

# The intracellular portion of GTR enhances NGF-promoted neurite growth through an inverse modulation of Erk and NF- $\kappa$ B signalling

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## Summary

**NF- $\kappa$ B transcription factors play a key role in regulating the growth of neural processes in the developing PNS. Although several secreted proteins have been shown to activate NF- $\kappa$ B to inhibit the growth of developing sympathetic neurons, it is unknown how the endogenous level of NF- $\kappa$ B activity present in these neurons is restricted to allow neurite growth to occur during their normal development. Here we show that activation of the glucocorticoid-induced tumour necrosis factor receptor (GTR) inhibits NF- $\kappa$ B activation while promoting the activation of Erk in developing sympathetic neurons. Conversely, inhibition of GTR results in an increase in NF- $\kappa$ B dependent gene transcription and a**

**decrease in Erk activation leading to a reduction in neurite growth. These findings show that GTR signalling can regulate the extent of sympathetic neurite growth through an inverse modulation of Erk and NF- $\kappa$ B signalling, which provides an optimal environment for NGF-promoted growth.**

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Key words: GTR, NF- $\kappa$ B, NGF, Growth, Neurite, Neuron

## Introduction

The growth of axons to and within their targets during development of the peripheral nervous system (PNS) is controlled by a variety of extrinsic and intrinsic signals (Glebova and Ginty, 2005; Honma et al., 2002; Markus et al., 2002; Thompson et al., 2004; O’Keeffe et al., 2008; Vizard et al., 2008; Bodmer et al., 2009; Armstrong et al., 2011). One of the key regulators of axonal growth in the PNS is nerve growth factor (NGF) (Levi-Montalcini, 1987). In the PNS, post-ganglionic sympathetic neurons require a constant supply of target-derived nerve growth factor (NGF), in order to survive, and maintain their connections (Glebova and Ginty, 2004). Within the target regions innervated by these neurons, NGF acts locally on axon terminals to promote their growth, branching and refinement of connections (Campenot, 1994; Edwards et al., 1989).

Recently the nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcription factor has been shown to be a key regulator of axonal growth in developing sympathetic and sensory neurons (Gallagher et al., 2007; Gutierrez et al., 2005; Gutierrez et al., 2008; Gavaldà et al., 2009; Gutierrez and Davies, 2011; Nolan et al., 2011). There are basal levels of NF- $\kappa$ B activity found in neonatal superior sympathetic ganglion (SCG) neurons (Gutierrez et al., 2008; Nolan et al., 2011), and any increase in its basal level of activity is known to inhibit axonal growth in these neurons (Gutierrez et

al., 2008). As there are factors present in the SCG and its targets that activate NF- $\kappa$ B (Nolan et al., 2011), thus inhibiting axonal growth, there must be other factors, produced in these target regions or indeed by the neurons themselves, that act to dampen NF- $\kappa$ B activation in order to provide a permissive niche for the facilitation of NGF-promoted axonal growth.

Recently a novel autocrine signalling loop involving the glucocorticoid-induced tumour necrosis factor receptor (GTR) and its ligand GITRL, has been shown to be crucial for NGF-promoted axonal growth and target innervation by post-ganglionic sympathetic neurons of the SCG (O’Keeffe et al., 2008). GTR, like the p75 neurotrophin receptor, is a member of the TNF receptor superfamily. GITRL and GTR are widely expressed in many tissues and a large body of evidence has shown that they are important for regulating innate and acquired immune responses (Krausz et al., 2007). In developing sympathetic neurons, GTR is required for the full growth promoting effects of NGF through a mechanism that facilitates NGF-promoted Erk activation (O’Keeffe et al., 2008). Interestingly, GTR has also been shown to promote or inhibit NF- $\kappa$ B signalling depending on the cellular context (Esparza and Arch, 2004; Esparza et al., 2006; Grohmann et al., 2007; Bae et al., 2008; Zhou et al., 2008). Because of its ability to regulate NF- $\kappa$ B activation and its positive role in NGF-promoted axonal

growth, we hypothesised that GITR could conceivably functions to dampen NF- $\kappa$ B activity in neonatal neurons, thus preventing NF- $\kappa$ B from actively inhibiting axonal growth. In this study we establish the importance of GITR signalling as a novel simultaneous regulator of both Erk and NF- $\kappa$ B signalling in sympathetic neurons and propose a mechanism whereby GITR provides an optimal environment for NGF-promoted growth during the stage of active target innervation.

## Materials and Methods

### Neuron culture and transfection

Dissociated cultures of SCG neurons from newborn C57/CD1 mice were grown in polyornithine/laminin coated 35 mm tissue culture dishes in defined medium and were transfected using Invitrogen Neon microporation kit prior to plating (Gutierrez et al., 2008). In all experiments, the neurons were transfected with a GFP expression plasmid to identify transfected neurons and visualise their neurite arbors for analysis. SCG neurons were grown in medium containing 10 ng/ml NGF (R&D systems).

### Quantification of NF- $\kappa$ B activity

To estimate the relative level of NF- $\kappa$ B activation, neurons were transfected with a plasmid expressing GFP under the control of an NF- $\kappa$ B promoter. Neurons were imaged with a Zeiss Axioplan confocal microscope and mean soma fluorescence intensity was obtained using LSM510 software (Gutierrez et al., 2005).

### Analysis of neuritic arbors

GFP-labelled neurons were visualised and digitally acquired using an Axioplan Zeiss laser scanning confocal microscope. Total neurite length and Sholl analysis was carried out as previously described (Gutierrez and Davies, 2007; O'Keefe et al., 2008).

### Immunocytochemistry

Cultures were fixed in 4% paraformaldehyde and washed with phosphate-buffered-saline (PBS), then blocked with 5% BSA in PBS. The cells were incubated overnight with primary antibody in 1% BSA at 4°C. The primary antibodies were:  $\beta$ -III tubulin (Promega, 1:1000); GITRL (R&D systems, 1:100); pSer536-p65 (Cell signalling 1:50) and phospho ERK 1/2 (Cell signalling 1:100). After washing, the cells were incubated with the appropriate secondary antibody (Alexa-Fluor, Invitrogen, 1:500) and were counterstained with DAPI (Chemicon).

### Plasmids

GITR full length was cloned in the pSecTag plasmid (Invitrogen) previously modified by removing the two original NsiI sites. All GITR transmembrane-cytoplasmic mutants have been firstly cloned by inserting PCR mutant products into the pCR3.1 plasmid (Invitrogen), then cut by NotI and NsiI restriction enzymes and subcloned into NotI and NsiI sites of GITR full length. The NotI site was present in the cloning site of the plasmids, the NsiI site was present in the GITR sequence (534–539 base pairs in the NCBI NM\_009400.2 sequence, corresponding to MA amino acids present in the GITR transmembrane domain). The cytoplasmic amino acid sequence of GITR is IWQLRRQHMCMPRET-QPFAEVQLSAEDACSFQFPPEERGEQTEEKCHLGGRWP and that of GITR $\kappa$ , GITR $\zeta$  and GITR $\omega$  was shorter, lacking part of the amino acids at the -COOH end. In particular, the cytoplasmic amino acid sequence of GITR $\kappa$  was IWQLRRQHMCMPRETQPFAEVQLSAEDACSFQFPPEERG, that of GITR $\zeta$  was IWQLRRQHMCMPRETQPFAEVQLS and that of GITR $\omega$  was IW.

## Results

The growth promoting effects of GITRL-GITR signalling are mediated by a juxtamembrane region of GITR

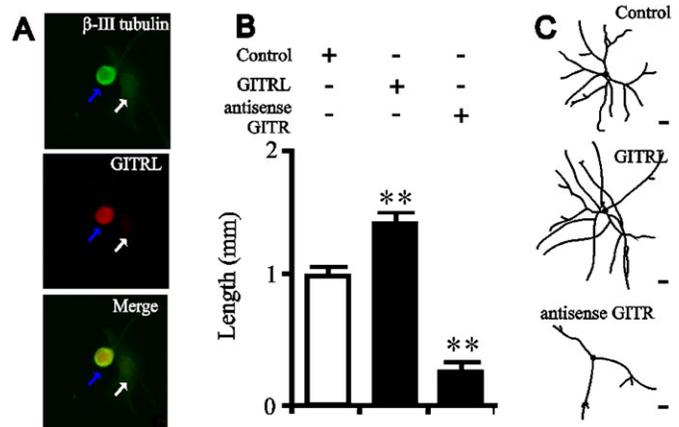
In the immune system, GITRL-GITR signalling has been shown to function through a transduction cascade downstream of GITR (Esparza and Arch, 2004; Esparza et al., 2006; Zhou et al., 2008). However, GITRL is also a transmembrane protein, and reverse signalling, through GITRL has recently been demonstrated upon its binding to GITR (Grohmann et al., 2007; Bae et al., 2008). In order to further explore how GITRL-GITR signalling regulates neurite growth in neurons, we created plasmids that express specific mutants of GITR that lack specific regions of the intracellular tail, GITR $\delta$ W, GITR $\delta$ K and GITR $\delta$ Z, as well as a

GITRL $\delta$ W mutant that lacks the intracellular tail of GITRL and examined their effects on neurite growth.

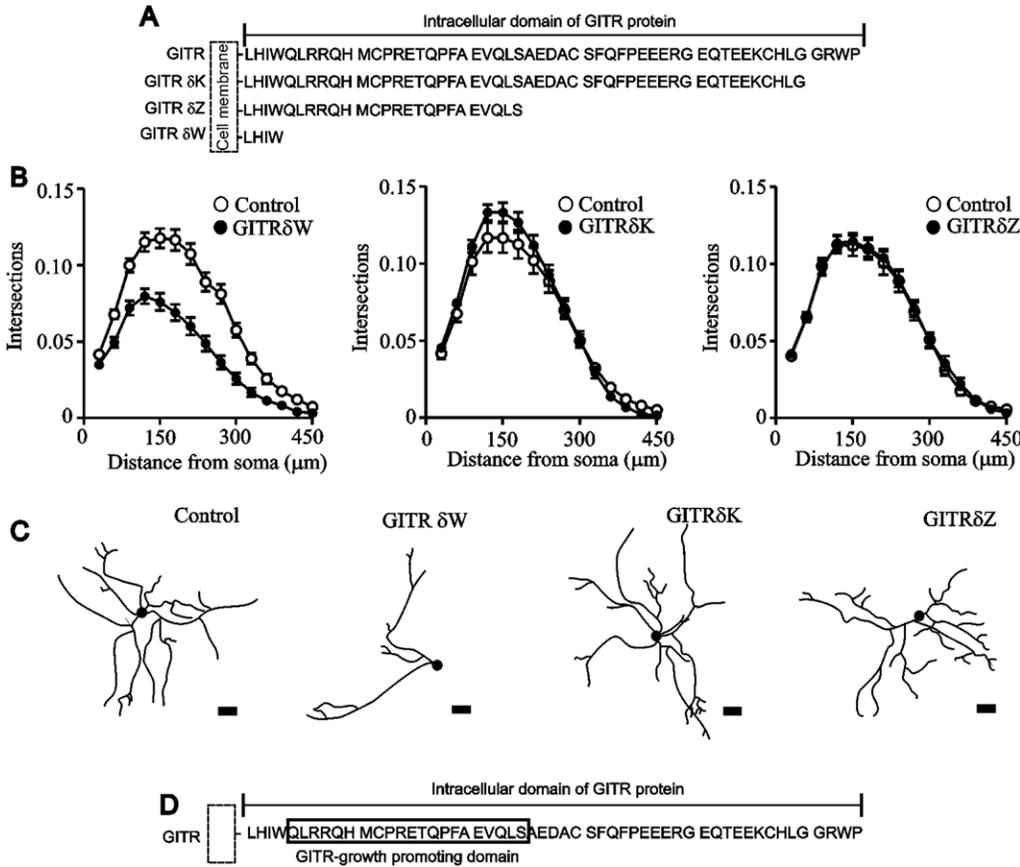
Firstly, using immunocytochemistry, we confirmed GITRL and GITR expression in cultured neonatal mouse SCG neurons, which is a period of development *in vivo* when these neurons are undergoing extensive growth and branching within their peripheral targets. GITRL (Fig. 1A) and GITR (not shown) were specifically expressed on neurons, as identified by  $\beta$ -III tubulin staining (Fig. 1A, blue arrow), and were not found on non-neuronal cells (Fig. 1A, white arrow). We next transfected P1 SCG neurons with expression vectors for GITRL or antisense GITR., Confirming a previous report (O'Keefe et al., 2008), neurons overexpressing GITRL displayed a significant increase in neurite length compared to their control counterparts (Fig. 1C), whereas those expressing antisense GITR had a significant decrease in neurite length (Fig. 1C).

To identify the specific region of GITR required for its growth promoting effects, we transfected P1 mouse SCG neurons with expression plasmids for GITR $\delta$ W, GITR $\delta$ K, GITR $\delta$ Z (Fig. 2A) and the GITRL $\delta$ W protein and examined their effects on neurite growth. To measure growth and elaboration of neurite arbours we used Sholl analysis, which provides a graphic illustration of neurite length and branching with distance from the cell body (Sholl, 1953; Gutierrez and Davies, 2007). Neurons overexpressing GITR $\delta$ W displayed a significant decrease in their Sholl profile (Fig. 2B,C) compared to their control counterparts. This reduction in growth was comparable to those neurons overexpressing anti-sense GITR (data not shown).

In marked contrast, neurons expressing the GITR $\delta$ K (Fig. 2A–C) and GITR $\delta$ Z (Fig. 2A–C) had no significant decrease in the Sholl profile compared to their control counterparts demonstrating the specific requirement of the immediate juxtamembrane segment of the receptor in the growth promoting effects of GITR. On the other hand, neurons overexpressing GITRL $\delta$ W displayed a significant increase in neurite growth compared to their control counterparts (data not shown), demonstrating that binding of the extracellular tail of GITRL to GITR is sufficient to trigger a growth promoting effect.



**Fig. 1. GITR signalling promotes the growth of developing sympathetic neurons.** (A) Photomicrographs of the same fields of P1 SCG dissociated cultures labelled for either GITRL or  $\beta$ -III tubulin (Neurons = blue arrows; Non-neuronal cells = white arrows). Prior to plating, P0 SCG neurons were transfected with a GFP expression plasmid together with either a GITRL or antisense GITR expression plasmid. After 24 hours incubation with 10 ng/ml NGF total neurite arbour, length was determined (B,C). Means  $\pm$  standard errors of > 150 neurons per condition are shown. \*\* $P$ <0.01. Scale bar = 50 $\mu$ m.

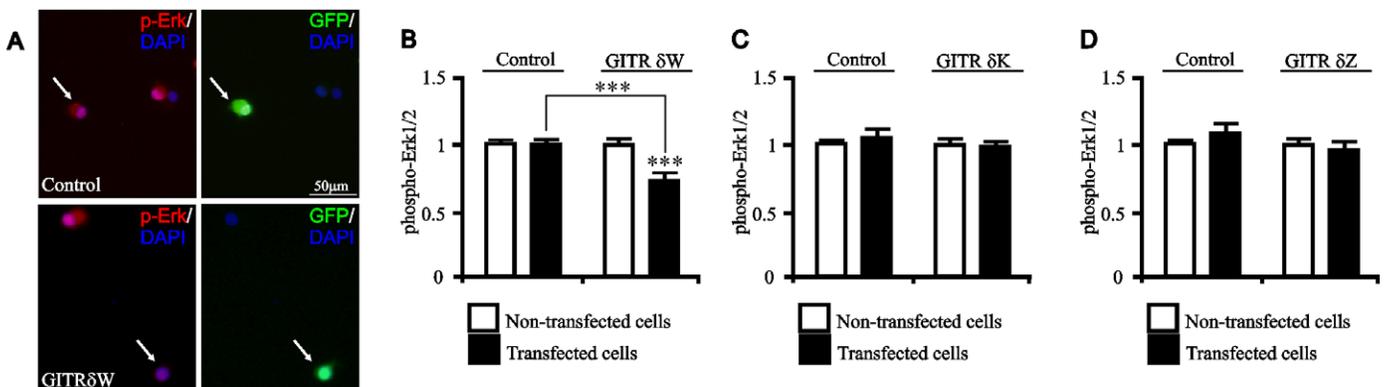


**Fig. 2. The growth promoting effects of GITRL-GITR signalling are mediated by a juxtamembrane region of GITR.** (A) Primary structure of the intracellular tail of GITR and the portion deleted in GITRδK, GITRδZ and GITRδW mutants. Prior to plating, P0 SCG neurons were transfected with a GFP expression plasmid together with either a GITRδW, GITRδK or a GITRδZ expression plasmid. After 24 hours incubation with 10 ng/ml NGF, total neurite arbour length was determined (B). (C) Line drawings of representative examples of the neurite arbors of SCG neurons transfected with GITRδW, GITRδK, GITRδZ and relevant controls 24 hours post transfection. (D) Schema showing the “growth promoting domain” of the intracellular portion of GITR. Scale bar = 50 μm. Means ± standard errors of > 150 neurons per condition are shown.

Collectively, these data show that GITRL-GITR signalling promotes process growth in neonatal SCG neurons through the GITR intracellular domain and identify the growth-promoting region responsible for mediating this effect (Fig. 2D).

The growth-promoting domain of GITR regulates NGF-induced Erk activation in developing SCG neurons  
Having identified the growth promoting domain of GITR, we next asked if this region could influence NGF-induced Erk

activation which is required for the growth promoting effects of NGF in developing SCG neurons (O’Keeffe et al., 2008). To this end, P1 mouse SCG neurons were transfected with expression plasmids for GITRδW, GITRδK and GITRδZ and grown for 24 h in medium containing NGF before immunocytochemically staining them for phospho-Erk. Densitometric analysis was conducted to assess relative levels of phospho-Erk on individual neurons. Transfected cells were identified by co-transfecting a GFP-expressing plasmid (Fig. 3A). Importantly,



**Fig. 3. The growth promoting domain of GITR regulates NGF-promoted Erk activation in developing SCG neurons.** Prior to plating, P1 SCG neurons were transfected with a GFP expression plasmid together with either a GITRδW, GITRδK or a GITRδZ expression plasmid. After 24 hours incubation with 10 ng/ml NGF, cultures were fixed and immunocytochemically stained for phospho-Erk. (A) Photomicrographs of control and GITRδW transfected P1 SCG dissociated cultures labelled for phospho-ERK and counterstained with DAPI. Transfected neurons were identified by their expression of GFP. (B–D) Graphical representation of the relevant differences in phospho-ERK expression in the different groups. Means ± standard errors of 50–90 neurons per condition are shown. Statistical comparisons: \*\*\*  $P < 0.001$ . Scale bar = 50 μm.

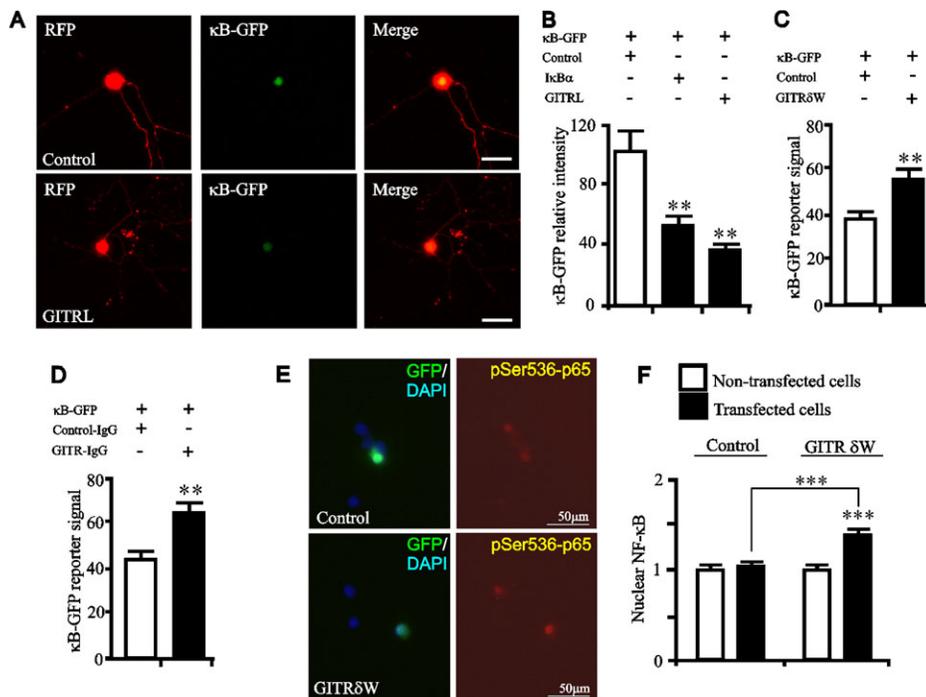
neurons transfected with a control empty plasmid displayed the same level of phospho-Erk as non-transfected neurons, indicating that the transfection procedure did not alter the relative activation of this pathway (Fig. 3B). Interestingly, neurons expressing GITR $\delta$ W had significantly lower levels of phospho-Erk when compared to non-transfected and control-transfected neurons ( $P < 0.001$ ) (Fig. 3A,B). There was no reduction, however, in the level of phospho-Erk staining in cells transfected with GITR $\delta$ K and GITR $\delta$ Z compared with non-transfected and control-transfected neurons (Fig. 3C,D). These results show that the GITR-mediated enhancement of NGF-induced Erk activation requires the intracellular growth promoting domain of GITR.

#### GITRL-GITR signalling down regulates NF- $\kappa$ B-dependent transcriptional activity in developing SCG neurons

Outside the nervous system, GITR has been shown to promote or inhibit NF- $\kappa$ B signalling depending on the cellular context (Esparza and Arch, 2004; Esparza et al., 2006; Grohmann et al., 2007; Bae et al., 2008; Zhou et al., 2008). As NF- $\kappa$ B, on the other hand, inhibits neurite growth in developing SCG neurons, we asked whether GITR regulated NF- $\kappa$ B in these neurons. Given their opposing effects, we hypothesised that GITRL-GITR signalling may function to maintain low levels of NF- $\kappa$ B activity in sympathetic neurons (Gutierrez et al., 2008; Nolan et al., 2011). To assess the regulatory influence of GITRL-GITR signalling on NF- $\kappa$ B signalling in developing sympathetic neurons, we transfected P1 SCG neurons with either a control plasmid or a GITRL plasmid and co-transfected these cells with a reporter plasmid expressing GFP under the control of an NF- $\kappa$ B promoter and compared the fluorescence intensity in experimental and control neurons (Gutierrez et al., 2005; Gallagher et al., 2007; Gutierrez et al., 2008). As a positive control, we also transfected P1 SCG neurons with a plasmid that expresses a mutated dominant negative I $\kappa$ B $\alpha$  protein with serine-to-alanine substitutions at

residues 32 and 36 that is known to inhibit NF- $\kappa$ B dependent transcriptional activity (Roff et al., 1996).

Interestingly, neurons transfected with GITRL displayed a marked decrease in NF- $\kappa$ B reporter signal to at least the same extent as neurons transfected with the S32A/S36A I $\kappa$ B $\alpha$  mutant when compared with control-transfected neurons (Fig. 4A,B). These results show that GITRL-GITR signalling inhibits NF- $\kappa$ B signalling in neonatal SCG neurons. In order to test if basal levels of GITRL-GITR signalling are required to restrict NF- $\kappa$ B activity in neonatal SCG neurons, we used three complementary approaches to inhibit GITRL-GITR signalling and assess the effect on NF- $\kappa$ B transcriptional activity. Firstly, we overexpressed the GITR $\delta$ W mutant receptor along with the NF- $\kappa$ B reporter plasmid and examined its effects on NF- $\kappa$ B transcriptional activity compared to that of an empty control plasmid. The overexpression of GITR $\delta$ W in neonatal SCG neurons resulted in a significant increase in NF- $\kappa$ B-dependent transcriptional activity (Fig. 4C). We next transfected P1 SCG neurons with the NF- $\kappa$ B reporter plasmid and determined its response to a GITR-IgG function blocking antibody, which has been previously shown to block GITR signalling and inhibit axonal growth (O'Keeffe et al., 2008). Treatment with GITR-IgG resulted in a significant increase in the NF- $\kappa$ B reporter signal in these neurons (Fig. 4D). Finally, in neurons transfected with either the GITR $\delta$ W mutant or an empty control plasmid, we examined the nuclear translocation of pS536-p65, which is the phosphorylated form of the NF- $\kappa$ B subunit known to actively inhibit neurite growth in these neurons (Gutierrez et al., 2008). As shown in Fig. 4E,F, there was a significant increase in nuclear pSer536-p65 NF- $\kappa$ B in neurons expressing GITR $\delta$ W compared with non-transfected and control-transfected neurons ( $P < 0.001$ ). Collectively, these data show that endogenous GITR signalling down regulates NF- $\kappa$ B-dependent transcriptional activity in P1 SCG neurons.



**Fig. 4. GITRL-GITR signalling regulates NF- $\kappa$ B dependent transcriptional activity in developing SCG neurons.** For analysis of NF- $\kappa$ B dependent transcriptional activity, neurons were co-transfected with the κB-GFP reporter for measuring transcriptional activity and RFP plasmid to identify the transfected cells, together with either GITRL, I $\kappa$ B- $\alpha$  or GITR $\delta$ W where indicated or alternatively treated with a GITR-IgG function blocking antibody (1  $\mu$ g/ml). (A,B) Overexpression of GITRL significantly inhibited NF- $\kappa$ B dependent transcriptional activity to the same extent as the SS-AA I $\kappa$ B- $\alpha$  super repressor. Conversely, cells transfected with (C) GITR $\delta$ W or (D) treated with the GITR-IgG function blocking antibody had a significantly increased level of NF- $\kappa$ B dependent transcriptional activity compared to control neurons. (E) Photomicrographs of dissociated P1 SCG cultures transfected with GITR $\delta$ W or control plasmid labelled for phosphoS536-p65 and counterstained with DAPI. (F) Graphical representation of relative nuclear levels of pS536-p65 in control transfected, GITR $\delta$ W transfected and non-transfected cells. All data were obtained 24 hours after transfection and analysis of mean nuclear fluorescence intensity was performed. Means  $\pm$  standard errors of 50–90 neurons per condition are shown. Statistical comparisons: \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Scale bar = 50  $\mu$ m.

To determine if preventing enhanced NF- $\kappa$ B activation could reverse the decrease in neurite growth following GITR inhibition, we co-transfected neonatal SCG neurons with either the GITR $\delta$ W or antisense GITR plasmids together with a plasmid expressing a dominant negative K44A IKK $\beta$  protein or a control plasmid. We chose to use the IKK $\beta$  dominant negative (DN) plasmid as we have previously shown that IKK $\beta$ , not IKK $\alpha$  activity is responsible for mediating the growth inhibitory effects of NF- $\kappa$ B in neonatal SCG neurons (Gutierrez et al., 2008). Neurons transfected with either an antisense GITR (Fig. 5A,B) or GITR $\delta$ W (Fig. 5A,C) displayed significant reductions in neurite length. However, neurons co-transfected with either antisense GITR or GITR $\delta$ W plasmids together with a K44A IKK $\beta$  expression plasmid displayed growth profiles identical to those of neurons transfected with an empty control plasmid. These data show that IKK $\beta$ -dependent NF- $\kappa$ B activity is responsible for the inhibition of neurite growth resulting from endogenous GITR inhibition.

#### Inverse modulation of NF- $\kappa$ B and Erk signalling by GITRL-GITR signalling is essential for NGF-promoted neurite growth

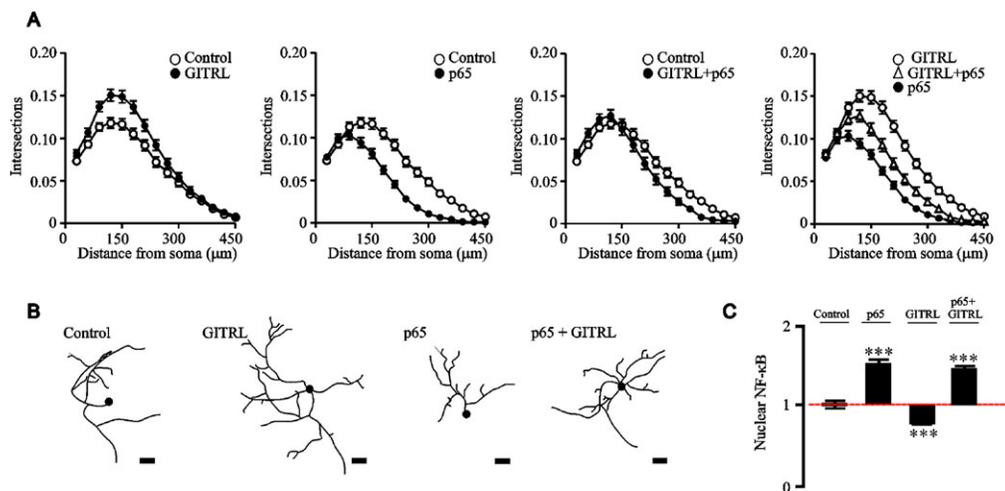
We have previously shown that GITRL/GITR signalling facilitates NGF mediated Erk activation, which is required for NGF-promoted neurite growth in developing SCG neurons (O'Keeffe et al., 2008). Given the observed inhibitory influence of GITRL/GITR on NF- $\kappa$ B signalling, we examined the potential link between NF- $\kappa$ B and Erk signalling in the promotion of neurite growth upon GITR stimulation in developing SCG neurons. Firstly, we transfected P1 SCG neurons with expression plasmids for the NF- $\kappa$ B p65 subunit and/or GITRL with the NF- $\kappa$ B reporter plasmid and examined their effects on neurite growth and NF- $\kappa$ B transcriptional activity. In agreement with our previous findings, p65 and GITRL-expressing neurons showed a significant increase and decrease in neurite growth respectively than control neurons (O'Keeffe et al., 2008; Gutierrez et al., 2005) (Fig. 6A,B). Interestingly, neurons

co-transfected with both GITRL and p65 were not significantly different in size when compared to controls, but were significantly larger than neurons transfected with p65 alone and significantly smaller than those expressing GITRL alone (Fig. 6A,B). When we analysed NF- $\kappa$ B transcriptional activity in these neurons, the overexpression of either p65 or GITRL in neonatal SCG neurons resulted in a significant increase and decrease respectively in NF- $\kappa$ B-dependent transcriptional activity (Fig. 6C). Interestingly, cells expressing both p65 and GITRL displayed the same degree of increase in NF- $\kappa$ B-dependent transcriptional activity as those expressing p65 alone. The latter result contrasts with the fact that, in terms of neurite growth, p65/GITRL expressing neurons are significantly larger than those expressing p65 alone, suggesting that this is due to GITRL engaging a second signal that opposes NF- $\kappa$ B and leads to NGF-promoted neurite growth.

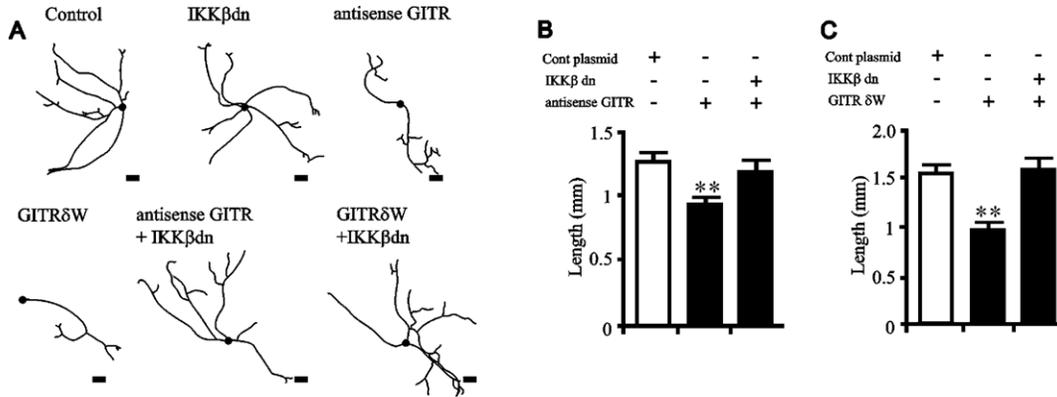
To determine if this is the case, we assessed the levels of phospho-Erk in neurons transfected with expression plasmids for p65 and/or GITRL. Surprisingly neurons expressing p65 had significantly lower levels of phospho-Erk than their control counterparts, whereas those expressing GITRL displayed significantly higher levels of active Erk when compared to control transfected neurons (Fig. 7A). However, neurons co-expressing both p65 and GITRL had the same degree of Erk activation as control transfected neurons (Fig. 7A). When we related this to the effects on neurite growth (Fig. 7B), it became clear that the extent of NGF-promoted neurite growth was related to the level of phospho-Erk in these neurons, which can in turn be modulated by altering NF- $\kappa$ B signalling (Fig. 7C). Taken together, these results show that GITRL-GITR signalling exerts an inverse modulation of NF- $\kappa$ B and Erk signalling which is essential for NGF-promoted neurite growth.

#### Discussion

NGF, the founding member of the neurotrophins family of proteins, was discovered over half a century ago by its ability to



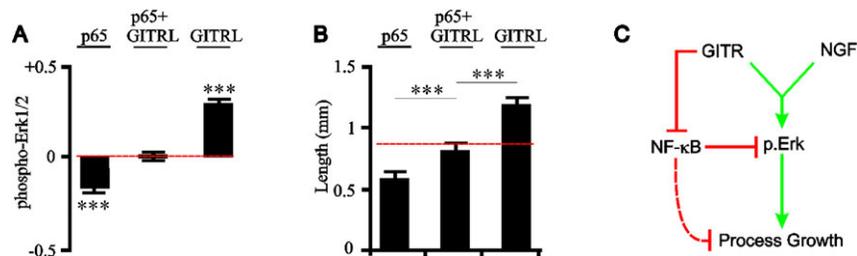
**Fig. 5. Inhibition of IKK $\beta$  prevents the effects of GITR inhibition on axonal growth.** P1 SCG neurons were transfected with expression plasmids for GFP together with either a control plasmid or plasmids encoding IKK $\beta$  dominant negative (IKK $\beta$  DN), antisense GITR, GITR $\delta$ W and the degree of neurite length was assessed 24 hours post transfection. (A) Photomicrographs showing neurons transfected with GITR $\delta$ W, antisense GITR and/or IKK $\beta$  DN and relevant controls 24 hours post transfection. (B) Quantification of neurite length in neurons transfected with antisense GITR and/or IKK $\beta$  DN where indicated compared to control transfected neurons. Antisense GITR induced significant decreases in neurite length, an effect prevented by co-expressing IKK $\beta$  DN. (C) Quantification of neurite length in neurons transfected with GITR $\delta$ W and/or IKK $\beta$  DN where indicated compared to control transfected neurons. GITR $\delta$ W induced significant decreases in neurite length, an effect prevented by co-expressing IKK $\beta$  DN. Statistical comparisons: \*\* $P$ <0.01. Scale bar = 50  $\mu$ m.



**Fig. 6. GITRL engages a second signal that opposes NF- $\kappa$ B and enhances NGF-promoted neurite growth.** Prior to plating, P1 SCG neurons were transfected with a GFP expression plasmid together with either a GITRL overexpression plasmid, p65 overexpression plasmid or both. (A) After 24 hours incubation with 10 ng/ml NGF total neurite arbour, length was determined. (B) Photomicrographs showing neurons transfected with GITRL, p65, GITRL + p65 and relevant controls 24 hours post transfection. (C) Graphical representation of the relevant differences in nuclear NF- $\kappa$ B expression between transfected and non-transfected cells. All data were obtained 24 hours after transfection and analysis of mean fluorescence intensity was performed. Means  $\pm$  standard errors of 50–90 neurons per condition are shown. Statistical comparisons: \*\*\* $P$ <0.001. Scale bar = 50  $\mu$ m.

promote neuronal survival and axonal growth, and is the paradigmatic target-derived neurotrophic factor on which the neurotrophic hypothesis is based. It recent years, a number of studies have identified new molecules involved in the NGF-promoted axonal growth of sympathetic neurons, including GITR (O’Keeffe et al., 2008), CaSR (Vizard et al., 2008), Wnt5a (Bodmer et al., 2009), Frizzled3 (Armstrong et al., 2011) and others. Our study highlights a previously unknown autocrine regulatory pathway in which autocrine expression of GITR in neonatal SCG neurons, is responsible for the simultaneous but inverse regulation of Erk and NF- $\kappa$ B activity in these neurons, bringing about the balance in their activation and inhibition required for optimal for neurite growth. We show that activating or inhibiting GITRL-GITR in neonatal SCG neurons promotes and inhibits axonal growth respectively. We demonstrate that activation of GITR signalling inhibits NF- $\kappa$ B-dependent transcriptional activity while facilitating Erk activation in these neurons. Conversely, inhibition of GITR signalling, results in increased NF- $\kappa$ B activity and a decrease in Erk activation leading to a decrease in neurite growth in these neurons.

The precise mechanism of how GITR inhibits NF- $\kappa$ B signalling in sympathetic neurons is unclear. In many cases, activation of a member of the TNFSF, family of receptors, results in recruitment of the receptor associated proteins TRADD and tumour necrosis factor receptor-associated factor (TRAF) 2, 5 or 6 (Hauer et al., 2005). This results in the phosphorylation of the IKK complex which, in turn, phosphorylates I $\kappa$ B $\alpha$ , targeting it for degradation, allowing phosphorylated NF- $\kappa$ B to translocate to the nucleus. Usually TRAF2 recruitment activates NF- $\kappa$ B; however, in primary lymphocytes, GITR has been shown to engage TRAF2 in a novel fashion as an inhibitor of NF- $\kappa$ B activation (Esparza and Arch, 2004). TRAF3 also serves as a negative regulator of the NF- $\kappa$ B pathway for many members of the TNF superfamily, including GITR (Hauer et al., 2005). However, a preliminary *in silico* analysis (unpublished observations) predicts that the TRAF binding region of GITR is located outside the growth promoting domain. The GITR $\delta$ K and GITR $\delta$ Z deletions disrupt this predicted TRAF binding region, but as neurons expressing these mutated forms of GITR grow normally, it would seem that GITR signalling in neurons is



**Fig. 7. Inverse modulation of NF- $\kappa$ B and Erk signalling by GITRL-GITR signalling regulates NGF-promoted neurite growth.** Prior to plating, P1 SCG neurons were transfected with a GFP expression plasmid together with either a p65 overexpression plasmid, GITRL overexpression plasmid or both. After overnight incubation in medium containing caspase inhibitors, cultures were treated with fresh medium containing 10 ng/ml NGF for 1 hour before being fixed for immunocytochemistry. (A) Graph illustrating fold-change of phospho-ERK1/2 expression in transfected cells compared to control transfected cells. Overexpression of p65 causes a significant decrease in ERK1/2 signalling while GITRL caused a significant increase. Cells transfected with both p65 and GITRL showed no change from control. (B) After 24 hours incubation with 10 ng/ml NGF total neurite arbour, length was determined. Transfection with p65 reduced total neurite length in contrast to the observed growth effect of GITRL but co-transfection of both had no effect compared to control cultures. (C) Schematic model showing the proposed mechanism underlying the role of GITR in the promotion of axonal growth. In a neuron receiving an adequate supply of NGF, ERK signalling is enhanced activated and NF- $\kappa$ B signalling is inhibited thus potentially providing an optimal cellular environment for neurite growth. Statistical comparisons: \*\*\* $P$ <0.001.

possibly TRAF independent. Further work will be required to ascertain the precise mechanism for GITR-mediated inhibition of NF- $\kappa$ B.

Although previous studies in the immune system have described the involvement of GITR in activating NF- $\kappa$ B and Erk (Esparza et al., 2006), to our knowledge this is the first report of an inverse regulation of these signalling pathways mediated by GITR. Interestingly, Erk and NF- $\kappa$ B signalling have been shown to be regulated by tumour progression locus 2 (TPL2), which is a serine threonine kinase that under homeostatic conditions is stoichiometrically associated with the NF- $\kappa$ B component p105, the precursor of p50 (Waterfield et al., 2003; Beinke et al., 2004). Upon stimulation of the IKK complex, p105 is phosphorylated and degraded resulting in the release of TPL2. TPL2 can then phosphorylate MEK1/2 which in turn activates ERK1/2 (Das et al., 2005). In the NF- $\kappa$ B pathway, TPL2 functions downstream of IKK- $\beta$ , and it does not regulate the degradation of I $\kappa$ B- $\alpha$  or the subsequent nuclear translocation of the p50 or p65 subunits but is instead involved in the phosphorylation of p65 (Waterfield et al., 2003). On the other hand, Tpl2 has been shown to be recruited by the CD40 receptor (a member of the TNF superfamily) upon its stimulation in a TRAF6-dependent manner (Eliopoulos et al., 2003). While it remains to be ascertained, Tpl2 could therefore provide a potential link between GITR and the simultaneous modulation of both Erk and NF- $\kappa$ B signalling.

The key role of NF- $\kappa$ B transcription factors in the negative regulation of axonal growth of developing sympathetic neurons has been previously described (Gutierrez et al., 2008). Experimental enhancement of NF- $\kappa$ B in neonatal sympathetic neurons, either by inflammatory cytokines (Gutierrez et al., 2008; Nolan et al., 2011) or by overexpressing its components (Gutierrez et al., 2008), leads to a strong inhibition of axonal growth. Our present findings show that NF- $\kappa$ B signalling results in a reduction in the level of NGF-induced Erk activation in those neurons. This suggests a potential mechanism whereby when neonatal sympathetic neurons innervate their targets, NF- $\kappa$ B activity is maintained at a relatively low level by GITR-GITRL signalling, while there is a high degree of activity of NGF-induced Erk-MAP kinase signalling as well as other downstream NGF-effectors (O'Keeffe et al., 2008; Bodmer et al., 2009), allowing an adequate rate of axonal growth and neurite arborisation to occur (Fig. 7). As GITR signalling only affects growth in the presence of NGF (O'Keeffe et al., 2008), this would only allow neurons receiving an adequate supply of NGF to achieve an adequate balance of NF- $\kappa$ B and Erk activation, thereby providing an optimal environment for neurite growth. In neurons that do not receive an adequate supply of NGF, this may result in a lack of autocrine GITRL-GITR signalling, which results in the inhibitory block on NF- $\kappa$ B being released, and a concurrent decrease in Erk activity leading to an inhibition in neurite growth (Fig. 7). This co-accessory role for GITR has been demonstrated in the immune system where CD3 stimulation in T-cells induces a robust proliferative response (Ronchetti et al., 2004), which is enhanced by their co-stimulation with GITRL (Ronchetti et al., 2004). However, activation of GITR in the absence of anti-CD3 does not induce any proliferative response (O'Keeffe et al., 2008). GITR-GITRL signalling in neurons may serve as an autocrine mechanism where active and permissive signals regulate axonal growth depending on the cellular environment. Neurons receiving an adequate supply of NGF can grow and branch normally as instructed by the active

influence of growth promoting NGF-TrkA signalling and permissively by GITR-mediated suppression of NF- $\kappa$ B.

The molecular events and downstream changes in gene expression following NF- $\kappa$ B activation that inhibit growth of neural processes are unclear. As NF- $\kappa$ B activation can induce or repress the expression of many genes in different cellular contexts (Hoffmann et al., 2006), it remains unclear whether NF- $\kappa$ B mediated growth inhibition is brought about by repression of those genes involved in the promotion of growth or the induction of genes that can in turn negatively regulate growth. Some genes that are regulated by NF- $\kappa$ B such as the neural cell adhesion molecule, tenascin-C and  $\beta$ 1 integrin may be potentially relevant in the control of neural process growth, (Mettouchi et al., 1997; Simpson and Morris, 2000; Wang et al., 2003). On the other hand, NF- $\kappa$ B-induced genes that can actively inhibit neurite growth have also been described (Nolan et al., 2011).

As GITR is expressed by other neurons in the developing nervous system, it will be important to determine how extensively it influences neurite growth during development. NF- $\kappa$ B has been shown to promote or inhibit neurite growth in different neuronal populations (Gutierrez et al., 2008). It is therefore conceivable that the GITR-mediated inverse modulation of NF- $\kappa$ B and Erk is only required in neurons in which NF- $\kappa$ B plays an inhibitory role in neurite growth. Indeed, GITR signalling does not affect neurite growth in sensory neurons of the nodose and trigeminal ganglia (data not shown) where NF- $\kappa$ B is known to promote (rather than inhibit) growth (Gutierrez et al., 2005; Gallagher et al., 2007; Gavalda et al., 2009).

In summary, our results demonstrate that autocrine GITRL-GITR signalling regulates Erk and NF- $\kappa$ B activation in order to provide an optimal environment for NGF-promoted neurite growth. This suggests a mechanism whereby axonal growth in neonatal sympathetic neurons may be regulated by a delicate cellular balance between active and permissive growth promoting and growth inhibiting signals depending on the availability of NGF. Our results will help to explain why neonatal sympathetic neurons have a basal level of NF- $\kappa$ B activity and raise intriguing questions for future research regarding the importance that regulating NF- $\kappa$ B signalling has for the correct formation of the sympathetic nervous system.

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### Competing Interests

The authors have no competing interests to declare.

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