Treating small fiber neuropathy by topical application of a small molecule modulator of ligand-induced GFRα/RET receptor signaling

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Small-fiber neuropathy (SFN) is a disorder of peripheral nerves commonly found in patients with diabetes mellitus, HIV infection, and those receiving chemotherapy. The complexity of disease etiology has led to a scarcity of effective treatments. Using two models of progressive SFN, we show that overexpression of glial cell line–derived neurotrophic factor (GDNF) in skin keratinocytes or topical application of XIB4035, a reported nonpeptidyl agonist of GFRα1 (a receptor tyrosine kinase), are effective treatments for SFN. We also demonstrate that XIB4035 is not a GFRα1 agonist, but rather it enhances GFRα family receptor signaling in conjunction with ligand stimulation. Taken together, our results indicate that topical application of GFRα/RET receptor signaling modulators may be a unique therapy for SFN, and we have identified XIB4035 as a promising candidate therapeutic agent.

Significance

Small-fiber neuropathy (SFN) is a disorder of peripheral nerves that affects millions of people around the world. Currently, there are no treatments for SFN, but target-derived neurotrophic factors have been considered potential therapeutics. Here, we show that topical application of XIB4035, a small molecule that enhances signaling by the glial-derived neurotrophic factor family of molecules, ameliorates SFN pathologies and symptoms in two mouse models. These findings imply that XIB4035 or related molecules could provide a new strategy for SFN treatment with potentially lower risk for side effects because of the topical delivery route and their limited signaling effects.


Conflict of interest statement: G.C. and J.C.M. are inventors on patent US7863295B2 associated with this work and owned by Children’s Medical Center Corporation.

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(22) and in animal models of SFN (23). Interestingly, line-D SFN correlates with a significant reduction of GDNF protein in peripheral nerves (7). We hypothesized that the reduced levels of GDNF contributes to the pathogenesis of the SFN, and that increasing the availability of target (i.e., skin)-derived GDNF could modify the SFN in line-D mice. To test this hypothesis, we crossed line-D mice with a transgenic line that overexpresses GDNF in the skin under the control of the keratin 14 promoter (K14-GDNF) (11). Importantly, K14-GDNF mice have normal thermoreception despite having increased IENF density (11). Behavioral analysis demonstrated that, as shown previously (7), line-D mice exhibit a dramatic loss in thermal nociception at 6 wk of age (Fig. 1A). In contrast, thermoreception in double-transgenic mice (line-D::K14-GDNF) is indistinguishable from that in wild-type and K14-GDNF mice (Fig. 1A). Microscopy showed that line-D::K14-GDNF mice have normal sciatric nerve Remak bundles (Fig. S24) and retain intraepidermal innervation (Fig. S2 B and C). IENF density in double-transgenic mice is lower than in K14-GDNF mice, but the magnitude of the reduction (40%) is much lower than in line-D mice compared with wild type (81%).

Topical Application of XIB4035 Curtails Progression of SFN in Two Animal Models. Transgenic GDNF overexpression served as a proof-of-concept that increasing skin GDNF levels could prevent progressive SFN in line-D mice. However, K14-GDNF mice overexpress GDNF during embryogenesis, altering sensory neuron development, which could contribute to the apparent therapeutic effects. Also, when considering clinical use, GDNF overexpression in the skin would require gene therapy, with its drawbacks and complications, and dermal GDNF application would not work, as peptides do not readily penetrate the skin. Therefore, we tested whether topical application of XIB4035, a nonpeptidyl small molecule reported to be an agonist for GFRα1 (20), could mimic the effects of GDNF overexpression in the skin. We applied a cream containing XIB4035 (1.5 mM) to the hindpaws of line-D and wild-type mice starting at weaning (P21), when mutant mice present with mild neuropathic symptoms (Fig. 1B) (7). For control, mice of both genotypes were also treated with the base cream containing no drug (vehicle). Thermoreception was tested using a hot plate before treatment and every 7 d thereafter. The behavior of wild-type mice remained normal, independent of treatment (Fig. 1B), whereas vehicle-treated line-D mice exhibited disease progression as reported for untreated line-D animals (7) (Fig. 1B). In contrast, reaction times of XIB4035-treated line-D mice were indistinguishable from wild type 1 wk after treatment began and for the duration of the experiment (Fig. 1B). Importantly, response thresholds to mechanical stimuli were normal in all groups after the treatment period, indicating XIB4035 treatment had no effect on mechanoreception. As with GDNF treatment, topical XIB4035 prevented IENF loss (Fig. S3) and degeneration of Remak bundles and C-fiber axons (Fig. 1C and Table 1). Thus, topical treatment with XIB4035 is as effective at preventing progressive SFN in line-D mice as GDNF skin overexpression.

To explore the utility of XIB4035 in a more clinically relevant model, we focused on diabetic peripheral neuropathy, as more than 50% of diabetic patients develop some form of peripheral neuropathy, particularly SFN (www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001713). We chose the streptozotocin (STZ)-induced diabetes model, in which STZ-induced pancreatic beta cell death causes diabetes (24) and produces SFN symptoms, including loss of thermal sensation (23). We initiated treatment early in the disease, i.e., after mice became hyperglycemic (>15 mmol/L) and continued it for 16 wk. Behavioral testing was initiated at week 8, a point at which diabetic mice showed significant hypoalgesia compared with normoglycemic mice (Fig. 1D). Remarkably, XIB4035-treated diabetic mice had better thermoreception than diabetic mice exposed to vehicle, and this improvement persisted throughout the experiment (Fig. 1D). Interestingly, diabetes induced a significant reduction in IENF density, which was not improved by XIB4035 treatment (Fig. S4), indicating that the behavioral improvement was not due to an effect on IENFs.

XIB4035 Improves Sensory Function in Animals with Advanced Neuropathy. Our results demonstrated that topically applied XIB4035 early in disease progression eliminates or attenuates SFN symptoms in the line-D and diabetic models, respectively.
However, many patients may begin treatment when they already present with advanced SFN symptoms. Therefore, we tested whether XIB4035 can act on line-D mice when treatment begins after animals have a more advanced SFN symptomatology (P28). Remarkably, neuropathic animals showed a significant reduction in symptoms 1 wk after treatment initiation (P35), and the improvement persisted as long as treatment was continued (Fig. 2). However, if treatment was interrupted at P35, neuropathic symptoms reappeared 2 wk later and persisted for the duration of the experiment (Fig. 2B), indicating that chronic XIB4035 treatment is necessary to maintain sensory recovery.

Given the difference in the IENF density results between XIB4035-treated line-D and diabetic mice, we felt it necessary to explore the anatomical basis for behavioral recovery in advanced neuropathy in more than one way. Therefore, we examined the structure of both peripheral and central C-fiber axons. These neurons have their sensory terminals in the skin and project axons into laminae I and II of the spinal cord. XIB4035 had no effect on IENF density by P35 if treatment of line-D mice was initiated at P28 (Fig. 2C; vehicle: 1.7 ± 0.5 IENF/100 μm; XIB4035: 1.8 ± 0.3 IENF/100 μm), similar to the IENF results from the diabetic mice. In contrast, XIB4035 had a positive effect on the central projections of some C fibers. To visualize central projections of peptidergic C fibers, considered the principal heat nociceptive cells, we stained for vanilloid receptor 1 (TrpV1) (25, 26). For lamina II terminals, we used isolectin-B4 (IB4) labeling (10), a marker for nonpeptidergic fibers, which is downregulated after injury (27). The IB4+ group of fibers includes those from GFRα2+ neurons that respond to nociceptive thermal stimuli (28-30). We found no discernible change in TrpV1+ lamina I terminals between wild-type and line-D mice (Fig. 2D), which was not unexpected as we previously showed that, at this age, line-D mice have no loss of dorsal root ganglion (DRG) peptidergic neurons (7).

In contrast, IB4 labeling was absent in mutant mice treated with vehicle, but was clearly present in XIB4035-treated mutant mice, albeit at lower levels than wild types [Fig. 2D; 83% of XIB4035-treated mice (n = 6) with IB4+ lamina II terminals vs. 0% (n = 4) in vehicle-treated mice]. These data demonstrate that XIB4035 is an effective therapy for SFN and suggest functional recovery involves the improved health and structure of nonpeptidergic C-fiber central projections.

Finally, given the potential need for chronic application of XIB4035 for treatment, we examined whether XIB4035 produces side effects in mutant and wild-type mice. XIB4035 did not induce thermal hyperalgesia in wild types (Fig. S5), and we did not detect any changes in appearance, or other outward negative signs in animals treated twice a day for 7 wk. Together, these results support the idea that XIB4035 is an effective therapy for SFN.

Table 1. Treatment with XIB4035 preserves Remak bundle structure in line-D mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C-fiber area (μm²)</th>
<th>C-fibers/Remak bundle</th>
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<tbody>
<tr>
<td>WT + vehicle</td>
<td>1.78 ± 0.10</td>
<td>9.90 ± 0.66</td>
</tr>
<tr>
<td>WT + XIB4035</td>
<td>1.90 ± 0.06</td>
<td>8.90 ± 0.52</td>
</tr>
<tr>
<td>Line-D + vehicle</td>
<td>1.01 ± 0.07</td>
<td>4.71 ± 0.42</td>
</tr>
<tr>
<td>Line-D + XIB4035</td>
<td>1.64 ± 0.07</td>
<td>8.40 ± 0.83</td>
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Animals were treated with XIB4035 or vehicle from P21 to P49. Sciatic nerves were prepared for EM, and the cross-sectional area of individual unmyelinated axons and the number of axons pre-Remak bundles were quantified. *P < 0.01; ANOVA Newman–Keuls post hoc. 

3 animals per genotype; ≥50 axons per animal. 

n = 3 animals per genotype; ≥16 bundles per animal.

Fig. 2. XIB4035 treats line-D mice with advanced SFN symptoms. (A) Hot-plate (54 °C) test shows XIB4035 improves the SFN symptoms of line-D mice when treatment is initiated after severe symptoms are apparent (P28). Mutant mice showed significant improvement in thermal nociception after 1 wk of treatment (P35), and this was maintained as long as the treatment continued [red line indicates treatment duration; one-way ANOVA Newman–Keuls post hoc test, n ≥ 7; line-D + vehicle vs. line-D + XIB4035; n.s., not statistically significant; ***P < 0.01, ***P < 0.001; WT + vehicle vs. line-D + vehicle, P < 0.001, WT + vehicle vs. line-D + XIB4035, P < 0.001 (P28, P35, P42); P < 0.01 (P49); P > 0.05 (P56); error bars indicate SEM]. (B) Chronic XIB4035 treatment is necessary to maintain gains. If mice were treated beginning at P28 (as in A), but treatment was interrupted at P35, neuropathic symptoms returned [red line indicates treatment duration; one-way ANOVA Newman–Keuls post hoc test, n ≥ 7; line-D + vehicle vs. line-D + XIB4035; n.s., not statistically significant; ***P < 0.01, ***P < 0.001; WT + vehicle vs. line-D + vehicle, P < 0.001; WT + vehicle vs. line-D + XIB4035, P < 0.001 (P28, P35, P42, and P49), P < 0.01 (P49), P > 0.05 (P56); error bars indicate SEM]. (C) If XIB4035 treatment is initiated at P28, the sensory recovery at P35 is not accompanied by recovery of IENF density. Representative images of PGP9.5-positive IENF staining at P35 in line-D hindpaw skin. (Scale bar: 100 μm.) (D) XIB4035 treatment initiated at P28 results in improved IB4 staining in the dorsal spinal cord. (Left) Representative images of spinal cord sections from wild-type mice treated with vehicle from P28 to P35 show the normal appearance of TrpV1+ (red) and IB4+ (green) nerve terminals. (Center) Images of line-D mice treated with vehicle show complete absence of IB4 staining. (Right) Images from line-D mice treated with XIB4035-containing cream show clear presence of IB4+ fibers. No obvious difference in TrpV1 labeling was observed between wild-type and mutant animals regardless of treatment. (Scale bar: 50 μm.)
demonstrate that topical XIB4035 can be used to treat SFN arising from diverse neurological insults without any overt side effects.

Mechanism of XIB4035 Action: It Is Not an Agonist for GFRα/RET Receptors. Tokugawa et al. (20) reported XIB4035 as a competitive agonist for the GDNF receptor GFRα1 in the Neuro2A (N2A) murine neuroblastoma cell line. Considering the ultimate goal is to use XIB4035 for treating SFN in humans but all data regarding XIB4035 have been obtained using murine models and cells, we measured the effects of XIB4035 on GFRα/RET signaling in the human neuroblastoma cell line SH-SY5Y. We performed two types of assays; immunoblots measuring RET phosphorylation and a reporter assay using the TH promoter to drive luciferase expression. For the latter, we used two paradigms, either overnight treatment immediately followed by luciferase activity measurements or 10-min treatments followed by washout and overnight incubation before measurements. As positive controls, we tested GDNF, NRTN, and ARTN, which induced TH-luciferase activity in a dose-dependent manner (Fig. S6). Contrary to expectations of a GFRα1 agonist, XIB4035 had no effect in the TH-luciferase assay in either the overnight (6.25–500 nM) or 10 min (1–15 μM) treatment paradigms (Fig. 3A) and failed to induce RET phosphorylation in these cells (Fig. 3B).

XIB4035 Is a Positive Modulator of Ligand-Induced GFRα/RET Signaling. Because the original report argued that XIB4035 displaces GDNF binding to GFRα1-expressing cells (Fig. 2 in ref. 20), we anticipated that cotreatment would reduce the effects of GDNF or other GFLs on TH-luciferase activity and RET phosphorylation. We tested this hypothesis on GDNF and ARTN, which induced strong responses in these cells (Fig. S6). Surprisingly, XIB4035 cotreatment significantly potentiated the effects of both ligands on TH-luciferase activity over a range of doses (Fig. 3C), resulting in a significant shift in the nonlinear regression of the dose–response curve, reduced minimal ligand dose necessary to induce luciferase activity above control, and increased maximal effect. Moreover, Western blot assays revealed that cotreatment with XIB4035 prolongs the GDNF-induced RET phosphorylation. In this experiment, SH-SY5Y cells were treated with GDNF with or without XIB4035 for 10 min and cell lysates were either collected immediately (0 min) or treatment was washed out and replaced with growth media for 30 or 60 min before lysates were collected. Both treatments

![Fig. 3](image-url)

**Fig. 3.** XIB4035 potentiates ligand-induced RET signaling but does not act as a GFRα/RET receptor agonist. (A) XIB4035 does not activate a TH-Luciferase reporter in SH-SY5Y cells. SH-SY5Y cells transfected with the reporter construct were treated with either 2 nM GDNF or increasing concentrations of XIB4035 either for 10 min and then incubated overnight in growth media or exposed to treatments overnight. Measurements of luciferase activity after the treatments show that GDNF treatment increases TH promoter activity in both conditions, but XIB4035 has no effect (one-way ANOVA Newman–Keuls post hoc test, n = 3; vs. control, *** = P < 0.001; error bars indicate SEM). (B) XIB4035 does not induce RET phosphorylation. SH-SY5Y cells were treated with either 2 nM GDNF or various concentrations of XIB4035 for 2 or 10 min, and cells were lysed immediately. Anti-phosphotyrosine Western blot analysis shows that RET phosphorylation (arrow) induced by GDNF is inhibited by XIB4035 but not by XIB4035. (C) XIB4035 enhances GFL-induced RET signaling. SH-SY5Y-THpGL3 stable cells were treated with increasing concentrations of GDNF or ARTN with or without 20 μM XIB4035 for 10 min. Treatments were replaced with growth media, and cells were maintained overnight before being assayed for luciferase activity. For both ligands, XIB4035 cotreatment caused a shift in the nonlinear regression of the dose–response curve (F test: GDNF vs. GDNF + 20 μM XIB4035, P = 0.000006; ARTN vs. ARTN + 20 μM XIB4035, P = 0.000005), reduced minimal ligand dose necessary to induce luciferase activity above control (Student’s t test vs. control: GDNF = 75 pm (P = 0.0063), GDNF + 20 μM XIB4035 = 2.7 pm (P = 0.038), ARTN = 75 pm (P = 0.0271), ARTN + 20 μM XIB4035 = 2.7 pm (P = 0.0124)), and increased maximal effect (Student’s t test: fold over control: 18 nM GDNF = 2.76 ± 0.32 vs. 18 nM GDNF + 20 μM XIB4035 = 3.41 ± 0.31, P = 0.0189; 18 nM ARTN = 2.78 ± 0.44 vs. 18 nM ARTN + 20 μM XIB4035 = 3.61 ± 0.47, P = 0.0241). (D) XIB4035 prolongs GFL-induced RET phosphorylation. SH-SY5Y cells were treated with 2 nM GDNF or 1 nM ARTN with or without 10 or 20 μM XIB4035 for 10 min. Cell lysates were either collected immediately or treatment was washed out and replaced with growth media for 30 or 60 min. Cell lysates were subjected to phosphotyrosine Western blot. RET phosphorylation (arrow) in the GDNF– or ARTN-treated samples returns to baseline by 60 min but not with addition of XIB4035.
produced equal levels of RET phosphorylation at 0 min, but ρ-RET signals were higher in the cotreated cells at 30 and 60 min posttreatment (Fig. 3D and Fig. S7). XIB4035 had the same effect on ARTN-induced RET phosphorylation (Fig. 3D).

Because SH-SY5Y cells express GFRα1, 2, and 3, and there is significant cross talk between the different GFLs and GFRα receptors, the experiments described above did not define the specific ligand–receptor pairs for which XIB4035 serve as a positive modulator. This is an important issue because different C-fiber neuron populations express different GFRα receptors (31, 32). Therefore, we tested cells expressing only one GFRα using either N2A cells (which express RET) transfected with GFRα1 or GFRα3 expression constructs, or B(E)2-C cells, which express only GFRα2 together with RET. First, to verify the lack of endogenous GFRα activity in the parental N2A line, we treated control transfected (mGFP) cells with either GDNF or ARTN, and demonstrated that neither ligand induced RET phosphorylation (Fig. S8A). In contrast, GDNF and ARTN induced RET phosphorylation when GFRα1 or GFRα3 receptors were expressed, respectively (Fig. S8A). We then treated the GFRα1- or GFRα3-transfected N2A cells with XIB4035 alone, the appropriate ligand for the expressed receptor alone, or XIB4035 and ligand in combination for 10 min, and collected lysates immediately after treatment or 60 min after washout. XIB4035 alone did not induce RET phosphorylation in either GFRα1- or GFRα3-transfected cells (Fig. S8 B and C), but RET phosphorylation was clearly prolonged at the 60-min time point when cotreated with XIB4035 and ligand compared with ligand alone (Fig. S8 B and C). Similar results were obtained with GFRα2 and NRTN using B(E)2-C cells (Fig. SSD). Importantly, XIB4035 did not influence the activity of another receptor tyrosine kinase pathway, NGF-induced activation of TrkA signaling in PC12 cells (Fig. S9), suggesting that XIB4035 is a specific signaling modulator of GDNF family ligands. Thus, the data indicate that XIB4035 is a specific, positive modulator of signaling by GFLs and their receptors, not an agonist for GFRα1 as previously reported (20).

Discussion

Delivery of neurotrophic factors has been considered a potential strategy for the treatment of a variety of neurological disorders, including neuropathic pain (33). GFLs exhibited great promise in animal models, but have yet to result in clinical therapies (34). Two major barriers for moving these therapies to the clinic have been the delivery methods and the high systemic doses necessary for efficacy. Previous studies demonstrated effectiveness of neurotrophic factors delivered via systemic or intrathecal injection in animal models (35–37). Nevertheless, these delivery routes proved ineffective and/or caused severe side effects in human patients. For example, intracerebroventricular administration of GDNF to Parkinson disease patients resulted in weight loss, anorexia, and nausea with little clinical benefit (38), and trials examining the efficacy of NGF treatment in diabetic patients with peripheral neuropathy or patients with HIV neuropathy have shown some improvement in the patient’s perception of symptom severity, but side effects including myalgia, peripheral edema, and hyperalgesia were also observed (39–41). Our finding that topical application of XIB4035 mitigates symptomatic pathology in two models of progressive SFN, acting even after SFN symptoms are severe, indicates that local directed delivery of small molecules that enhance GFL/GFRα/RET signaling could be an effective treatment for SFN without the side effects associated with generalized delivery. Furthermore, given that XIB4035 shows effectiveness in two models of SFN with different pathogenic mechanisms, it may be useful across a broad spectrum of SFNs.

Using cell-based assays, we show that XIB4035 is not a GFRα1 agonist as previously reported (20), but functions by specifically augmenting ligand-stimulated GFRα/RET signaling. Furthermore, the remarkable similarity between the effects of topical XIB4035 treatment and GDNF skin overexpression on SFN pathologies indicates that XIB4035 acts as an enhancer of GFL/GFRα/RET signaling in vivo. These findings introduce a unique paradigm for therapeutic intervention for SFN, i.e., use of a topical receptor tyrosine kinase signaling modulator rather than systemic delivery of an agonist. This therapeutic approach has the potential to minimize the side effects observed with systemic neurotrophic factor delivery; i.e., by not being an agonist, the drug will not activate GFRα/RET signaling in cells that express the receptors but are not normally exposed to the ligand; rather it will only enhance signaling where all of the endogenous components (ligands and receptors) are present. These findings also raise the possibility that small-molecule positive modulators of other receptor tyrosine kinase signaling pathways could be used as therapeutics for other neurological disorders.

We found that XIB4035-induced improvement of thermal sensation in diabetic mice and line-D mice with advanced symptoms is not accompanied by recovery of IENF density, a common clinical indicator of neuropathy severity (22). However, the absence of a simple linear association between IENF density and thermal sensitivity has been noted previously (23, 42). This phenomenon may reflect the specificity of sensory neuron subpopulations for heat sensation and growth factors (43) and/or a step function between IENF loss and behavioral response to noxious thermal stimuli (44). We also did not detect differences in Trpv1+ peptidergic terminals between mutant and wild-type animals; however, we cannot rule out the possibility that Trpv1+ cell function is disrupted. In contrast, IB4 labeling of nonpeptidergic C-fiber projections was lost in line-D mice and partially rescued by XIB4035 treatment. Because line-D mice have no DRG loss at the age analyzed (7), the recovery of IB4 labeling is likely an indicator that XIB4035 treatment improves the health of nonpeptidergic neurons (27). Further studies will be necessary to define which neuronal populations are responsible for the observed behavioral recovery and the more detailed mechanisms by which it occurs. The finding that application of the therapeutic to the skin, where sensory nerve terminals are located, improves overall sensory neuron structure, including projections into the spinal cord, shows that, in this disorder, neurons improve with very limited exposure to a drug that enhances trophic support for sensory neurons, as it has been done previously. This approach could have implications for the treatment of other neurological disorders as well.

Finally, our results concerning the molecular mechanism of XIB4035 action differ from the only published study pertaining to this molecule and the GFRα/RET complex. We found that XIB4035 functions as a positive modulator of GFL signaling by prolonging ligand-induced RET receptor activation and enhancing downstream effects of this receptor, in both rodent and human cells. We propose that XIB4035 acts by enhancing signaling of endogenous GFLs, e.g., NRTN produced by basal keratinocytes (45). Taken together, our data suggest that small-molecule modulators of GFL receptors and their coreceptor, RET, can be used to treat SFNs of different origins, and that XIB4035 has potential as a therapeutic molecule for treating SFN.

Materials and Methods

Animals. Transgenic mouse lines were used as previously described (7, 11). Paw withdrawal latency was measured using hot-plate analgesia meters: Columbus Instruments for Fig. 1 A and B; IITC for Fig. 2 A and B and Fig. S4. Mechanical sensitivity was tested by simulation of the plantar surface of the hindpaw with a series of von Frey filaments. The use of animals was approved by the Animal Care and Use Committee of Boston Children’s Hospital. Detailed methods are provided in SI Materials and Methods.

Diabetic Neuropathy. Adult female mice were made diabetic and paw thermal response latency was measured using a modified Hargreaves test, as described (23). Detailed methods are provided in SI Materials and Methods.
Preparation and Use of XIB4035. The cream containing XIB4035 (1.5 mM; Zenerex Molecular) consisted of N-methyl-pyrrolidone (6.25%), isopropyl myristate (6.25%), and petroleum jelly (87.5%)(vol/vol). Control cream had the same ingredients without XIB4035. Cream was applied twice daily to the hindpaws of mice. The molecular structure of the compound was confirmed by NMR spectroscopy and mass spectrometry.

Immunohistochernistry. Footpad skin was dissected from hindpaws and stained as floating sections with PGP9.5 rabbit polyclonal antibody (Ultraclone; 1:1,000). Spinal cord sections were stained, mounted on slides, and incubated with a rabbit polyclonal antibody (Abcam; 1:2,000) and Alexa 488-conjugated control. Nuclei were stained with DAPI. Detailed methods are provided in SI Materials and Methods.

Immunohistochernistry Analysis. Skin section images were acquired as 30-μM Z stacks and processed as maximum-intensity projections using a Zeiss LSM 700 microscope. Detailed methods are provided in SI Materials and Methods.

Promoter Cloning and Stable Cell Generation. The TH promoter was cloned into the pGL3 basic vector (Promega), as previously described (14). Detailed methods are provided in SI Materials and Methods.

Cell Assays. For luciferase assays, SH-SY5Y-ThpGL3 cells were treated for indicated times and assayed for firefly luciferase 16 h later (Promega). For phosphorylated Ret immunoblot experiments, cells were treated as indicated and lysates were subjected to Western blot analysis using anti-phosphorytrosine (4G10) mouse monoclonal antibody (EMD Millipore; 1:4,000). N2A and PC-12 cells were starved in basal media containing 1% FBS overnight before treatments. GDNF, NRTN, and ARTN were purchased from PeproTech. j-NFG (R&D Systems, Inc.) at 50 μg/mL was used to stimulate TrkA receptor phosphorylation in PC-12 cells. Detailed methods are provided in SI Materials and Methods.

Statistical Analyses. All statistical analyses were performed using Prism 4 (GraphPad Software). SEM was used to indicate error in all analyses as n ≥ 3. For Fig. 3C, comparison of the nonlinear curve regression was performed using an F test. Analysis of the minimal ligand dose necessary to induce luciferase activity (Fig. 3C) was performed by Student’s t test comparing the average fold luciferase induction from three experiments to control (nontreated).

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Supporting Information

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SI Materials and Methods

Animals. Transgenic mouse lines used as previously described (1, 2). The hot-plate test was performed using hot-plate analgesia meters; Columbus Instruments for Fig. 1 A and B; IITC for Fig. 2 A and B and Fig. S4. Paw withdrawal latency was measured as the time required for the mouse to visibly respond to the thermal stimulus, e.g., licking paws, shaking hindpaws, or jumping. Mechanical sensitivity was tested by simulation of the plantar surface of the hindpaw with a series of von Frey filaments. The threshold was determined as the lowest force that evoked a visible withdrawal response. The use of animals was approved by the Animal Care and Use Committee of Boston Children’s Hospital.

Diabetic Neuropathy. Adult female C57BL/6J mice were made diabetic (blood glucose, >15 mmol/L) by injection of streptozotocin (STZ) (90 mg/kg, i.p.) on 2 consecutive days, with confirmation of hyperglycemia made 7 d after STZ delivery. Paw thermal response latency of the right paw was measured every 2 wk from weeks 8–16 of diabetes using a modified Hargreaves test, as described (3).

Preparation and Use of XIB4035. The cream containing XIB4035 (1.5 mM; ZereneX Molecular) consisted of N-methyl-pyrolidone (6.25%), isopropyl myristate (6.25%), and petroleum jelly (87.5%). Control cream had the same ingredients without XIB4035. Cream was applied twice daily to the hindpaws of mice for lengths of time indicated.

Plastic Embedding and Electron Microscopy. Tissue was prepared as in ref. 1. Photographs were taken using the Tecnai G2 Spirit BioTWIN transmission electron microscope.

Tissue Collection and Immunostaining. Mice were anesthetized with 2.5% Avertin. For experiments involving line-D mice, hindpaws were removed, immersion fixed in 2% paraformaldehyde, 14% picric acid in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C, and cryoprotected in 20% sucrose overnight at 4 °C. Footpad skin was dissected from hindpaws, embedded in OCT, sectioned at 30 μm, and stained as floating sections with protein gene product 9.5 (PGP9.5) rabbit polyclonal antibody (Abcam; 1:2,000), and developed for HRP reaction (1:1,000; Biogenesis) (4). Spinal cords were collected from mice anesthetized with 2.5% Avertin, immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. Samples were embedded in paraffin and cut at a thickness of 6 μm. Sections were collected onto glass slides and incubated with an antibody against the panneuronal marker PGP9.5 (PGP9.5) rabbit polyclonal antibody (Ultraclone; 1:1,000) overnight at 4 °C followed by incubation with goat anti-rabbit Alexa 488 (Invitrogen) at 1:1,000 for 1 h at room temperature. For experiments involving diabetic mice, hindpaws were removed and fixed for 24 h in 4% paraformaldehyde in 0.1 M phosphate buffer. Samples were embedded in paraffin and cut at a thickness of 6 μm. Sections were collected onto glass slides and incubated with an antibody against the panneuronal marker PGP9.5 and developed for HRP reaction (1:1,000; Biogenesis) (4). Spinal cords were collected from mice anesthetized with 2.5% Avertin, immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4 °C, and cryoprotected in 20% sucrose overnight at 4 °C. Tissue was embedded in OCT, sectioned at 15 μm, and stained, mounted on slides, and incubated with TrpV1 rabbit polyclonal antibody (Abcam; 1:2,000) and Alexa 488-conjugated isoelectin-B4 overnight at 4 °C followed by incubation with goat anti-rabbit Alexa 594 (Invitrogen) at 1:1,000 for 1 h at room temperature. In all experiments, nuclei were stained with DAPI during secondary antibody incubations.

Immunostaining Analysis. For experiments involving line-D mice, skin section images were acquired as 30-μm Z stacks (1-μm intervals) and processed as maximum-intensity projections using a Zeiss LSM 700 microscope and ZEN software. Acquisition measures were set using control-treated wild-type sections and used for all sections imaged. A measured line was drawn using the DAPI channel to delineate the border between basal keratinocytes and outer layers of the epidermis. All PGP9.5-positive intraepidermal nerve fibers (IENFs) crossing the grid lines were counted and expressed as a number per 100-μm length. For experiments involving diabetic mice, the total numbers of IENFs were counted blinded to group identity. The length and area of each section were determined using point-counting methods and a grid reticle containing 100 squares, each 25 μm². The number of intersections between the stratum granulosum and the grid lines were counted and length calculated using a previously derived equation (4). Analysis of IENF density was blinded to genotype and treatment.

Promoter Cloning and Stable Cell Generation. The tyrosine hydroxylase (TH) promoter was cloned into the pGL3 basic vector (Promega), as previously described (5) (TH-pGL3). Briefly, a 2-kb promoter region upstream from the transcription initiation site of the rat TH gene was cloned from genomic DNA using the primer sequences TGACCGTAGGCACACCTCTCTCTATCCCCGT and AGAAGCTTCCTGCGAGCCGCGGCCCCCT. SH-SY5Y cells were cotransfected with the TH-pGL3 and the pBabe-puromycin expression vectors at a molar ratio of 10:1.

Cell Assays. SH-SY5Y-TH(pGL3) cells were maintained in DMEM/F12, 5% FBS, and 1% penicillin/streptomycin on collagen-coated plates (4 μg/mL). Neuro-2A cells were grown in MEM, 5% FBS, and 1% penicillin/streptomycin. BE(2)-C cells were grown in DMEM/F12 media containing 5% FBS and 1% penicillin/streptomycin. PC-12 cells were maintained in media containing RPMI 1640, 10% horse serum, 5% FBS, and 1% penicillin/streptomycin on collagen-coated plates. For luciferase assays, cells were treated for either 10 min with washout or overnight with various molecules and assayed for firefly luciferase 16–24 h post-treatment initiation using the luciferase assay system (Promega).

For phosphorylated Ret immunoblot experiments, cells were treated with various combinations of molecules for 10 min and either collected immediately or had treatment washed out and returned to control treatment media for the times indicated before cell collection. Lysates were collected in lysis buffer containing the following: 1% TX-100, 0.25% deoxycholic acid, 0.1 mM sodium fluoride, 0.1 mM sodium pyrophosphate, 0.02 mM sodium orthovanadate, and protease inhibitors. Immunoblots were blocked in 5% BSA in TBST plus 0.1 mM sodium fluoride, 0.1 mM sodium pyrophosphate, and 0.02 mM sodium orthovanadate, and probed in blocking solution using anti-phosphotyrosine (4G10) mouse monoclonal antibody (1:4,000; EMD Millipore). Secondary detection was performed in blocking solution using HRP-conjugated goat anti-mouse antibodies (1:1,000; MP Biomedicals). N2A cells were transfected with expression plasmids expressing a membrane bound form of EGFP (mGFP), rat GFRα1, or human GFRα3 (GFRα constructs were kind gifts from Dr. Jefferey Milbrandt at Washington University School of Medicine, St. Louis, MO). N2A and PC-12 cells were starved in basal media containing 1% FBS overnight before treatments. Glial
cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), and artemin (ARTN) were purchased from PeproTech. β-NGF (R&D Systems) at 50 μg/mL was used to stimulate TrkA receptor phosphorylation in PC-12 cells.

**Immunoblot Quantification.** Blots were imaged using an Image Quant LAS4000 mini (GE Healthcare Life Sciences). Densitometry was performed using ImageJ software over four individual experiments. Mean pixel intensity was measured over the GDNF-stimulated band at 0 min. All other phosphorylated RET bands within an experiment were measured using the identical area selection. Blot background was measured and subtracted from all values within each experiment before normalization. All measurements were normalized to the average mean pixel intensity of their respective GDNF-stimulated band at 0 min. Average normalized mean pixel intensity was calculated and plotted. Statistical significance was determined between untreated, GDNF alone, and GDNF plus 20 μM XIB4035 within each time point.

**Statistical Analyses.** All statistical analyses are as indicated and were performed using Prism 4 (GraphPad Software). SEM was used to indicate error in all analyses as n ≥ 3. For Fig. 3C, comparison of the nonlinear curve regression was performed using an F test. Analysis of the minimal ligand dose necessary to induce luciferase activity (Fig. 3C) was performed by Student t test comparing the average fold luciferase induction from three experiments to control (nontreated).


**Fig. S1.** IENF density loss in line-D mice correlates with deteriorating thermnociception. (A) Immunolabeling of PGP9.5-positive nerve terminals demonstrates a loss of IENFs in footpads of line-D mutant mice between P21 and P35. Nuclei are stained with DAPI. (Scale bar: 100 μm.) (B) Quantification of nerve terminals in the footpad skin of wild-type mice at P21 and line-D mice at P21, P28, and P35 shows that line-D mice have IENF loss already by P21, which worsens with age concurrent with declining sensory function (wild-type P21 vs. line-D P21, t test, **P < 0.01; line-D P21, P28, and P35, ANOVA Newman–Keuls post hoc, n = 4; *P < 0.05; error bars indicate SEM).
Fig. S2. Overexpression of GDNF in the skin rescues the small-fiber pathology of line-D mice. (A) Electron micrographs of transverse sections from sciatic nerves show that Remak bundle structure is lost in line-D mice but preserved in line-D::K14-GDNF mice at P30. (Scale bar: 4 μm.) (B) The density of PGP9.5-positive nerve terminals in footpads at P30 is increased by GDNF overexpression (K14-GDNF) and reduced in line-D mice compared with double-transgenic mice. Skin innervation in K14-GDNF::line-D mice is not different from in wild types. (Scale bar: 25 μm.) (C) IENF density is maintained in line-D mice by GDNF skin overexpression. Quantification of nerve terminals in footpad skin of line-D::K14-GDNF mutants at P30 show that GDNF overexpression maintains IENF density at wild-type levels (one-way ANOVA with Newman–Keuls post hoc test, n = 3; depicted significance vs. wild type; n.s., not statistically significant; *P < 0.05; other comparisons: line-D vs. line-D::K14-GDNF and K14-GDNF vs. line-D::K14-GDNF, P < 0.05; K14-GDNF vs. line-D, P < 0.001; error bars indicate SEM).
Fig. S3. Treatment of line-D mice with XIB4035 during early SFN progression prevents IENF loss in the skin. (A) The number of PGP9.5-positive nerve terminals in footpads is increased after 4 wk of treatment with XIB4035. The fiber loss seen in line-D mice treated with control cream is prevented by XIB4035 treatment. (Scale bar: 25 μm.) (B) Quantification of nerve terminals in the footpad skin after 4 wk of treatment (P49) shows that the number of PGP9.5-positive endings in both wild type and line-D treated with XIB4035 is greater than vehicle-treated animals of the same genotype (one-way ANOVA with Newman–Keuls post hoc test, n = 5; *P < 0.05; ***P < 0.001; other comparisons, WT + vehicle vs. line-D + XIB4035 and WT + XIB4035 vs. line-D + XIB4035, not statistically significant; WT + vehicle vs. line-D + vehicle and WT + XIB4035 vs. line-D + vehicle, P < 0.001; error bars indicate SEM).

Fig. S4. XIB4035 treatment of diabetic mice does not rescue IENF loss. Quantification of IENF density in footpad skin shows that diabetes leads to IENF loss that is not altered by XIB4035 treatment (one-way ANOVA with Newman–Keuls post hoc test, n ≥ 6; n.s., not statistically significant; *P < 0.05; error bars indicate SEM).
Fig. S5. XIB4035 does not alter thermal nociceptive function in wild-type mice. Wild-type mice treated with XIB4035 beginning at 4 wk of age (P28) and continuing to 6 wk of age (P42) showed no hypersensitivity to a milder noxious thermal stimulus (51 °C) than the one used in all other hot-plate testing (54 °C) [Student t test, n ≥ 10; P28 (p = 0.0671), P35 (p = 0.8343, P42 (p = 0.8492); error bars indicate SEM].
Fig. S6. GDNF-family ligands induce activation of the TH promoter luciferase reporter in a dose-dependent fashion. SH-SY5Y human neuroblastoma cells carrying a TH-luciferase reporter (SH-SY5Y-THpGL3) show dose-dependent luciferase activity responses to various ligand (GDNF, NRTN, or ARTN) concentrations (one-way ANOVA Newman–Keuls post hoc test, n = 3; vs. control, *P < 0.05, **P < 0.01, ***P < 0.001; error bars indicate SEM).
Fig. S7. Cotreatment with XIB4035 prolongs ligand-induced RET phosphorylation. Quantification of RET tyrosine phosphorylation levels by Western blot shows that cotreatment with XIB4035 does not alter the level of RET phosphorylation induced by 10-min treatment by GDNF (0 min), but prolongs RET phosphorylation after treatment is washed out. One-way ANOVA with Newman–Keuls post hoc test; n = 4 independent experiments. ANOVAs were calculated for each time point together with the untreated sample. p-RET levels in the untreated samples are not different from that in the 30- and 60-min GDNF treatment alone. n.s., not statistically significant; *P < 0.05; error bars indicate SEM.
Fig. S8. XIB4035 prolongs RET phosphorylation in cells expressing either GFRα1, 2, or 3 upon stimulation with cognate ligands. (A) Control N2A cells (transfected with mGFP construct) lack RET activation upon stimulation with GFLs. Transfection with GFRα1 or GFRα3 confers ligand-induced RET activation to N2A cells and demonstrates ligand/receptor pair specificity (GFRα1/GDNF or GFRα3/ARTN). (B and C) XIB4035 prolongs ligand-induced RET phosphorylation in either GFRα1 (B)- or GFRα3 (C)-expressing cells. RET phosphorylation in GDNF (B)- or ARTN (C)-treated samples returns to baseline by 60 min, whereas addition of 40 μM XIB4035 prolongs the ligand-induced RET phosphorylation to at least 60 min. (D) XIB4035 prolongs ligand-induced RET phosphorylation in GFRα2-expressing cells. RET phosphorylation in NRTN-treated samples returns to baseline by 60 min, whereas addition of 20 μM XIB4035 prolongs the ligand-induced RET phosphorylation to at least 60 min.

Fig. S9. XIB4035 does not change NGF-induced TrkA phosphorylation. NGF-induced TrkA phosphorylation in PC12 cells is not prolonged by addition of XIB4035.