NF-κB signalling regulates the growth of neural processes in the developing PNS and CNS

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Summary

The proper growth and elaboration of neural processes is essential for the establishment of a functional nervous system during development and is an integral feature of neural plasticity throughout life. Nuclear factor-kappa B (NF-κB) is classically known for its ubiquitous roles in inflammation, immune and stress-related responses and regulation of cell survival in all tissues, including the nervous system. NF-κB participation in other cellular processes remains poorly understood. Here we report a mechanism for controlling the growth of neural processes in developing peripheral and central neurons involving the transcription factor NF-κB. Inhibiting NF-κB activation with super-repressor IκB-α, BAY 11 7082 (IκB-α phosphorylation inhibitor) or N-acetyl-Leu-Leu-norleucinal (proteosomal degradation inhibitor), or inhibiting NF-κB transcriptional activity with κB decoy DNA substantially reduced the size and complexity of the neurite arbors of sensory neurons cultured with brain-derived neurotrophic factor while having no effect on their survival. NF-κB exerted this effect during a restricted period of development following the phase of naturally occurring neuronal death when the processes and connections of the remaining neurons are extensively modified and refined. Inhibiting NF-κB activation or NF-κB transcriptional activity in layer 2 pyramidal neurons in postnatal somatosensory cortical slices reduced dendritic arbor size and complexity. This function of NF-κB has important implications for neural development and may provide an explanation for reported involvement of NF-κB in learning and memory.

Key words: NF-κB, Axon, Dendrite, BDNF, Pyramidal neuron, Sensory neuron, Mouse

Introduction

Nuclear factor-κB (NF-κB) is a ubiquitously expressed transcription factor that plays a key role in regulating the expression of genes involved in a variety of cellular processes, including innate and adaptive immune responses, stress responses and cell survival and proliferation (Baldwin, Jr, 1996; Karin, 1999). It consists of homodimers or heterodimers of a family of five structurally related proteins: p65 (RelA), RelB, c-Rel, p50 and p52, of which the p50/p65 heterodimer is predominant in many cell types (Karin and Lin, 2002; Li and Verma, 2002). NF-κB is held in an inactive form in the cytosol by interaction with a member of the IκB family of inhibitory proteins: IκBα, IκBβ, IκBε, IκBγ, Bcl-3, p100 and p105, of which IκBα and IκBβ are the most abundant. Upon stimulation by extracellular inducers, IκB is phosphorylated by an IκB kinase complex, which leads to ubiquitination and proteosome-mediated degradation of IκB. This exposes a nuclear localization signal on the liberated NF-κB, which translocates to the nucleus, where it binds to consensus κB sequences in the promoter and enhancer regions of responsive genes (Baldwin, Jr, 1996; Karin, 1999).

In the nervous system, NF-κB is widely expressed (Bhakar et al., 2002; O'Neill and Kaltschmidt, 1997; Yalcin et al., 2003) and is activated by a variety of neurotrophic factors, cytokines and neurotransmitters (Carter et al., 1996; Guerrini et al., 1995; Hamanoue et al., 1999; Kaltschmidt et al., 1995; Yalcin et al., 2003). NF-κB can promote neuronal survival and protect neurons from toxic insults (Bhakar et al., 2002; Bui et al., 2001; Daily et al., 2001; Digicaylioglu and Lipton, 2001; Fridmacher et al., 2003; Lipsky et al., 2001; Mattson et al., 2000; Piccioli et al., 2001; Tamatani et al., 2000; Yu et al., 2000) but can also play a role in bringing about neuronal death (Pizzi et al., 2002; Shou et al., 2002). It has been implicated in adaptive responses to inflammatory conditions and neurodegenerative disorders (Bhakar et al., 2002; Blondeau et al., 2001; Clemens et al., 1997; Fridmacher et al., 2003; Gabriel et al., 1999; Gentry et al., 2000; Grilli and Memo, 1999; Guerrini et al., 1995; Mattson et al., 2000), and participates in peripheral nerve myelination (Nickols et al., 2003). Recent studies have shown that NF-κB might also play an important role in neural-specific functions. NF-κB is activated in hippocampal neurons and cerebellar granule cells in response to N-methyl-d-aspartate receptor occupancy by glutamate (Guerrini et al., 1995; Meffert et al., 2003; Scholzke et al., 2003). Treatment of hippocampal slices with κB decoy DNA prevents induction of long-term depression and significantly reduces long-term potentiation (Albensi and Mattson, 2000). Pharmacological inhibition of NF-κB activation interferes with memory

formation in the crab (Merlo et al., 2002), and mice lacking p65 exhibit a deficit in spatial learning (Meffert et al., 2003). NF-κB is also required for consolidation of fear conditioning in the amygdala (Yeh et al., 2002).

In addition to activity-induced changes in synaptic function, the elaboration and modification of neuronal processes is thought to be an important anatomical change underlying learning and memory. To investigate if NF-κB is involved in regulating the growth and morphology of neural processes in development, we studied the activation and role of NF-κB in two well-characterized populations of developing peripheral and central neurons. Sensory neurons of the embryonic and postnatal mouse nodose ganglion survive and extend neurites in dissociated cell culture supplemented with brain-derived neurotrophic factor (BDNF) (Davies et al., 1993) and layer 2 pyramidal neurons of the mouse somatosensory cortex elaborate extensive dendritic arbors in organotypic slice cultures established in the postnatal period (Gutierrez et al., 2004; Niblock et al., 2000). Here we show that preventing NF-κB activation or inhibiting NF-κB transcriptional activity reduces process growth and complexity in both neuronal models.

Materials and methods

Dissociated neuron cultures

Dissociated cultures of nodose ganglion neurons were set up from embryos and early postnatal mice obtained from overnight matings of CD1 mice. Dissected ganglia were trypsinized (0.25% trypsin for 25 minutes at 37°C) and dissociated by trituration. The neurons were plated in defined, serum-free medium on a poly-ornithine/laminin substratum in 35 mm tissue culture dishes (Davies et al., 1993).

Cortical slice cultures

Vibrotome slices (300 μm) of P6 or P7 CD1 mouse cerebrum were cut in the coronal plane in cold (4°C) artificial cerebrospinal fluid (160 mmol/l NaCl, 200 mmol/l KH_2PO_4, 5 mmol/l KCl, 1 mmol/l $MgSO_4$, 33 mmol/l glucose, 10 mmol/l HEPES, 1 mmol/l $CaCl_2$). The slices were cultured in 35 mm Petri dishes on 0.4 μm Millicel inserts (Millipore) floating on 1 ml of culture medium (50% Dulbecco's Minimal Essential Medium, 25% heat-inactivated horse serum, 25% Hank's Balanced Salt Solution, 6.5 mg/ml glucose and 100 U/ml streptomycin and penicillin). The cultures were incubated in 5% CO_2 at 37°C.

Ballistic transfection

Ballistic transfection of dissociated neurons and cortical slices was carried out using a hand-held gene gun (Helios Gene-gun, BioRad Hercules, CA USA). Gold particle cartridges were prepared using the manufacturer's protocol. Briefly, 20 mg of 1.6 μm gold particles were suspended in 100 μl of 50 mmol/l spermidine and 20 μg of pYFP (Clontech) together with either pSR-IκB-α or pCDNA control plasmid. The gold particles were precipitated with 100 μl of 2 mol/l $CaCl_2$, washed three times with 100% ethanol, resuspended in 1.2 ml of 100% ethanol plus 0.01 mg/ml polyvinylpirrolidone and loaded into Teflon tubing.

Double-stranded κB Decoy DNA was prepared by annealing complementary single stranded oligonucleotides of the following sequences: 5′-GAGGGGACTTTCCCT-3′ and 5′-AGGGAAAGTCC-CCTC-3′. Control DNA with a scrambled sequence was prepared by annealing the following sequences: 5′-GATGCGTCTGTCGCA-3′ and 5′-TGCGACAGACGCACT-3′. Double-stranded DNA solutions were prepared at a concentration of 50 mmol/l and ethanol precipitated onto the gold microcarriers along with either the pYFP or pRFP reporter plasmid. In the case of cortical slice cultures, control and κB decoy DNA-coated particles were mixed prior to loading into the Teflon tubing.

For transfecting nodose neurons, between 1000 and 3000 neurons were plated in a 50 μl droplet of defined medium in the centre of a 35 mm diameter tissue culture dish that had been pre-coated with poly-ornithine (0.5 mg/ml, overnight) and laminin (20 μg/ml for 3 hours). Neurons were incubated at 37.5°C in a humidified 3.5% CO_2 incubator for 3-4 hours to allow the cells to attach, and the medium was removed from the dish just before transfection. The coated gold particles were shot into the cultured neurons with the gun pressurized at 200 psi. A 70 μm nylon mesh screen was placed between the gun and the culture to protect the cells from the shock wave. After transfection, 2 ml of defined medium with or without 10 ng/ml BDNF was added to the culture dishes.

For transfecting cortical neurons, gold particles were shot into slices at a pressure 250 to 300 psi. A 70 μm nylon mesh screen was also used to protect the tissues from the shock wave. After transfection, the slices were incubated in medium with or without 10 ng/ml BDNF for 48 hours before the dendritic arbors of transfected pyramidal neurons were imaged.

Quantification of fluorescence

To estimate the relative level of NF-κB activation in cultured neurons under different experimental conditions, the neurons were transfected with a plasmid expressing GFP under the control of an NF-κB promoter. Neurons were imaged with a Zeiss Axioplan laser scanning confocal microscope. The mean fluorescence intensity for each soma was obtained using LSM510 software, based on the standard 255 intensity level scale after subtraction of background intensity. Between 40 and 60 neurons were imaged for each experimental condition and all imaging and quantification was performed blind. Statistical comparisons were performed using simple ANOVA followed by Fisher's post-hoc test.

Estimates of neuronal survival in dissociated cultures

For estimating the survival of transfected neurons, cultures were shot with the gene gun 3 hours after plating and YFP-labelled neurons were counted 12 hours after plating and again at 48 hours. The number of labelled neurons surviving at 48 hours was expressed as a percentage of the initial number of labelled neurons. The area counted was defined by the area in which gold particles could be seen to be embedded in the bottom of the culture dish. Triplicate cultures were set up for all conditions and the data shown are compiled from two to four separate experiments for each age.

Estimates of neuronal survival in cultures that were not transfected with the gene gun were made by counting the number of neurons in a 12 × 12 mm grid in the centre of the dish 3 to 6 hours after plating and again at 48 hours. The number of neurons surviving at 48 hours was expressed as a percentage of the initial number of neurons.

Analysis of nodose neuritic arbors

Transfected YFP-labelled neurons were visualized and digitally acquired using an Axioplan Zeiss laser scanning confocal microscope. For experiments in which neurons were not transfected with YFP, they were first fluorescently labelled for expression of the neuron-specific marker β-III tubulin. For this, the cells were rinsed with PBS at room temperature, fixed in 4% paraformaldehyde/PBS for 30 minutes at room temperature, rinsed twice with PBS and subsequently permeabilized and blocked with 5% bovine serum albumin and 0.1% Triton X-100 in PBS for 60 minutes at room temperature. The cells were incubated with monoclonal anti-βIII tubulin antibodies (1:1000, Promega) overnight at 4°C, rinsed three times with PBS and incubated with FITC-labelled rabbit anti-mouse IgG secondary antibody (Molecular Probes, Inc, Eugene, OR, USA) for 1 hour at room temperature.

For every condition studied, between 40 and 70 neurons were captured, and neuritic arbors were traced using LSM510 software.

These traces were used to ascertain total neurite length and number of branch points. Sholl analysis was also carried out on these traces. For this, concentric, digitally generated rings, 30 μm apart were centred on the cell soma, and the neurites intersecting each ring were counted (Sholl, 1953). Pair-wise comparisons were made using the Student *t*-test. For multiple comparisons, ANOVA was performed followed by Fisher's post-hoc test.

Analysis of pyramidal dendrites

Layer II/III pyramidal neurons of the somatosensory cortex were studied with an Axioplan Zeiss laser scanning confocal microscope 48 hours after transfection. The slices were fixed for 30 minutes with 4% paraformaldehyde in PBS, and DAPI counterstaining was used to confirm the laminar localization. For every experimental condition studied, the dendritic organization of between 50 and 60 neurons was reconstructed and analysed. For each neuron, 15 and 20 optical sections were obtained using 20× and 40× water immersion objectives. Three-dimensional projections were generated by merging the resulting Z stacks, and the dendritic arbors were traced using LSM510 software. Neurons tracings were analysed using a customized Matlab script for the automatic counting of branching points, number of primary dendrites, dendritic length and other topological parameters. Sholl analysis was carried out directly on the Z-stack images. In this case, concentric, digitally generated rings, 15 μm apart were centred on the cell soma, and the dendrites intersecting each ring were counted (Sholl, 1953). Statistical analysis was carried out as described above.

Results

Super-repressor IκB-α reduces neurite growth from nodose neurons

We began our investigation of the potential role of NF-κB signalling in regulating neurite growth and morphology by transfecting cultured neonatal nodose ganglion neurons with a plasmid that expresses a super-repressor form of the NF-κB inhibitor IκB-α. This IκB-α protein associates normally with NF-κB, but carries serine-to-alanine mutations at residues 32 and 36, which prevent signal-promoted phosphorylation and proteosome-mediated degradation, thereby preventing release and translocation of NF-κB to the nucleus (Rodriguez et al., 1996; Roff et al., 1996).

Given the well-known involvement of NF-κB in regulating cell survival, we first assessed the effect of the super-repressor IκB-α on the survival of neonatal nodose ganglion neurons. Three hours after plating, P1 nodose neurons were transfected with either the super-repressor IκB-α plasmid or control plasmid and half the culture dishes were supplemented with BDNF. Transfection was carried out by firing plasmid-coated gold particles into cultures using the Helios gene gun. To identify the transfected neurons and outline their neurite arbors, the gold particles were also coated with a YFP expression plasmid. Twelve hours after plating, when all transfected neurons had become clearly recognizable by the expression of YFP, the total numbers of transfected neurons were counted. Forty-eight hours after plating, the surviving labelled neurons were counted and expressed as a percentage of the numbers counted at 12 hours. Fig. 1 shows a representative sample of reconstructed newborn nodose neurons grown in the presence of BDNF. The illustrated neurons in the upper row represent the range of morphologies observed in P1 control transfected cultures. A corresponding sample of super-repressor IκB-α transfected cultures is shown in the lower row. Fig. 2A shows that 80% of neurons were surviving with BDNF at this time and that there was no significant difference in the numbers of super-repressor IκB-α transfected neurons and control transfected neurons surviving with this neurotrophin. Only 40% of the neurons survived for 48 hours without BDNF (Fig. 2A), and transfection of these neurons with super-repressor IκB-α did not further reduce their survival (data not shown). These results indicate that preventing NF-κB activation with super-repressor IκB-α does not affect the survival of P1 nodose neurons, either in the presence or in the absence of BDNF.

The lack of effect of super-repressor IκB-α on the survival of nodose neurons grown with BDNF made analysis of its influence on neurite growth straightforward, as any differences in the neurite arbors of IκB-α-transfected and control-transfected cultures could not be ascribed to differences in the subpopulations of neurons surviving in these cultures. To assess the effect of the super-repressor IκB-α on neurite arbor size and morphological complexity, P1 nodose neurons were transfected 3 hours after plating and grown for 24 hours with or without BDNF before the resulting YFP-labelled neurons

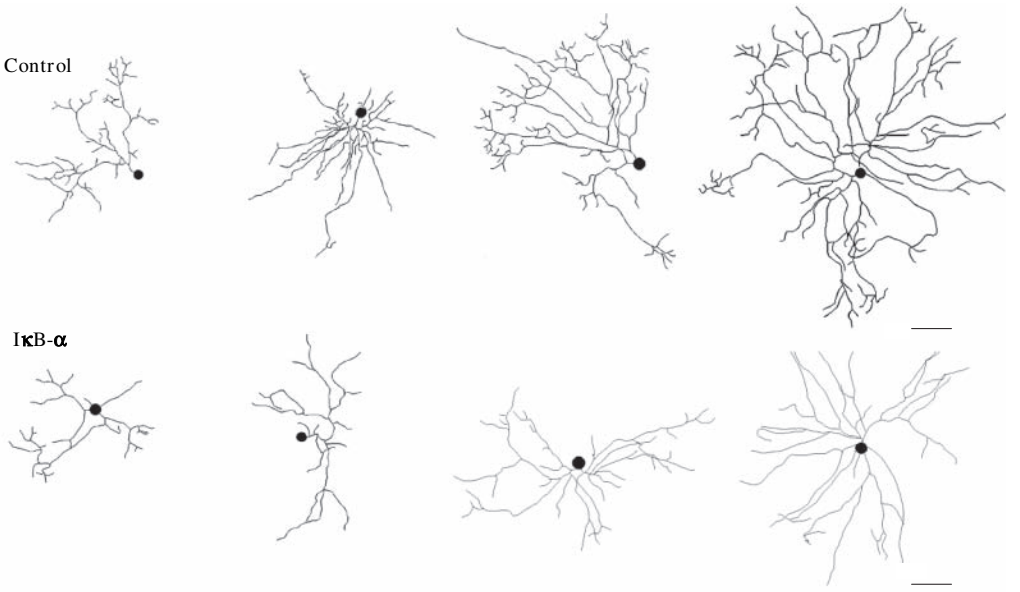

Fig. 1. Representative sample of reconstructed newborn nodose neurons. The illustrated neurons represent the range of morphologies observed in P0 control transfected cultures (upper row) and super-repressor IκB-α transfected cultures (lower row) after 24 hours incubation with 10 ng/ml BDNF. The neurons shown correspond to percentiles 25, 50, 75 and 100 of the sampled populations in terms of total neurite length. Scale bar: 50 μm.

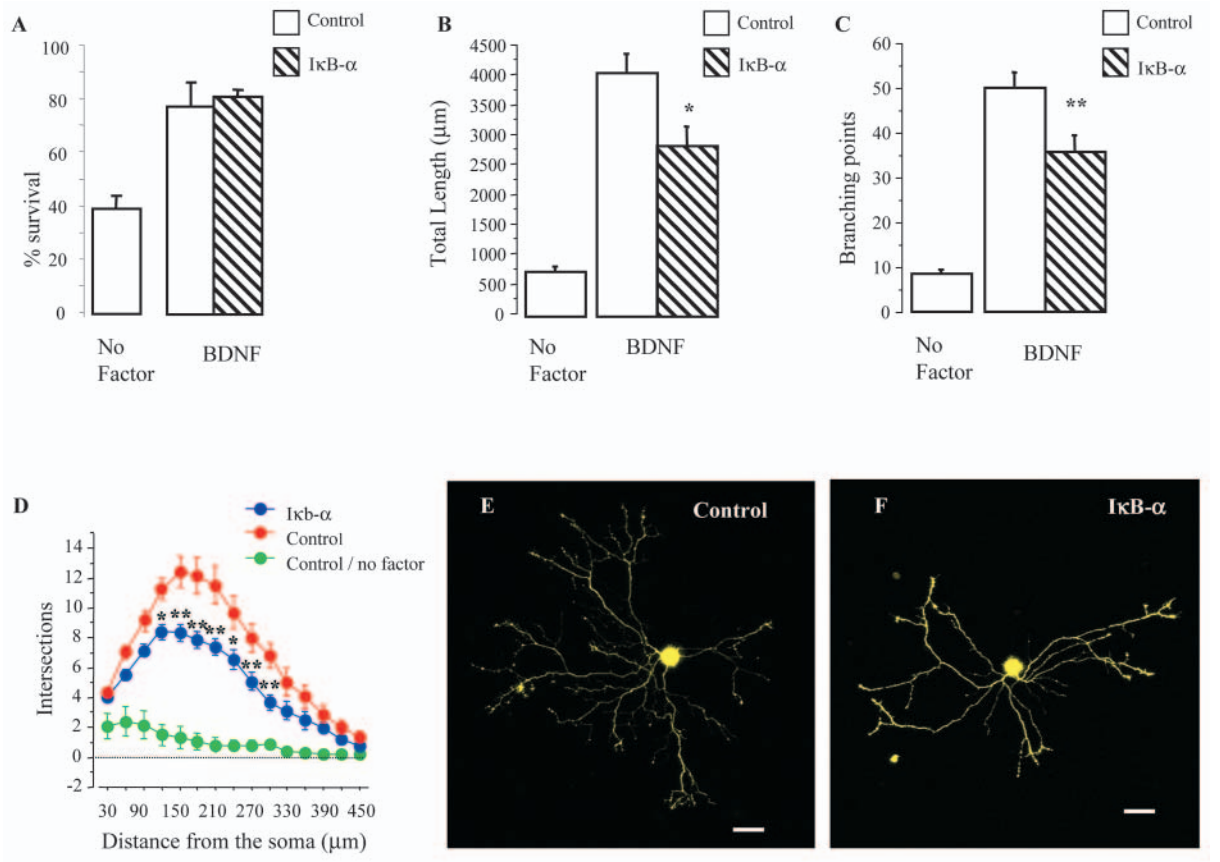

Fig. 2. Super-repressor IκB-α reduces neurite growth from neonatal nodose neurons but does not affect survival. P1 nodose neurons were transfected with a YFP expression plasmid together with either a super-repressor IκB-α plasmid or an empty control plasmid and were incubated in medium containing 10 ng/ml BDNF. Control transfected neurons were also grown without BDNF. (A) Percent survival 48 hours after transfection. (B) Total neurite length 24 hours after transfection. (C) Number of branch points in neurite arbors 24 hours after transfection. (D) Sholl analysis of neurite arbor morphology 24 hours after transfection. (E) Photomicrograph of a typical control transfected neuron grown for 24 hours with BDNF. (F) Photomicrograph of a typical super-repressor IκB-α transfected neuron grown for 24 hours with BDNF. Scale bar: 50 μm. The means and standard errors of data obtained from 40-70 neurons in each experimental condition shown. Statistical comparisons shown are with respect to the control transfected neurons, **P*<0.05, ***P*<0.001.

were visualized and digitally acquired with a confocal microscope. Total neurite length, number of branch points and Sholl analyses were carried out on the digitized images. Fig. 2B shows that in BDNF-supplemented cultures there was a significant 30.1% reduction in total neurite length in IκB-α-transfected compared with control-transfected neurons (*P*<0.05, *t*-test). The neurite arbors of the smaller numbers of neurons surviving in control-transfected cultures that were not supplemented with BDNF were very much smaller than those growing with BDNF. Fig. 2C shows that there was also a significant 28.4% reduction in the number of branch points in the neurite arbors of IκB-α-transfected, BDNF-supplemented neurons compared with control-transfected BDNF-supplemented neurons (*P*<0.001). Sholl analysis provided complementary quantitative data on the morphological changes brought about by the super-repressor IκB-α. Fig. 2D plots the number of neurite intersections on a series of concentric rings centred on the cell body, which is indicative of neurite branching over distance. In BDNF-supplemented cultures, the number of dendritic intersections initially increased with distance from the cell body to reach a maximum at between 150 and 180 μm. Beyond this distance, there was a gradual decrease in the number of intersections, reaching fewer than two intersections at 450 μm. There were fewer intersections at all circles in super-repressor IκB-α-transfected neurons compared with control-transfected neurons, and these differences reached statistical significance between 120 and 300 μm. Control-transfected neurons that were grown without BDNF showed a small number of neurite intersections that decreased with distance from the cell body, with no peak. The typical appearances of IκB-α-transfected and control-transfected neurons grown with BDNF are shown in Fig. 2E,F. Taken together, these results show that blocking NF-κB activation with super-repressor IκB-α significantly impairs neurite growth from neonatal nodose neurons. To exclude the possibility that reduction in neurite growth was secondary to a global reduction in cellular metabolism affecting overall cell growth, we measured cell soma size in super-repressor IκBα-transfected and control-transfected cells. Quantification of somal cross sectional area by confocal microscopy revealed no significant difference between super-repressor IκBα-transfected (680±113 μm^2, *n*=50) and control-transfected neurons (715±34 μm^2, *n*=51) after 24 hours incubation with BDNF.

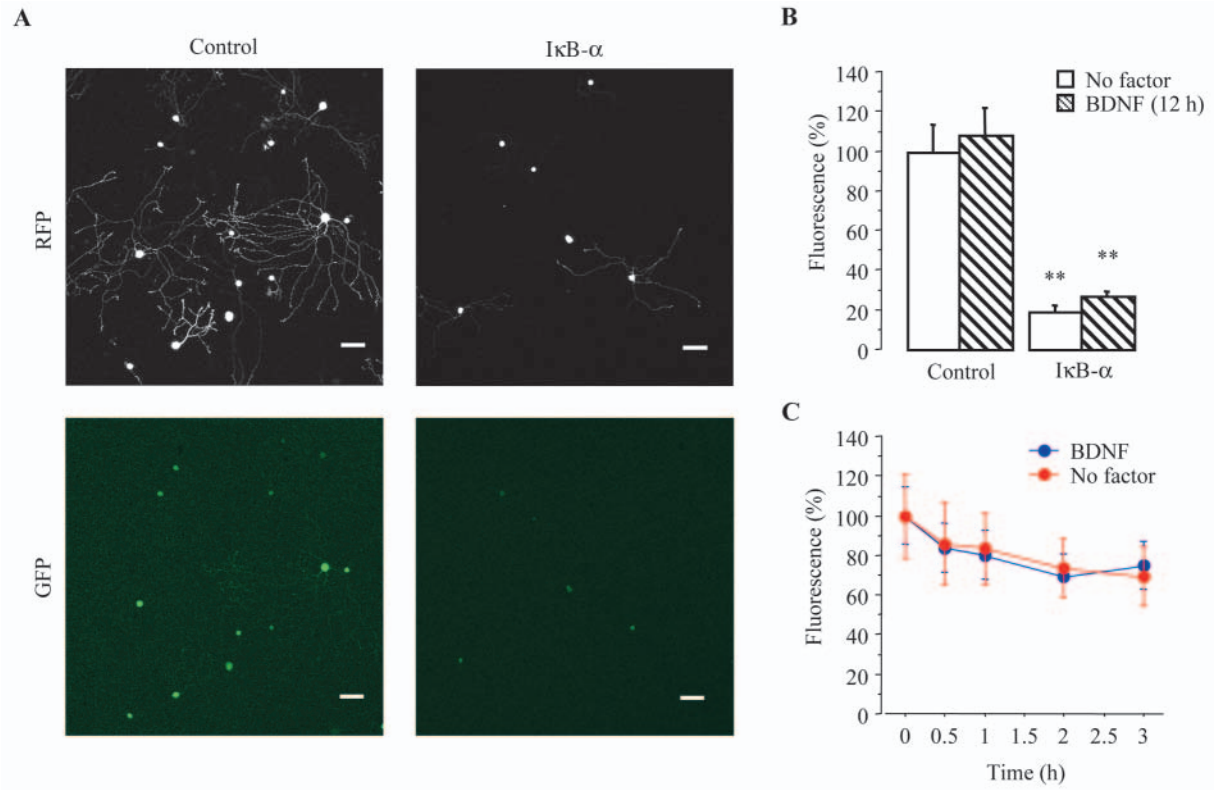

Fig. 3. NF-κB-dependent gene expression in newborn nodose neurons is not affected by BDNF. (A) Photomicrographs of representative fields of neurons co-transfected with the GFP NF-κB reporter plasmid, RFP plasmid and either the super-repressor IκB-α plasmid (right panels) or corresponding control plasmid (left panels) and cultured with 20 ng/ml BDNF for 12 hours. Transfected neurons and their dendritic morphology are outlined by the RFP (shown with a white filter) and can be seen in the upper panels. The effect of super-repressor IκB-α on neuronal morphology, seen in the previous figures, is also evident in these images. GFP fluorescence in the same fields (lower panels) shows the marked reduction caused by super-repressor IκB-α. (B) Quantification of the level of NF-κB-driven GFP fluorescence in super-repressor IκB-α-transfected and control-transfected neurons after the neurons have been incubated for 12 hours with and without 20 ng/ml BDNF. Fluorescence measurements were made from 40 to 60 neurons in each experimental condition, and the data are expressed as a percentage of the mean fluorescence of the No factor/Control vector-transfected group (mean and standard error are shown). Statistical comparisons shown are with respect to the control transfected neurons, $^{**}P<0.001$. (C) Timecourse of NF-κB-driven GFP fluorescence after BDNF stimulation. Neurons were co-transfected with the GFP NF-κB reporter plasmid and the RFP plasmid, and were cultured without factors for 8 hours. Neurons were then imaged immediately before and at 0.5, 1, 2 and 3 hours after the addition of BDNF to the medium (20 ng/ml). An untreated group of neurons (No Factor) was imaged at the same times. The fluorescence of each neuron was quantified and expressed as a percentage of the initial (0 hour time point) measurement for each group. Scale bars: 50 μm.

NF-κB transcriptional activity in nodose neurons is unaffected by BDNF

To determine if NF-κB transcriptional activity is influenced by BDNF, we transfected newborn nodose neurons with a reporter construct in which GFP is under the control of an NF-κB promoter. Transfected neurons were positively identified by co-transfection with an RFP expression plasmid. Neurons were shot 3 hours after plating with gold particles coated with both these plasmids together with either the super-repressor IκB-α plasmid or corresponding empty control plasmid and were incubated for a further 12 hours with or without BDNF. Twelve hours were chosen because at this time most neurons still survive even in the absence of BDNF. Fig. 3A shows that neurons transfected with the control plasmid exhibited a clear GFP signal, whereas neurons transfected with the super-repressor IκB-α plasmid exhibited a weak GFP signal. Quantification (Fig. 3B) revealed a statistically significant five-fold reduction in average GFP fluorescence in super-repressor IκB-α-transfected neurons compared with control-transfected neurons, confirming that super-repressor IκB-α effectively reduces NF-κB-dependent gene expression. However, there was no significant difference in the level of GFP fluorescence in control-transfected neurons grown with or without BDNF, and the GFP signal was reduced to the same extent by super-repressor IκB-α in the presence and absence of BDNF.

We carried out additional experiments to test more rigorously whether BDNF treatment activates NF-κB-dependant transcription. Neurons were plated in defined medium without BDNF and co-transfected with the GFP NF-κB reporter and the RFP plasmid. Eight hours later, the neurons were treated with BDNF and the GFP signal was quantified at intervals in these and untreated parallel cultures. Fig. 3C shows that there was no difference in the GFP signal between BDNF-treated and untreated neurons at time intervals of up to 3 hours after BDNF addition to the medium. These results suggest that BDNF does not obviously affect the level of NF-κB-dependent gene transcription in these neurons.

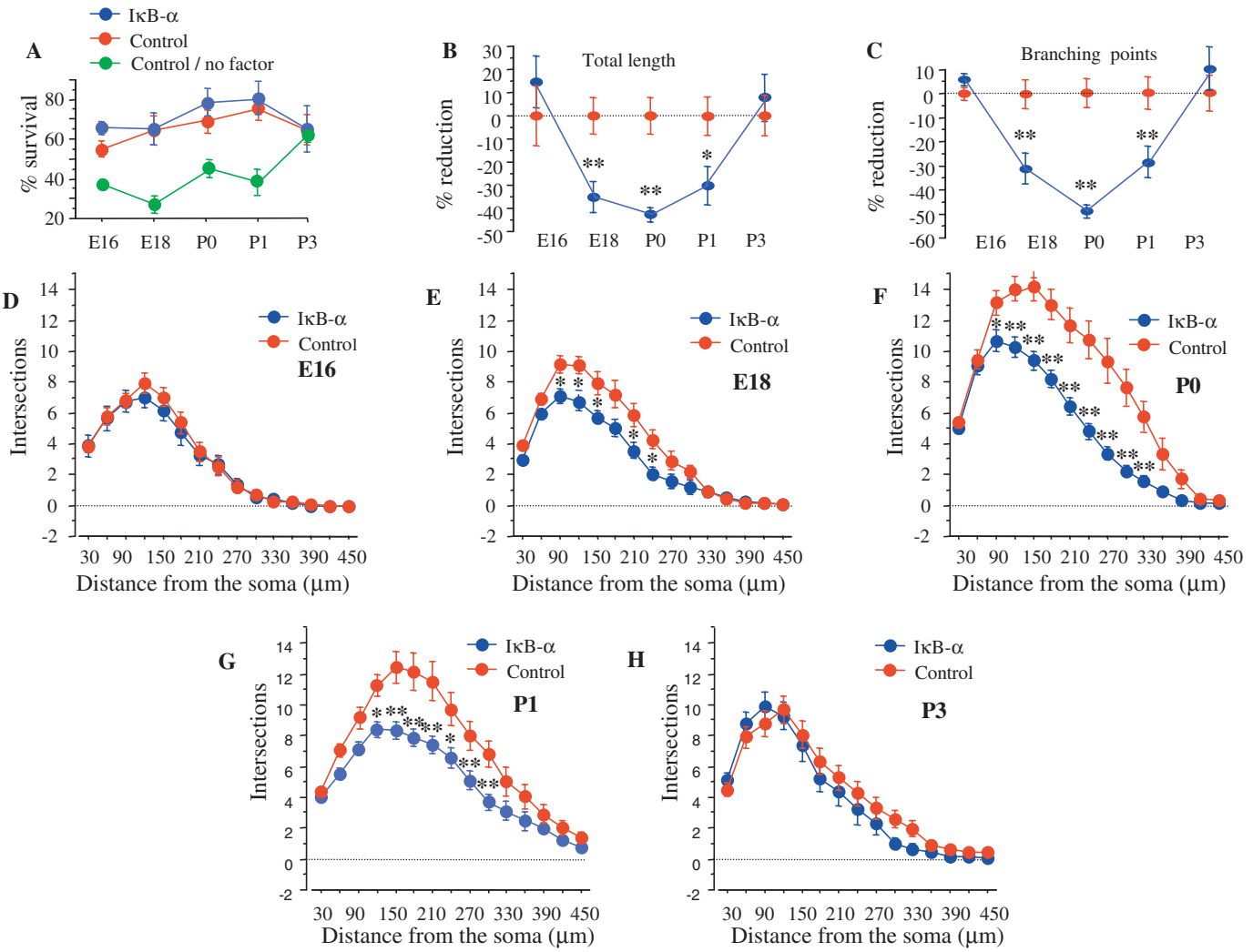

Fig. 4. Super-repressor IκB-α reduces neurite growth from nodose neurons in a developmentally dependent manner. E16, E18, P0, P1 and P3 nodose neurons were transfected with a YFP expression plasmid together with either a super-repressor IκB-α plasmid or an empty control plasmid and were incubated in medium containing 10 ng/ml BDNF. (A) Percent survival 48 hours after transfection. In addition to showing survival data for super-repressor IκB-α transfected and control transfected neurons grown with BDNF, survival data for control transfected neurons grown without BDNF are also shown at each age. (B) Total neurite length 24 hours after transfection. (C) Number of branching points in neurite arbors 24 hours after transfection. (D-H) Sholl analysis of neurite arbor morphology 24 hours after transfection at E16, E18, P0, P1 and P3, respectively. Statistical comparisons shown are with respect to the control transfected neurons, **P*<0.05, ***P*<0.001.

Super-repressor IκB-α reduces neurite growth in a developmentally dependent manner

To ascertain whether the inhibitory effect of the super-repressor IκB-α on neurite growth occurs over a particular stage of development, we established cultures of nodose neurons over a range of embryonic and postnatal stages. These neurons were grown with BDNF and transfected with either the super-repressor IκB-α plasmid and pYFP or the control plasmid and pYFP. From the youngest age at which neurons could be effectively transfected using the gene gun (E16) to the latest age studied (P3), neuron counts after 48 hours incubation demonstrated that the super-repressor IκB-α did not affect the ability of the neurons to survive with BDNF (Fig. 4A). Quantification of total neurite length and branch point number (Fig. 4B,C) revealed that super-repressor IκB-α had no significant effect on neurite growth at E16 and P3. However, at intermediate stages it caused highly significant reductions in length and branching that were maximal at P0, where 43 and 48% reductions were observed, respectively. Likewise, Sholl analysis revealed that super-repressor IκB-α caused significant reductions in the size and complexity of neurite arbors at E18, P0 and P1, with a maximal effect at P0 (Fig. 4D-H). To investigate if NF-κB activation plays any role on neurite growth at stages prior to E16, we crossed mice that were heterozygous for a null mutation in the *p65* gene to generate p65-deficient and wild-type embryos. Cultures were established from these embryos at E14, the oldest age to which p65-deficient embryos survive, and neurite growth was quantified after 24 hours incubation in medium containing BDNF. These experiments revealed no significant differences in neuronal survival, neurite length, branch number and branching with distance from the cell body between wild-type

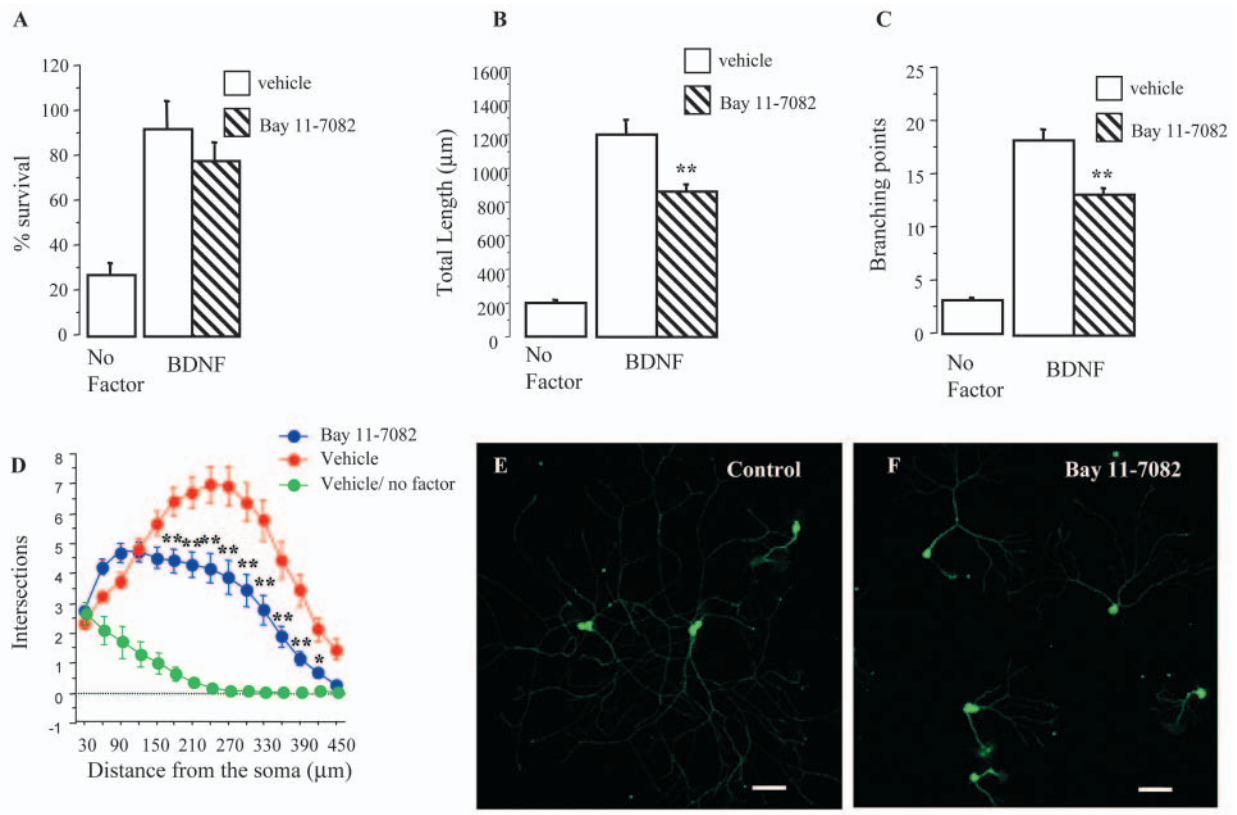

Fig. 5. BAY 11-7082 reduces neurite outgrowth from nodose neurons. P0 nodose neurons were incubated in medium containing 10 ng/ml BDNF and either the IκB-α phosphorylation inhibitor BAY 11-7082 at a concentration of 10 μmol/l or vehicle control. Vehicle-treated neurons were also grown without BDNF. (A) Percent survival after 48 hours incubation. (B) Total neurite length after 24 hours incubation. (C) Number of branching points in neurite arbors after 24 hours incubation. (D) Sholl analysis of neurite arbor morphology after 24 hours incubation. (E) Photomicrograph of a typical βIII tubulin stained, vehicle-treated neuron grown for 24 hours with BDNF. (F) Photomicrograph of a typical βIII tubulin stained, BAY 11-7082 treated neuron grown for 24 hours with BDNF. The means and standard errors of data obtained from at least 40 neurons in each experimental condition shown. Statistical comparisons shown are with respect to the control transfected neurons, **P*<0.05, ***P*<0.001. Scale bars: 50 μm.

and p65-deficient neurons (data not shown). Taken together, the above results suggest that NF-κB activation impairs neurite growth only during a restricted window of development encompassing late embryonic and early neonatal stages.

BAY 11-7082 reduces neurite growth from nodose neurons

To further substantiate the involvement of IκB-α phosphorylation in the regulation of neurite growth, we studied the effect of BAY 11-7082, a selective, irreversible inhibitor of IκB-α phosphorylation. These experiments were carried out at P0 because the above studies on super-repressor IκB-α suggested that the involvement of NF-κB signalling in enhancing neurite growth was maximal at this age. Fig. 5A shows that there was no significant difference in the numbers of neurons surviving after 48 hours incubation of BDNF-supplemented cultures treated with either 10 μmol/l BAY 11-7082 or vehicle. To investigate if the same dose of BAY 11-7082 affects neurite growth, BDNF-supplemented cultures were treated with this drug or vehicle, and were fixed and stained for βIII tubulin after 24 hours incubation. Analysis of confocal images of the stained neurons showed that BAY 11-7082 caused a statistically significant 19.6% reduction in total neurite length compared with vehicle-treated neurons (*P*<0.001, Fig. 5B). BAY 11-7082 also caused a statistically significant 28.2% reduction in branch point number (*P*<0.001, Fig. 5C). Accordingly, Sholl analysis revealed that in BDNF-supplemented cultures BAY 11-7082 caused significant reductions in neurite branching between 150 and 420 μm from the cell body (Fig. 5D). The typical appearances of BAY 11-7082-treated and vehicle-treated P0 nodose neurons grown with BDNF are shown in Fig. 5E,F. These results provide complementary evidence for the involvement of IκB-α-dependent activation of NF-κB in regulating neurite growth in neonatal nodose neurons.

Proteosome inhibition reduces neurite growth from nodose neurons

Upon phosphorylation, IκB-α is recognized by the ubiquitin ligase complex leading to its ubiquitination and degradation by the proteosome. Accordingly, proteosome inhibition has been shown to impair NF-κB activation. To provide additional supporting evidence for the involvement of NF-κB signalling in regulating neurite growth, we investigated the effects of N-acetyl-Leu-Leu-norleucinal (ALLN), a widely used proteosomal degradation inhibitor. Fig. 6A shows that ALLN did not impair the survival of P0 nodose neurons grown with BDNF. At the same dose, however, ALLN caused marked and

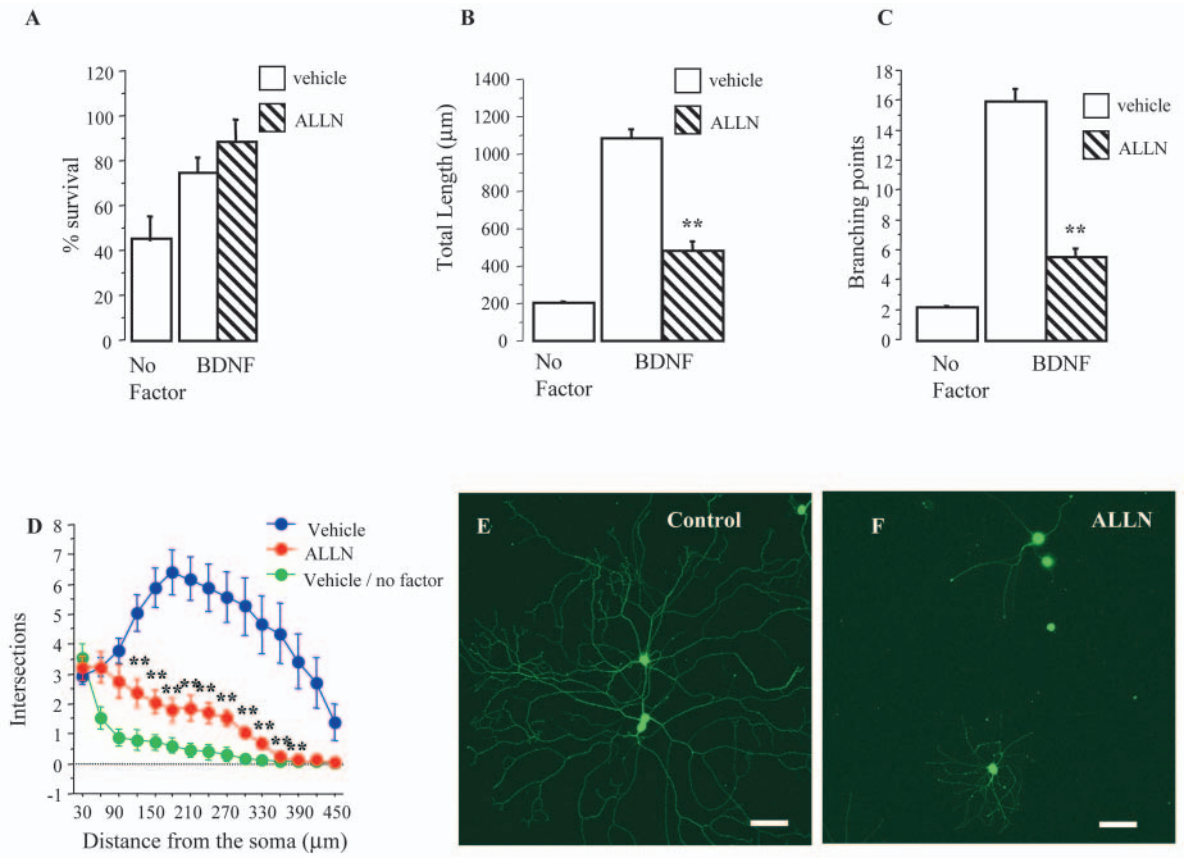

Fig. 6. Proteosome inhibition reduces neurite growth from nodose neurons. P0 nodose neurons were incubated in medium containing 10 ng/ml BDNF and either the proteosomal degradation inhibitor ALLN at a concentration of 10 μmol/l or vehicle control. Vehicle-treated neurons were also grown without BDNF. (A) Percent survival after 48 hours incubation. (B) Total neurite length after 24 hours incubation. (C) Number of branching points in neurite arbors after 24 hours incubation. (D) Sholl analysis of neurite arbor morphology after 24 hours incubation. (E) Photomicrograph of a typical βIII tubulin-stained, vehicle-treated neuron grown for 24 hours with BDNF. (F) Photomicrograph of a typical βIII tubulin-stained, ALLN-treated neuron grown for 24 hours with BDNF. Statistical comparisons shown are with respect to the control transfected neurons, **P*<0.05, ***P*<0.001. Scale bars: 50 μm.

highly significant decreases in the length and branching of neurites (Fig. 6,C). Likewise, Sholl analysis revealed marked decreases in neurite branching and length (Fig. 6D). ALLN completely eliminated the increase in branching that is normally observed within the first 180 μm from the cell body in neurons grown with BDNF. Indeed, the gradual decrease in the number of neurite intersections with distance from the cell body in ALLN-treated neurons grown with BDNF resembles more closely that of neurons grown without BDNF than neurons grown with BDNF. The diminutive neurite arbors of ALLN-treated neurons grown with BDNF and the normal sized neurite arbors of vehicle-treated neurons grown with BDNF are shown in Fig. 6E,F. These results show that proteosomal activity influences neurite growth in newborn nodose neurons, and although this experimental approach is not specific for NF-κB, the findings are consistent with the above demonstration that NF-κB activation influences neurite growth.

κB decoy DNA reduces neurite growth from nodose neurons

In addition to demonstrating the importance of key steps in the activation of NF-κB for neurite growth regulation, we also studied the significance of NF-κB transcriptional activity itself by delivering to cultured nodose neurons double-stranded DNA oligonucleotides containing the κB consensus binding sequence found in the promoters of NF-κB target genes. This κB decoy DNA has been successfully used both in vitro and in vivo to inhibit NF-κB transcriptional activity selectively and efficiently by sequestering transcriptionally active NF-κB complexes (Yeh et al., 2002). P0 nodose neurons were transfected with gold particles coated with either a double-stranded oligonucleotide containing the p50/65 consensus sequence or a scrambled control oligonucleotide. Each oligonucleotide was co-precipitated with the reporter plasmid (pYFP) to visualize the transfected cells. As in the above experiments, the survival of transfected cells was quantified to rule out any detrimental effect of these treatments on neuronal viability. Fig. 7A shows that after 48 hours incubation with BDNF there was no significant difference in survival between the decoy κB and control transfected neurons. κB decoy DNA did, however, cause a statistically significant reduction in total neurite ($P<0.001$, Fig. 7B) and branch point number ($P<0.001$, *t*-test, Fig. 7C) compared with neurons transfected with the scrambled control oligonucleotide. Sholl analysis also revealed that κB decoy DNA caused significant reductions in neurite branching between 150 and 360 μm from the cell body

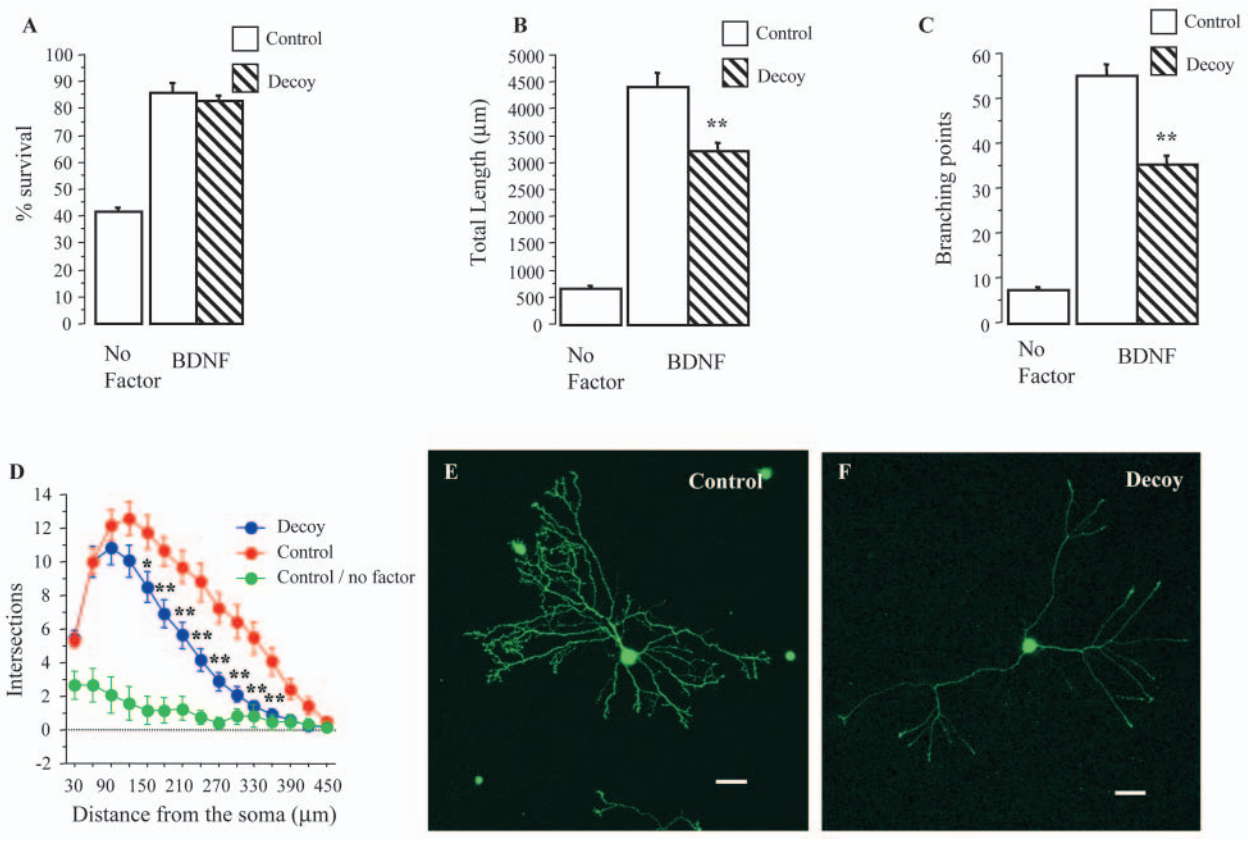

Fig. 7. κB decoy DNA reduces neurite growth from nodose neurons. P0 nodose neurons were transfected with a YFP expression plasmid together with either a κB decoy DNA or a scrambled control variant and were incubated in medium containing 10 ng/ml BDNF. Control transfected neurons were also grown without BDNF. (A) Percent survival 48 hours after transfection. (B) Total neurite length 24 hours after transfection. (C) Number of branching points in neurite arbors 24 hours after transfection. (D) Sholl analysis of neurite arbor morphology 24 hours after transfection. (E) Photomicrograph of a typical neuron transfected with the scrambled control oligonucleotide and grown for 24 hours with BDNF. (F) Photomicrograph of a typical neuron transfected with the κB decoy oligonucleotide and grown for 24 hours with BDNF. Statistical comparisons shown are with respect to the control transfected neurons, $*P<0.05$, $**P<0.001$. Scale bars: 50 μm.

in BDNF-supplemented medium (Fig. 7D). The typical appearances of BDNF-supplemented neurons transfected with κB decoy DNA and scrambled control DNA are shown in Fig. 7E,F, respectively. These results demonstrate that NF-κB transcriptional activity plays a key role in regulating neurite growth in newborn nodose neurons.

NF-κB gain of function promotes neuronal survival but does not enhance neurite growth

To investigate whether augmenting NF-κB activation in cultured nodose neurons would further enhance neurite growth, we transfected the neurons with a p65 expression plasmid that has been shown to activate NF-κB in other sensory neurons (Hamanoue et al., 1999). Co-transfection with the GFP NF-κB reporter revealed that overexpression of p65 caused a twofold increase in NF-κB-dependent gene transcription in these neurons, as assessed by measuring the GFP signal 24 hours after transfection (Fig. 8A,B). Overexpression of p65 did not enhance the growth and complexity of the neurite arbors. No significant differences were observed in total neurite length, number of branch points and in the Sholl analysis of neurite arbors of p65 transfected, compared with control transfected, neurons grown either with or without BDNF (Fig. 8D-F). Overexpression of p65 did, however, enhance the survival of nodose neurons grown without neurotrophic factors to the same extent as BDNF treatment. These results indicate that the basal level of NF-κB activity in cultured nodose neurons is maximal for neurite growth but suboptimal for survival.

Super-repressor IκB-α and κB decoy DNA reduce the size and complexity of pyramidal dendritic arbors in cortical slice cultures

To investigate if NF-κB signalling participates in regulating process growth and morphology elsewhere in the developing nervous system, we studied the effect of inhibiting NF-κB activation in pyramidal neurons of layers 2 and 3 of the somatosensory cortex, a well-characterized population of neurons that show active dendritic growth during the first postnatal week. Cortical slices were prepared from P3 and P4 mice, and were transfected with either super-repressor IκB-α plasmid or κB decoy DNA using the gene gun within 1 hour of the explant culture being established. Given the complexity of cortical slice cultures, we introduced a modification in the transfection technique to minimize variations across slice cultures. The cultures were shot with a mixture of two different gold microcarriers: one coated with a YFP plasmid, the other with an RFP plasmid. In one set of experiments, the YFP

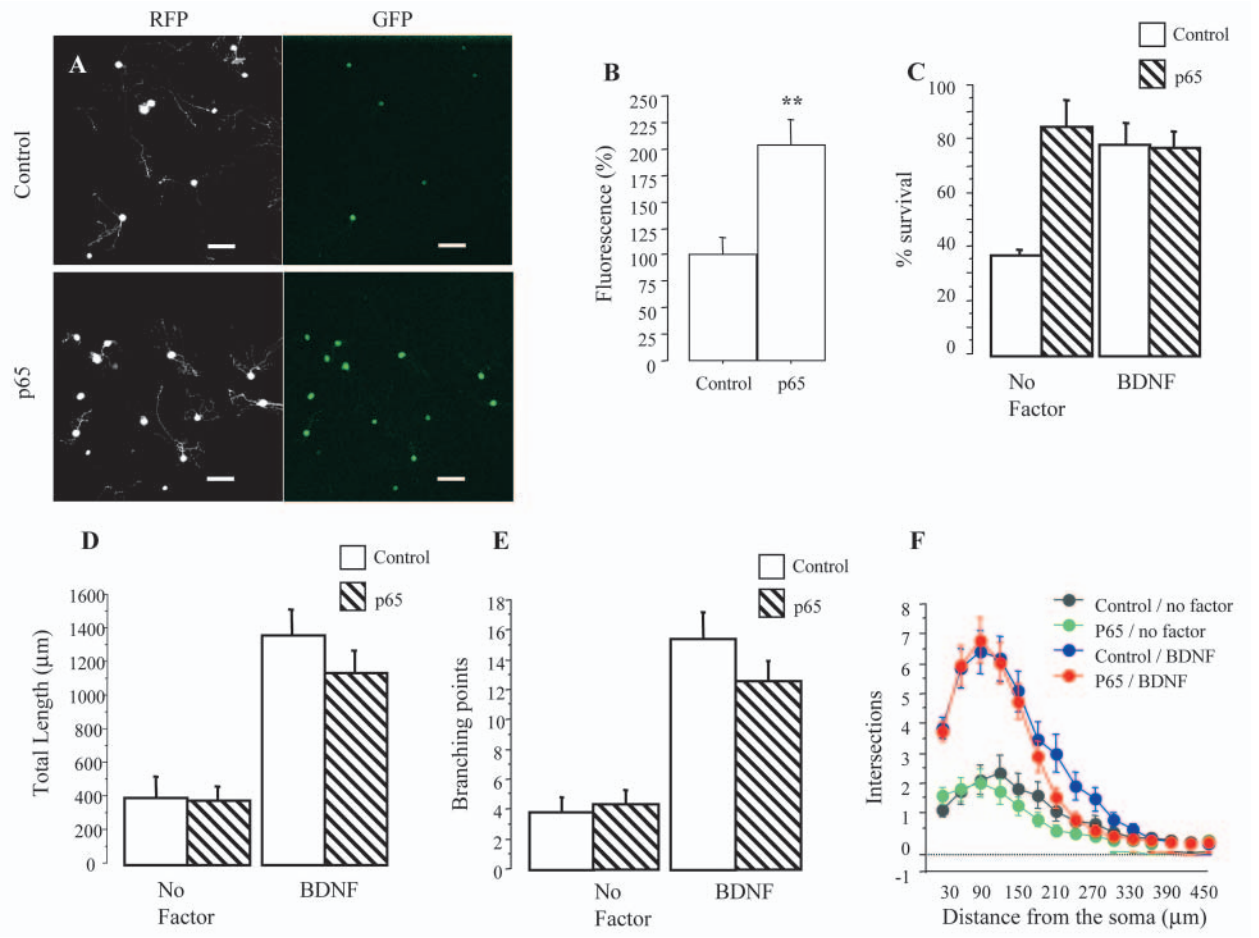

Fig. 8. Overexpression of p65 augments NF-κB activation and promotes neuronal survival but does not enhance neurite growth. (A) Photomicrographs of representative fields of P0 nodose neurons co-transfected with the κB-dependent GFP reporter plasmid, RFP plasmid and either the p65 overexpression plasmid (bottom) or the corresponding empty plasmid (top) and cultured with BDNF for 24 hours. Transfected neurons are outlined by the RFP (shown with a white filter, left panels). GFP fluorescence in the same fields (right panels) shows the marked increase caused by p65 overexpression. (B) Quantification of the level of NF-κB-driven GFP fluorescence in p65 transfected and control transfected neurons after 24 hours incubation with BDNF. Fluorescence measurements are expressed as a percentage of the mean fluorescence of the control vector-transfected group (mean and standard error are shown). (C) Percent survival 48 hours after transfection. (D) Total neurite length after 24 hours incubation. (E) Number of branching points in neurite arbors after 24 hours incubation. (F) Sholl analysis of neurite arbor morphology after 24 hours incubation. Statistical comparisons shown are with respect to the control transfected neurons, $^{**}P<0.001$. Scale bars: 100 μm.

microcarriers were co-coated with an NF-κB inhibitory system (either the super-repressor IκB-α plasmid or κB decoy DNA) and the RFP microcarriers were co-coated with the appropriate control (empty IκB-α plasmid and scrambled κB oligonucleotide, respectively). In a separate set of experiments, the order of the visual reporters was reversed to control for any effect resulting from the choice of the reporter. After the DNA precipitation step, both sets of carriers were pooled into a single suspension and loaded into the Teflon tubing for ballistic transfection. The resulting transfected slices possessed two clearly distinguishable fluorescently labelled cells, one expressing the NF-κB inhibitory treatment, the other the control. Very rarely, some cells were co-transfected with both kinds of gold microcarriers. These cells were easily recognized by their double labelling, and were discarded from the analysis. After 48 hours incubation in BDNF-supplemented medium, 40 to 50 layer 2/3 pyramidal neurons per labelled group were scanned and the resulting Z-stack images of the dendritic trees were analysed as described for the above experiments.

As in studies of cultured nodose neurons, we wished to ascertain whether inhibiting NF-κB activation in pyramidal neurons affected their survival in cortical slices. For this, we transfected cortical slices with a YFP plasmid together with either the super-repressor IκB-α plasmid or an empty plasmid and counted the total number of labelled neurons in layers 2 and 3 of the somatosensory cortex 24 hours after transfection (when labelled pyramidal neurons were clearly discernible) and again 48 hours after transfection. There was no significant difference in the number of labelled neurons 48 hours after transfection, expressed as a percentage of the number of labelled neurons 24 hours after transfection in control-transfected slices (60.1±7.9%, n=11 separate cultures) and super-repressor IκB-α-transfected slices (70.0±5.6%, n=16 separate cultures). Similar results were obtained when NF-κB-

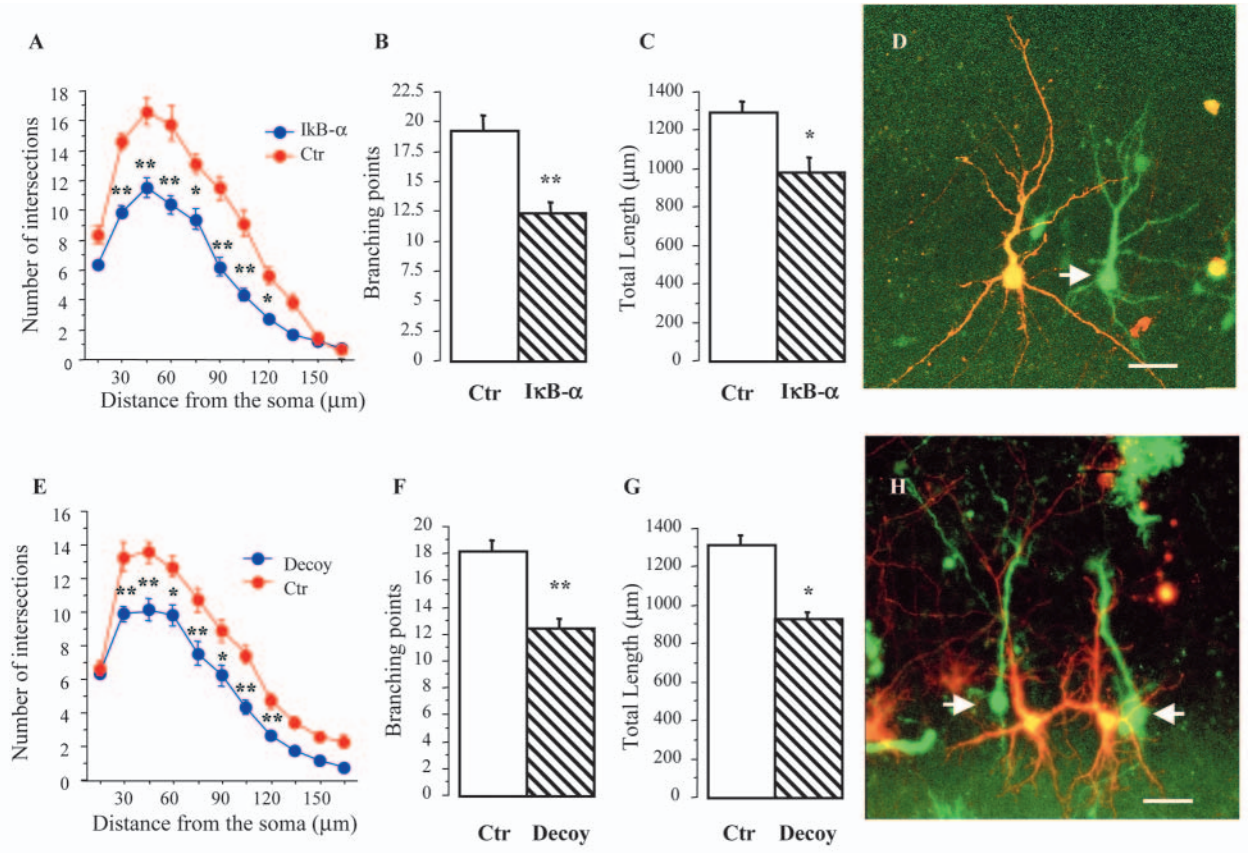

Fig. 9. Super-repressor IκB-α and κB decoy DNA reduce the size and complexity of pyramidal dendritic arbors in cortical slice cultures. Slice cultures of the somatosensory cortex of P3/P4 neonatal mice were simultaneously bombarded with a mixture of two sets of gold particles, each carrying a different reporter (YFP or RFP) together with either an NF-κB inhibitory system (super-repressor IκB-α plasmid or κB decoy DNA) or the appropriate corresponding control (empty plasmid or scrambled κB DNA, respectively). Forty-eight hours after transfection, between 40 and 50 individual neurons expressing each reporter were scanned, and the resulting Z-stack images of the dendritic trees were traced and analysed. (A,B,C) Sholl analysis, number of branching points and, total dendritic length, respectively, for neurons expressing super-repressor IκB-α or control plasmid. (D) Photomicrograph of representative pyramidal neurons transfected with the super-repressor IκB-α plasmid (arrow) or the control plasmid. (E,F,G) Sholl analysis, number of branching points and, total dendritic length, respectively, for neurons expressing κB decoy DNA or control scrambled κB DNA. (H) Photomicrograph of representative pyramidal neurons transfected with κB decoy DNA (arrows) or the control scrambled DNA. The means, standard errors and statistical comparisons (*P<0.05, **P<0.001) are shown. Scale bars: 50 μm.

dependent transcription was blocked with κB decoy DNA (79.5±4.3,% survival, n=18 separate cultures) compared with control DNA transfected neurons (83.6±5.6% survival, n=14 separate cultures). These data suggest that inhibiting NF-κB activation does not affect the survival of pyramidal neurons in cortical slices.

Fig. 9B,C shows that the super-repressor IκB-α caused significant reductions in the number of branch points of pyramidal neuron dendritic arbors and the overall length in these arbors compared with control-transfected neurons. Accordingly, Sholl analysis revealed that the super-repressor IκB-α caused significant reductions in dendritic branching between 30 and 120 μm from the cell body (Fig. 9A). The typical appearances of IκB-α-transfected and control-transfected pyramidal neurons are shown in Fig. 9D. Neurons expressing κB decoy DNA showed very similar significant decreases in overall dendritic branching (Fig. 9F) and length (Fig. 9G) compared with neurons expressing the scrambled κB control DNA, and Sholl analysis likewise revealed significant reductions in dendritic branching between 30 and 120 μm from the cell body in κB decoy-expressing neurons (Fig. 9E). The typical appearances of pyramidal neurons transfected with κB decoy DNA and scrambled control DNA are shown in Fig. 9H. Taken together, these results show that blocking NF-κB activation with either super-repressor IκB-α or κB decoy DNA significantly impairs the growth layer 2/3 pyramidal neuron dendrites in neonatal cortical slice cultures.

Discussion

In this study we have characterized a mechanism for regulating the growth of neural processes in the developing nervous system involving the transcription factor NF-κB. We have detected NF-κB-dependent transcriptional activity in neonatal mouse nodose ganglion neurons in culture and have demonstrated that inhibiting NF-κB activation and NF-κB-dependent transcription in these neurons by a variety of complementary approaches significantly reduces the size and complexity of their neurite arbors. Inhibiting IκB-α phosphorylation with super-repressor IκB-α or BAY 11-7082,

inhibiting proteosomal function with N-acetyl-Leu-Leu-norleucinal or blocking NF-κB-dependent transcription with κB decoy DNA all caused highly significant reductions in total neurite length, total branch number and branching with distance from the cell body (data from Sholl analysis). The effects of inhibiting NF-κB signalling on neurite growth and branching cannot be attributed to any detrimental effect on neuronal viability, because none of the treatments affected the survival of nodose neurons in BDNF-supplemented medium. Furthermore, inhibiting NF-κB signalling had no effect on soma size, suggesting that reduction in neurite growth was not secondary to a global reduction in cellular metabolism.

Analysis of the effect of super-repressor IκB-α at different developmental stages revealed that NF-κB affects neurite growth and morphology only during a restricted period of development between E18 and P1. This period occurs at the latter end of the phase of naturally occurring neuronal death in cranial sensory ganglia when the peripheral and central axons of the remaining neurons are establishing and refining their terminal arborizations (Davies and Lumsden, 1984). Thus it is possible that NF-κB plays a role in modulating the growth and branching of axonal terminals in the target fields of sensory neurons during this critical period of development when functional connections are being established.

In addition to its influence on peripheral sensory neurites, NF-κB signalling also regulates the size and complexity of the dendritic arbors of layer 2-3 pyramidal neurons in the postnatal mouse somatosensory cortex. Both super-repressor IκB-α and κB decoy DNA each caused highly significant reductions in the overall dendrite length, total branch number and branching with distance from the cell body in cortical slices established from P3 and P4 mice. This was observed at a stage in development when the dendritic arbors of these neurons are growing rapidly and establishing functional connections. Taken together, these findings reveal that NF-κB signalling influences the growth of neural processes in at least two populations of neurons in the developing peripheral nervous system (PNS) and central nervous system (CNS).

We have shown that NF-κB is activated at a certain basal level in cultured nodose neurons independently of BDNF. This basal level of NF-κB activity is maximal for neurite growth because augmenting NF-κB transcriptional activity by overexpressing p65 causes no further increase in neurite growth. Overexpression of p65 does, however, enhance the survival of nodose neurons grown without neurotrophic factors as effectively as BDNF. Likewise, overexpression of p65 in trigeminal sensory neurons grown without neurotrophic factors promotes their survival just as well as NGF (Hamanoue et al., 1999). Taken together, these findings suggest that different thresholds of NF-κB activity enhance neurite growth and neuronal survival. What drives the basal level of NF-κB transcriptional activity in cultured nodose neurons is unclear. This may be constitutive or secondary to activation of receptors for neurotrophic factors or cytokine receptors. While exogenous factors can be excluded because it occurs in defined medium, neurotrophic factors or cytokines could be produced in culture by the neurons themselves or by some residual non-neuronal cells.

In addition to intrinsic developmental programmes that establish certain characteristic morphological features of the dendritic and axonal architecture of different kinds of neurons, a wide variety of extrinsic signals regulate the growth of neural processes, including neurotransmitters, growth factors, extracellular matrix proteins and an assortment of guidance molecules. These extrinsic signals variously engage several intracellular signalling pathways to influence process growth, including Ras-MEK-ERK, PI 3-kinase-Akt and calcium/calmodulin kinases. Activation of these pathways influences the dynamics of the neuronal cytoskeleton in a variety of ways to control axonal and dendritic growth. For example, by phosphorylating microtubule-associated proteins and by regulating the activity of the Rho family of GTPases and their effectors, which in turn regulate the structure and dynamics of the actin cytoskeleton (Fink and Meyer, 2002; Lundquist, 2003; Miller and Kaplan, 2003). CREB and NeuroD have also been implicated in mediating the effects of calcium/calmodulin kinases on dendritic growth (Gaudilliere et al., 2004; Redmond et al., 2002), although the target genes that mediate the effects of these transcription factors on dendrites are not known. It has also been reported that inhibiting NF-κB in PC12 cells decreases the proportion of cells that possess neurites following TrkA activation (Foehr et al., 2000). However, it is unclear whether this result reflects a direct influence of NF-κB signalling on neurite growth per se or represents one of the downstream consequences of TrkA-induced differentiation of these tumour cells.

Of the multitude of genes induced by NF-κB in various cell types, several encode cell adhesion molecules and other proteins that influence cell migration (Pahl, 1999), some of which could be potentially relevant for process growth in neurons. For example, activity-dependent upregulation of NCAM on cultured striatal neurons depends on NF-κB activation (Simpson and Morris, 2000). NCAM is widely expressed on the dendrites and axons of a variety of neurons in the developing brain, and becomes progressively localized to synapses with age (Butler et al., 1998; Chung et al., 1991; Fox et al., 1995; Persohn and Schachner, 1990). NCAM stimulates neurite growth from many kinds of neurons in vitro (Skaper et al., 2001). Clustering of NCAM on cultured cerebellar neurons leads to activation of MAP kinase (Schmid et al., 1999), and MAP kinase activation has been shown to promote the growth of hippocampus and sympathetic dendrites (Vaillant et al., 2002; Wu et al., 2001). Tenascin-C is also an NF-κB-regulated protein (Mettouchi et al., 1997) that is a prominent component of the neural extracellular matrix, where it plays important roles in regulating neurite outgrowth and guidance during development (Joester and Faissner, 2001). Although tenascin-C is expressed predominantly by glial cells in the PNS and CNS, several populations of neurons also express tenascin-C during development, when its regulation might influence process growth. For example, tenascin-C is transiently expressed by subsets of neurons in the embryonic hippocampus (Ferhat et al., 1996) and postnatal spinal cord (Zhang et al., 1995). β1 integrin is another NF-κB-regulated protein (Wang et al., 2003). β1 integrin dimerizes with several different α integrin subunits to form receptors for many extracellular matrix components, including laminins (Previtali et al., 2001). Interaction between laminin and α1β1 or α3β1 integrins expressed on the growth cone promotes the growth of sensory and sympathetic neurites in culture (DeFreitas et al., 1995; Schmidt et al., 1995; Tomaselli et al., 1993), and anti-integrin β1 antibody in combination with anti-L1 and anti-N-

cadherin reduces the growth of ciliary ganglion neurons on Schwann cells (Bixby et al., 1988).

In summary, we have characterized a function of NF-κB signalling that differs markedly from its well-established ubiquitous role in immune and stress responses and regulation of apoptosis. Our demonstration that NF-κB transcriptional activity promotes the growth and branching of axonal and dendritic processes in the developing PNS and CNS furthers our understanding of the molecular mechanisms that establish and refine neural connections during development and has potentially important implications for the involvement of NF-κB in learning and memory.

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