Developmental switch in NF-κB signalling required for neurite growth

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For a given cell type, particular extracellular signals generate characteristic patterns of activity in intracellular signalling networks that lead to distinctive cell-type specific responses. Here, we report the first known occurrence of a developmental switch in the intracellular signalling network required for an identical cellular response to the same extracellular signal in the same cell type. We show that although NF-κB signalling is required for BDNF-promoted neurite growth from both foetal and postnatal mouse sensory neurons, there is a developmental switch between these stages in the NF-κB activation mechanism and the phosphorylation status of the p65 NF-κB subunit required for neurite growth. Shortly before birth, BDNF activates NF-κB by an atypical mechanism that involves tyrosine phosphorylation of IκBα by Src family kinases, and dephosphorylates p65 at serine 536. Immediately after birth, BDNF-independent constitutive activation of NF-κB signalling by serine phosphorylation of IκBα and constitutive dephosphorylation of p65 at serine 536 are required for BDNF-promoted neurite growth. This abrupt developmental switch in NF-κB signalling in a highly differentiated cell type illustrates an unsuspected plasticity in signalling networks in the generation of identical cellular responses to the same extracellular signal.

KEY WORDS: NF-κB, Neurite growth, Signalling, BDNF, Sensory neuron

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of secreted signalling proteins that plays a crucial role in regulating both neuronal survival and the growth of neural processes in the developing nervous system (Bibel and Barde, 2001). It binds to the common neurotrophin receptor p75[NTR] and the TrkB receptor tyrosine kinase that are expressed by subsets of developing neurons, including the sensory neurons of the nodose ganglion (Reichardt, 2006). In recent years, nuclear factor kappa-B (NF-κB) signalling has been recognised as an important novel regulator of axonal and dendritic growth in the developing nervous system (Sole et al., 2004; Gutierrez et al., 2005; Gallagher et al., 2007; Gutierrez et al., 2008), and is the focus of the current investigation.

The NF-κB transcription factor system has been studied most extensively in the immune system, where it plays a central role in regulating the expression of genes involved in innate and adaptive immune responses, inflammatory responses, cell survival and cell proliferation (Liang et al., 2004). In the developing nervous system, NF-κB is emerging as an important regulator of axonal and dendritic growth. NF-κB is a dimer of five possible subunits, of which the most abundant and ubiquitous is the p50-p65 heterodimer. This is held in a latent form in the cytoplasm by interaction with a member of the IκB inhibitory family of proteins, of which IκBα is the most prevalent. NF-κB is activated by mechanisms that disrupt its interaction with IκB, resulting in translocation of the liberated NF-κB to the nucleus where it binds to κB elements in the promoter and enhancer regions of responsive genes, leading to gene induction or gene repression (Perkins, 2006). The canonical activation mechanism involves the phosphorylation of IκBα on serines 32 and

36, which marks IκBα for ubiquitylation and proteasome-mediated degradation. An alternative activation mechanism involves the phosphorylation of IκBα on tyrosine 42 (Tyr42), which results in the dissociation of IκBα from NF-κB without proteasome-dependent degradation (Koong et al., 1994; Imbert et al., 1996). Both activation mechanisms have been shown to contribute to neurite growth promoted by different neurotrophic factors (Gutierrez et al., 2005; Gallagher et al., 2007). Recently, NF-κB signalling has been show to inhibit, as well as promote, neurite growth, depending on the phosphorylation status of the p65 subunit. Phosphorylation at serine 536 (Ser536) confers a profound neurite growth-inhibitory effect on activated NF-κB (Gutierrez et al., 2008).

In the present study, we investigated the role of NF-κB signalling in regulating neurite growth from a well-characterised population of peripheral nervous system neurons during development. The sensory neurons of the nodose ganglia of foetal and postnatal mice survive and extend neurites in culture in response to BDNF (Gutierrez et al., 2005). We report that although NF-κB signalling makes a major contribution to BDNF-promoted neurite growth throughout development, there is an acute switch in the NF-κB signalling network close to birth that involves changes in both the activation mechanism and p65 phosphorylation. Our findings provide a clear illustration of a surprising degree of plasticity in signalling networks in achieving the same cellular response under unchanged extracellular conditions.

MATERIALS AND METHODS
Neuron culture and transfection
Dissociated cultures of nodose ganglion neurons were established from embryonic day 15 (E15), E16, E17, postnatal day 0 (P0) and P3 CD1 mice, and were plated in defined, serum-free medium on a poly-ornithine/laminin substratum (Davies et al., 1993). For transfection experiments, neurons were electroporated before plating, using a Microporator (NanoEnTek, Seoul, Korea), with plasmids of interest plus a YFP expression plasmid to identify transfected neurons and visualise their neurite arbors for analysis by confocal microscopy (Gutierrez et al., 2005). In non-transfection experiments, neurite arbors were labelled with the fluorescent dye calcein-AM (Invitrogen) at the end of the experiment. Neuron survival was

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quantified as previously described (Gutierrez et al., 2005). For every condition in each experiment, images of at least 50 neurons were digitally acquired by confocal microscopy and analysed to obtain total neurite length, number of branch points and Sholl profiles (Gutierrez and Davies, 2007). Pair-wise comparisons were made using Student's *t*-test. For multiple comparisons, ANOVA was carried out, followed by Fisher's post-hoc test.

Quantification of NF-κB activity

To estimate the relative level of NF-κB activation, neurons were transfected with a plasmid expressing GFP under the control of an NF-κB promoter (Gutierrez et al., 2008). Neurons were imaged with a Zeiss Axioplan confocal microscope 4 hours after transfection (unless otherwise stated), and the number of GFP-positive neurons was counted in a standard 12×12 mm grid. All imaging and quantification was performed blind. Statistical comparisons were performed by ANOVA followed by Fisher's post-hoc test.

Western blotting

Protein for western blotting was extracted from either cultured neurons or whole ganglia. Neurons were plated on poly-ornithine/laminin-coated 96-well plates in medium without BDNF for 18 hours. To prevent the death of these neurons during this period, the irreversible caspase inhibitor Boc–D–FMK (25 μM, Calbiochem, UK) was added to the medium after plating. The cultures were then treated with 10 ng/ml BDNF for the indicated times. The cells were lysed in RIPA buffer and insoluble debris was removed by centrifugation. Whole ganglia were triturated and lysed in RIPA buffer. Samples were transferred to PVDF membranes using Bio-Rad TransBlot (Bio-Rad, CA, USA). The membranes were blocked with 5% dried milk in PBS with 0.1% Tween-20, and were incubated with anti-IκBα (1:1000; Cell Signaling), anti-phospho-Ser IκBα (1:1000; Cell Signaling), anti-phospho-Tyr42 IκBα (1:200, Abcam), anti-phospho-Lck (1:1000, Santa Cruz), anti-phospho-Src (1:1000, Santa Cruz), anti-phospho-Ser536-p65 (1:1000, Cell Signaling), anti-p65 (1:1000, Cell Signaling) or anti-β-III tubulin (1:10000; Promega). The appropriate peroxidase-linked secondary antibody (1:2000; Promega) was used to detect each primary antibody on the blots and staining was visualised using ECL plus (Amersham). Densitometry was carried out using the Gel-Pro Analyzer 32 program (Media Cybernetics, USA). To avoid potential proteosome-mediated degradation of IκBα complicating the analysis of phospho-Ser32/phospho-Ser36 IκBα levels, the cultures used for these studies were pre-treated with the proteosome inhibitor ALLN (1.5 μM, Calbiochem, UK) 30 minutes prior to BDNF treatment.

RESULTS

BDNF activates NF-κB in late foetal but not postnatal nodose neurons

We have previously demonstrated that although basal NF-κB signalling makes a major contribution to BDNF-promoted neurite growth from neonatal nodose neurons, BDNF does not directly activate NF-κB at this stage of development (Gutierrez et al., 2005). However, when we studied NF-κB activation at earlier stages, we found a pronounced increase in NF-κB transcriptional activity in response to BDNF. Dissociated cultures of nodose neurons were established over a range of ages, and NF-κB transcriptional activity was quantified in neurons grown with and without BDNF by transfecting the neurons with a reporter construct in which GFP is under the control of an NF-κB promoter. All cultures received the broad-spectrum, irreversible caspase inhibitor Boc-D-FMK to prevent neuronal death in the absence of BDNF and to ensure similar number of neurons under both experimental conditions (cell counts revealed no significant differences in survival, data not shown). Quantification of the NF-κB reporter signal 4 hours after transfection revealed marked, highly significant increases in BDNF-treated cultures established from mice at late foetal stage (E16 and E17), but no significant differences between control and BDNF-treated cultures established at an earlier foetal stage (E15) and at postnatal stages (P0 and P3) (Fig. 1A). Highly significant elevations in reporter signal were maintained in BDNF-treated E16 and E17
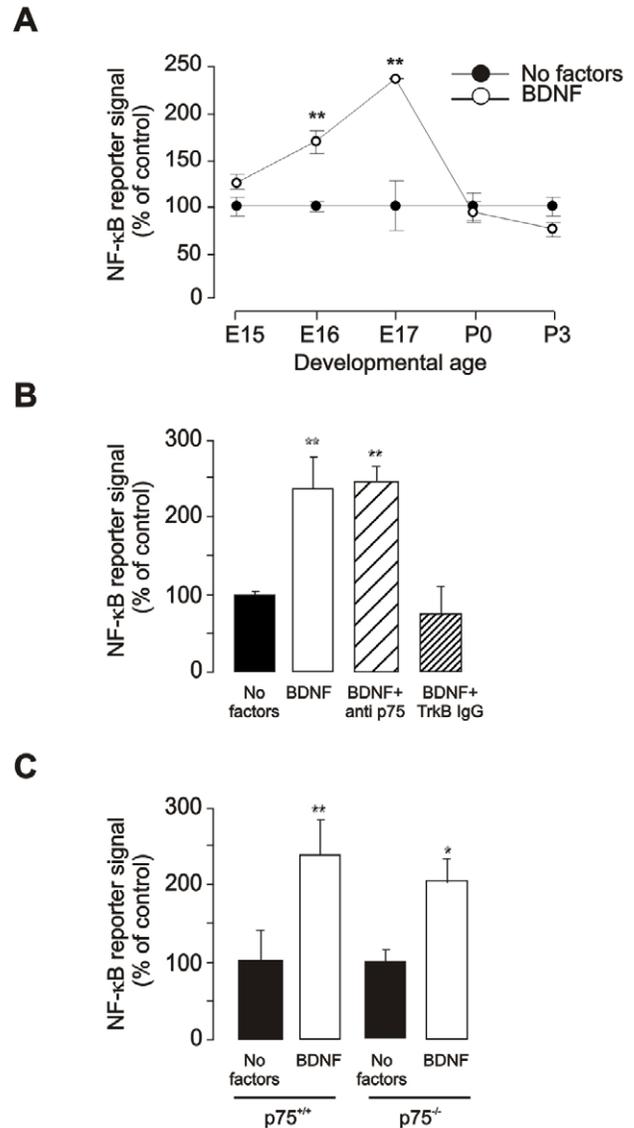

Fig. 1. BDNF activates NF-κB in nodose neurons via TrkB during a narrow developmental window. (**A**) NF-κB reporter signal in E15 to P3 nodose neurons grown with and without 10 ng/ml BDNF for 4 hours. (**B**) NF-κB reporter signal in E17 nodose neurons grown for 4 hours with no factors, with BDNF alone, or with BDNF plus function-blocking antibodies against either p75[NTR] or TrkB. (**C**) NF-κB reporter signal in nodose neurons of E17 *p75[NTR+/+]* and *p75[NTR–/–]* embryos grown with or without BDNF for 4 hours. Each data point (mean±s.e.m. from three separate experiments) shows the number of GFP-positive neurons expressed as a percentage of the mean number in the control group (no factors). Statistical comparisons shown are with the respective controls; *P<0.02, **P<0.01. All cultures received 25 μM Boc-D-FMK.

neurons for at least 18 hours after transfection, and the presence of caspase inhibitor did not affect NF-κB reporter signal in BDNF-treated foetal neurons (data not shown). These findings indicate that BDNF activates NF-κB in nodose neurons during a narrow developmental window shortly before birth.

To determine which receptor mediates the induction of NF-κB by BDNF, E17 neurons were transfected with the NF-κB reporter and were pre-incubated with functional blocking antibodies to either p75[NTR] or TrkB (also known as Ntrk2) for 1 hour prior to BDNF

treatment. Quantification of the NF-κB reporter signal 4 hours later revealed that BDNF-promoted NF-κB activation was completely unaffected by anti-p75[NTR] and totally blocked by anti-TrkB antibodies (Fig. 1B), suggesting that BDNF activates NF-κB via its receptor tyrosine kinase, TrkB. To confirm the non-involvement of p75[NTR] in BDNF-promoted NF-κB activation, we compared the NF-κB reporter signal in E17 nodose neurons from p75-deficient embryos and wild-type littermates following BDNF treatment. BDNF promoted highly significant, >2-fold increases in NF-κB reporter signal in neurons from both $p75^{NTR+/+}$ and $p75^{NTR-/-}$ embryos (Fig. 1C), confirming that BDNF does not activate NF-κB via p75[NTR]. Interestingly, nerve growth factor (NGF), which activates NF-κB via p75[NTR] in Schwann cells and NGF-responsive trigeminal ganglion neurons (Carter et al., 1996; Hamanoue et al., 1999), did not activate NF-κB in either late foetal or postnatal nodose ganglion neurons (data not shown). Also, despite the expression of tumour necrosis factor (TNF) receptors on these neurons, TNF-α did not cause any detectable change in NF-κB reporter signal at any of the ages analysed (data not shown).

NF-κB activation in late foetal neurons is required for BDNF-promoted neurite growth

We have previously shown that NF-κB signalling in neonatal nodose neurons is selectively required for BDNF-promoting neurite growth but not survival (Gutierrez et al., 2005). To ascertain whether NF-κB activation has a similar role in nodose neurons at late foetal stage, we studied the effect of blocking NF-κB activation with the specific NF-κB inhibitor SN50, or by transfecting the neurons with double-stranded decoy DNA oligonucleotides containing the κB consensus-binding sequence (Morishita et al., 1997). Both SN50 and the κB-decoy caused marked, highly significant reductions in the overall length (Fig. 2A,D) and number of branch points (Fig. 2B,E) in the

neurite arbors of E17 nodose neurons cultured with BDNF, and caused clear shifts in the Sholl profiles (Fig. 2C,F), which provide graphic illustrations of neurite length and branching with distance from the cell body. The typical appearances of SN50-treated neurons and κb decoy DNA-transfected neurons together with the corresponding control neurons are shown in Fig. 2G. Despite the pronounced effect of SN50 and κB-decoy DNA on BDNF-promoted neurite growth, they had no significant effect on BDNF-promoted neuronal survival (data not shown). These findings show that, as with neonatal nodose neurons, NF-κB signalling is required for BDNF-promoted neurite growth from late foetal-stage nodose neurons, but does not participate in BDNF-promoted neuronal survival.

BDNF activates NF-κB in late foetal neurons by promoting tyrosine but not serine phosphorylation of IκBα

We investigated the mechanism by which BDNF activates NF-κB in E17 nodose neurons by using western blotting to study the pattern of IκBα phosphorylation and the stability of IκBα protein at intervals following BDNF treatment. The neurons were initially incubated for 18 hours in medium containing the caspase inhibitor Boc-D-FMK to prevent their death in the absence of BDNF, and were subsequently exposed to BDNF for different time intervals prior to protein extraction and western blot analysis. There was no change in total IκBα protein and no change in the level of phosphoserine IκBα following BDNF treatment (Fig. 3A), which implies that BDNF does not enhance canonical NF-κB signalling in these neurons.

Because ciliary neurotrophic factor (CNTF) activates NF-κB in neonatal nodose neurons by an alternative mechanism that involves the phosphorylation of IκBα at Tyr42 without subsequent protein
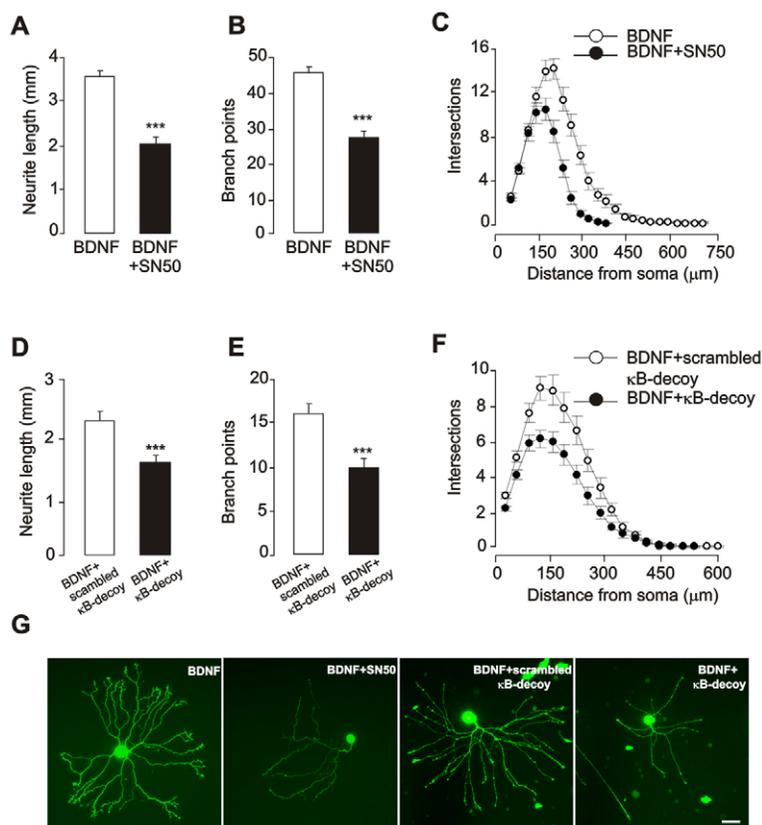

Fig. 2. NF-κB activation is required for BDNF-promoted neurite growth from E17 nodose neurons. (**A-C**) Length (A), branch point number (B) and Sholl profiles (C) after 24 hours with 10 ng/ml BDNF or BDNF plus 20 μg/ml SN50. (**D-F**) Length (D), branch point number (E) and Sholl profiles (F) of neurons grown with BDNF for 24 hours after transfection with either the κB-decoy oligonucleotide or a scrambled sequence control oligonucleotide. Each data point shows the mean±s.e.m. of at least 150 neurons in each condition from three independent experiments (***$P<0.001$, statistical comparison with control, ANOVA with Fisher's post-hoc test). (**G**) Photomicrographs of typical neurons grown for 24 hours with BDNF and either treated with SN50, or transfected with either κB decoy or control oligonucleotide. Scale bar: 50 μm.
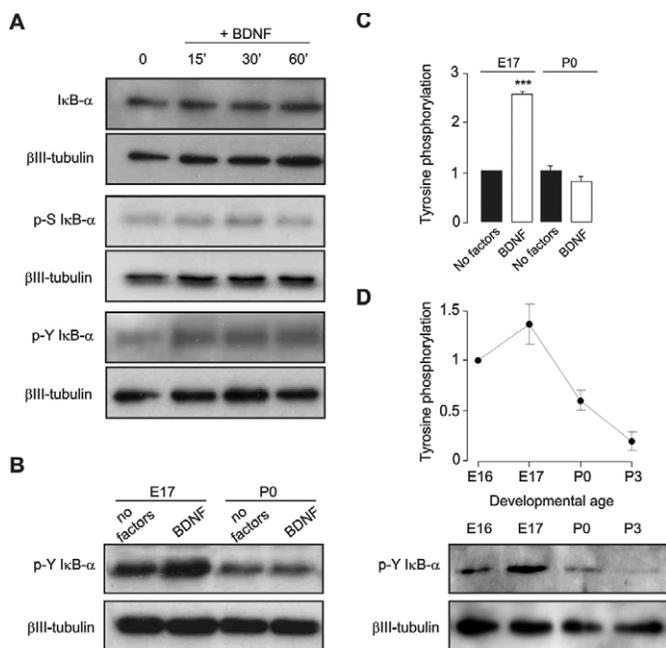
Fig. 3. BDNF activates NF-κB via tyrosine phosphorylation of IκBα in foetal nodose neurons. (**A**) Representative western blots of total IκBα, phospho-Ser32/phospho-Ser36 IκBα and phospho-Tyr42 IκBα in protein extracts of E17 nodose neurons treated with 10 ng/ml BDNF for 15, 30 and 60 minutes (0=untreated neurons) 18 hours after plating. The blots were reprobed with antibodies to the neuron-specific protein βIII-tubulin to compare the relative protein levels in the lanes of each blot. (**B**) Representative western blots of phospho-Tyr42 IκBα in protein extracts of E17 and P0 nodose neurons treated with 10 ng/ml BDNF for 15 minutes (no factors=untreated neurons) 18 hours after plating. (**C**) Quantification of the relative levels of phospho-Tyr42 IκBα from densitometric scans of three separate experiments at each age (***$P<0.01$, statistical comparison with no factors control, ANOVA with Fisher's post-hoc test). (**D**) Quantification of the relative levels of phospho-Tyr42 IκBα from the whole nodose ganglia dissected at different ages. All cultures received 25 µM Boc-D-FMK at the time of plating.

Fig. 4. Developmental switch in the NF-κB activation mechanism required for BDNF-promoted neurite growth. (**A**) E17 and P0 nodose neurons were co-transfected with a YFP expression plasmid together with either a plasmid expressing the Y42F IκBα mutant (black circles) or an empty control (white circles) plasmid. BDNF (10 ng/ml) was added to the medium after transfection, and Sholl analysis was carried out 24 hours later. (**B**) E17 and P0 nodose neurons were treated immediately after plating with either 1.5 µM ALLN (black circles) or the vehicle control (white circles). Sholl analysis was carried out after 24 hours incubation with BDNF. Each data point shows the mean±s.e.m. from 50-90 neurite arbors.

degradation (Gallagher et al., 2007), we investigated whether BDNF treatment promotes tyrosine phosphorylation of IκBα in nodose neurons. Western blot analysis revealed a clear increase in the level of phospho-Tyr42 IκBα in E17 but not P0 nodose neurons within 15 minutes of BDNF treatment (Fig. 3A-C), which suggests that BDNF activates NF-κB in late foetal nodose neurons by an alternative pathway that involves the phosphorylation of IκBα at Tyr42. Furthermore, western blot analysis of protein directly extracted from nodose ganglia dissected from foetal and postnatal mice revealed a 10-fold reduction in the in vivo level of phospho-Tyr42 IκBα from E17 to P3 (Fig. 3D). This result suggests the involvement of tyrosine phosphorylation of IκBα in NF-κB activation in foetal nodose neurons in vivo, and the attenuation and loss of this activation mechanism postnatally.

Developmental switch in the NF-κB activation mechanism required for BDNF-promoted neurite growth

Our demonstration that BDNF treatment promotes phosphorylation of IκBα at Tyr42 and enhances NF-κB transcriptional activity in nodose neurons at late foetal stages, but not after birth, raised the question of whether BDNF-promoted
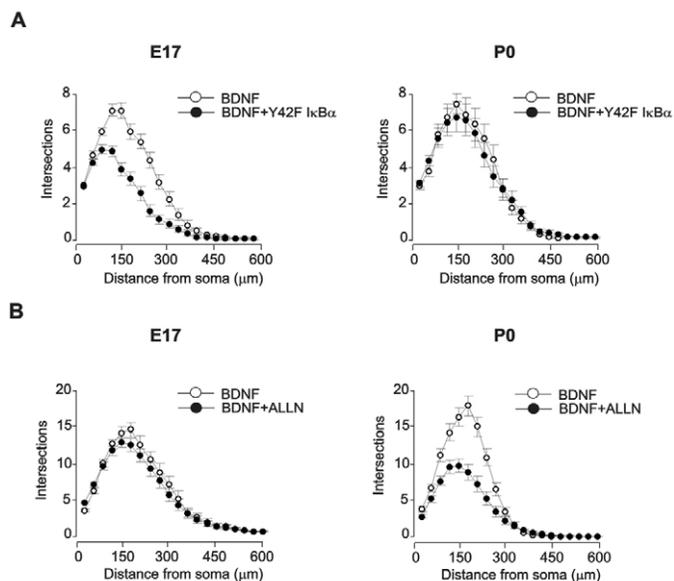
NF-κB signalling in foetal neurons is required for its capacity to promote neurite growth at this stage of development. NF-κB activation by Tyr42 phosphorylation of IκBα can be effectively and specifically blocked with an IκBα protein that has a tyrosine to phenylalanine substitution at residue 42 (Y42F) (Imbert et al., 1996). E17 nodose neurons transfected with a plasmid that expresses this Y42F IκBα mutant had markedly smaller and less-branched neurite arbors than control-transfected neurons grown with BDNF (Fig. 4A). However, this Y42F IκBα mutant had no effect on BDNF-promoted neurite growth from P0 neurons (Fig. 4A). BDNF-promoted neuronal survival was unaffected by this mutant at either age (data not shown). These results show that BDNF-promoted tyrosine phosphorylation of IκBα plays an important role in the neurite growth-promoting effects of this neurotrophin on nodose neurons at E17 but not at P0.

We have previously demonstrated that constitutive, proteosome-dependent NF-κB activation makes an important contribution to BDNF-promoted neurite growth in newborn nodose neurons (Gutierrez et al., 2005). To determine whether this activation pathway plays any role in BDNF-promoted neurite growth in E17 nodose neurons, we studied the effect of the proteasome inhibitor *N*-acetyl-Leu-Leu-norleucinal (ALLN) on the neurite arbors of these neurons incubated with BDNF. After 24 hours, Sholl analysis showed that ALLN had no effect on neurite arbor size and complexity (Fig. 4B). However after birth, ALLN caused a marked decrease in neurite arbor size, confirming our previous observations (Fig. 4B). ALLN did not affect BDNF-promoted neuronal survival at either age (data not shown). These findings indicate that BDNF-promoted neurite growth from developing nodose neurons is

dependent on NF-κB signalling that switches from a proteosome-independent mechanism at E17 to a proteosome-dependent mechanism by P0.

Src tyrosine kinases phosphorylate IκBα in BDNF-treated late foetal-stage nodose neurons

To delineate further the NF-κB-related signalling events required for BDNF-promoted neurite growth in nodose neurons at late foetal stage, we investigated the potential involvement of several tyrosine kinases that have been reported to phosphorylate IκBα. Because the tyrosine kinase SYK phosphorylates IκBα on Tyr42 in nodose neurons treated with CNTF and is required for CNTF-promoted neurite growth (Gallagher et al., 2007), we used western analysis and functional blockade of SYK to investigate whether this tyrosine kinase plays any role in BDNF-promoted IκBα phosphorylation and neurite growth in nodose neurons at late foetal stage. Western blotting using a specific anti-phospho-SYK antibody failed to detect phospho-SYK in E17 nodose neurons following BDNF treatment (data not shown). The potent selective SYK inhibitor piceatannol (Oliver et al., 1994) did not affect the BDNF-induced elevation in phospho-Tyr42 IκBα and neither piceatannol nor transfection of E17 neurons with a plasmid expressing a dominant-negative SYK protein (Gallagher et al., 2007) affected BDNF-promoted neurite growth (data not shown). These results suggest that SYK plays no

role in BDNF-promoted IκBα phosphorylation and neurite growth from E17 nodose neurons. In addition to SYK, Src and a member of the Src family of tyrosine kinases called Lck have been shown in a variety of cell lines to phosphorylate IκBα on Tyr42 (Abu-Amer et al., 1998; Livolsi et al., 2001; Fan et al., 2003; Mahabeleshwar and Kundu, 2003; Kang et al., 2005; Lluis et al., 2007). Western blot analysis using specific phospho-Src and phospho-Lck antibodies revealed clear increases in the levels of the active phosphorylated forms of both proteins in E17 nodose neurons within 15 minutes of BDNF treatment (Fig. 5A). Confirming the importance of Src-family tyrosine kinases in BDNF-promoted IκBα phosphorylation and subsequent NF-κB activation in E17 nodose neurons, we showed that PP2, a selective inhibitor of these kinases, but not a non-functional structural analogue, PP3 (Hanke et al., 1996), completely prevented BDNF-promoted IκBα phosphorylation (Fig. 5B) and NF-κB activation (Fig. 5D) in these neurons. Although BDNF also activated Src and Lck in P0 neurons just as effectively as in E17 neurons (Fig. 5C), PP2 had no effect on the level of NF-κB transcriptional activity in P0 neurons (Fig. 5D). These findings, together with our demonstration that BDNF does not promote IκBα phosphorylation in P0 neurons, suggest that even though Src and Lck are activated by BDNF in both P17 and P0 neurons, these kinases are only able to phosphorylate IκBα and activate NF-κB in E17 neurons.
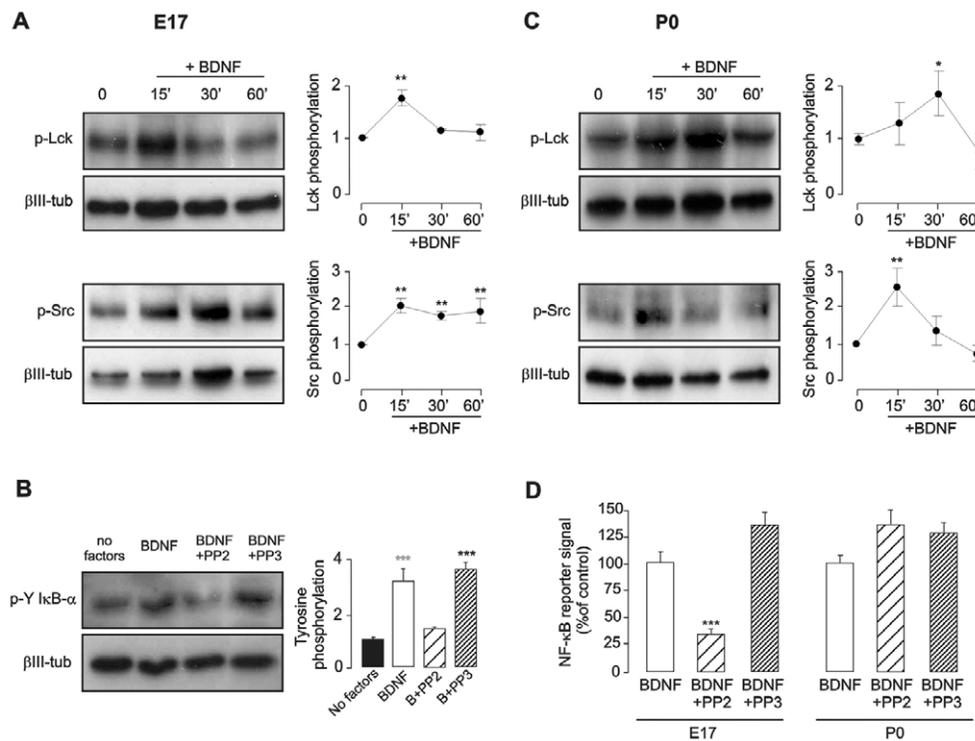

Fig. 5. Src tyrosine kinases are required for BDNF-promoted IκBα phosphorylation and NF-κB activation. (**A**) Representative western blots and densitometric quantification (mean±s.e.m. of three separate experiments) of phospho-Lck and phospho-Src in protein extracts of E17 nodose neurons treated with 10 ng/ml BDNF for 15, 30 and 60 minutes (0=untreated neurons) 18 hours after plating. (**B**) Representative western blots and densitometric quantification (mean±s.e.m. of three separate experiments) of phospho-Tyr42 IκBα in protein extracts of E17 nodose neurons grown for 18 hours without BDNF (no factors), or treated with BDNF for 30 minutes with or without pre-treatment for 30 minutes with either 2 μM PP2 or 2 μM PP3. (**C**) Representative western blots and densitometric quantification (mean±s.e.m. of three separate experiments) of phospho-Lck and phospho-Src in protein extracts of P0 nodose neurons treated with BDNF for 15, 30 and 60 minutes (0=untreated neurons) 18 hours after plating. (**D**) NF-κB reporter signal from E17 and P0 nodose neurons transfected with the GFP NF-κB reporter and cultured for 4 hours with BDNF alone or with BDNF with either 2 μM PP2 or 2 μM PP3. All of the above cultures received 25 μM Boc-D-FMK at the time of plating. Statistical comparison with no factors control (A,B,C) or BDNF alone (D), ANOVA with Fisher's post-hoc test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

We investigated the functional significance of Src kinase-dependent changes in NF-κB signalling in nodose neurons by studying the consequences of inhibiting these kinases with either PP2 or by overexpressing the inhibitory protein C-terminal Src kinase (Csk) (Nada et al., 1991). PP2, but not PP3, and Csk overexpression each brought about clear reductions in neurite arbor size and complexity in E17 neurons but not in P0 neurons (Fig. 6A,B). In these experiments, neither PP2, PP3 nor Csk had any detrimental effect on neuronal survival (data not shown). Taken together, these findings show that Src kinases mediate BDNF-promoted tyrosine phosphorylation of IκBα, leading to increased NF-κB transcriptional activity and enhanced neurite growth and branching in late foetal-stage but not postnatal nodose neurons.

Regulation of p65 phosphorylation at serine 536 by BDNF in foetal nodose neurons

We have recently shown that NF-κB can either promote or inhibit neurite growth depending on the phosphorylation status of the p65 NF-κB subunit at Ser536; whereas dephospho-Ser536-p65 is growth promoting, phospho-Ser536-p65 is growth inhibitory (Gutierrez et al., 2008). To investigate if there are age-related differences in the regulation of p65 phosphorylation in nodose neurons, we used western blotting to quantify the level of phospho-Ser536-p65 in E17 and P0 neurons following BDNF treatment. Whereas BDNF caused a marked decrease in the level of phospho-Ser536-p65 in E17 neurons within 15 minutes of treatment (Fig. 7A), the level of phospho-Ser536-p65 was completely unaffected by BDNF in P0 neurons (Fig. 7B). Moreover, direct comparison of the levels of phospho-Ser536-p65 in untreated E17 and P0 neurons revealed significantly lower levels in P0 neurons (Fig. 7B). These findings indicate that BDNF causes a rapid decrease in phospho-Ser536-p65 in E17 nodose neurons, but does not cause any further decrease in the already lower levels of this growth-inhibitory phosphoprotein in P0 nodose neurons.

To investigate the functional significance of the changes in p65 phosphorylation brought about by BDNF in foetal neurons, we studied the consequences of experimentally mimicking or manipulating changes in p65 phosphorylation independently of BDNF. First, we transfected E17 nodose neurons with a plasmid expressing a dominant-positive phosphomimetic p65 mutant (S536D) that behaves like constitutively active phospho-Ser536-p65 (Sasaki et al., 2005). Sholl analysis carried out 24 hours after transfection of S356D indicated that expression of this mutant caused a marked decrease in the size and complexity of neurite arbors in nodose neurons in BDNF-supplemented medium compared with in control transfected neurons (Fig. 7C). Second, because we have previously shown that IKKβ, in addition to activating NF-κB, is capable of phosphorylating p65 on Ser536 in nodose neurons (Gutierrez et al., 2008), we transfected E17 neurons with a plasmid expressing IKKβ. Overexpression of this kinase also markedly reduced BDNF-promoted neurite growth and branching compared with in control-transfected neurons (Fig. 7D). Neither the S536D-p65 plasmid nor the IKKβ plasmid affected the survival of E17 neurons grown with BDNF (data not shown). These results demonstrate that either mimicking Ser536 phosphorylation of p65 or counteracting BDNF-promoted dephosphorylation of p65 at Ser536 with IKKβ inhibits BDNF-promoted neurite growth from E17 nodose neurons, suggesting that BDNF-promoted dephosphorylation of p65 at Ser536 is required for BDNF to promote neurite growth at this stage of development.
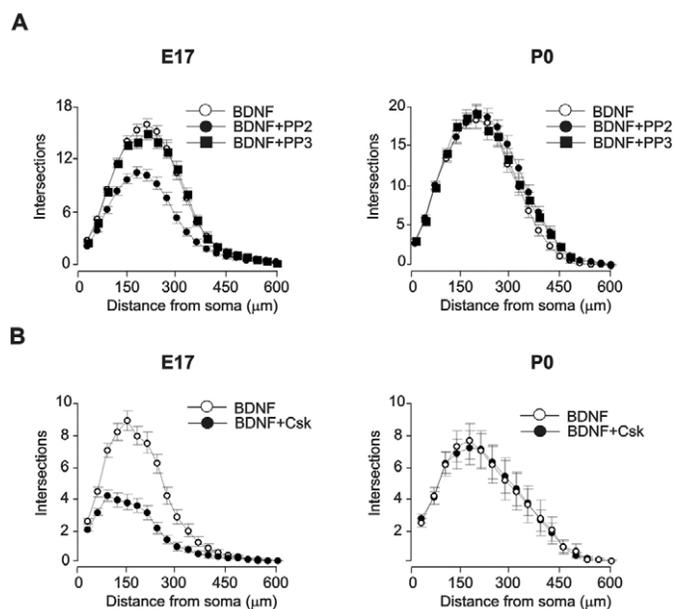

Fig. 6. Src tyrosine kinases contribute to BDNF-promoted neurite growth from E17 nodose neurons. (**A**) Sholl plots of E17 and P0 nodose neurons grown for 24 hours with 10 ng/ml BDNF alone or with BDNF with either 2 μM PP2 or 2 μM PP3. (**B**) Sholl plots of E17 and P0 nodose neurons grown for 24 hours with BDNF after transfection with either a Csk-expression plasmid or an empty control plasmid. Shown is the mean±s.e.m. of data from 50-90 neurite arbors in each experimental group.

DISCUSSION

We have discovered an abrupt developmental switch in the NF-κB signalling network of BDNF-dependent sensory neurons. While NF-κB signalling is required for BDNF-promoted neurite growth from both late foetal-stage and early postnatal neurons, there are clear and distinctive differences both in the mechanism of NF-κB activation and in a functionally important post-translational modification of the p65 NF-κB subunit at these stages. During a narrow developmental window from E16 to E17, BDNF acting via the TrkB receptor tyrosine kinase activates NF-κB by an atypical mechanism that involves the phosphorylation of IκBα on Tyr42 and the dissociation of this phosphoprotein from NF-κB without proteosome-mediated degradation. The relatively high in vivo levels of phospho-Tyr42-IκBα in late foetal nodose ganglia and the subsequent marked decrease in the level of this phosphoprotein during the early postnatal period are consistent with the in vivo operation of this atypical NF-κB activation mechanism prenatally and its subsequent absence postnatally.

Several observations suggest that two members of the Src family of protein tyrosine kinases, Lck and Src, mediate BDNF-promoted tyrosine phosphorylation of IκBα during this stage of development. IκBα is a known substrate of these kinases, BDNF activates both kinases and a selective inhibitor of these kinases prevents IκBα tyrosine phosphorylation and NF-κB activation in response to BDNF. It has been reported in other cells that the myristoylated docking protein FRS2 constitutively interacts with Src, is tyrosine phosphorylated and binds directly to TrkB following activation of the latter by BDNF (Easton et al., 1999; Meakin et al., 1999). Our demonstration that the selective inhibition of Src family tyrosine kinases or the blocking of tyrosine phosphorylation of IκBα in late foetal neurons significantly reduces BDNF-promoted neurite growth

Fig. 7. Regulation of p65 phosphorylation at serine 536 by BDNF in foetal nodose neurons. (**A**) Representative western blots of phospho-Ser536-p65 and total p65 in protein extracts of E17 nodose neurons treated with 10 ng/ml BDNF for 15, 30 and 60 minutes (0=untreated neurons) 18 hours after plating. Densitometric quantification of the levels of phospho-Ser536-p65 relative to βIII-tubulin (means±s.e.m. of three separate experiments; **P<0.01, statistical comparison with no factors control, ANOVA with Fisher's post-hoc test). (**B**) Representative western blot and quantification of phospho-Ser536-p65 in protein extracts of E17 and P0 nodose neurons treated for 30 minutes with BDNF or untreated 18 hours after plating (**P<0.01, statistical comparison with no factors control). All cultures in A and B received 25 μM Boc-D-FMK at the time of plating. (**C**) E17 neurons were co-transfected with a YFP expression plasmid together with either a plasmid expressing the S536D-p65 mutant or an empty control plasmid. BDNF (10 ng/ml) was added to the medium after transfection, and Sholl analysis was carried out 24 hours later. (**D**) E17 neurons were co-transfected with a YFP expression plasmid together with either a plasmid expressing IKKβ or an empty control plasmid. BDNF (10 ng/ml) was added to the medium after transfection and Sholl analysis was carried out 24 hours later. C and D show the mean±s.e.m. of data from 50-90 neurite arbors in each experimental group.
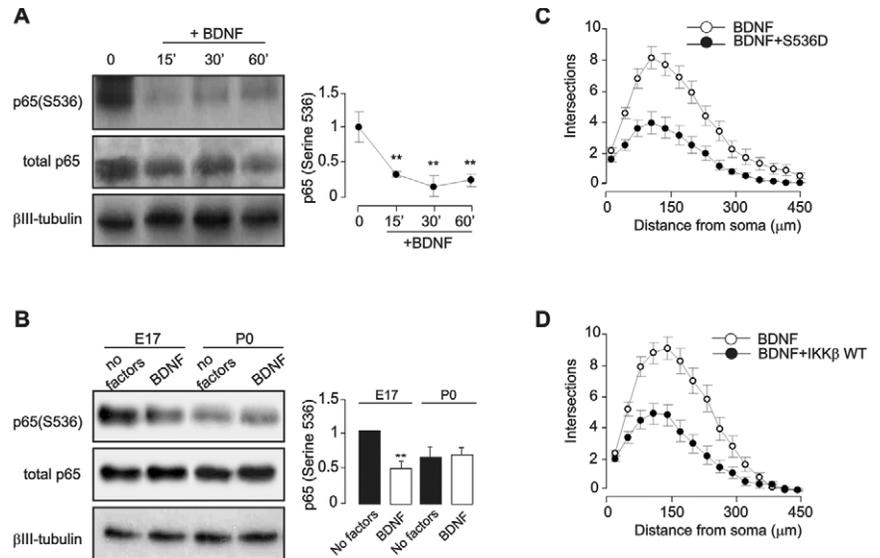
from foetal neurons suggests that Src/Lck-dependent IκBα phosphorylation and proteosome-independent NF-κB activation plays an important role in mediating the effects of BDNF on neurite growth at this stage of development.

In addition to promoting NF-κB activation by an atypical mechanism in late foetal nodose neurons, BDNF also causes the dephosphorylation of p65 at Ser536. We have previously shown that phosphorylation of p65 at Ser536 exerts a marked inhibitory effect on neurite growth in postnatal sensory and sympathetic neurons (Gutierrez et al., 2008). Our finding in late foetal nodose neurons that enhancing p65 phosphorylation at Ser536 either by overexpressing IKKβ or by mimicking p65 phosphorylation at Ser536 with a phosphomimetic mutant inhibits BDNF-promoted neurite growth suggests that phospho-Ser536-p65 is also growth inhibitory at this stage of development. This implies that, in addition to enhancing NF-κB activation, BDNF promotes neurite growth from nodose neurons at late foetal stage by promoting the dephosphorylation of p65 at Ser536. How BDNF signalling causes the dephosphorylation of p65 at Ser536 is unknown, but it could be due to the inhibition of a kinase acting at this residue or to the activation of an opposing phosphatase.

Immediately following birth, the two NF-κB-dependent mechanisms by which BDNF promotes neurite growth from nodose neurons at late foetal stage no longer operate. In P0 neurons, BDNF no longer promotes tyrosine phosphorylation of IκBα and NF-κB activation, even though there is an activation of Lck and Src kinases, and it no longer affects p65 phosphorylation at Ser536. BDNF-promoted neurite outgrowth from P0 neurons is completely unaffected by inhibiting Src family tyrosine kinases and by preventing IκBα tyrosine phosphorylation, demonstrating that atypical NF-κB activation plays no role in BDNF-promoted neurite growth following birth. The endogenous level of p65 phosphorylation at Ser536 is significantly lower in neonatal neurons than in foetal neurons, which indicates a shift towards a neurite growth-promoting form of NF-κB in neonatal neurons that is independent of BDNF. Although BDNF becomes disengaged from NF-κB signalling in neonatal nodose neurons, we have previously

shown that constitutive NF-κB activation by the classical, proteosome-dependent mechanism nonetheless makes a major contribution to BDNF-promoted neurite growth at this stage of development (Gutierrez et al., 2005). These findings, together with our demonstration that inhibiting proteosome function in nodose neurons at late foetal stage has no effect whatsoever on BDNF-promoted neurite growth, reveal a clear developmental switch in the NF-κB activation mechanism required for BDNF-promoted neurite growth from an atypical, proteosome-independent mechanism in late foetal-stage neurons to the classical, proteosome-dependent mechanism in neonatal neurons. It is worth mentioning that this switch is specific for BDNF, as CNTF, which is known to activate NF-κB in postnatal nodose neurons (Gallagher et al., 2007), was also able to activate NF-κB and promote neurite arborisation at late foetal stages (data not shown).

Our developmental study provides evidence for a surprising and unexpected level of plasticity in the intracellular signalling network. Over the course of a few days of development, there is a complete switch in the activation mechanism and functional modulation of a key signalling pathway required for an identical cellular response to the same extracellular signal in the same cells. This provides a clear illustration of how alternative patterns of activity in the intracellular network can generate the same end result in cells exposed to an identical set of extracellular cues. Why such plasticity exists is unclear. Perhaps these developmental changes in signalling while maintaining by alternative mechanisms a particular cellular response to a particular unchanging set of external cues might be necessary for developmental changes in other cellular responses to take place. Perhaps BDNF or other extracellular signals might affect cell physiology in distinctive ways in late foetal-stage and neonatal nodose neurons for which the developmental changes in NF-κB signalling may be required. Following birth, neurons of the peripheral nervous system, in particular, undergo important adaptive changes to a free-living existence, and also are subject to perinatal changes in the extracellular milieu that may impinge upon their development. For example, the relative hypercalcaemia of the foetus facilitates sympathetic axon growth and branching by activating an

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extracellular calcium-sensing receptor expressed on the neurons at this stage of development (Vizard et al., 2008). While an exhaustive and comprehensive analysis of developmental changes in intracellular signalling, gene expression and cellular responses might provide a clearer understanding of the purpose of intracellular signalling plasticity in sensory neurons, our work provides a clear demonstration of the occurrence of such plasticity, and illustrates the value of studying intracellular signalling in well-characterised primary cell systems at closely staged developmental intervals to gain unexpected, novel insights.

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