Interleukin-17A Increases Neurite Outgrowth from Adult Postganglionic Sympathetic Neurons

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Inflammation can profoundly alter the structure and function of the nervous system. Interleukin (IL)-17 has been implicated in the pathogenesis of several inflammatory diseases associated with nervous system plasticity. However, the effects of IL-17 on the nervous system remain unexplored. Cell and explant culture techniques, immunohistochemistry, electrophysiology, and Ca^{2+} imaging were used to examine the impact of IL-17 on adult mouse sympathetic neurons. Receptors for IL-17 were present on postganglionic neurons from superior mesenteric ganglia (SMG). Supernatant from activated splenic T lymphocytes, which was abundant in IL-17, dramatically enhanced axonal length of SMG neurons. Importantly, IL-17-neutralizing antiserum abrogated the neurotrophic effect of splenocyte supernatant, and incubation of SMG neurons in IL-17 (1 ng/ml) significantly potentiated neurite outgrowth. The neurotrophic effect of IL-17 was accompanied by inhibition of voltage-dependent Ca^{2+} influx and was recapitulated by incubation of neurons in a blocker of N-type Ca^{2+} channels (ω -conotoxin GVIA; 30 nM). IL-17-induced neurite outgrowth *in vitro* appeared to be independent of glia, as treatment with a glial toxin (AraC; 5 μM) did not affect the outgrowth response to IL-17. Moreover, application of the cytokine to distal axons devoid of glial processes enhanced neurite extension. An inhibitor of the NF- κB pathway (SC-514; 20 μM) blocked the effects of IL-17. These data represent the first evidence that IL-17 can act on sympathetic somata and distal neurites to enhance neurite outgrowth, and identify a novel potential role for IL-17 in the neuroanatomical plasticity that accompanies inflammation.

Introduction

The adult sympathetic nervous system (SNS) displays plasticity of form and function in response to many distinct types of inflammation, both acute and chronic. Acute inflammatory states such as myocardial infarction (Hasan et al., 2006), ozone-induced pulmonary inflammation (Graham et al., 2001), interstitial cystitis (Peeker et al., 2000), and intestinal parasitic infection (Kannan et al., 1996) increase sympathetic innervation density in the afflicted tissues. Chronic autoimmune diseases are also associated with remodeling of sympathetic axons; recent studies of inflammatory bowel disease (IBD) patients and animal models of IBD provided anatomical evidence of increased sympathetic innervation of colonic smooth muscle (Lourenssen et al., 2005), mesenteric blood vessels (Birch et al., 2008), and dorsal root ganglia (Xia et al., 2011). Models of chronic

arthritis have demonstrated similar effects on the SNS (Lorton et al., 2009; Straub et al., 2011).

Anatomical remodeling of the SNS often leads to important functional alterations in the system involved. In models of myocardial infarction, the sympathetic outflow to the heart is increased as a result of inflammation-induced SNS hyperinnervation, which has been suggested to increase susceptibility to arrhythmia postinfarction (Hasking et al., 1986; Himura et al., 1993; Eisenhofer et al., 1996). Animal models of neuropathic and inflammatory pain implicate sympathetic sprouting in dorsal root ganglia in the onset and maintenance of mechanical and thermal allodynia (Gibbs et al., 2008). Increased sympathetic axonal sprouting has also been linked to aberrant vascular regulation in obese rats (Haddock and Hill, 2011). Finally, recent evidence from studies of vagally mediated neuroimmunomodulation strongly suggest that sympathetic postganglionic neurons comprise the final motor pathway in the autonomic regulation of inflammation severity in inflammatory diseases (The et al., 2007; Ghia et al., 2008; Rosas-Ballina et al., 2008, 2011; Wong et al., 2011). Given the functional consequences of sympathetic neuroanatomical plasticity during inflammation, a better understanding of the molecular mechanisms and candidate mediators of these effects is required.

Mounting evidence suggests a role for the inflammatory cytokine network in promoting sympathetic axonal remodeling during inflammatory disease (Kannan et al., 1996; Rapalino et al., 1998; Xie et al., 2006; Hyatt Sachs et al., 2010). The extent of sympathetic axonal sprouting following myocardial infarction, for example, is positively correlated with the degree of infiltrating immune cells, which synthesize and secrete proinflammatory

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cytokines (Hasan et al., 2006; Wernli et al., 2009). Interleukin-17A (IL-17), the principal cytokine secreted from the novel T_H17 lineage of helper T lymphocytes, has recently emerged as a key proinflammatory cytokine that orchestrates immune responses during infection, acute inflammation, allergy, and autoimmune disease (Nakae et al., 2003; Bettelli et al., 2007; Korn et al., 2007; Oboki et al., 2008; Littman and Rudensky, 2010). However, little is known about the effect of IL-17 on the nervous system. Given that IL-17 is implicated in the pathogenesis of the types of inflammation that lead to sympathetic neuroanatomical plasticity, we hypothesized that IL-17 contributes to sympathetic remodeling during inflammation. In the current study, we used immunohistochemistry, neurite outgrowth assays, electrophysiology, and Ca²⁺ imaging to investigate whether IL-17 induces axon outgrowth from adult mouse sympathetic neurons and identify the signaling mechanisms involved.

Materials and Methods

Animal care

All animal care protocols were developed in accordance with the Guidelines of the Canadian Council of Animal Care and were approved by the Queen's University Animal Care Committee. Adult male CD1 mice weighing between 25 and 35 g were purchased from Charles River and maintained on a 12:12 h light:dark cycle, with access to standard lab chow and tap water *ad libitum*.

Tissue collection

Following deep anesthesia by an overdose of inhaled isoflurane, mice were killed by cervical dislocation. The superior mesenteric ganglion (SMG) was dissected and cleaned of all visible adipose tissue before transfer to a vial of prewarmed HBSS. Unless stated otherwise, drugs and reagents were obtained from Sigma.

SMG dissociation

The SMG was dissociated to yield single neurons as previously described (Motagally et al., 2009a). Briefly, the SMG was subjected to enzymatic dissociation in collagenase solution (Type 1A; 1547 U/ml) with bovine serum albumin (BSA; 6 mg/ml) in HBSS, followed by trypsin solution (type XII-S; 1500 U/ml) with BSA at 37°C. The SMG was then mechanically dissociated into a suspension of single cells. Cell suspensions of 100–200 μ l were plated onto laminin (10 μ g/ml)-coated glass coverslips in a 24-well plate and allowed to adhere for 2.5 h, at which time Leibowitz's (L-15) media supplemented with 24 mM NaHCO₃, 10% fetal bovine serum (FBS), 38 mM D-glucose, 5000 IU penicillin/streptomycin, and 50 ng/ml nerve growth factor (NGF; Cedarlane Laboratories) was added. Recombinant mouse interleukin-17 (Peprotech) was reconstituted in PBS. To study neurite extension in a compartmented culture system, SMG neurons were loaded into a 1 CC syringe fitted with a 28-gauge Teflon needle (World Precision Instruments) and 50–80 μ l of cell suspension were injected into the proximal chamber of a compartmented culture dish.

Explant cultures

Collagen gels were formed using eight parts acidified rat tail collagen (Roche Molecular Biochemical) reconstituted with one part 10 $\times$ concentrated DMEM and one part 10 $\times$ concentrated HBSS, and diluted to 1 mg/ml with DMEM containing 5% FBS and 50 ng/ml NGF, as previously described (Barrett et al., 2005). SMG were placed in collagenase solution for 20 min to enhance the permeability of the ganglion capsule, rinsed, and cut into four pieces under a dissecting microscope, then each piece was embedded into semisolidified collagen gels. For directional neurite extension assays, Cibicron agarose beads were soaked in either IL-17 (100 ng/ml) in HBSS or HBSS alone for 4 h at 4°C. A single bead was then placed 800–900 μ m from the ganglion explant, and the explants were cultured for 3 d. Bead position was marked by punching a hole in the gel before immunohistochemical processing.

Table 1. Primary antibodies

Reactivity	Host	Source	Concentration	Reference
β -3 tubulin	Rabbit	Sigma	1:500	Leiss et al., 1988
HuD	Mouse	Invitrogen	1:500	Antunes et al., 2000
IL-17RA	Goat	R & D Systems	1:50	Kao et al., 2005
SNAP-25	Rabbit	Invitrogen	1:4000	Geddes et al., 1990
SOX-10	Goat	Santa Cruz Biotechnology	1:500	Liu et al., 2002
TH	Rabbit	Millipore	1:500	Lourenssen et al., 2005

Table 2. Secondary antibodies

Reactivity	Host	Source	Concentration
Mouse (488)	Goat	Invitrogen	1:1000
Rabbit (555)	Goat	Invitrogen	1:2000
Rabbit (549)	Donkey	Jackson ImmunoResearch	1:800
Goat (488)	Donkey	Jackson ImmunoResearch	1:800

Immunohistochemistry

Tissue cryosections. Dual-label immunohistochemistry was performed on sections of SMG to examine whether cells in the ganglion expressed the IL-17 receptor A (IL-17R) and tyrosine hydroxylase (TH), a marker of sympathetic neurons. Mice were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer while under deep anesthesia (ketamine/xylazine at 0.166 mg/g). The SMG were removed and fixed overnight in 4% PFA at 4°C, then transferred to 30% sucrose solution for 24 h. SMG were subsequently embedded in freezing media (Shandon Cryomatrix; Fisher Scientific), frozen in chilled 2-methylbutane, and stored at -80°C . Sections (10 μ m) were cut using a cryostat (Thermo Shandon; Fisher Scientific) and collected on Superfrost Plus microscope slides.

Cryosections were washed in PBS-Tween (0.1%) three times before incubation in 10% normal donkey serum for 1 h in a humidified chamber (used for all incubations). Slides were washed three times in PBS-Tween (0.1%), and primary antiserum to IL-17R (Table 1) was added to slides overnight. Following removal of primary antiserum, slides were washed an additional three times and incubated in secondary antiserum for 2 h. Slides were then washed three times in PBS-Tween (0.1%), immunofluorescently labeled with TH using the same protocol described above, then coverslipped with fluorescence mounting media (DAKO).

Isolated cells and explant cultures

Cells and tissue were fixed using 4% PFA in 0.1 M sodium phosphate buffer. Dissociated cells were fixed for 10 min at room temperature; collagen gels containing explants were fixed overnight at 4°C. Following removal of the fixative and washes in PBS, the tissues were blocked for 1 h at room temperature in 5% normal goat, horse, or donkey serum in PBS with 0.1% Tween, depending on the species in which the secondary antiserum was raised. Primary antisera to SNAP-25, HuD, β -3 tubulin, or IL-17R were then added overnight at 4°C (for dilutions, see Table 1). Primary antiserum was removed, tissues were washed a further three times in PBS, and an appropriate secondary antibody (Table 2) was added for 1 h at room temperature. Following three washes in PBS, coverslips were mounted with fluorescence mounting medium (DAKO) and visualized using an epifluorescence microscope (Olympus BX51). Photomicrographs were taken using a Coolsnap CCD camera and ImagePro software.

Quantification

For HuD/SNAP-25-stained coverslips, every third field of view in the horizontal and vertical axes using a 40 $\times$ objective lens was analyzed. The number of HuD-positive neurons in the entire field of view and the number of SNAP-25-positive neurites crossing either the vertical or horizontal midline of the field of view were determined. For real time outgrowth assays, the number of neurons, neurites, branch points, and neurons with neurites on half of each coverslip were recorded. A neurite was included for analysis if it was >1 cell body in length.

For β -3-tubulin-stained explant cultures in collagen gels, regions of interest (ROI) adjacent to each ganglion were identified on thresholded black-and-white images. An experimental ROI 750×750 pixels in size was selected on the side of the explant facing the bead. Using ImageJ software (rsbweb.nih.gov/ij/), the total immunofluorescence within the experimental ROI was calculated and compared with a control ROI of the same size on the opposite side of the bead.

To analyze effects of IL-17 on neurite branching complexity, the Sholl analysis (Gutierrez and Davies, 2007) was performed on dissociated SMG neurons cultured for 5 d in the presence or absence of IL-17. Isolated SMG neurons and neurites were fluorescently labeled by incubation in calcein-AM (1 μ M; Invitrogen) for 15 min at 37°C and visualized using a Zeiss axiovert inverted fluorescence microscope. Only neurons with clearly distinguishable processes that were separated from nearby cells to prevent neurite overlapping were analyzed. Micrographs of fluorescently labeled neurons and neurite arbors were acquired using AxioVision camera and software, and subsequently analyzed using a MATLAB 6.5 script (Gutierrez and Davies, 2007) to generate a Sholl plot.

Production of compartmented neuronal cultures

Dissociated SMG neurons were plated in the central chambers of compartmented cultures using a previously described protocol (Campenot et al., 2009). Briefly, compartmented cultures were assembled in laminin-coated (10 μ g/ml) 35 mm Petri dishes using a three-chambered Teflon divider (Tyler Research Corporation) that fluidically isolates neuron cell bodies and proximal axons (PAX) from their distal axons (DAX). The floor of the tissue culture dish was scored into a series of 20 parallel scratches 225 μ m apart, each of which exposed the bare plastic beneath. Twenty-four hours after the plating of neurons, 3–4 ml of L-15 culture medium with NGF (50 ng/ml) and cytosine arabinoside (1 μ M) was added to the perimeter of the PAX compartment. Medium was removed from the DAX compartments and replaced with fresh medium containing NGF (50 ng/ml) plus IL-17 or spleen cell supernatants, or supernatants plus neutralizing antiserum.

Axonal growth into DAX compartments was measured after 5 d in culture. Axons were fluorescently labeled with calcein-AM (1 μ M) and visualized using the 20 $\times$ objective of an inverted fluorescence microscope (Zeiss Axiovert). An ocular micrometer was used to measure the distance from the edge of the silicone grease barrier to the tip of the longest axon in a track. A minimum of 10 tracks per culture were quantified.

Collection of supernatant from activated splenocytes

Spleens were isolated from normal mice and quickly transferred to a vial of chilled RPMI-1640 medium (Invitrogen). Spleens were passed through a 70 μ m pore-size mesh cell strainer (Fisher Scientific) and centrifuged at $400 \times g$ for 10 min. Following removal of supernatant, the red blood cells were lysed (RBC Lysis Buffer) and splenocytes were centrifuged at $400 \times g$ for 10 min. The supernatant was discarded and the cell pellet resuspended in RPMI supplemented with 10% FBS and 5000 IU penicillin and streptomycin. Viable cells were identified by a trypan blue dye exclusion assay, counted using a hemocytometer and plated at 5×10^6 cells/well in a 24-well tissue culture plate. To stimulate release of cytokines of the adaptive immune system, cells were cultured in the presence of the T-lymphocyte mitogen concanavalin A (ConA, 2.5 g/ml) (Gantner et al., 1995; Palacios, 1982; Sharon and Lis, 1989) for 72 h, at which point cell-free supernatants were harvested and stored at -20°C for subsequent assay. Distal axons of SMG neurons in compartmented cultures were incubated in DAX medium containing either supernatant from ConA-stimulated spleen cells or RPMI with ConA (2.5 μ g/ml), NGF, FBS, penicillin, and streptomycin as a vehicle control, at a final dilution of 1:6 for 5 d.

ELISA measurement of IL-17

IL-17 content of activated spleen cell supernatants was determined using a mouse IL-17 sandwich ELISA kit (R & D Systems), according to the manufacturer's instructions. Samples and standards were assayed in duplicate.

Patch-clamp electrophysiology

Dissociated SMG neurons were cultured overnight in the presence or absence of IL-17 (1 ng/ml) and/or other drugs. All electrophysiological recordings were performed at room temperature. To record current through voltage-gated Ca^{2+} channels (I_{Ca} ; carried by Ba^{2+}), coverslips of dissociated neurons were superfused with an external solution of the following composition (mM): 140 tetraethylammonium (TEA)-Cl, 2 MgCl_2 , 5 BaCl_2 , 10 glucose, 10 HEPES (pH 7.4 with TEA-OH). Fire-polished glass pipettes with resistances between 3 and 8 M Ω were back-filled with internal solution containing the following (in mM): 120 CsCl, 1 MgCl_2 , 4 MgATP , 0.3 NaGTP , 10 EGTA, 10 HEPES, and 50 μ g/ml amphotericin B (pH 7.2 with CsOH). Patch pipettes were lowered onto cells visualized with a phase contrast microscope, and once a seal was established (1 G Ω or greater) and perforation had taken place (access resistance <20 M Ω), series resistance was compensated at least 80%. Data were acquired using a Digidata 1440A analog-to-digital converter and pClamp 10.1 software (MDS Analytical Technologies). Current recordings were normalized to cell capacitance and expressed as current density.

Ca^{2+} imaging

Dissociated SMG neurons were incubated with the fluorescent Ca^{2+} -indicator dye Fura-2 AM (5 μ M) for 30–45 min at 37°C. Cells were washed three times and left to cool to room temperature in HEPES buffer [containing the following (in mM): 140 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose, pH adjusted to 7.4 with NaOH] for 20–30 min before imaging to remove extracellular dye and allow for intracellular de-esterification of the Fura. Coverslips were continuously superfused with the HEPES-buffered solution throughout the experiment. ROI were defined either within the cell body of the neuron or within the growth cone for experiments on Day 2. Growth cones were identified visually by their fan-like morphology (Ibarretxe et al., 2007). The cells were illuminated at 340 and 380 nm once per second using a DeltaRamV high-speed random access monochromator (Photon Technology International). Images were recorded using a Photometrics Cascade 512B CCD camera and ImageMaster 5.0 software. The ratio of fluorescence at 340 nm (f_{340}) versus 380 nm (f_{380}) excitation within ROI was calculated and used to estimate $[\text{Ca}^{2+}]_i$. The amplitude of depolarization-induced Ca^{2+} entry, evoked by superfusion of high K^+ external solution of the following composition (mM): 105 NaCl, 40 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose, pH adjusted to 7.4 with NaOH, was compared in control cells and cells cultured in IL-17.

Data analysis

All morphological analyses were performed by observers blind to the treatment groups. Current-voltage relationships were analyzed using Clampfit, Version 10 (MDS Analytical Technologies). Statistical analyses were performed and graphs were generated using GraphPad Prism 5 software. Population data were analyzed by Mann–Whitney or Kruskal–Wallis tests followed by Dunn's multiple comparison posttests for nonparametric data. A two-way ANOVA was used when analyzing current-voltage relationships and Sholl plots, with Bonferroni's *post hoc* test to determine significant differences between the group means. Population data are presented as means $\pm$ SEM. Statistical significance was reached at $p < 0.05$.

Results

Sympathetic neurons express the receptor for IL-17

IL-17 receptor immunoreactivity was localized to the somata of sympathetic neurons in sections of mouse SMG (Fig. 1A). Ninety-five $\pm$ 2% of TH-IR SMG neurons were immunoreactive for IL-17R, and 84 $\pm$ 3% of IL-17R-immunoreactive cells were TH-IR ($n = 6$ mice for each count). Furthermore, isolated SMG neurons cultured for 3 d exhibited IL-17R expression within the soma and along neurites (Fig. 1B).

Figure 1. Neurons from SMG express the receptor for IL-17. **A**, Immunofluorescent labeling of cryostat sections of SMG revealed abundant IL-17 receptor expression within neuronal perikarya. Receptor immunoreactivity was also observed in cultured SMG neurons **B**, IL-17 receptor expression was localized to cell bodies, neurites, and growth cones. Scale bars, 50 μ m.

Supernatant from activated T lymphocytes evokes neurite outgrowth that is inhibited by neutralization of IL-17

Neurons within the SMG project axons to the spleen, where they are thought to play an immunomodulatory role (Rosas-Ballina et al., 2011). We therefore determined the effect of exposing SMG neurons to the inflammatory mediators released from activated splenocytes. *In vitro* restimulation of isolated spleen cells by ConA induces polyclonal T cell differentiation, leading to the release of cytokine mediators of the adaptive immune response (Palacios, 1982; Schulz et al., 2008). We incubated distal SMG neurites in supernatant obtained from activated T lymphocytes for 5 d and measured neurite extension in compartmented cultures. Sympathetic neurites exposed to the supernatant were significantly longer than neurites incubated in the vehicle control (Fig. 2). Undiluted supernatant collected from activated T lymphocytes contained a high concentration of IL-17 (48.32 ± 14.41 ng/ml, $n = 5$ supernatants from 5 mice), as determined by ELISA. Given a 1:6 dilution of the supernatant in media, the final concentration of IL-17 in the media was ~ 8 ng/ml. Neutralization of IL-17 activity by addition of an IL-17 function-blocking antibody [2.5 μ g/ml, a concentration previously reported to block IL-17 signaling (Yao et al., 1995a,b); R & D Systems] blocked neurite outgrowth in response to splenocyte supernatant (Fig. 2). Splenocyte supernatant still exerted a significant neurotrophic

Figure 2. Supernatants from cultured splenocytes exerted an IL-17-dependent neurotrophic effect on SMG neurons. Concanavalin A-stimulated splenocyte supernatant was added to distal compartments of SMG neurons in compartmented cultures and markedly enhanced levels of neurite extension relative to neurites cultured in the vehicle control for 5 d. Blocking IL-17 activity with a neutralizing antibody had no effect on neurite outgrowth when added alone but eliminated the ability of splenocyte supernatants to promote sympathetic axon outgrowth. An isotype-matched control antiserum did not block the neurotrophic effect of splenocyte supernatant. $N > 40$ lanes per treatment group. *** $p < 0.0001$ versus all other treatment groups except for supernatant plus control IgG; # $p < 0.05$ versus all other treatment groups except for supernatant; Kruskal–Wallis test with Dunn's multiple comparison posttest.

effect on neurites cultured in the presence of an isotype control IgG (Abcam) relative to control cultures.

Interleukin-17 enhances neurite outgrowth and arborization

We next investigated whether IL-17 could induce sympathetic axon remodeling using several *in vitro* assays of neurite outgrowth (Fig. 3). The ratio of neurites to neurons was significantly increased among neurons exposed to recombinant mouse IL-17 (1 ng/ml) compared with control neurons at Days 2 and 3. Incubation in IL-17 did not have any effect on the number of neurons, identified by HuD immunoreactivity (control: 211 ± 58 neurons per coverslip, $n = 8$; IL-17: 190 ± 89 neurons per coverslip, $n = 7$; $p > 0.05$, Mann–Whitney test). Similarly, the number of glial cells per coverslip (identified by SOX-10 immunoreactivity) was not affected by incubation in IL-17 for 7 d (control: 5116 ± 975 cells, $n = 5$; IL-17: 4677 ± 266 cells, $n = 5$; $p > 0.05$, Mann–Whitney test). These data indicate that IL-17 does not affect neuronal or glial survival. Sholl analysis of neurite arborization was performed on neurons cultured for 5 d in the presence or absence of IL-17. This time point was chosen to allow significant neurite outgrowth without resulting in overlap between the neurites of adjacent neurons. Sholl analysis demonstrated that neurons incubated in IL-17 had increased morphological complexity relative to control neurons (Fig. 3D). The number of intersections between neurites and a series of concentric rings superimposed on the neural soma were statistically increased between 75 and 210 μ m from the cell body in IL-17-treated cultures compared with control cultures.

Enhanced neurite growth does not depend on glial cells

Glial cells present in the SMG cultures may mediate the effect of IL-17 on SMG neurite outgrowth. To create enriched neuronal cultures with little to no glia, 5 μ M cytosine arabinoside (AraC) (Besirli et al., 2003) was added after 24 h of culture. AraC depleted

SOX-10-immunoreactive glia by $65 \pm 7\%$ at Day 3 and $89 \pm 2\%$ at Day 7 ($n = 4$ coverslips for control, 6 for IL-17). IL-17 significantly increased the number of neurites per neuron at Days 2, 3, and 7 in the presence of AraC (Fig. 4) to an extent that was not different from IL-17 alone.

Inhibition of Ca^{2+} currents by IL-17

An important mechanism of neurite growth regulation is the control of intracellular $[\text{Ca}^{2+}]$ (Bolsover, 2005; Spitzer, 2006). Electrophysiological and Ca^{2+} imaging studies were therefore undertaken to determine whether IL-17 caused alterations in I_{Ca} . Overnight incubation with IL-17 significantly attenuated inward voltage-gated Ca^{2+} current, as measured by voltage-clamp electrophysiology (Fig. 5*A,B*). Intracellular Ca^{2+} imaging in both the cell bodies (Fig. 5*C*) and growth cones (Fig. 5*D*) revealed that the peak f340:f380 increase as a percentage of baseline during depolarization with 40 mM extracellular K^+ was significantly lower in neurons cultured overnight in the presence of IL-17.

Attenuating I_{Ca} causes enhanced growth

If I_{Ca} inhibition is involved in the neurite growth induced by IL-17, then the effects of IL-17 on neurite outgrowth should be replicated by an I_{Ca} blocker. To inhibit inward I_{Ca} , we chose a concentration of ω -conotoxin GVIA (30 nM) that would partly block the current through the N-type VGCC, which is the predominant type of VGCC in SMG neurons (Motagally et al., 2009a,b). Incubation of SMG neurons for up to 3 d recapitulated the enhanced neurite outgrowth seen with IL-17 treatment (Fig. 5*E*).

Electrophysiological and neurotrophic effects of IL-17 are reversed by an NF- κB inhibitor

The NF- κB intracellular signaling pathway plays a key role in the induction of many inflammatory signaling cascades (Shen and Gaffen, 2008). Using SC-514, which blocks the $\text{I}\kappa\text{B}$ kinase activation and subsequent activity in the NF- κB signaling cascade, we investigated whether the effects of IL-17 on I_{Ca} and neurite outgrowth were dependent on NF- κB signaling. Preincubation of cultured neurons in SC-514 (20 μM) 2 h before addition of IL-17, and throughout incubation with IL-17, almost completely prevented I_{Ca} inhibition by IL-17 (Fig. 6*A*). We have previously shown that SC-514 alone has no effect on I_{Ca} (Motagally et al., 2009a), suggesting that the effect of SC-514 was on intracellular signaling downstream of IL-17 receptor activation, rather than a direct effect on I_{Ca} . Consistent with these voltage-clamp data, Ca^{2+} imaging analysis showed that cultures pretreated with SC-514 before IL-17 had significantly larger increases in f340:f380 compared with IL-17 treatment alone (data not shown). Importantly, inhibition of NF- κB also mitigated the effects of IL-17 on neurite outgrowth (Fig. 6*B*).

Figure 3. IL-17 enhanced neurite outgrowth and branching. *A,B*, Representative images of control (*A*) and IL-17 (1 ng/ml)-treated (*B*) SMG cultures at Day 7 *in vitro*. Cultures were fixed and immunostained for HuD (a neuronal marker, green) and SNAP-25 (an axonal marker, red). Scale bars, 25 μm . *C*, Cultures incubated in IL-17 had significantly more neurites per neuron compared with controls after 3 d in culture. * $p < 0.05$, Kruskal–Wallis test plus Dunn’s multiple comparison posttest; $N = 6$ coverslips from at least three mice per treatment group. *D*, Sholl analysis of neurite morphology after 5 d of IL-17 treatment revealed that neurite arborization was significantly greater in neurons incubated in IL-17 compared with controls. $N = 40$ –60 neurons per treatment group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, two-way ANOVA with Bonferroni’s posttest.

Figure 4. Neurotrophic effects of IL-17 did not depend on glial cells. When the glial cell population was depleted with AraC (5 μM), IL-17 still significantly enhanced the number of neurites per neuron after 3 and 7 d in culture. $N =$ at least 6 coverslips per treatment group, * $p < 0.05$ AraC versus AraC plus IL-17; # $p < 0.05$ AraC versus IL-17; Kruskal–Wallis test with Dunn’s multiple comparison posttest.

Figure 5. IL-17 inhibited voltage-gated Ca^{2+} current. **A**, Representative voltage-clamp recordings of I_{Ca} from a control neuron and a neuron incubated in IL-17 overnight. The voltage-clamp protocol is illustrated in inset. **B**, Mean $\pm$ SEM current voltage relations of control and IL-17-treated neurons. Incubation of neurons in 1 ng/ml IL-17 significantly reduced I_{Ca} between -10 and 10 mV. $N = 21$ control cells, 13 IL-17; $*p < 0.05$, two-way ANOVA with Bonferroni's posttest. **C**, Ca^{2+} -imaging data demonstrate a significant decrease in the peak intracellular Ca^{2+} transient during a 40 mM KCl-induced depolarization in neurons incubated in IL-17 compared with controls. $N = 34$ cells per treatment group; $***p < 0.0001$, Mann–Whitney test. **D**, IL-17 significantly attenuated depolarization-induced Ca^{2+} influx in SMG neuronal growth cones. $N = 8$ cells for control, 10 cells for IL-17; $*p < 0.05$, Mann–Whitney test. **E**, Treatment of SMG neurons with the N-type Ca^{2+} channel blocker ω -conotoxin GVIA (30 nM) significantly enhanced the ratio of neurites to neurons compared with controls. $*p < 0.05$ relative to control; two-way ANOVA with Bonferroni's *post hoc* test; $N = 6$ coverslips per treatment group.

Figure 6. Inhibition of NF- κ B signaling blocked the effects of IL-17 on I_{Ca} and neurite outgrowth. **A**, Current voltage curve depicting the effect of blocking the NF- κ B pathway on I_{Ca} inhibition by IL-17. With SC-514 pretreatment, control and experimental values were not significantly different ($N = 21$ for control, 13 for IL-17, 12 for SC-514; $p > 0.05$ between control and SC-514, two-way ANOVA; $p < 0.05$ between control and IL-17 from -10 to $+10$ mV; Fig. 4). **B**, Pretreatment with SC-514 prevented the enhanced neurite outgrowth caused by IL-17. In SC-514-treated coverslips, the ratio of neurites to neurons in the presence of IL-17 was not significantly different from control ($*p > 0.05$, Mann–Whitney test).

IL-17 is chemotactic for extending neurites

Using SMG explants seeded onto collagen gels, the growth of neurites toward an IL-17 (100 ng/ml)-soaked agarose bead was examined. Directional growth toward the cytokine-soaked bead was significantly increased relative to an HBSS-soaked negative

control bead (Fig. 7A–C). In addition, we used the compartmented culture system to further localize the effects of IL-17 on SMG neurite proliferation by selectively applying the cytokine to cell bodies and PAX or DAX for 5 d (Fig. 7D). Addition of IL-17 to either the PAX or DAX compartments resulted in significantly increased axonal outgrowth relative to control cultures. Interestingly, selective application of IL-17 to distal terminals significantly enhanced axonal length relative to neurons exposed to the cytokine in the cell body compartment.

Discussion

The $\text{T}_{\text{H}}17$ lineage of T lymphocytes is increasingly recognized as playing a central role in autoimmune diseases that are accompanied by neuronal dysfunction, including IBD, rheumatoid arthritis, and multiple sclerosis (MS). An elegant study of the experimental autoimmune encephalitis model of MS in mice found compelling evidence of a contribution of $\text{T}_{\text{H}}17$ lymphocytes to neuronal dysfunction within brainstem plaques (Siffrin et al., 2010). IL-17 is the canonical cytokine released from $\text{T}_{\text{H}}17$ lymphocytes, yet little was known before the current study about its effects on neurons. Our findings identify a novel role for IL-17 in increasing neurite outgrowth from adult sympathetic postganglionic neurons by activation of NF- κ B signaling and suppression of voltage-dependent Ca^{2+} influx.

IL-17-secreting immune cells contribute to the pathogenesis of inflammatory disease by amplifying effector immune responses that ultimately give rise to tissue damage (Langrish et al., 2005; Park et al., 2005; Yen et al., 2006; Siffrin et al., 2010). In contrast to these damaging effects, supernatant from activated T cells had significant neurotrophic effects when applied to the axons of SMG neurons. A neutralizing antibody that blocked IL-17 function (Yao et al., 1995a,b) abolished axon extension in the presence of T lymphocyte supernatants. This is consistent with a recent study of corneal nerve regeneration following corneal abrasion, where restoration of sub-basal nerve plexus density was attenuated by $>50\%$ following systemic treatment with

an IL-17 function-blocking antibody (Li et al., 2011). However, the neurotrophic effects of IL-17 in the cornea were attributed to the arrival of trophic factor-secreting neutrophils at the wound site, whereas our data suggest that IL-17 can exert a direct neurotrophic effect on SMG neurons. Thus, our current findings, along with those of Li et al. (2011), suggest that IL-17 can participate in neural tissue remodeling and repair via direct or indirect signaling mechanisms.

Mechanisms of IL-17 effects on SMG neurons

The link between Ca^{2+} influx and regulation of neurite outgrowth is well established (Bolsover, 2005; Spitzer, 2006), and it appears that in many systems, a rise in $[\text{Ca}^{2+}]_i$ precludes neurite sprouting. A recent study of *Xenopus* motor neurons implicated N-type VGCCs in the ability of the growing neurite to transduce a stop signal upon encountering the appropriate target extracellular matrix (Sann et al., 2008). Similarly, Ryan et al. (2007) demonstrated that depolarization inhibits neurite outgrowth from descending lamprey brain neurons, and that this inhibition is due to inward current through N-type VGCCs. Consistent with these findings, incubation of SMG neurons in IL-17 inhibited Ca^{2+} currents through VGCCs in cell bodies and growth cones. Interestingly, incubation of SMG neurons with ω -conotoxin-GVIA increased the growth of neurites to a level similar to that seen following IL-17 incubation. These data suggest that inhibition of VGCC may contribute to neurite outgrowth from SMG neurons in response to IL-17. VGCC inhibition alone is not sufficient to explain how IL-17 promotes neurite outgrowth from adult SMG neurons, because $\text{TNF}\alpha$ also inhibits VGCC in these neurons (Motagally et al., 2009a) but has no impact on neurite outgrowth (data not shown). However, $\text{TNF}\alpha$ has inconsistent effects on Ca^{2+} influx and neurite outgrowth in postganglionic sympathetic neurons. In contrast to adult SMG neurons, VGCC is enhanced by $\text{TNF}\alpha$ in neonatal superior cervical ganglion (Soliven and Albert, 1992), a population of neurons whose neurite outgrowth is inhibited by $\text{TNF}\alpha$ (Gutierrez et al., 2008). These inconsistencies may be related to the differences in growth responses between adult and neonatal neurons or the status of NF- κ B signaling. Nonetheless, it is noteworthy that, similar to the effect of IL-17 on adult SMG neurons, sympathetic neurite outgrowth from neonatal SCG neurons in response to $\text{TNF}\alpha$ is associated with changes in Ca^{2+} influx pathways.

Inhibition of the NF- κ B pathway prevented both the IL-17-induced neurite outgrowth and alterations in I_{Ca} . These data suggest mechanistic commonality between the two effects of IL-17. Although IL-17 may inhibit inward Ca^{2+} current and enhance neurite growth through separate pathways downstream of NF- κ B activation, the likeliest explanation for these results is that

Figure 7. Neurites of SMG neurons grew preferentially toward sources of IL-17. Analysis of β 3-tubulin immunoreactivity in SMG explants cultures revealed enhanced neurite outgrowth in the direction of an IL-17-soaked bead relative to control ($N = 5$ per group). Bead location is marked with a circle on the representative micrographs. **A, B**, Control (HBSS-soaked) bead (**A**) and IL-17 (100 ng/ml)-soaked bead (**B**). Scale bars, 25 μm . **C**, Mean $\pm$ SEM ratio of fluorescence from regions of interest in the quadrant of the micrograph closest to the bead relative to the quadrant on the opposite side of the micrograph (opposite quadrants not shown in **A** and **B**). * $p < 0.05$, Mann–Whitney test. **D**, Dissociated SMG neurons were added to compartmented cultures to examine the effect of adding IL-17 (1 ng/ml) to either the cell bodies and PAX, or the DAX. Application of IL-17 to distal axon compartments significantly enhanced axonal extension after 5 d relative to neurons cultured in control medium and neurons exposed to the cytokine in the cell body compartment (*** $p < 0.0001$ vs control; ### $p < 0.0001$ vs PAX; Kruskal–Wallis test with Dunn’s multiple comparison posttest; $N > 30$ lanes per treatment).

NF- κ B-dependent regulation of neurite outgrowth and Ca^{2+} signaling are interrelated. We propose a model wherein NF- κ B-dependent inhibition of N-type VGCC relieves a constitutive stop signal and results in increased growth and elaboration of sympathetic neurites. The involvement of NF- κ B signaling in regulating neurite outgrowth is consistent with reports on neonatal neurons from cortex, nodose ganglion, and superior cervical ganglion (Gutierrez et al., 2005, 2008; Gutierrez and Davies, 2011) and suggests an important role for this intracellular signaling network in neuroanatomical plasticity that extends beyond the developing nervous system.

Certain cytokines (IL-4, IL-6, and interferon- γ) enhance the growth of neurites initiated by endogenous and recombinant neurotrophins (Gölz et al., 2006; O’Keeffe et al., 2008). NGF was present in all culture media, as postganglionic neurons cannot survive without it. Therefore, it is possible that IL-17 is acting synergistically by enhancing the actions of NGF. We examined this possibility using real-time reverse transcription PCR to quantify mRNA encoding TrkA and p75 receptors for NGF. Overnight incubation of SMG neurons with IL-17 had no effect on TrkA or p75 expression (data not shown). Furthermore, since

inhibition of NF- κ B reversed the effect of IL-17, and NGF does not predominantly act via this pathway (Neet and Campenot, 2001), it is plausible that IL-17 enhances neurite outgrowth via a novel mechanism that may or may not overlap with NGF signaling. However, structural homologies between NGF and IL-17 dimers exist in locations corresponding to receptor-binding domains (Hymowitz et al., 2001), suggesting similar receptor–ligand signaling properties between the two molecules. NGF-mediated neuronal survival is dependent on retrograde axonal signaling pathways following NGF binding to cognate receptors on distal axon terminals, though the mechanistic nature of this signal remains to be fully understood (Neet and Campenot, 2001; Barker et al., 2002). In a similar fashion, IL-17 acting at distal sympathetic terminals may support axonal growth by retrograde transport mechanisms that involve downstream activation of NF- κ B at the neuronal cell body. Additional studies will be required to fully characterize the precise molecular events mediating IL-17-induced changes in sympathetic axon architecture.

IL-17 can act directly on sympathetic axons to drive outgrowth

Evidence from the current study suggests that IL-17 can act directly on SMG neurons, rather than glial cells, to drive neurite elongation *in vitro*. The receptor for IL-17 is localized to TH-immunoreactive neuronal cell bodies and neurites. The effects of IL-17 on SMG neurite growth *in vitro* were most pronounced when IL-17 was applied selectively to distal axons in the complete absence of glia. Furthermore, the directional outgrowth of sympathetic axons in explant cultures toward a source of IL-17 is consistent with a direct action of IL-17 on sympathetic axons. Together, these data suggest that, *in vivo*, sympathetic axons that innervate inflamed tissues may be exposed to elevated levels of IL-17 that can act directly on nerve terminals to drive outgrowth, which may contribute to sympathetic neuroanatomical remodeling during inflammation. However, it is still possible that circulating IL-17 acts in part via glial cells within sympathetic ganglia to enhance axon outgrowth.

Comparison with the conditioning lesion effect

One of the best characterized examples of cytokine involvement in sympathetic neuroanatomical plasticity is their participation in neurite outgrowth following a conditioning lesion, such as axotomy (McQuarrie and Grafstein, 1973; McQuarrie et al., 1978). The conditioning lesion enhances the expression of cytokines of the gp130 family, which includes leukemia inhibitory factor, IL-6, IL-11, and oncostatin M, in tissues innervated by the axotomized sympathetic neurons and in the sympathetic neurons themselves (Hyatt Sachs et al., 2010). These cytokines then contribute to regeneration of damaged axons following nerve injury. While the enhancement of sympathetic neurite outgrowth by IL-17 is reminiscent of the conditioning response, several observations suggest they are two independent processes. First, IL-17 does not contain the gp130 protein motif, which is a prerequisite for mediators of the conditioning lesion effect on sympathetic neurons (Hyatt Sachs et al., 2010). Second, the conditioning lesion response predominantly uses the janus kinase-signal transducer and activator of transcription 3 pathways (Rajan et al., 1995; Habecker et al., 2009; Hyatt Sachs et al., 2010), in contrast to the NF- κ B-dependent effect of IL-17. Therefore, we propose that IL-17 mediates a neuroanatomical response distinct from the effect of conditioning lesions.

Interleukin 17, T_H17 diseases, and neuronal alterations

The discovery of the T_H17 lineage of T helper lymphocytes, defined by their production and secretion of IL-17, has led to an appreciation of the role that this cytokine plays in several immune-mediated diseases. While the general consensus is that IL-17 is a damaging, proinflammatory cytokine, there is emerging evidence that it plays a role in homeostasis and tissue repair (Littman and Rudensky, 2010). Our findings demonstrate a novel neurotrophic effect of IL-17 on adult sympathetic neurons that appears to be dependent on NF- κ B activation and inhibition of intracellular Ca²⁺ dynamics. The finding that IL-17 promotes neurite arborization may provide a mechanism for some of the morphological and functional alterations in the SNS that occurs in inflammatory diseases dominated by a T_H17 cytokine profile. For example, in murine models of arthritis and colitis, both of which are mediated by IL-17-producing CD4⁺ lymphocytes (Murphy et al., 2003; Nakae et al., 2003; Roark et al., 2007), sympathetic innervation density is enhanced (Almarestani et al., 2008). It is also interesting to consider these findings in the context of recent reports of sympathetic suppression of the innate immune response (Rosas-Ballina et al., 2008; Vida et al., 2011) and enhancement of IL-17 secretion from T lymphocytes (Kim and Jones, 2010; Manni et al., 2011). If IL-17 does attract axons toward a cellular source of IL-17, it may create a positive feedback loop that drives further IL-17 secretion. Together, our findings identify IL-17 as a potentially important mediator of sympathetic neuroanatomical plasticity during inflammation and emphasize that sympathetic axons are not static entities, but rather dynamic structures capable of adaptive responses to changes in immune system activation.

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