TNFα reverse signaling promotes sympathetic axon growth and target innervation

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Reverse signaling via members of the tumor necrosis factor (TNF) superfamily controls multiple aspects of immune function. Here we document TNFα reverse signaling in the nervous system to our knowledge for the first time and show that it has a crucial role in establishing sympathetic innervation. During postnatal development, sympathetic axons express TNFα as they grow and branch in their target tissues, which in turn express TNF receptor 1 (TNFR1). In culture, soluble forms of TNFR1 act directly on postnatal sympathetic axons to promote growth and branching by a mechanism that depends on membrane-integrated TNFα and on downstream activation of ERK. Sympathetic innervation density is substantially lower in several tissues in postnatal and adult mice lacking either TNFα or TNFR1. These findings reveal that target-derived TNFR1 acts as a reverse-signaling ligand for membrane-integrated TNFα to promote growth and branching of sympathetic axons.

RESULTS

TNFα and its receptors in SCG neurons and targets

To clarify the role of TNFα in development of sympathetic neurons, we analyzed expression of TNFα and its receptors, TNFR1 and TNFR2, in SCG neurons and tissues innervated by these neurons. In low-density dissociated cultures of SCG neurons from a postnatal day 0 (P0) mice, we observed intense TNFα immunoreactivity in the cell bodies and throughout the neurite arbors of the neurons. In marked contrast, intense TNFR1 immunoreactivity was restricted to the neuron cell bodies, and the neurites were completely unlabeled (Fig. 1a). Accordingly, in histological sections of the SCG, neuron cell bodies were labeled by both anti-TNFα and anti-TNFR1, and post-ganglionic sympathetic fiber bundles, identified by tyrosine hydroxylase immunostaining, were labeled by anti-TNFα but not by anti-TNFR1 (Fig. 1b). We observed weak TNFR2 immunoreactivity in cell bodies and neurites of the neurons (data not shown). Quantification of Tnf and Tnfrsf1a transcripts in dissected SCG revealed a gradual increase in the amounts of these mRNAs from embryonic day 13 (E13) to reach a peak in expression by postnatal day 5 (P5), followed by a decline to low or negligible levels in the adult (Fig. 1c,d).

In the SCG target tissues analyzed in this study (submandibular salivary gland, nasal turbinate tissue and iris), tyrosine hydroxylase–positive sympathetic fibers were clearly labeled by anti-TNFα, LIGHT and RANKL decrease neurite growth8–11. TNF superfamily members act as reverse signaling receptors for their respective TNFR superfamily partners in cell lines and several cell types of the immune system12. In this study, we demonstrate that TNFα acts as a ligand for membrane-integrated TNFα expressed along the axons of postnatal SCG neurons and that TNFR1-activated TNFα reverse signaling promotes axon growth and tissue innervation by a mechanism dependent on ERK1 and ERK2.
from Tnf<sup>+/–</sup> and Tnfrsf1a<sup>−/−</sup> mice were not labeled by anti-TNFα and anti-TNFR1, respectively (data not shown).

**TNFα reverse signaling promotes sympathetic axon growth**

Our finding that TNFG is expressed on postnatal sympathetic axons ramifying in TNFFR1-expressing targets raised the possibility that target-derived TNFR1 might act as a reverse-signaling ligand for TNFG. To examine the possibility that TNFα reverse signaling might influence growth of sympathetic axons, we cultured P0 SCG neurons with NGF to sustain their survival with and without a divalent TNFR1-Fc chimera (in which the extracellular domains of two TNFR1 molecules are linked to the Fc part of a human IgG1 antibody) that has been shown to be a potent reverse-signaling ligand for TNFG<sup>14</sup>. After a 24-h incubation, the neurite arbors of neurons treated with the TNFR1-Fc were larger than those grown with NGF alone (Fig. 2a,b). Quantification of the size and complexity of the neurite arbors revealed that the TNFR1-Fc chimera had highly significantly greater neurite length and number of branch points (Fig. 2c,d; statistical details in figure legends), and the Sholl profiles, which plot neurite branching with distance from the cell body, were larger in the presence of TNFR1-Fc (Fig. 2e). NGF-supplemented neurons treated with an Fc protein fragment did not have significantly larger neurite arbors than neurons grown with NGF alone (Fig. 2c–e), indicating that the Fc fragment of the TNFR1-Fc chimera did not affect neurite growth. Dose-response analysis (data not shown) revealed that the effect of the TNFR1-Fc chimera on neurite growth reached a plateau at 10 ng ml<sup>−1</sup>. Soluble recombinant monovalent TNFR1 (sTNFR1), which activates TNFα reverse signaling at much higher concentrations<sup>3–11</sup>, also significantly enhanced neurite growth and branching (Fig. 2c–e). Cell counts showed that neither the TNFR1-Fc chimera nor sTNFR1 significantly affected neuron survival in these NGF-supplemented cultures (neurons surviving 24 h after plating expressed as a percentage of the number plated (mean ± s.e.m.): NGF alone, 79.4% ± 8.4%; NGF with TNFR1-Fc, 83.9% ± 7.8% (P = 0.674 versus NGF alone); NGF with sTNFR1, 97.2% ± 9.4% (P = 0.142 versus NGF alone); n = 3 experiments, t-test).

To verify that TNFα is essential for enhancement of neurite growth by TNFR1-Fc, we compared the effects of TNFR1-Fc on cultures of SCG neurons obtained from Tnf<sup>+/+</sup>, Tnf<sup>−/−</sup> and Tnfrsf1a<sup>−/−</sup> mice. For these studies, we crossed Tnf<sup>+/−</sup> mice and established separate SCG neuron cultures from each littermate. The Sholl profiles of SCG neurons of newborn Tnf<sup>+/+</sup> and Tnf<sup>−/−</sup> mice grown with TNFR1-Fc plus NGF were markedly larger than those grown with NGF alone, whereas the Sholl profiles of SCG neurons of newborn Tnf<sup>−/−</sup> mice grown with NGF and TNFR1-Fc and without TNFR1-Fc were very similar (Fig. 2f). Whereas TNFR1-Fc caused highly significant increases neurite length and branch point number in the neurite arbors of neurons cultured from newborn Tnf<sup>+/+</sup> and Tnf<sup>−/−</sup> mice (P < 0.001, n = 150 neurons per condition from three separate experiments, Fisher’s post hoc test; data not shown), TNFR1-Fc had no significant effect on neurite arbor size in cultures established from newborn Tnf<sup>−/−</sup> mice (P = 0.14 for length, P = 0.21 for branch number, n = 150 neurons per condition from three separate experiments, Fisher’s post hoc test; data not shown). These results demonstrate that TNFG is essential for the effect of TNFR1-Fc on neurite growth. In contrast to TNFα-deficient neurons, TNFR1-deficient neurons responded, just as wild-type neurons did, to TNFR1-Fc and sTNFR1 with enhanced neurite growth (Fig. 2g).

To determine whether membrane-integrated TNFα is required for enhancement of neurite growth by TNFR1-Fc, we pretreated P0 SCG neurons with TNFG-convert enzyme TACE, also known as ADAM17, a metalloproteinase that cleavages and releases the ectodomain of membrane-integrated TNFG<sup>18</sup>, thereby preventing reverse signaling. Sholl analysis revealed that neurite arbors of neurons cultured with TACE plus TNFR1-Fc were smaller than those of neurons cultured with TNFR1-Fc alone (Fig. 2h). Culture with TACE resulted in significantly smaller neurite length and number of branch points relative to neurons treated with TNFR1-Fc (P < 0.01, 234 control neurons, 243 TNFR1-Fc–treated neurons, 247 TACE-treated neurons and 258 TACE plus TNFR1-Fc–treated neurons; Kruskal-Wallis test, data not shown). These findings are consistent with transduction of the TNFR1-Fc signal by intact membrane-integrated TNFG.
these neurons were much smaller than those of neurons grown with NGF. Sholl analysis revealed that TNFR1-Fc increased the size and complexity of neurite arbors (Fig. 2). We carried out similar experiments using SCG neurons from wild-type neurons treated with the cell-permeant broad-spectrum caspase inhibitor Q-VD-OPh to prevent apoptosis in the absence of NGF. In these experiments, treatment with TNFR1-Fc likewise significantly increased the size and complexity neurite arbors ($P < 0.001$, $n = 196$ control neurons and 175 TNFR1-Fc–treated neurons, Fisher’s post hoc test; data not shown).

To determine whether TNFR1-Fc enhances neurite growth during a particular phase of sympathetic neuron development, we examined the effect of TNFR1-Fc on SCG neuron cultures established over a range of ages. Sholl analysis revealed that TNFR1-Fc did not enhance neurite growth from either embryonic day 18 (E18) or P10 SCG neurons, but enhanced neurite arbor size and complexity from P0 and P5 neurons (Fig. 3a). This indicates that TNFα reverse signaling enhances neurite growth from SCG neurons during a restricted period of postnatal development when the axons of these neurons are ramifying in their target tissues.

**TNFR1-Fc acts locally on axons to enhance their growth**

Expression of TNFR1 in sympathetic target tissues raises the possibility that it acts as a reverse-signaling ligand on the TNFα-expressing sympathetic axon terminals to promote their growth locally as they ramify in these tissues. To test whether TNFR1 can act locally on sympathetic axon terminals to promote growth, we cultured sympathetic neurons in microfluidic devices in which the cell soma and growing axon terminals are cultured in different compartments separated by a barrier (Fig. 3b). We seeded P0 SCG neurons into one compartment (the soma compartment) of a two-compartment device that contained NGF in both compartments to sustain neuronal survival and encourage axon growth from the soma compartment into the axon compartment. We added TNFR1-Fc to either the soma or axon compartment and used an Fc protein fragment as control. After a 24-h incubation, we labeled the axons in the axon compartment with the fluorescent vital dye calcein-AM, which also retrogradely labels cell bodies of neurons that project axons into the axon compartment. We used a stereological method to quantify the extent of axon growth in the axon compartment relative to the number of neurons projecting axons into this compartment. Addition of TNFR1-Fc to the axon compartment resulted in a marked and highly significant increase in axon growth in this compartment compared to Fc-treated controls.
Diminished innervation density in Tnf^{-/-} and Tnfrsf1a^{-/-} mice

To ascertain whether the increase in sympathetic axon growth and branching brought about by TNFR1-activated TNFα reverse signaling in vitro is physiologically relevant for the establishment of sympathetic innervation in vivo, we used tyrosine hydroxylase immunofluorescence to identify sympathetic fibers and quantify sympathetic innervation density in mice that are homozygous or heterozygous for targeted deletions of Tnf^{+/-} and Tnfrsf1a^{+/-} and in their wild-type littermates. We chose iris, nasal turbinate tissue and submandibular gland for this analysis because they receive a dense innervation of sympathetic fibers from the SCG. We initially carried out this analysis at P10, which is immediately after the period of development when TNFR1-activated TNFα reverse signaling enhances neurite growth in vitro and is at a stage in vivo when the sympathetic innervation of these tissues has become well established.

We crossed Tnf^{+/+}, Tnf^{+/+} and Tnf^{-/-} pups and crossed Tnfrsf1a^{+/-} mice to generate litters of Tnfrsf1a^{+/-} and Tnfrsf1a^{-/-} mice had less tyrosine hydroxylase immunofluorescence compared those of with wild-type littermates (Fig. 4). Tyrosine hydroxylase immunofluorescence was significantly lower in these tissues in both Tnf^{-/-} mice (Fig. 4c) and Tnfrsf1a^{-/-} mice (Fig. 4f-h) compared to wild-type littermates. Tyrosine hydroxylase immunofluorescence was also lower in the tissues of Tnf^{-/-} and Tnfrsf1a^{-/-} mice compared with wild-type mice, although these decreases were only significant in the nasal turbinate tissue of Tnf^{-/-} mice (Fig. 4c) and the submandibular gland of Tnfrsf1a^{-/-} mice (Fig. 4f).

To exclude the possibility that the decreases in tyrosine hydroxylase immunofluorescence in the tissues of Tnf^{-/-} and Tnfrsf1a^{-/-} mice were secondary to downregulation of tyrosine hydroxylase expression in the innervating neurons, we used western blotting to quantify the amounts of tyrosine hydroxylase protein in the SCG of these mice and the corresponding wild-type mice. This analysis revealed no significant differences in the levels of tyrosine hydroxylase protein relative to the level of the neuron-specific β-III tubulin protein in the SCG of Tnf^{-/-} mice and Tnfrsf1a^{-/-} mice (Supplementary Fig. 1) compared with the ganglia of wild-type mice.
at P10 ($P = 0.914$ for Tnf$^{−/−}$ versus Tnf$^{+/+}$ and $P = 0.697$ for Tnfrsf1a$^{−/−}$ versus Tnfrsf1a$^{+/+}$; $n = 3$ mice of each genotype, t-test).

To determine whether the diminished density of sympathetic innervation observed in the tissues of Tnf$^{−/−}$ and Tnfrsf1a$^{−/−}$ mice was secondary to the smaller size of the innervating population of neurons, we counted the neurons in the SCG of Tnf$^{−/−}$ and Tnfrsf1a$^{−/−}$ mice and in their wild-type littermates at P10. These counts revealed no significant differences in the numbers of SCG neurons between Tnf$^{−/−}$ and Tnf$^{+/+}$ mice and between Tnfrsf1a$^{−/−}$ and Tnfrsf1a$^{+/+}$ mice (Supplementary Fig. 1.c,f). $P$ = 0.624 for Tnf$^{−/−}$ versus Tnf$^{+/+}$ and $P = 0.902$ for Tnfrsf1a$^{−/−}$ versus Tnfrsf1a$^{+/+}$; $n = 3$ mice of each genotype, t-test). Taken together, these results demonstrate that TNFα and TNFRI have a crucial role in establishing sympathetic innervation in vivo and suggest that the influence of TNFR1-activated TNFα reverse signaling on axon growth and branching is physiologically relevant.

To ascertain whether the diminished density of sympathetic innervation observed in Tnf$^{−/−}$ and Tnfrsf1a$^{−/−}$ mice at P10 is maintained in the mature nervous system, we quantified tyrosine hydroxylase immunofluorescence in sections of the anatomically circumscribed tissues of the iris and submandibular salivary gland of adult mice. This analysis not only revealed highly significant decreases in tyrosine hydroxylase immunofluorescence in both tissues in Tnf$^{−/−}$ and Tnfrsf1a$^{−/−}$ adult mice compared to age-matched wild-type mice (Fig. 5a–f), but the proportion of decrease was greater than that observed at P10, especially in the submandibular gland, where tyrosine hydroxylase immunofluorescence was 70–80% lower in Tnf$^{−/−}$ and Tnfrsf1a$^{−/−}$ mice, 7 Tnfrsf1a$^{+/+}$ mice, 7 Tnfrsf1a$^{−/−}$ mice, 6 Tnfrsf1a$^{+/−}$ mice and 6 Tnfrsf1a$^{−/−}$ mice (**$P < 0.01$ and ***$P < 0.001$ compared to control, Fisher’s post hoc test).
Figure 5  Sympathetic innervation density in adult Tnf−/− and Tnfrsf1a−/− mice, and nerve fiber branching in P10 Tnf−/− and Tnfrsf1a−/− mice.  
(a) Representative micrographs of sections of the iris and submandibular gland parenchyma of adult Tnf−/− and Tnfrsf1a−/− mice labeled with anti-tyrosine hydroxylase (TH) immunofluorescence. Relative TH immunofluorescence in the iris (b) and submandibular gland (c) of adult Tnf−/−, Tnf+/− and Tnfrsf1a−/− mice expressed as a percentage of the mean level in Tnf+/− mice. Representative micrographs of sections of the iris and submandibular gland parenchyma of adult Tnfrsf1a−/− mice labeled with anti-TH (images were selected from 106 Tnfrsf1a−/− and 181 Tnfrsf1a−/− iris images and 20 Tnfrsf1a−/− and 31 Tnfrsf1a−/− submandibular gland images). Relative TH immunofluorescence in the iris (d) and submandibular gland (e) of adult Tnfrsf1a−/−, Tnfrsf1a+/− and Tnfrsf1a−/− mice expressed as a percentage of the mean level in Tnfrsf1a+/− mice. Mean ± s.e.m. of data from three mice of each genotype are shown. Representative images whole-mount preparations of the submandibular gland hilus of P10 Tnf+/−, Tnf−/−, Tnfrsf1a−/− and Tnfrsf1a+/− mice labeled with anti-TH (images were selected from 4 Tnfrsf1a−/−, 3 Tnfrsf1a+/−, 6 Tnfrsf1a−/− and 7 Tnfrsf1a+/− mice). Scale bars, 100 µm. **P < 0.01 and ***P < 0.001, compared with wild type; Fisher’s post hoc test.

sympathetic nerves reach this gland, many fibers did not grow into and ramify in this tissue (Fig. 5g). Using a stereological method to estimate the extent of branching near the hilus, we found highly significant decreases in the number of nerve branches in Tnf−/− mice (37.3% ± 7.0% decrease in nerve branches in Tnf−/− mice relative to Tnf+/− mice (mean ± s.e.m.; P < 0.0001, n = 4 Tnf−/− mice and n = 3 Tnf+/− mice, Fisher’s post hoc test)) and Tnfrsf1a−/− mice (17.9% ± 5.1% decrease in nerve branches in Tnfrsf1a−/− mice relative to Tnfrsf1a+/− mice (mean ± s.e.m., P < 0.003, n = 6 Tnfrsf1a−/− mice, n = 7 Tnfrsf1a+/− mice, Fisher’s post hoc test)).

To corroborate our conclusion based on tyrosine hydroxylase immunohistochemistry experiments that Tnf−/− and Tnfrsf1a−/− mice have lower density of sympathetic innervation compared with wild-type mice, we used dopamine β-hydroxylase (DBH) immunohistochemistry as another marker of sympathetic fibers in tissue sections of separate sets of P10 mutant and wild-type mice. Although this analysis was based on a smaller number of mice, DBH immunofluorescence was nonetheless highly significantly lower in the iris, nasal turbinate tissue and submandibular gland of both Tnf−/− mice compared to wild-type mice (P = 0.0014 for iris, P = 0.0146 for nasal turbinate tissue and P = 0.0014 for submandibular gland; n = 3 of each genotype, Fisher’s post hoc test) and Tnfrsf1a−/− mice compared to wild-type mice (P = 0.00143 for iris, P = 0.00144 for nasal turbinate tissue and P = 0.00026 for submandibular gland, n = 3 of each genotype, Fisher’s post hoc test; Supplementary Fig. 2). Furthermore, we observed significant decreases in DBH immunofluorescence in the nasal turbinate tissue of Tnf−/− and Tnfrsf1a−/− mice relative to wild type mice (P = 0.024 and 0.0016, respectively, n = 3 of each genotype, Fisher’s post hoc test).

TNFR1-Fc enhances axonal growth by activating ERK1 and ERK2

To study the molecular mechanism underlying the enhancement of neurite growth from SCG neurons by TNFα reverse signaling, we explored a common link in intracellular signaling between the control of neurite growth and TNFα reverse signaling in the immune system. ERK1 and ERK2 have been shown to be activated both by TNFα reverse signaling in monocytes23 and by NGF in PC12 cells and in SCG neurons, and to contribute to growth of neurites in response to NGF24–26.

To investigate whether ERK signaling contributes to TNFR1-promoted neurite growth from SCG neurons, we first tested whether TNFR1-Fc activates ERK1 and ERK2 in these neurons. In these experiments, we cultured P0 SCG neurons for 12 h with NGF before treating them with TNFR1-Fc. The initial period of culture permitted phospho-ERK1 and phospho-ERK2 to reach a basal level so that any subsequent increase could be observed more easily. Treatment with TNFR1-Fc after 12 h caused rapid increases in the amounts of phospho-ERK1 and phospho-ERK2 within 5 min, which returned to basal levels within 30–45 min (Fig. 6a).

To determine whether treatment with TNFR1-Fc can result in activation of ERK1 and ERK2 independently of NGF, we initially cultured P0 SCG neurons for 12 h with the caspase inhibitor Q-VD-OPh to prevent apoptosis in the absence of NGF before treatment with TNFR1-Fc. As with neurons maintained with NGF, treatment with TNFR-Fc caused a rapid transient increase in the amounts phospho-ERK1 and phospho-ERK2 (Fig. 6b). Likewise, Bax-deficient SCG neurons cultured in the absence of NGF exhibited very similar changes in the amounts of phospho-ERK1 and phospho-ERK2 after treatment with TNFR1-Fc after 12 h of culture (Fig. 6c). To confirm that TNFα is needed for the activation of ERK1 and ERK2 by TNFR1-Fc, we treated SCG neurons obtained from Tnf−/− mice with TNFR1-Fc. We observed no significant changes in phospho-ERK1 and phospho-ERK2 relative to total ERK1 and total ERK2 protein in TNFα-deficient neurons after treatment with TNFR1-Fc (Fig. 6d). Taken together, these results show that TNFα reverse signaling causes rapid, transient activation of ERK1 and ERK2 independently of NGF.

To investigate whether ERK1 and ERK2 activation is responsible for the enhanced neurite growth brought about by TNFR1-Fc,
we examined whether U0126, a selective MEK1 and MEK2 inhibitor that interferes with MEK1 and MEK2-dependent activation of ERK1 and ERK2 (ref. 27), could prevent the increase in neurite growth. In these experiments, we plated P0 SCG neurons in NGF-free medium containing Q-VD-OPh and pretreated these neurons for 2 h with either U0126 or the inactive analog U0124 before TNFR1-Fc addition and incubation for 24 h in medium containing Q-VD-OPh (mean ± s.e.m., >200 neurons per condition from four independent experiments). (g) Change in cytosolic free-Ca\textsuperscript{2+} after addition of either Fc fragment or TNFR1-Fc in either Ca\textsuperscript{2+}-free medium or medium containing 1.2 mM Ca\textsuperscript{2+} (mean ± s.e.m. of three experiments, >200 neurons imaged per condition per experiment). (h) Sholl profiles of P0 SCG neurons cultured for 24 h with either NGF (control) or NGF plus 1 μM BAPTA-AM, TNFR1-Fc or BAPTA-AM plus TNFR1-Fc (mean ± s.e.m., >150 neurons per condition from three independent experiments, **P < 0.01 and ***P < 0.001, comparison with control, Fisher’s post hoc test). (i) Representative western blots of lysates of P0 SCG neurons grown for 12 h with Q-VD-OPh without NGF before treatment with either 1 μM BAPTA-AM or BAPTA-AM plus TNFR1-Fc (images selected from three separate experiments of each kind). Full-length western blots are shown in Supplementary Figure 3.

**DISCUSSION**

Reverse signaling via membrane-integrated members of the TNF superfamily controls multiple aspects of immune function\textsuperscript{13}. Although there is a burgeoning literature on the roles of the TNF superfamily in the nervous system in development, physiology and pathology\textsuperscript{30,31}, these roles have been investigated and interpreted in the framework of conventional forward signaling. Here we show that TNF superfamily reverse signaling also occurs in the nervous system. We found that soluble monovalent TNFR1 and divalent TNFR1-Fc chimera, proteins that initiate TNFα reverse signaling in a variety of TNFα-expressing cell lines and cells of the immune system\textsuperscript{14–17},
enhanced axonal growth and branching from TNFα-expressing postnatal SCG in culture. Expression of membrane-integrated TNFα was essential for the effect of TNFR1-Fc on neurite growth because TNFα-converting enzyme, which cleaves the extracellular domain of TNFα, impaired the ability of TNFR1-Fc to enhance neurite growth.

The neurite growth-enhancing effect of TNFR1-Fc was restricted to P0 to P5, when sympathetic axons are growing and ramifying in their targets in vivo under the influence of target-derived NGF. TNFR1-Fc not only greatly enhanced the size and complexity of the neurite arbors of postnatal SCG neurons beyond what was seen with maximally effective concentrations of NGF but it promoted neurite growth and branching in the absence of NGF in cultures in which neuronal apoptosis was prevented by caspase inhibition or deletion of the pro-apoptotic Bax protein. This indicates that TNFα reverse signaling enhanced neurite growth and branching independently of NGF and could therefore affect axonal growth at various NGF concentrations that developing SCG neurons encounter in vivo.

Although SCG neurons express both TNFα and TNFR1, TNFα is distributed along axons both in vitro and in vivo but TNFR1 expression is restricted to the soma. Expression of TNFR1 in cells in tissues innervated by SCG neurons, together with our demonstration in compartment cultures that TNFR1-Fc enhances axon growth when applied to the axon terminals, but not to the cell soma, suggests that target-derived TNFR1 acts locally on axons to promote growth. TNFR1 expressed in target tissues could act on the membrane-integrated TNFα of the innervating axons either as a soluble, diffusible protein after its release from the cells that synthesize it or as a membrane-integrated protein. Although we do know whether either or both of these alternatives pertain in vivo, the restriction of TNFR1 to the cells that produce it as membrane-integrated protein could potentially have a more precise influence on regulating the local growth, branching and disposition of axons in target tissues. Although SCG neurons express both TNFR1 and TNFα, our observation that the magnitude of NGF-promoted neurite growth from cultured postnatal SCG neurons lacking either TNFR1 or TNFα is similar to that from that from wild-type neurons suggests that any potential autocrine signaling between these membrane proteins does not influence on neurite growth.

The physiological relevance of the axon growth-promoting effect of target-derived TNFR1 on TNFα-expressing sympathetic axons in vivo is amply demonstrated by extensive, blinded quantification of the sympathetic innervation density of several tissues of Tnfα and Tnfrsf1a mutant mice and wild-type littermates. This analysis revealed substantial decreases in tyrosine hydroxylase immunofluorescence in the irides, nasal tissue and submandibular gland of Tnfrsf1a−/− mice at P10 compared with wild-type littermates. The smaller decreases in tyrosine hydroxylase immunofluorescence in the nasal tissue of Tnf+/– mice and the submandibular gland of Tnfrsf1a−/– mice compared to wild-type littermates hint at a gene-dosage effect in heterozygous mice. Quantification of tyrosine hydroxylase protein amounts and numbers of neurons in the SCG of Tnf+/– and Tnfrsf1a−/– mice and corresponding wild-type mice indicated that decreases in sympathetic innervation density were not secondary to decreases in either tyrosine hydroxylase expression or the size of the innervating population of sympathetic neurons. Quantification of sympathetic innervation density using DBH immunofluorescence as an alternative marker of sympathetic fibers, likewise revealed substantial decreases in immunofluorescence in the iris, nasal turbinale tissue and submandibular gland of both Tnf−/− and Tnfrsf1a−/– mice compared to wild-type mice. Furthermore, analysis of whole-mount preparations suggests that whereas sympathetic nerves reach their targets in Tnf−/– and Tnfrsf1a−/– mice, many fibers do not grow into and ramify in these targets. Sympathetic innervation density in adult mice was even lower in Tnf−/– and Tnfrsf1a−/– than at P10. Taken together, these studies suggest that TNFR1-activated TNFα reverse signaling substantially contributes to the establishment of sympathetic innervation of several cranial tissues in postnatal mice and the maintenance of sympathetic innervation in throughout life.

In common with TNFα reverse signaling in monocytes, we found that TNFα reverse signaling in postnatal SCG neurons led to a rapid, pronounced and transient activation of ERK1 and ERK2. Like the enhanced neurite growth promoted by TNFR1-Fc, the activation of ERK1/ERK2 by TNFR1-Fc depended on the expression of TNFα and occurred independently of NGF. Several studies have shown that activation of ERK1/ERK2 by NGF in SCG neurons contributes to its ability to promote neurite growth and that phosphorylation and activate ERK1 and ERK2, completely prevented neurite growth, enhancing action of TNFR1-Fc, suggests that ERK signaling mediates the effect of TNFα reverse signaling on neurite growth. As in macrophages, activation of TNFα reverse signaling in SCG neurons caused Ca++ influx and rapid elevation of cytosolic Ca++ levels. Our demonstration that the intracellular Ca++ chelator BAPTA prevented both ERK1 and ERK2 activation and enhanced neurite growth in response to TNFR1-Fc, suggests that elevation of cytosolic Ca++ is necessary in mediating the effect of TNFα reverse signaling on neurite growth. The identity of the channels that mediate Ca++ influx and how TNFα reverse signaling gates these channels remain intriguing questions.

We previously reported that soluble TNFα decreases the extent of NGF-promoted neurite growth from cultured SCG neurons from newborn mice by a mechanism dependent on IKKβ-activated NF-κB signaling. Our demonstration that SCG neurons cultured from Tnfrsf1a−/– mice lack this inhibitory response suggests that it is mediated by TNFR1. In accordance with the restriction of TNFR1 to the soma of SCG neurons, we only observed the growth-inhibitory effect of soluble TNFα in compartment cultures when TNFα was added to the soma compartment, not the axon compartment. This suggests that target-derived TNFα is unlikely to regulate axon growth in vivo. Moreover, if the growth-inhibitory effect of soluble TNFα observed in vitro were the predominant, physiologically relevant influence of TNFα in vivo, one might predict an increase in sympathetic innervation density in Tnf−/– and Tnfrsf1a−/– mice, then the decrease we observed.

Expression of TNFR1 and TNFR2 by neurons has been implicated in the regulation of neuronal death and survival. There is evidence that TNFα forward signaling via TNFR1 in fetal mouse SCG and trigeminal sensory neurons has a role in accelerating neuronal apoptosis after NGF deprivation. Conversely, TNFα forward signaling via TNFR2 has been implicated in the survival effects of NGF on P5 rat DRG sensory neurons. However, by P10 we observed similar numbers of SCG neurons in Tnf−/–, Tnfrsf1a−/– and wild-type littermates, indicating that by this late stage of development compensatory changes have taken place in these mice for any effects of TNFα on neuronal survival and the timing and magnitude of naturally occurring neuronal death observed earlier in development.

Our discovery of TNF reverse signaling in the nervous system increases our appreciation of the diversity and complexity of signaling between cells in the nervous system and may necessitate a re-evaluation of the mechanistic explanation for previous in vivo and in vitro studies of TNFα function in the nervous system. For example, the phenotypic consequences of deleting the Tnf gene may result from...
eliminating either forward or reverse signaling. Likewise, the addition of soluble TNFα to cultured cells could either activate forward signaling or interfere with potential endogenous reverse signaling by competing for TNFR1 binding. In addition to providing important new insights into the regulation of the growth of sympathetic axons and the establishment of sympathetic innervation in vivo, our findings highlight the importance of evaluating the relative contributions of forward and reverse signaling in different developmental systems and experimental models.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

L.K. conducted the majority of the cell culture experiments, quantitated submandibular gland and iris innervation and did the western blot analysis. C.O. quantified nasal tissue and adult submandibular gland innervation. C.E. undertook the immunocytochemical and immunohistochemical localization of TNFα and TNFR1 and the whole-mount studies. T.V. contributed to studies of iris innervation. S.W. carried out the QPCR. L.K. and A.M.D. wrote the manuscript. A.M.D. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Real-time quantitative PCR. Amounts of mRNAs encoding TNFR and TNFR1 were quantified by real-time (RT) quantitative (Q)PCR relative to a geometric mean of mRNAs for glyceraldehyde phosphate dehydrogenase (GAPDH) and succinate dehydrogenase (SDHA). Total RNA was extracted from SCG with the RNeasy Mini extraction kit (Qiagen), and 5 μl was reverse-transcribed for 1 h at 45 °C using the AffinityScript kit (Agilent) in a 25 μl reaction according to the manufacturer's instructions. In a 20 μl reaction volume, 2 μl of cDNA was amplified using Brilliant III ultrafast QPCR master mix reagents (Agilent). QPCR products were detected using dual-labeled (FAM/BHQ1) hybridization probes specific to each of the cDNAs (MWG/Eurofins). The PCR primers were: TNFR forward, 5′-TAC TTA GAC TTT GCG GAG-3′ and reverse, 5′-AGA GTA AAG GGG TCA GAG CAT CAA ATT CAT-3′; TNFR1 forward, 5′-TTC CCA GAA TTA CCT CAG-3′ and reverse, 5′-AAC TGT TGC TCC TTA CAG-3′; GAPDH forward, 5′-GAG AAA CCT GCC AAG TAT G-3′ and reverse, 5′-GGA GTT GCT GTT GAA GTC-3′; SDHA forward, 5′-GGA ACA GTC CAA AAA CAG-3′ and reverse, 5′-CCA CAG CATCAA ATT CAT-3′. Dual-labeled probes were: TNFa, 5′-FAM-CAG GTC TAC TTT GGA GGT ATT CTT G-BHQ1-3′; TNFR1, 5′-FAM-CAG CTC GGT TTT CTT GGC TGC-CTT GTC AGC-BHQ1-3′; GAPDH, 5′-FAM-AGA CCA CCT GGT CCT CAG TGT BQH1-3′; SDHA, 5′-FAM-CCT GGC GCT TCT ACT CCT CTBQH1-3′. Forward and reverse primers were used at a concentration of 150 nM each and dual-labeled probes were used at a concentration of 300 nM. PCR was performed using the MX3000P platform (Agilent) using the following conditions: 45 cycles of 95 °C for 12 s and 60 °C for 35 s. Standard curves were generated in every mice. The neurons were then treated with TNFR1-Fc for times ranging from 5 min to 120 min.

The majority of cultures were established from CD1 mice. Bax−/- mice were obtained from Tgfb/− mice backcrossed into a CD1 background. Tgfr1a1− mice were maintained in a c57bl6 background. Neutrons of different genotypes were generated by crossing heterozygous mice. Separate cultures were established from each littermate resulting from these crosses, and the genotypes were only determined after the cultures had been analyzed by a PCR based approach using tissue samples obtained at the time the cultures were set up. All animal experiments were conducted in accordance with the 1986 Animal Procedures Act approved by the Home Office (UK). Mice of both sexes were used and at the ages specified in the text.

Purified recombinant NGE, TNFR1-Fc, soluble TNFR1, TNFα and caspase inhibitor Q-VD-OPh were obtained from R&D Systems and the human Fc fragment was obtained from Abcam.

Quantification of the sympathetic innervation of SCG targets. Batches of tissue from littermates of all three genotypes of each mouse mutant were processed at the same time to ensure they were stained in an identical manner. For the iris, all sections were imaged. For the nasal turbinate tissue and submandibular gland, every fifth section was imaged. The outline of the iris and the core tissue of the nasal turbinate (that is, turbinate tissue excluding the nasal mucosa, which displays some non-specific staining) in these images was traced using Adobe Photoshop CS. Total iris and core nasal turbinate area and the area containing intense immunoreactive tyrosine hydroxylase–positive fibers were estimated by automatic pixel counts using identical settings for all sections and all genotypes, and the ratio tyrosine hydroxylase–positive area to total iris area and core turbinate area was calculated. For the submandibular gland, multiple random images were analyzed in which the ratio of immunoreactive tyrosine hydroxylase–positive fibers to total image area was estimated. Background staining was subtracted from all images before quantification. Background staining was obtained by imaging sections of the tissues that were incubated with secondary antibody alone. The data are expressed as a percentage of the mean for data in wild-type mice for each tissue. This analysis was carried out by multiple authors and was done blind.

For whole-mount studies, the submandibular glands of P10 of Tgfb/++, Tgfb−/−, Tgfr1a1+/+ and Tgfr1a1−/− pups were fixed in 4% paraformaldehyde for at least 24 h. The tissue was dehydrated in 50% methanol for 1 h at room temperature and 80% methanol for a further 1 h. Endogenous peroxidase activity was quenched by placing tissue in a solution of 80% methanol, 20% DMSO and 3% H2O2 overnight at 4 °C. Tissue was rehydrated by placing in 50% methanol for 1 h, 30% methanol for 1 h, PBS for 1 h, all at room temperature, and was blocked overnight at 4 °C with 4% BSA containing 1% Triton X-100 in PBS. The tissue was then incubated with polyclonal antibody to tyrosine hydroxylase (1:200, Millipore) in blocking solution for 72 h at 4 °C. After washing three times for 2 h in 1% Triton X-100 in PBS at room temperature, the tissue was kept at 4 °C overnight in a fourth wash.
before being incubated with rabbit, HRP-conjugated antibody (1:300, Promega) in blocking solution at 4 °C overnight. The tissue was then washed for 2 h at room temperature with PBS containing 1% Triton X-100. Tyrosine hydroxylase-positive fibers were visualized by DAB-HRP staining: the tissue was incubated with 1× DAB for 20 min at room temperature and then with 1× DAB containing 0.006% H2O2 for 2–5 min to develop the staining. After washing with PBS, the tissue was and incubated at 4 °C overnight in PBS. BABB (one part benzyl alcohol: two parts benzyl benzoate) was used as a clearing solution. The tissue was placed in 50% methanol for 10 min, washed 3 times with 100% methanol (once for 30 min and twice for 15 min) at room temperature and was incubated in 50% BABB for 5 min before being placed in BABB. To compare the extent of sympathetic nerve branching near the gland hilus, a modified line-intercept method was used. Using ImageJ, a grid of 24 squares (4 × 6 squares) of side length 158 µm per square was aligned in a standard orientation next to the hilus of each gland. The number of fiber bundles intersecting the sides of squares in the grid was scored blind for the glands from each animal. Fiber density was estimated using the formula πD2/I, where D is the interline interval (158) and I the mean number of intersections along one side of each square in the grid. The data are expressed as a percentage of the mean data for wild-type mice.

Quantification of neuron numbers in SCG. Estimates of the numbers of neurons in the SCG of P10 Tnfrsf1a+/−, Tnfrsf1a−/−, Tnf−/− and Tnf+/+ pups were carried out by stereological analysis of ganglia serially sectioned at 8 µm and immunolabeled for H2O2 for 2–5 min to develop the staining. After washing with PBS, the tissue was and incubated at 4 °C overnight in PBS. BABB (one part benzyl alcohol: two parts benzyl benzoate) was used as a clearing solution. The tissue was placed in 50% methanol for 10 min, washed 3 times with 100% methanol (once for 30 min and twice for 15 min) at room temperature and was incubated in 50% BABB for 5 min before being placed in BABB. To compare the extent of sympathetic nerve branching near the gland hilus, a modified line-intercept method was used. Using ImageJ, a grid of 24 squares (4 × 6 squares) of side length 158 µm per square was aligned in a standard orientation next to the hilus of each gland. The number of fiber bundles intersecting the sides of squares in the grid was scored blind for the glands from each animal. Fiber density was estimated using the formula πD2/I, where D is the interline interval (158) and I the mean number of intersections along one side of each square in the grid. The data are expressed as a percentage of the mean data for wild-type mice.

Immunoblotting. Immunoblotting was carried out using the BioRad TransBlot (BioRad) as previously described. The blots were probed with antibodies to phospho-ERK1/ERK2 (1:1,000, Cell Signaling, 9101), total ERK1/ERK2 (1:1,000, Cell Signaling, 9102) or β-III tubulin (1:10,000, R&D systems, MAB1195). Binding of the primary antibodies was visualized with an HRP-conjugated secondary antibody (1:2,000; Promega, either W4021 or W4011) and ECL-plus reagent (Amersham). Densitometry was carried out using Gel-Pro Analyzer 32 program (Media Cybernetics). The levels of phospho-ERK1 and phospho-ERK2 were normalized to the levels of total ERK1 and ERK2.

Calcium imaging. SCG neurons were cultured at high density (50,000 cells per 35-mm dish) in medium containing 10 ng/ml NGF plus an inhibitor of TNFα processing (TAPI-0, 300 nM, Enzo Life Sciences) to inhibit cleavage of membrane-integrated TNFα. Twelve hours after plating, the medium was changed to Ringel’s solution (125 mM NaCl, 4 mM KCl, 1.2 mM CaCl2, 0.5 mM MgCl2, 10 mM glucose and 10 mM HEPES, pH 7.4) or containing 0.1% BSA and Fura-2 AM (1 mg/ml, Invitrogen). After another 30 min at room temperature, the cells were washed twice for 10 min with Ringer’s solution or Ca2+-free Ringer’s solution (Ca2+-free Ringer’s solution (125 mM NaCl, 4 mM KCl, 0.5 mM MgCl2, 10 mM glucose and 10 mM HEPES, pH 7.4)) and were treated with either 5 µg/ml TNFR1-Fc in Ringer’s solution or Ca2+-free Ringer’s solution or 5 µg/ml Fc fragment in Ringer’s solution. Time-lapse imaging of the 340/380 nm ratio was carried out at intervals using a Zeiss Axiovert 200 fluorescence microscope. At least 20 neurons were imaged per condition in each experiment, and the mean percentage change in the 340/380 nm ratio (minus background) was calculated.

Statistical analysis. Data are expressed as mean and standard errors. No statistical methods were used to predetermine sample sizes but our sample sizes are similar to those generally used in the field. Following normality test and homogeneity of variance (F-test), group comparison were made using a t-test or one-way ANOVA as appropriate followed by Fisher’s post hoc test for normally distributed data. A nonparametric test, the Kruskal-Wallis test, was performed for data that were not normally distributed. Differences were considered significant for P < 0.05. The experiments were not randomized. Data from all experiments are included, none were excluded.

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