Growth and Turning Properties of Adult Glial Cell–Derived Neurotrophic Factor Coreceptor α1 Nonpeptidergic Sensory Neurons

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Abstract
An overlapping population of adult primary sensory neurons that innervate the skin express the glial cell–derived neurotrophic factor coreceptor α1 (GFRα1), the lectin IB4, and the “regenerative brake” phosphatase and tensin homolog deleted on chromosome 10. Using an adapted turning and growth assay, we analyzed the growth cone behavior of adult immunoselected GFRα1 sensory neurons. These neurons had less robust baseline growth and reluctant responsiveness to individual growth factors but responded to synergistic types of input from glial cell–derived neurotrophic factor, hepatocyte growth factor, a phosphatase and tensin homolog deleted on chromosome 10 inhibitor, or a downstream Rho kinase inhibitor. Hepatocyte growth factor and the phosphatase and tensin homolog deleted on chromosome 10 inhibitor were associated with growth cone turning. A gradient of protein extracted from skin samples, a primary target of GFRα1 axons, replicated the impact of synergistic support. Within the skin, glial cell–derived neurotrophic factor was expressed within epidermal axons, indicating an autocrine role accompanying local hepatocyte growth factor synthesis. Taken together, our findings identify unique growth properties and plasticity of a distinct population of epidermal axons that are relevant to neurologic repair and skin reinnervation.

Key Words: Epidermis, Glial cell–derived neurotrophic factor (GDNF), Growth cones, Growth cone turning, Hepatocyte growth factor (HGF), Nonpeptidergic sensory neurons, PTEN, Regeneration.

INTRODUCTION
In adults, the ongoing growth and plasticity of sensory axons in the epidermis allow repair after normal keratinocyte sloughing, wounding, or nearby axon loss. Epidermal axons are highly irregular, with turns and twists, and are intimately inserted in between individual keratinocytes that undergo turnover (1). The trajectories of these axons betray ongoing growth. A high proportion also expresses the growth protein growth-associated protein 43, which is usually associated with regenerating axons (2, 3). Simple interventions, such as hair removal, are linked to local cutaneous axon growth—a property associated with hepatocyte growth factor (HGF) and activated Rac1 (2).

Two major classes of unmyelinated axons innervate the epidermis: TrkA nerve growth factor (NGF)–responsive axons and a nonpeptidergic group of axons that express receptors for glial cell–derived neurotrophic factor (GDNF), GDNF coreceptor α1 (GFRα1), and Ret. Sensory neurons expressing GFRα1 have extensive overlap with neurons binding to Griffonia simplicifolia lectin (IB4) (4). The proportions of skin innervation are estimated to be 40% to 70% IB4 and 43% to 51% TrkA or calcitonin gene–related peptide (5–9). Both axon types are important mediators of nociceptive information, but their growth and plasticity behavior seem to differ (10–16). TrkA axons in adults exhibit ongoing growth and turning responsiveness to a gradient of NGF (17). In contrast, IB4 neurons have been reported to exhibit attenuated growth in vitro and in vivo (18, 19). Moreover, this population expresses high levels of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a brake on regenerative outgrowth and phosphorylatedinositol 3-kinase (PI3K) signaling that might account for some of their reluctant growth properties (11, 20).

In the skin epidermis, entering axons lose their intimate association with Schwann cells, a source of trophic support (21). Without it, axons might fail to advance beyond the dermis. The property of collateral sprouting is thought to relate to increases in nearby NGF levels in denervated skin, which in turn attract neighboring axons (22, 23). However, a progressive loss of keratinocytes, as they move outward and are shed, may restrict the growth factor supply. It is also possible that the plasticity of epidermal axons is self-limited.

In this work, we explored the short-term growth and behavioral properties of adult neurons that express GFRα1. Dorsal root ganglion (DRG) neurons were immunoselected and analyzed using the growth cone turning assay described by Lohof et al (24) and Zheng et al (25) in developing neurons. We confirmed not only that these neurons show attenuated growth in a short-term assay but also that they require a
combination of stimuli to advance and exhibit limited turning, properties that may be accounted for by GDNF and HGF expression in the epidermis.

MATERIALS AND METHODS

Animals and Preconditioning

Adult male Sprague-Dawley rats (Charles River Laboratories, Sherbrooke, Quebec, Canada) with an initial weight of 170 to 200 g (aged 2 months) were housed in plastic cages with a normal light/dark cycle and had free access to rat chow and water. All protocols were reviewed and approved by the University of Calgary Animal Care Committee using Canadian Council on Animal Care guidelines. Experiments were designed to minimize the number of animals required and to minimize animal suffering as per Canadian Council on Animal Care guidelines. Rats were anesthetized with isoflurane (Halocarbon Laboratories, River Edge, NJ). Under sterile conditions, the left sciatic nerve was cut at the midthigh level, the skin wound was sutured with 3-0 silk suture (Ethicon Inc, Irvine, CA), and the rats were caged individually for 3 days, with buprenorphine jello provided once a day after the injury for analgesia.

Adult Sensory Neuron Cultures

Before tissue harvesting, the rats were anesthetized using isoflurane and then killed using an overdose of the same agent. Dorsal root ganglion neurons were dissociated and maintained in vitro using the method of Lindsay (26), with modification. Briefly, L4 to L5 DRG were removed from the rats and placed in L15 medium (Invitrogen, Burlington, Canada), where the axon roots and dural tissue were manually removed. The DRG were rinsed 3 times in L15 medium and then transferred to a tube containing 2 mL of 0.1% collagenase (Invitrogen) in L15 medium. After a 90-minute incubation at 37°C, the DRG neurons were placed into single-cell suspension by trituration 10 to 15 times every 7 minutes through three 1-mL pipette tips. The single-cell suspension was spun for 5 minutes at 800 rpm at 4°C, the cell pellet was washed 3 times in 2 mL of L15. After the final 5,800 rpm spin, the cells were resuspended in L15 medium and passed through a 70-km mesh (VWR International Co, Mississauga, Canada) to isolate the neuron–glial cell population from myelin and tissue debris, spun 1 more time, and then placed in 500 μL of L15 medium enriched with a 1:100 dilution of N2 supplement and 0.1% bovine serum albumin (BSA; Sigma Aldrich, Oakville, CA) for Dynal bead isolation of GFRα1-positive neurons.

GFRα1-Bound Dynal bead Production and Isolation

To prepare Dynal magnetic beads, we modified the following procedure from Dynal Biotech (Invitrogen) along the lines published by Tucker et al (10). Briefly, for each isolation, 25 μL of beads was transferred to a 2-mL tube; after the tubes had been placed in the Dynal magnetic particle concentrator (MPC), their buffer was removed. The beads were washed once with 1 mL of 0.1% BSA in Dulbecco phosphate-buffered saline (PBS; Invitrogen) and then resuspended in 100 μL of 0.1% BSA in PBS. To link the GFRα1 antibody with the magnetic beads, we added 0.5 μg of mouse anti-GFRα1 antibody (R&D Systems, Minneapolis, MN) and incubated the tubes at room temperature (RT) for 60 minutes with gentle rotation. After incubation, the tubes were placed in the Dynal MPC for 1 minute, the buffer was removed, and the beads were washed 3 times with 1 mL of 0.1% BSA in PBS. Finally, the GFRα1-bound magnetic beads were resuspended in 100 μL of 0.1% BSA in PBS and stored at 4°C for up to 1 month. The precoated beads were washed with 0.1% BSA in 1 mL of PBS, the buffer was removed after the tubes had been placed in the magnet for 1 minute, and 0.5 mL of DRG neurons (suspended in a 1:100 dilution of N2 supplement and 0.1% BSA in L15 medium) was added to each tube. To promote GFRα1 receptor–GFRα1 antibody interaction, we incubated the cells at RT for 60 minutes, with gentle tilting every 3 to 5 minutes. To isolate the GFRα1-expressing DRG neurons, we placed the tubes in the Dynal MPC for 2 minutes and washed the cells 3 times in 1 mL of 0.1% BSA in L15 medium (the neurons not expressing GFRα1 did not bind to the beads or subsequently to the magnet and were thus removed). The isolated DRG neurons were resuspended in 200 μL of L15 medium containing a 1:100 dilution of N2 supplement and 0.1% BSA, and 4 μL of DNAse releasing buffer was added to cleave the DNA linker of the magnetic beads and thus isolate the DRG neurons, which were still bound to the mouse GFRα1 antibody. To encourage the breaking of the interaction between the magnetic beads and the DRG neuron/GFRα1 antibody complex, we placed the cells at RT for 15 minutes, with gentle tilting every 2 to 3 minutes, and then gently triturated them through a 200-μL pipette tip 15 times; finally, we placed the tube in the Dynal MPC for 2 minutes. The supernatant (containing the GFRα1-expressing DRG neurons still bound to the GFRα1 antibody) was plated onto acid-washed coverslips coated with poly-1-lysine and 10 μg/mL laminin; after a 10-minute incubation to promote cell seeding, 2 mL of growth medium—consisting of DMEM/F12 (Invitrogen) with 2% B27, 0.5% BSA, 10 μg/mL Ca2+ (Sigma Aldrich), 50 U/mL penicillin (Invitrogen), and 50 U/mL streptomycin (Invitrogen)—was added and changed at 1 day in vitro. We confirmed that the remaining anti-GFRα1 antibody was absent after immunoselecting by labeling with a secondary antibody only (immunocytochemistry discussed later) and failing to identify any labeling. Second, we noted that almost all isolated neurons (>90%) were immunolabeled with the anti-GFRα1 primary antibody (as discussed later).

Growth Cone Turning Assay

The procedure was modified for adult mammalian DRG neurons from previous embryonic neuron cultures in Xenopus laevis (24, 25, 27), as previously described in adults (17, 28). To acquire sufficient immunoselected GFRα1 neurons, we harvested 8 DRG samples from 4 rats to generate 6 plates for analysis at a given setting. For unselected neurons, 2 DRG samples were harvested from 1 rat to generate 6 plates. Briefly, different gradients were created and maintained with a Picospritzer II (Parker Hannafin, Fairfield, NJ) ejecting at a pressure of 3 psi and a frequency of 2 Hz for 20 milliseconds using a pulse generator (SD9; Grass Instrument Co, Quincy, MA) from a micropipette with an opening of 0.5 to 1 μm. The micropipette...
was defined as a branch arising from a primary branch. The main advancing growth cone process, and secondary branch numbers in a given experimental day to the total growth cones examined (ratio of branches to growth cones as percentage). The ratio (in percentage) of primary branches was measured. For branches, we calculated the percentage of total growth cones examined that exhibited a branch (percentage of growth cones with branches) and the ratio of primary or secondary branches (percentage of branches with respect to the last 10-μm segment of the axon shaft). By this method, stable concentration gradients can be established from the micropipette, and at a distance of 50 to 100 μm, the growth cones encounter a concentration approximately 100- to 1,000-fold lower than in the pipette. The turning angles of turning from the initial trajectory made during the 60-minute exposure to the concentration of gradients were measured. For branches, the calculated percentage of total growth cones examined that exhibited a branch (percentage of growth cones with branches) and the ratio of primary or secondary branch numbers in a given experimental day to the total growth cones examined (ratio of branches to growth cones as percentage). The ratio (in percentage) of primary branches was higher than the percentage of growth cones with branches because a number of neurons had more than 1 primary branch. Primary branch was defined as a branch originating from the main advancing growth cone process, and secondary branch was defined as a branch arising from a primary branch.

**Immunocytochemistry and Immunohistochemistry**

Neurons were fixed with 4% warmed paraformaldehyde for 15 minutes followed by PBS, given twice for 15 minutes each. Next, the dishes were blocked with 10% goat serum (0.3% Triton X100/PBS) for 60 minutes at RT. Neurons and neurites were stained with 1-hour incubation of primary antibodies listed in the Table at RT. After washing with PBS twice for 15 minutes each, secondary antibodies (Table) were added for 60 minutes at RT. To label the mouse anti-GFRα1 antibody used for GFRα1 DRG cell isolation (which might still be bound to GFRα1 receptor on the DRG neurons), we incubated the cultures for 1 hour with Cy3 sheep anti-mouse secondary antibody 1:100 (Sigma Aldrich). Subsequently, the unbound GFRα1 receptor was labeled for 1 hour with rabbit polyclonal/anti-GFRα1 antibody 1:10. The chambers were then washed 3 times with 1× PBS and incubated with Alexa Fluor 488 goat anti-rabbit secondary antibodies 1:200 for 1 hour at RT. After the slides had been washed 3 times with PBS, they were mounted with DAPI and viewed with a fluorescent microscope (Zeiss Axioskope) at 400×.

For immunohistochemistry, published methods were followed (20). In brief, DRG were harvested and fixed in modified Zamboni fixative (2% paraformaldehyde, 0.5% picric acid, and 0.1% PBS) overnight at 4°C and then cryoprotected in 20% sucrose/PBS. After DRG had been embedded in optimum cutting temperature compound (Miles), 14-μm-thick sections were placed onto poly-l-lysine-coated glass slides. Primary and secondary antibodies used are given in the Table. Primary antibodies applied were incubated at 4°C for 24 hours. The following day, the slides were washed with PBS and incubated with secondary antibodies, anti-mouse immunoglobulin G (IgG) CY3 conjugate (1:100; Sigma, St. Louis, MO), or Alexa Fluor 488 goat anti-rabbit IgG (H + L) conjugate (1:200; Cedarlane, Burlington, Canada) for 1 hour at RT. Finally, cover slips were mounted on the slides with bicomponent-buffered glycerol (pH 8.6), and the slides were viewed with a fluorescent microscope (Zeiss Axioskope). Negative controls included omission of primary antibodies on parallel sections (data not shown). Pixel intensity measurements and analysis

### TABLE. Antibodies

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were performed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA). Foot pad (FP) skin samples were harvested using a skin punch and fixed in 2% PLP (2% paraformaldehyde, l-lysine, and sodium periodate) for 18 hours at 4°C and cryoprotected overnight in 20% glycerol/0.1 mol/L Sorenson phosphate buffer at 4°C. Foot pad samples were sectioned at 25 μm and blocked in 10% goat serum for 1 hour at RT. Sections were incubated with unjugated IB4 lectin (10 μg/mL; Vector Laboratories, Burlingame, CA) for 1 hour and then incubated with primary antibodies for 48 hours at 4°C. Secondary antibodies were used were goat anti-rabbit Cy3 (1:100; Jackson ImmunoResearch, West Grove, PA) and donkey anti-goat 488 (1:200; Invitrogen) for 1 hour at RT. Images were captured using an Olympus laser scanning confocal microscope (100× magnification and 1 μm step size). Epidermal fibers labeled with GDNF and IB4 were imaged.

**Quantitative Reverse Transcription–Polymerase Chain Reaction**

Quantitative reverse transcription–polymerase chain reaction was performed according to previous descriptions (20). Briefly, total RNA was extracted using Trizol reagent (Invitrogen), and first-strand DNA was synthesized using SuperScript II First-Strand Kit (Invitrogen). Real-time quantitative polymerase chain reaction was performed on the Step One Plus sequence detection system (Applied Biosystems, Foster City, CA). Primers of interest were designed with Primer Express 2.0 (Applied Biosystems) and synthesized by One Plus sequence detection system (Applied Biosystems, Foster City, CA). Primer sequences were as follows:

- RPLP0 forward: 5′-ATGCCCGAACACCCGCTT-3′
- RPLP0 reverse: 5′-GCACATCGCTCAGGATTTCAA-3′
- HPRT forward: 5′-TTATGTCGGCCTTTGACTGT-3′
- HPRT reverse: 5′-TTATGTCCCCCGTTGACTGT-3′
- GDNF forward: 5′-ATGCCCGAACACCCGCTT-3′
- GDNF reverse: 5′-AAGTGCCCGCCGCTTGT-3′
- GDNFRa forward: 5′-AGGCAGGACGGGCTTGTGTA-3′
- GDNFRa reverse: 5′-GGCTGAAGTTGGTTT-3′
- C-met (HGF receptor) forward: 5′-AGATCATCTGCTG-3′
- C-met (HGF receptor) reverse: 5′-AAAGTGTTTGGA-3′
- HGF forward: 5′-AACACAGCTTTTTGCCTTGAGC-3′
- HGF reverse: 5′-CTGGAATGATTGTAAGACCAA-3′

**Analysis**

Results are presented as mean ± SE. For outgrowth analysis, we analyzed results from all neurons/neurites examined in all days. For percentage of change, we considered growth cones analyzed on a given day (usually 4–8 separate growth cones calculated as a mean percentage) as an independent value and calculated the mean percentage for the group from the total number of days (n) analyzed. Given an extensive series of independent assays (>20), statistical comparisons were focused on specific and limited questions comparing growth and behavior after carrier (PBS) exposure to the intervention of interest. In a number of experiments, the hypothesis of the experimental design was an expected direction of change (a growth response to each intervention), allowing use of unpaired 1-tailed Student t-tests for these comparisons, including Welch correction and nonparametric comparisons for unequal variations and distributions. Paired Student t-tests were used for comparisons of branching after bisperoxovanadium (BPV) soma application before and after intervention.

**RESULTS**

**Growth Cones of Adult Sensory Neurons Express the Substrates for GDNF and HGF Signaling**

We first tested whether immunoselected GFRα1 adult sensory neuron growth cones expressed critical substrates required to make them responsive to local pharmacologic manipulation. We confirmed that DRG populations of sensory neurons from which we immunoselected had a substantial overlap in their expression of GFRα1 and IB4, yielding an enriched study group of these neuronal subtypes (Fig. 1). We next identified that the receptors for GDNF (GFRα1) and HGF (c-met) were present on the growth cones of these harvested neurons (Figs. 1D, 2A). Growth cones also expressed pAkt, the downstream growth-related kinase, and PTEN (Figs. 2B, C).

**Nonpeptidergic Neurons Extend Growth Cones More Slowly Than Unselected Neurons**

In previous dissociated neuronal cultures, evidence has suggested that nonpeptidergic IB4 neurons have lesser baseline plasticity than other sensory neuron populations. To test this possibility, we evaluated outgrowth of preconditioned sensory neurons selected for their expression of GFRα1, a population that largely overlaps with nonpeptidergic IB4 neurons. Their length of outgrowth in response (more than 60 minutes after Picospritzer exposure) to PBS without added growth factors was compared with a population of unselected DRG sensory neurons. The length of outgrowth was reduced in the nonpeptidergic neuron–enriched neuron population compared with unselected neurons (Fig. 3; Video, Supplemental Digital Content 1, http://links.lww.com/NEN/A623; Video, Supplemental Digital Content 2, http://links.lww.com/NEN/A624). The percentages of neurons extending primary branches were similar between the 2 populations as were the percentages of neurons extending axons at least 10 μm. Similarly, the percentages of neurons with secondary branches were similar in the neuron groups. There was a nonsignificant trend toward fewer growth cone branches in the nonpeptidergic neurons (data not shown). Nonpeptidergic neurons were more likely to retract (Fig. 3; Video, Supplemental Digital Content 3, http://links.lww.com/NEN/A625).

**GDNF and HGF Combined, But Not Separately, Increase Growth in Adult Nonpeptidergic Neurons**

We hypothesized that GFRα1-selected neurons would respond to a GDNF gradient. To test this idea, we applied...
FIGURE 1. Adult sensory neurons and their growth cones express GFRα1. Dorsal root ganglion sensory neurons immunolabeled for GFRα1 and IB4 (A), neurofilament (Nf) (B), or PGP 9.5 (C). Note the close overlap between GFRα1 neurons and IB4 neurons. This population was immunoselected for outgrowth and turning studies. Smaller GFRα1 neurons have markedly less overlap with larger sensory neurons with high Nf expression. PGP 9.5 labels all sensory neurons in the DRG. The arrow points to a larger-caliber sensory neuron with PGP 9.5 labeling, but not GFRα1 labeling. (D) In addition, GFRα1 labels growth cones, indicated by colabeling with βIII tubulin. Scale bars = (A, B) 50 μm; (C) 100 μm; (D) 20 μm.
FIGURE 2. Growth cones express substrates for HGF signaling and PTEN inhibition. Growth cones from dissociated adult DRG sensory neurons express c-met (the receptor for HGF) (A), pAkt (the downstream effector of PTEN inhibition) (B), and PTEN (C). Growth cones (arrows) are double labeled with anti-βIII tubulin and anti-neurofilament (Nf). Scale bar = 20 μm.
FIGURE 3. Outgrowth properties of GFRα1 neurons are restrained. (A, B) Individual growth cones imaged by brightfield microscopy before (left) and after (right) 60 minutes of growth. Asterisks indicate the tip of the Picospritzer. Here, only PBS carrier was applied to the growth cones. Two examples of unselected “normal” adult DRG neurons are shown (A) illustrating advancement of the growth cone after 60 minutes. The outgrowth in neurons immunoselected for GFRα1 expression (B) is attenuated in comparison. (C) The length of axon outgrowth (change in axon length, labeled as “axon length”) was significantly lower in immunoselected GFRα1 neurons than in unselected neurons (* p = 0.026, unpaired Student t-test, 2-tailed, n = 124 GFRα1, 44 unselected). (D, E) Retraction (in percentage) of growth cones was more common in immunoselected GFRα1 neurons (* p = 0.051, unpaired Student t-test, 2-tailed, n = 31 GFRα1, 8 unselected). Scale bar = 50 μm.
A Selective PTEN Inhibitor Enhances GDNF- and HGF-Induced Outgrowth

In previous work, we have shown that the nonpeptidergic IB4 neuron population expresses high levels of PTEN, a regenerative roadblock (20). We wondered whether short-term inhibition of PTEN directed specifically at the growth cone using a relatively selective pharmacologic antagonist of PTEN, bpV(pic), would alter their responsiveness. Low doses of bpV(pic) (50 nmol/L) had no impact on overall axon length or on the percentage of axons that grew at least 10 μm in length. Interestingly, low doses of bpV(pic) were more likely to be associated with growth cone retraction than high doses (data not shown). High-dose bpV(pic) (1,000 nmol/L) alone similarly did not significantly increase axon growth and was associated with more growth cones having outgrowth less than 10 μm (data not shown). Both of these negative outcomes were abrogated when combined with HGF or GDNF. Doses of bpV(pic) (50 nmol/L), when combined with low doses (7 nmol/L) of GDNF, increased the distance of axon outgrowth and the percentage of axons that grew longer than 10 μm compared with those exposed to PBS carrier or to low doses of GDNF alone. Pretreating neurons with bpV(pic) (200 nmol/L) in the bath before GDNF treatment of the growth cone did not confer this benefit. Adding bpV(pic) (200 nmol/L) to HGF (700 nmol/L) or combining bpV(pic) (1,000 nmol/L) with HGF (350 nmol/L) and GDNF (350 nmol/L) also increased the length of axon outgrowth (Figs. 5A–C; Video, Supplemental Digital Content 7, http://links.lww.com/NEN/A630).

Overall, the findings indicate that a PTEN inhibitor alone, either applied in the growth media of the neuron or directed toward the growth cone, did not restore the attenuated growth of nonpeptidergic neurons. However, the findings did suggest that GDNF in surprisingly low doses and PTEN inhibition directed together toward the growth cones of nonpeptidergic GFRα1 neurons provided synergistic short-term growth support. Similar benefits were identified when PTEN inhibition was combined with HGF.

The formation of branches from growth cones was more probable if bpV(pic) was added in a gradient with either GDNF or HGF. High doses of bpV(pic) (200 nmol/L) combined with GDNF (700 nmol/L) or bpV(pic) at a dose of 1,000 nmol/L with both GDNF and HGF (350 nmol/L each) was more likely to exhibit branches than PBS (Fig. 5D). This impact was not observed with either the PTEN inhibitor or the growth factors alone, including low-dose and high-dose GDNF or HGF and GDNF combined. A primary branch, but not a secondary branch, was more probable if HGF (700 nmol/L), GDNF (700 nmol/L; higher dose), or both (350 nmol/L each) were combined with bpV(pic) (Figs. 5E–G). Neither bpV(pic) nor GDNF (higher or lower dose) nor HGF alone increased the number of primary branches more than PBS carrier. In contrast, after exposure of growth cones to bpV(pic) alone, there was a nonsignificant trend toward fewer secondary branches. Growth cones exposed to GDNF alone trended toward having more secondary branches compared with growth cones exposed to GDNF combined with bpV(pic). Taken together, these findings suggest that local PTEN inhibition supported primary growth cone branching but only when combined with a growth factor. The lack of a direct branching action from PTEN inhibition alone contrasted with its actions when applied to perikarya (discussed later).

Nonpeptidergic Axons Turn Selectively

The angle of growth cone advancement in GFRα1 neuron variants varied widely whether they were exposed to PBS, HGF, GDNF, or bpV(pic), indicating the absence of robust directional guidance in this neuron population. Growth cones turned toward or away from gradients or were indifferent. Growth cones exposed to GDNF + bpV(pic) were not more likely to turn in a consistent direction but instead were more likely to turn in either direction toward or away from a gradient compared with PBS or GDNF alone (data not shown). Both high and low doses of GDNF + bpV(pic) were likely to turn 30° or more away from the gradient. Similarly, GDNF added to HGF was also associated with this behavior, unlike HGF alone. Attractive (≥30°) turning was only observed toward a gradient of HGF, high doses of bpV(pic) alone, or HGF combined with bpV(pic), but not toward growth cones exposed to GDNF with or without bpV(pic) (Fig. 6; Video, Supplemental Digital Content 8, http://links.lww.com/NEN/A630; Video, Supplemental Digital Content 9, http://links.lww.com/NEN/A631). Groups not shown did not have consistent directions of change. Overall, these findings indicated neurotropism with HGF and PTEN inhibition but no impact of GDNF.

Rho Kinase Inhibition Provides Support for Growth Cone Behavior

The GFRα1 neurons had increased growth when they were exposed to 100 μmol/L HA-1077 (fascilid), a relatively selective Rho kinase (ROK) inhibitor. Although they were no more likely to grow neurites 10 μm or more than carrier when exposed to a gradient of HA-1077, they had a higher number of branches as a proportion (expressed as percentage) of total growth cones and a higher percentage of primary branches. The ROK inhibitor was...
FIGURE 4. Combined GDNF and HGF exposure is required for growth cone advancement. (A–C) Individual growth cones imaged by brightfield microscopy before (left) and after (right) 60 minutes of growth. Growth cone advancement is limited (with formation of branches in these examples) after either GDNF (700 nmol/L) (A) or HGF (700 nmol/L) (B) but is more pronounced after a combination of GDNF and HGF (350 nmol/L each) (C). (D) Axon outgrowth (change in axon length) after application of GDNF or HGF was not significantly greater than that of carrier but was significantly higher in response to both GDNF and HGF (*p = 0.022, unpaired Student t-test, 2-tailed, n = 124 PBS, 47 G, 59 H, 44 H + G). Combined HGF and GDNF were less likely to be associated with growth cone retraction (E) (*p = 0.043, unpaired Student t-test, 2-tailed, n = 31 PBS, 9 G, 11 H, 8 G + H) or growth less than 10 μm in length (F) (*p = 0.048, unpaired Student t-test, 1-tailed, n = 31 PBS, 9 G, 11 H, 8 G + H). (G) The proportion of neurites more than 10 μm in length was not significantly different among the groups, although there was a trend toward higher numbers with combined treatment. Scale bar = 50 μm.
FIGURE 5. Responses to HGF and GDFN are enhanced by the PTEN inhibitor BPV. (A–C) Neurite outgrowth in response to low-dose GDFN (LGDNF; 7 nmol/L), high-dose GDFN (HGDFN; 700 nmol/L), HGF (700 nmol/L), or BPV alone (50 or 1,000 nmol/L) did not differ from carrier control. Outgrowth (change in axon length) was significantly increased when HGF (700 nmol/L) or LGDNF was combined with a PTEN inhibitor bp(V)pic (BPV), or when HGF (350 nmol/L) + GDNF (350 nmol/L) were combined with high-dose bp(V)pic (1,000 nmol/L) (A) (* p = 0.006 for LGDNF + BPV, * p = 0.047 for HGF + BPV, * p = 0.046 for PBS vs GDNF + HGF + BPV, Student t-test, 1-tailed test, n = 124 PBS, 52 LGDNF, 47 HBVPN, 47 HGDFN, 62 LG + B, 50 H + B, 43 G + H + B, 49 LBVPN; unpaired Student t-test, 2-tailed except as indicated; dose for BPV is 50 nmol/L for the third and sixth bars, 200 nmol/L for the seventh and eighth bars, and 1,000 nmol/L for the ninth and tenth bars). (B, C) Individual growth cones imaged by brightfield microscopy before (left) and after (right) 60 minutes of growth illustrate increased outgrowth in response to GDNF (B) or HGF (C) combined with BPV. (D) The percentage of growth cones with branches was increased in response to the combination of HGDFN and BPV (* p = 0.034, 1-tailed Student t-test, n = 31 PBS, 9 hi G, 8 H + G, 11 H, 9 H + B, 12 hi G + B, 12 lo G + B) and the combination of HGDFN + BPV + HGF (* p = 0.03, 2-tailed Student t-test), but not in response to GDNF (high dose or low dose), HGF, or the combination of HGF and GDFN (doses as described previously). (E) The ratio of primary branch numbers to growth cones (expressed as percentage) was also increased with HGDFN and BPV (* p = 0.043, 1-tailed Student t-test, n = 31 PBS, 9 hi G, 8 H + G, 11 H, 9 H + B, 12 hi G + B, 12 lo G + B), HGF and BPV (* p = 0.043, 1-tailed Student t-test), or all 3 agents (* p = 0.008, Student t-test, 2-tailed), but not with the other interventions. (F, G) Individual growth cones imaged by brightfield microscopy before (left) and after (right) 60 minutes of growth illustrate branching in response to GDNF + BPV (F) or HGF + BPV (G). Scale bar = 50 μm.
FIGURE 6. Directional responses of GFRα1 neurons. (A) The percentage of neurons that turned 30° or more in response to the gradients indicated is indicated and did not exceed 20%. Turning was observed with HGF (700 nmol/L), BPV (1,000 nmol/L), and a combination of HGF (700 nmol/L) and BPV (200 nmol/L) or HGF (350 nmol/L) plus GDNF (350 nmol/L) with high-dose BPV (1,000 nmol/L) (* p = 0.012 PBS vs HGF; * p = 0.05 PBS vs BPV; * p = 0.037 PBS vs H + B [1-tailed]; * p = 0.03 PBS vs GDNF + HGF + BPV [1-tailed], n = 31 PBS, 11 HGF, 9 BPV, 9 H + B, 9 G + H, 7 G + H + B; unpaired Student t-test, 2-tailed, except as indicated). (B) Responsiveness to HGF or BPV was lost when combined with GDNF, and these neurons were more likely to turn more than 30° away from the gradient (* p = 0.035, PBS vs GDNF + BPV; * p = 0.009, PBS vs HGF + GDNF, n = 31 PBS, 9 BPV, 12 G + B, 8 H + G, 9 H = B, 11 HGF, 7 G = H + B; unpaired Student t-test, 2-tailed). (C–G) Individual growth cones imaged by brightfield microscopy before (left) and after (right) 60 minutes of growth are illustrated in response to HGF (attraction; C, D), BPV (attraction (E), repulsion from GDNF (F), and indifference to GDNF + BPV (G). Combined tracings of all neurite trajectories in response to PBS (H), HGF (I), BPV (J), GDNF (K), GDNF + BPV (L), GDNF + HGF (M), and GDNF + HGF + BPV (N) are illustrated. Scale bar = 50 μm.
not associated with turning behavior (Fig. 7; Video, Supplemental Digital Content 10, http://links.lww.com/NEN/A632). The impact of ROK inhibition alone on growth and branching suggested a downstream impact of Rho-ROK in attenuating growth properties of GFRα1 neurons.

**FP Protein Extracts Replicate Synergistic Support**

Sensory neurons enter and ramify within the epidermis. To identify whether nonpeptidergic axons are likely to be supported by proteins within this microenvironment, we generated protein fractions from the epidermis of rat FPs, matched the overall protein concentration with that of growth factors applied previously, and tested their impact on short-term growth and trajectory in both selected GFRα1 sensory neurons and unselected sensory neurons.

The FP protein extract was associated with a mild rise in the axon length of IB4 neurons, but not in the axon length of unselected neurons (Figs. 8A, B; Video, Supplemental Digital Content 11, http://links.lww.com/NEN/A633). The percentage of axons that grew at least 10 μm was not altered in either neuron population. The extract had no impact on the percentage of growth cones that were unchanged, retracted, or grew less than 10 μm in the GFRα1-selected or unselected neurons. Foot pad protein was associated with a rise in the number of branches among total growth cones (expressed as percentage) and increased the percentage of primary neurites among growth cones with branches (Figs. 9C–E). There was no impact on the percentage of secondary neurites. Foot pad extract application was associated with a rise in the percentage of growth cones that turned 30° or more toward the pipette (Figs. 8F, G).

Analysis of FP samples using quantitative reverse transcription–polymerase chain reaction confirmed the presence of GDNF, HGF, c-met, and GFRα1 messenger RNA within the skin, indicating that both the ligand and receptors for growth signaling are endogenously expressed in this target tissue (data not shown). Immunohistochemistry identified a striking colocalization of GDNF ligand and IB4 axon profiles within the dermis and epidermis (Fig. 8H). Although the staining did not exclude low background levels of GDNF in keratinocytes or other skin cells, axon staining was very prominent. Taken together, these results indicate that the skin does offer support to adult sensory neurons that travel within it. As observed with combinations of GDNF, HGF, or PTEN inhibition described previously, these extracts replicated an impact on both growth and turning. In the case of GDNF, its striking localization to the same neurons that express its receptors suggests a local form of autocrine support for axon branches within the skin.

**PTEN Inhibitors Applied to the Neuron Soma Can Induce Distal Branching**

We wondered whether inhibiting PTEN with a gradient directed to the soma of an immunoselected GFRα1 neuron, rather than the growth cone, might influence short-term growth cone behavior. For this experiment, we directed the pipette to piconspitz bpV(pic) toward the neuron soma rather than a growth cone. Doses of PTEN were compared with carrier. This intervention had no impact on the overall growth (in length) of distal axon growth cones (data not shown). However, after bpV(pic) application, there was an interesting and significant rise in the outgrowth of distal primary and secondary branches of neurites. This differed from the lack of impact on branching observed when bpV(pic) was applied directly to growth cones, as described previously. Moreover, the pattern of change was interesting in that the changes developed (in the absence of simple outgrowth) quite some distance from the pipette source (Fig. 9; Video, Supplemental Digital Content 12, http://links.lww.com/NEN/A634). In some neurons, branching occurred in neurites on the opposite side of the cell body from where the Picospritzer application was provided. Although not observed after exposure to PBS carrier at the cell body, some neurons exposed to bpV(pic) developed new tertiary branches as well. Finally, 1 neuron exposed to 50 nmol/L bpV(pic) and 2 neurons exposed to 200 nmol/L developed a new neurite from the cell body, which was not seen after carrier. Overall, these results indicate that perikarya retain the capacity to alter distal growth cone behavior in the short term and that PTEN factors into this property.

**DISCUSSION**

The plasticity of nonpeptidergic peripheral neurons relates widely to issues of pain, skin sensory loss, and regeneration after injury or disease. In this study, we focused on GFRα1 receptor–expressing sensory neurons (also known as GDNF-‘‘responsive’’) that heavily overlap with the IB4 population (5–9). Recently, we showed that this class of neurons has high expression of PTEN, an endogenous brake on PI3K-pAkt–associated neurite outgrowth (20). Here, we sought to understand how responsive this population might be in adults, providing insight into aspects of skin innervation.

Our major findings were as follows: i) Neurons expressing GFRα1 extended shorter distances in vitro than unselected neurons; ii) despite their expression of GFRα1, these sensory neurons did not exhibit growth or turning responses to GDNF alone; iii) a synergistic combination of inputs, either with HGF or with a PTEN inhibitor, supported outgrowth; iv) HGF or a PTEN inhibitor offered tropism for turning; v) inhibition of ROK increased growth cone advancement but not turning; vi) FP protein extracts replicated the impact of synergistic stimuli on growth cones; and vii) a PTEN inhibitor, directed instead to sensory neuron perikarya, induced changes in growth cones in distant branches.

The original rationale and exploitation of short-term growth and turning assays of neurons centered around neural development, using much more highly plastic embryonic or neonatal neurons (24, 25). In contrast, similar studies in adult neurons have been rare. Any conclusions about the plasticity of sensory neurons in adults based on embryonic or neonatal work may be misleading. Moreover, collateral sprouting of epidermal axons in response to sensory neuron dropout or proximal lesions is a common mechanism of neurologic recovery that has considerable clinical relevance and justifies direct analysis of the behavior of adult neurons.

The reluctant short-term regrowth of nonpeptidergic neurons is significant but cannot be explained by heightened PTEN expression alone because the inhibitor failed to increase
FIGURE 7. Inhibition of ROK enhances growth and branching, but not turning. (A) A directional gradient of HA-1077 (fasudil; 100 µmol/L) is associated with increased axon outgrowth (change in axon length) (* p = 0.0052, unpaired Student t-test, 2-tailed, n = 124 PBS, 37 fasudil) compared with PBS carrier. (B) An individual growth cone imaged by brightfield microscopy before (left) and after (right) 60 minutes of growth is illustrated in response to fasudil. The growth cone is largely indifferent to the directional gradient, with early branch formation at the growth tip. Inhibition of ROK increased the number of branches as a proportion of total growth cones (expressed as percentage) (* p = 0.027, unpaired Student t-test, 2-tailed, n = 31 PBS, 6 fasudil) (C) and increased the percentage of primary, but not secondary, branches (* p = 0.04, unpaired Student t-test, 1-tailed, n = 31 PBS, 6 fasudil) (D). The ROK inhibitor gradient was not associated with an increased percentage of growth cones turning 30° or more toward the pipette (E), and all of the neurite trajectories are illustrated (F). Scale bar = 50 µm.
FIGURE 8. An extract of FP skin proteins replicates the growth, branching, and turning properties of GFRα1 neurons. (A) An equivalent protein concentration (equivalent to GDNF and HGF concentrations applied previously) extracted from adult rat FP skin is associated with increased outgrowth of axons (change in axon length) from immunoselected GFRα1 neurons (* p = 0.008, unpaired Student t-test, 2-tailed, n = 124 PBS, 44 FP). (B, C) Individual growth cones imaged by brightfield microscopy before (left) and after (right) 60 minutes of growth are illustrated in response to skin FP protein. Note that the axon turns in the direction of the pipette (B) or branches (C). Scale bar = 50 µm. The extract was associated with an increased number of branches as a proportion of total growth cones (expressed as percentage) (D) (* p = 0.053, unpaired Student t-test, 1-tailed, n = 31 PBS, 7 FP) and increased the percentage of primary (* p = 0.038, unpaired Student t-test, 2-tailed, n = 31 PBS, 7 FP), but not secondary, branches (E). (F) Skin FP extract was associated with an increased percentage of neurons with growth cones that turned toward the protein gradient from the pipette (* p = 0.036, unpaired Student t-test, 1-tailed, n = 31 PBS, 7 FP). (G) All neurite trajectories in response to the extract are illustrated. (H) Transverse sections of rat FP skin immunostained for GDNF (red; left) identify close colocalization in epidermal axons labeled with IB4 lectin (green; middle). Merged image on the right shows almost complete colocalization. Scale bar = 20 µm.
FIGURE 9. Perikarya-directed PTEN inhibition induces distal neurite branching. (A–I) The PTEN inhibitor bp(v)pic (BPV) was directed by the Picospritzer to the perikarya, instead of the growth cone, of immunoselected GFRα1 adult sensory neurons. Trajectories of growth are illustrated by drawings of neurons and their branches after application of PBS carrier (A–C), BPV at 50 nmol/L (D–F), and BPV at 200 nmol/L (G–I). The original configuration of the neurons is illustrated in blue. New primary branches are illustrated in yellow, new secondary branches are illustrated in green, and tertiary branches are illustrated in pink. Outgrowth data indicate a significant rise in primary branches (J) (* p = 0.015, paired before vs after 50 nmol/L treatment, n = 22, paired Student t-test; * p = 0.007, paired before vs after 200 nmol/L, n = 24, paired Student t-test) and secondary branches (K) (* p = 0.047, paired before vs after 200 nmol/L treatment, n = 24, paired Student t-test), and, in one case, tertiary branches (L) when perikarya were exposed to 200 nmol/L BPV. An individual neuron imaged by brightfield microscopy before (M) and after (N) 60 minutes of growth is illustrated in response to 200 nmol/L BPV applied to its perikarya. Note the rise in branches in neurites distant from the side of application of the PTEN inhibitor. Scale bar = 50 μm.
growth when directed at growth cones. Similarly, it seems improbable that in vivo regrowth is limited by the availability of GDNF because even high doses failed to increase advancement. We argue that a requirement for at least 2 forms of stimulation to coax limited growth indicates cooperative growth factor signaling for growth in adults, a property not extensively examined. Glial cell–derived neurotrophic factor and HGF do seem to cooperate and—because they are both synthesized and expressed in the skin—are important candidates. Moreover, our FP skin protein extracts replicated this form of cooperative action, as indicated by increased growth but also by turning and branching. That more than one mechanism is involved in the support of IB4 neurons is also supported by the work of Tucker et al (29), who demonstrated that IB4 sensory neurons depended on signaling through PI3K-pAkt and MEK. These investigators also identified an important role for integrin signaling through a laminin substrate to support IB4 neurons; in view of this requirement, all of our experiments were carried out on laminin. In their work, IB4 neurons failed to grow on laminin in the absence of GDNF, whereas IB4-negative neurons grew readily on laminin even in the absence of NGF to support them. It may be that additional growth factors, such as fibroblast growth factors, similarly costimulate this population of axons to activate both intracellular growth pathways. Finally, more recent work indicates that the patterns of gene expression in both naive and injured sensory neurons differ between the IB4 and the TrkA population (30).

An important caveat of our work was that the assays were short term (i.e., not more than 60 minutes). Whereas these conditions are ideal for analyzing rapidly growing neuron populations (usually embryonic or neonatal), they may not characterize slowed graduated responses in the adult. Early responses may depend less on local axon translation than pharmacologic inhibition, although rapid changes in gene expression, beyond the resolution of this work, may yet be possible (31). In the case of GFRα1 neurons, both early outgrowth (as confirmed here) and later outgrowth (previously described) are attenuated. The short-term impairment that we observed suggests that GFRα1 neurons possess a regenerative roadblock intrinsic to their short-term growth cone behavior. In previous work, we noted that preconditioning was required for NGF turning of TrkA neurons (17). Here we show that GDNF short-term growth responses are attenuated after preconditioning, in keeping with findings by Kalous and Keast (32).

The overall impacts of our interventions were unlikely to be technical, given a more striking impact of NGF on TrkA neurons that we documented using identical experimental methods (17). Similarly, Murray and Shewan (33) showed attraction of unselected nonconditioned adult sensory neurons to eAMP, mediated by exchange protein activated by cAMP (Epac), a guanine nucleotide exchange factor for Rap1. That ROK inhibition encouraged limited but significant nondirected growth may indicate that RhoA-ROK acts downstream of other signals and accounts for significant growth reluctance. RhoA and ROK expressions are features of adult sensory neurons, and ROK inhibition enhances outgrowth of peripheral neurons in vivo and in vitro (34).

In the present study, we did not find evidence that growth and directional turning of adult nonpeptidergic neurons are inextricably linked. HGF and bpV(pic), alone or together, could induce turning in the direction of an applied gradient. This finding confirms previous work that HGF offers directional support, as studied using neonatal neurons in stripe assays or by growth cone turning in unselected neonatal neurons (2, 35). Here we show that adult neurons also possess this property. Moreover, HGF-induced turning is proof of principle that our approach would have been sensitive enough to detect directionality from GDNF gradients had they existed, but neither a low dose nor a high dose of GDNF had even minor evidence of this property; in fact, many growth cones seemed to turn away from these gradients. Furthermore, adding GDNF to HGF abolished any evidence of turning. It is of interest to speculate why this might be the case. For example, it may be argued that the 2 growth factors normally support epidermal axons in different ways: GDNF by autocrine support released from axons and HGF by skin keratinocytes. It is possible that GDNF, expressed and released by epidermal axons, induces repulsion rather than attraction to prevent skin hyperinnervation. In contrast to the present findings, IB4 neurons do respond to GDNF in vitro across longer periods, a feature confirmed by others (10, 11, 19). Longer-term changes in gene expression may allow for the delivery of intracellular signals that can suppress RhoA-ROK inhibition and allow graduated growth (34).

Here we show that PTEN inhibition at the soma, rather than the growth cone, was associated with significant changes in outgrowth behavior, including neurite initiation and distal changes in the branching patterns of neurites. This interesting finding supports work indicating that directed interventions to the cell body can induce substantial changes in the behavior of remote axons (36, 37). It is also consistent with the idea that PI3K-pAkt signaling operates as a short-term “switch” that can influence widespread sites within the neuron tree (29). In some cases, the outgrowth occurred on the opposite side of the soma from the direction of application. In contrast, direct application of PTEN inhibitors alone, applied to growth cones, did not alter their growth cone branching. The findings do fit with more long-term assays of neurons with the PTEN inhibitor in their media for 18 hours, where substantial outgrowth is identified (20).

In this work, we explored how endogenous dermal and epidermal support might influence adult axon behavior. The growth cones possessed the necessary receptors and substrates to respond to HGF and GDNF, which are synthesized in skin. GDNF expression was unexpectedly localized to axons, matching the expression data of GDNF in IB4 DRG neurons described by Fang et al (38). Although the findings do not exclude some keratinocyte synthesis, its localization suggests an autocrine cell autonomous action perhaps supporting outgrowth rather than directionality. In contrast, HGF is also extensively expressed by epidermal keratinocytes (2); there, it may might attract and support axons that have shed their Schwann cell partners. Finally, the growth support offered by protein extracts of skin supports the idea that, in adults, even reluctant nonpeptidergic axons require local and intimate support. Because the impact of this extract differed from the simple combination of HGF and GDNF, however, it is highly probable that additional forms of support and attraction are provided within the skin. Taken together, the results indicate a pattern of restrained outgrowth in nonpeptidergic GDNF-responsive neurons. Unlike
TrkA NGF-responsive neurons, these growth cones do not respond to GDNF immediately but require synergistic support.

ACKNOWLEDGMENTS

The authors acknowledge the support of Dr Christine Webber (now at the University of Alberta) in setting up the adult sensory neuron growth cone turning assay. Mr Jose Martinez performed quantitative reverse transcription–polymerase chain reaction assays of skin samples and assisted with protein extraction.

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