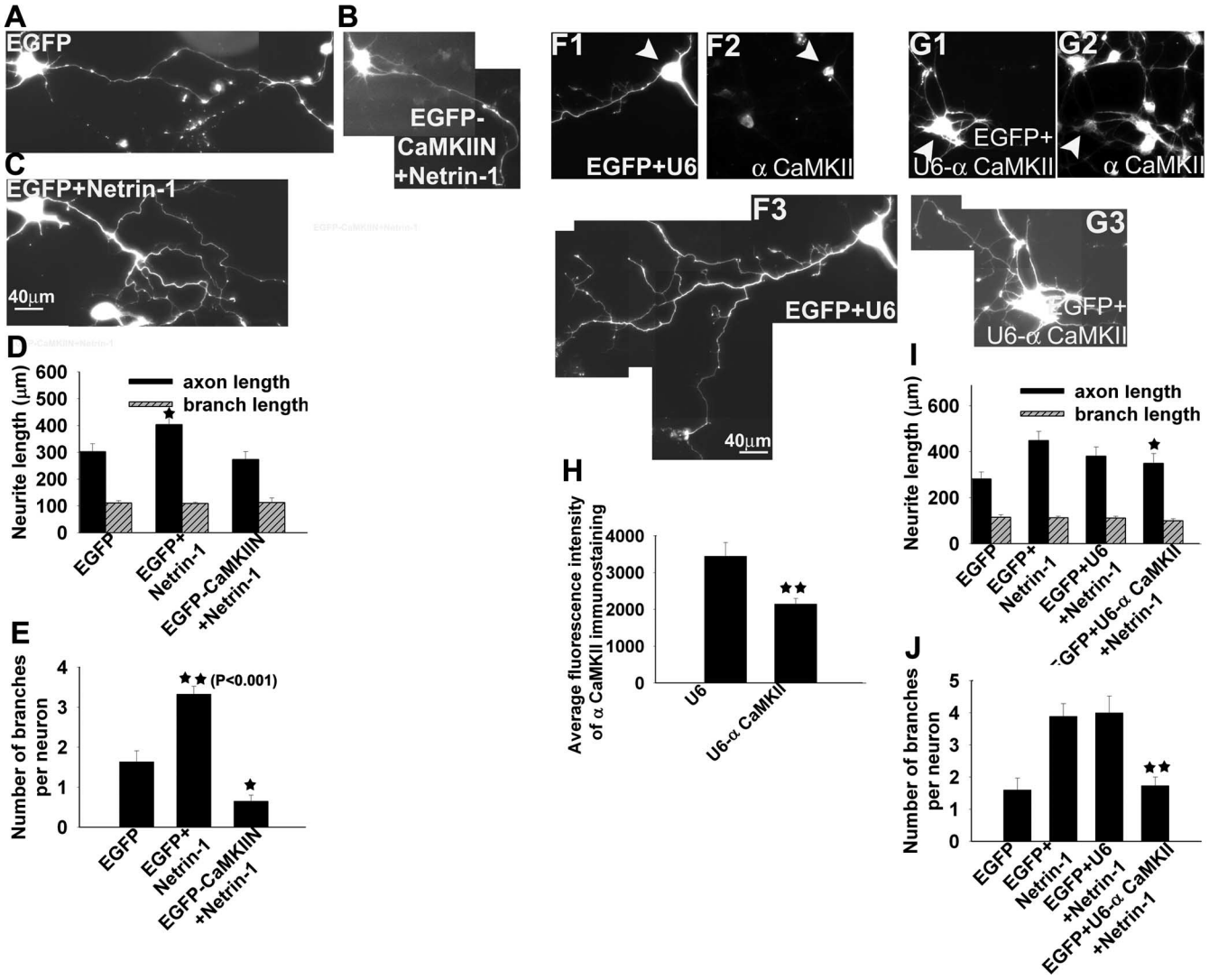

Figure 8. Molecular and RNAi knockdown of CAMKII abolishes netrin-1-induced axon branching. ***A–C***, Examples of neurons transfected EGFP (***A***), EGFP and treated with netrin-1 (***B***), and EGFP–CaMKIIN and treated with netrin-1. ***D***, ***E***, Bar graphs comparing axon length and branch length (***D***) and number of branches per neuron (***E***) in all conditions. Netrin-1 cannot promote branching in neurons overexpressing the inhibitory CaMKIIN protein. ***F1–F3***, Example of a neuron (arrowheads) at 1 d in culture cotransfected with EGFP and a control DNA vector U6 (***F1***) and immunostained with an antibody to αCaMKII at 3 d in culture (***F2***). This same neuron, when stimulated with netrin-1, is able to branch extensively (***F3***). Thus, the control U6 vector does not affect levels of CaMKII or the ability of the axon to branch in the presence of netrin-1. ***G1–G3***, Example of a neuron (arrowheads) at 1 d in culture cotransfected with EGFP and a U6 –αCaMKII RNAi vector (***G1***). The same neuron is shown in a different fluorescent channel (***G2***) demonstrating immunostaining with an antibody to αCaMKII at 3 d in culture. The neuron (arrowhead) after RNAi knockdown of CaMKII has reduced levels of αCaMKII compared with the neighboring cells and concomitantly few axon branches (***G3***) when stimulated with netrin-1. ***H***, Bar graph comparing fluorescence intensity of αCaMKII immunostaining in neurons transfected with the control U6 vector or the RNAi U6 –αCaMKII. ***I***, ***J***, Bar graphs comparing axon length and branch length (***I***) and number of branches per neuron (***J***) in all conditions.

cone guidance (Zheng et al., 1994; Kuhn et al., 1998; Wen et al., 2004). Wen et al. (2004) showed that pharmacological inhibition of CaMKII prevented netrin-1-induced repulsive growth cone turning and that local high increases in the amplitude of Ca^{2+} signals induced turning behaviors. Our results also show the involvement of CaMKII in the netrin-1 signaling pathway. However, we demonstrate a signaling mechanism that is dependent on the induction of high-frequency Ca^{2+} transients for axon branching. Others have found that CaMKI, but not CaMKII, promotes axon extension and growth cone motility (Wayman et al., 2004). Similarly, both positive (Fink et al., 2003; Jourdain et al., 2003; Gaudilliere et al., 2004) and inhibitory (Wu and Cline, 1998; Redmond et al., 2002) effects for CaMKII have been described for dendritic growth and branching. Although the reasons for these discrepancies are unclear, the present results obtained with a variety of techniques including pharmacological and molecular CaMKII inhibitors, overexpression of EGFP CaMKII, and RNAi knockdown provide strong evidence that αCaMKII is essential for netrin-1-induced cortical axon branching. Our results also suggest that CaMKII has a global effect on axon outgrowth, because CaMKII increases axon length as well as branching either with or without application of netrin-1. MAPKs have also been shown to influence netrin-1-induced axon guidance (Forcet et al., 2002; Ming et al., 2002; Campbell and Holt, 2003). Consistent with these studies, our results show that MAPK specifically promotes axon branching without affecting axon outgrowth. However, it is not clear why, in contrast to our results for cortical neurons, spinal commissural neurons require MAPK signaling for netrin-1-mediated axon outgrowth (Forcet et al., 2002). Interestingly, overexpression of CaMKII in the presence of

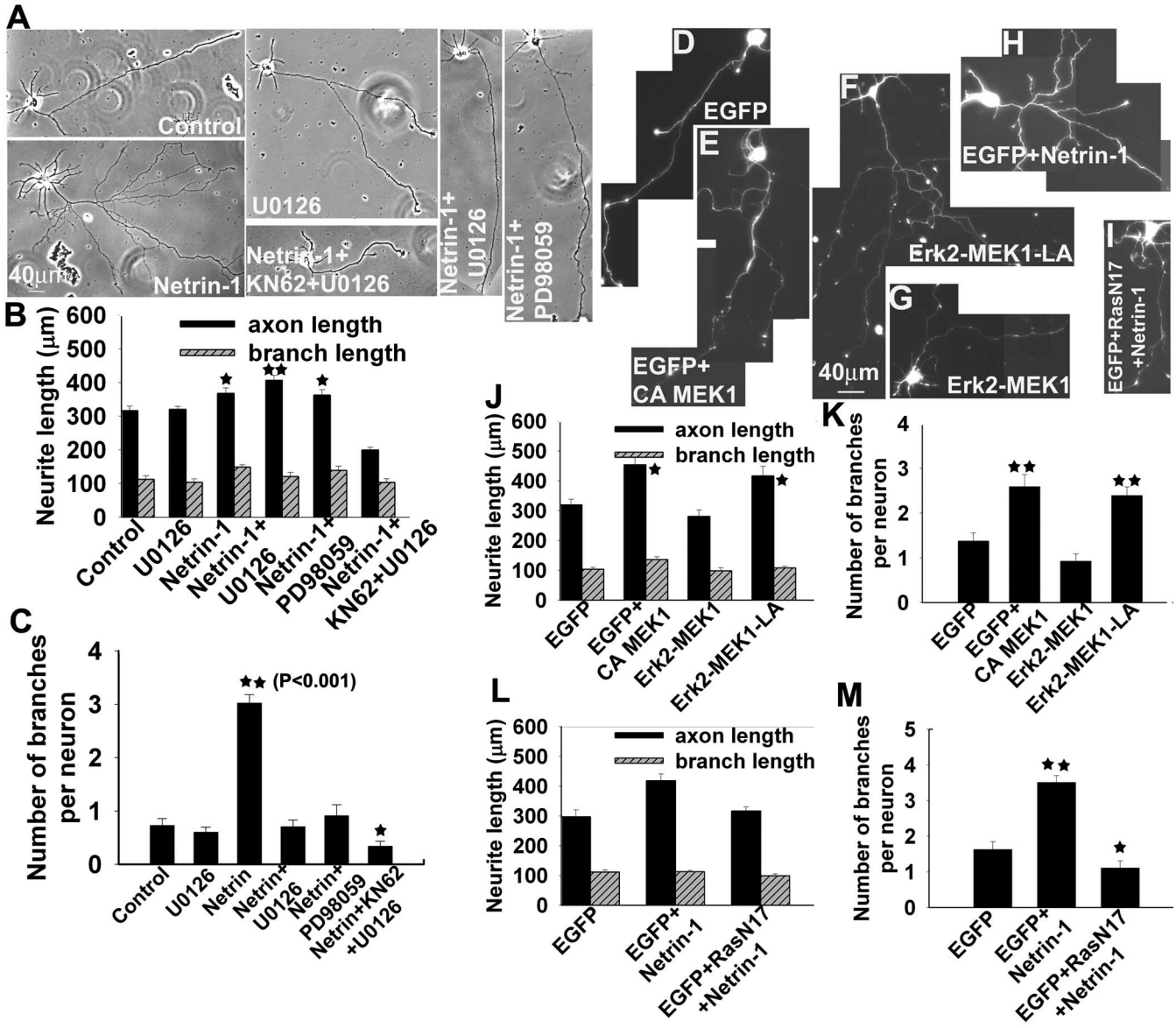

Figure 9. MAPK overexpression promotes and pharmacological or molecular inhibition of MAPK prevents netrin-1-induced axon branching without affecting axon outgrowth. ***A***, Examples of neurons treated with BSA (control), netrin, and the MAPK inhibitors U0126 and PD98059 in combination with netrin and the CAMKII inhibitor KN62. ***B***, ***C***, Bar graphs comparing axon length and branch length (***B***) and number of branches per neuron (***C***) in all conditions. Both MAPK inhibitors prevent netrin-1-induced branching, and the combination of U0126 with KN62 severely reduces axon outgrowth and abolishes netrin-1-induced branching. ***D–I***, Examples of neurons transfected with EGFP alone (***D***), EGFP and a constitutively active form of MEK1 (***E***), myc-tagged Erk2-MEK1-LA (***F***), a control myc-tagged Erk2-MEK1 (***G***), EGFP with the addition of netrin-1 (***H***), and EGFP with a dominant-negative form of Ras (RasN17) in the presence of netrin-1 (***I***). ***J–M***, Bar graphs comparing axon length and branch length (***J***, ***L***) and number of branches per neuron (***K***, ***M***) in all conditions.

MAPK inhibitors greatly reduces axon branching (Fig. 7*M*,*P*), suggesting that CaMKII can activate MAPKs (Chen et al., 1998). Interactions between these signaling pathways are not well understood. In preliminary experiments, however, we found that constitutively active CaMKII activates MAPK as determined by immunostaining with phospho-MAPK (our unpublished results). Second, we found that the CaMKII inhibitor KN62 suppresses netrin-1-induced phosphorylation of MAPK. Both results suggest that CaMKII can activate MAPK signaling. However, molecular mechanisms of interaction between these two signaling pathways are beyond the scope of this study.

Effects of calcium signaling pathways on the cytoskeleton

Ultimately, signaling pathways activated by guidance cues must converge on the actin–microtubule cytoskeleton to induce growth cone turning behaviors or axon branching (Dent and Gertler, 2003). Cortical axon branching has been shown to require dynamic actin–microtubule interactions (Dent and Kalil, 2001), and netrin-1 elicits filopodial protrusion through actin polymerization (Dent et al., 2004; Lebrand et al., 2004). Netrin-1 is known to activate Rho GTPases, which regulate actin polymerization (Li et al., 2002; Shekarabi and Kennedy, 2002), and also activate MAP 1B, which is thought to play a role in actin–microtubule interactions (Del Rio et al., 2004). Thus, netrin-1-induced calcium signaling could influence regulators of cytoskeletal dynamics required for axon branching. We found that CaMKII is a major target of netrin-1-induced calcium signaling. Studies of regulation of dendritic morphology and plasticity suggest that CaMKII may be an important link to the cytoskeleton. In dendritic filopodia, resembling spines, αCaMKII was shown to inter-

act with βCaMKII and translocate to dendritic spines, thereby targeting CaMKII to the actin cytoskeleton (Shen et al.,1998). This is consistent with the role of CaMKII in stabilizing dendritic arbors (Wu and Cline, 1998). Although these studies do not identify an exact role for CaMKII in regulating actin polymerization, there is evidence (Chen et al., 2003) that CaMKII can activate cdc42, which polymerizes actin during filopodia formation. The repetitive calcium transients induced by netrin-1 described here could provide a mechanism for translocating CaMKII to actin filaments in nascent axonal filopodia. In the future, it will be important to understand exactly how major targets of calcium signaling, such as CaMKII and MAPK, regulate cytoskeletal dynamics required for development of new branches.

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