Nerve growth factor–mediated photoablation of nociceptors reduces pain behavior in mice

Linda Nocchi, Carla Portulano, Federica Franciosa, Balint Doleschall, Mirela Panea, Nainika Roy, Mariano Maffei, Alessandra Gargano, Emerald Perlas, Paul A. Heppenstall*

Abstract
Nerve growth factor (NGF) and its receptors TrkA and p75 play a key role in the development and function of peripheral nociceptive neurons. Here, we describe novel technology to selectively photoablate TrkA-positive nociceptors through delivery of a phototoxic agent coupled to an engineered NGF ligand and subsequent near-infrared illumination. We demonstrate that this approach allows for on demand and localized reversal of pain behaviors in mouse models of acute, inflammatory, neuropathic, and joint pain. To target peripheral nociceptors, we generated a SNAP-tagged NGF derivative NGF R121W that binds to TrkA/p75 receptors but does not provoke signaling in TrkA-positive cells or elicit pain behaviors in mice. NGF R121W-SNAP was coupled to the photosensitizer IRDye700DX phthalocyanine (IR700) and injected subcutaneously. After near-infrared illumination of the injected area, behavioral responses to nociceptive mechanical and sustained thermal stimuli, but not innocuous stimuli, were substantially reduced. Similarly, in models of inflammatory, osteoarthritic, and neuropathic pain, mechanical hypersensitivity was abolished for 3 weeks after a single treatment regime. We demonstrate that this loss of pain behavior coincides with the retraction of neurons from the skin which then reinnervate the epidermis after 3 weeks corresponding with the return of mechanical hypersensitivity. Thus NGF R121W-SNAP–mediated photoablation is a minimally invasive approach to reversibly silence nociceptor input from the periphery, and control pain and hypersensitivity to mechanical stimuli.

Keywords: Nociceptor, TrkA, NGF, Acute pain, Chronic pain, Osteoarthritis, CFA-Induced pain, Spared nerve injury, Photoablation, Photosensitizer

1. Introduction
Current therapeutic strategies for the management of chronic pain have limited efficacy and may be associated with dependence, tolerance, and serious side effects. To avoid these issues, and develop more efficacious pain therapies, much effort has been made in developing strategies to target the peripheral nervous system (PNS) and molecules that are selectively expressed in nociceptors. Among the most promising of those, nerve growth factor (NGF) and its receptors TrkA and p75 have emerged as front-runners for the design of next-generation analgesics.13,15,41–43,53

Nerve growth factor (NGF) and its receptors TrkA and p75 play a key role in the development and function of nociceptive neurons. The recognition that NGF has a critical role in the generation and potentiation of pain has created a strong rationale for developing methods that interfere with its signaling. The most successful of these approaches has been the development of anti-NGF antibodies,13 which have advanced to Phase III trials for osteoarthritis (OA) of knee and hip. There are, however, a number of safety issues associated with antagonizing NGF, the most prominent of which is a rapidly progressive OA, leading to joint replacement.24

Rather than inhibiting NGF signaling, here we asked whether NGF may offer a means of accessing nociceptive neurons through their TrkA receptors to deliver a phototoxic agent to silence their activity. To test this hypothesis, we generated a SNAP-tagged NGF that could be conjugated to the photosensitizer IRDye700DX phthalocyanine (IR700). While injection of NGF SNAP IR700 and subsequent near-infrared (NIR) illumination reduced nociceptive behavior in mice, results were confounded by the fact that NGF is in itself proalgesic. We thus turned to “painless” derivatives of NGF which still bind to receptors, but provoke abrogated signaling, to deliver the photosensitizer. Such NGF mutants have been described in patients with Hereditary Sensory and Autonomic Neuropathy-type 5 (HSANV), who...
display loss of pain perception and other sensory and autonomic abnormalities, as a result of mutations in NGF. We selected the NGF p.R121W mutation as a delivery tool because this gave the highest yields in recombinant production when fused to the SNAP-tag. We found that NGF<sup>R121W-SNAP</sup> binds to TrkA-positive cells but did not elicit TrkA-mediated signaling or sensitization in vivo. Moreover, injection in mice of NGF<sup>R121W-SNAP</sup> IR700 followed by NIR illumination led to long-term reversal of nociceptive behavior that paralleled retraction of nerve fibers from the epidermis. Thus, through ligand-guided delivery of a small molecule photosensitizer, we are able to achieve selective disruption of nociceptor input and substantially reduce pain.

2. Results

2.1. Generation and characterization of NGF<sub>SNAP</sub>

Recombinant NGF was cloned and purified as a SNAP-tag fusion protein (NGF<sub>SNAP</sub>), which allows for covalent coupling to BG-derived photosensitizers and imaging fluorophores. We first tested the binding selectivity of NGF<sub>SNAP</sub> by labelling it with a BG-fluorophore and applying it to cells expressing neurotrophin receptors. NGF<sub>SNAP</sub> was coupled in vitro to BG-Surface549 and applied at a 100-nM concentration to Hek293T cells transiently transfected with TrkA, TrkB, or TrkC and p75. NGF<sub>SNAP</sub> bound its cognate receptor, decorating the surface of TrkA-/p75-transfected cells (Fig. 1A), while cells transfected with BDNF/NTR4 and NT3 receptors with p75 were not stained (Figs. 1B and C), demonstrating that binding occurs faithfully in a receptor-specific fashion.

Having confirmed that NGF<sub>SNAP</sub> specifically labels TrkA-expressing cells, we next asked whether a photosensitizer can be coupled to the ligand to ablate TrkA-positive cells. Hek293T cells transfected with TrkA/p75 were incubated with NGF<sub>SNAP</sub> conjugated to the NIR photosensitizer BG-IR700 and illuminated with NIR light for 2 minutes. Twenty-four hours later, cell death was analyzed using propidium iodide staining. In cells transfected with TrkA/p75, we observed concentration-dependent increase in cell death upon treatment with NGF<sub>SNAP</sub> IR700 and NIR illumination (Figs. 1D–F). In the absence of TrkA expression, cell viability was fully preserved after NGF<sub>SNAP</sub> IR700 application and illumination (Fig. 1G).

We next asked whether NGF<sub>SNAP</sub> IR700 can be used to photobleach nociceptors in vivo. NGF<sub>SNAP</sub> conjugated with BG-IR700 (Fig. 1H, filled circles) or unconjugated NGF<sub>SNAP</sub> (open circles) was injected into the hind paw of C57BL/6J male naive mice, and skin was illuminated for 2 minutes with IR light exposure on 3 consecutive days. Behavioral responses to calibrated von Frey filaments were monitored after each ablation at 24 hours and 5 days after the last treatment. As shown in Figure 1H, mechanical thresholds increased in ablated mice, suggesting that TrkA-expressing nociceptors were targeted by NGF<sub>SNAP</sub> IR700. However, we also observed a decrease in von Frey thresholds in control NGF<sub>SNAP</sub> mice (Fig. 1H), indicating that the ligand in itself was having a proalgesic effect and might constitute a confounding factor in the measurement of mechanical thresholds in ablated mice. In further experiments, we thus sought to exploit a “painless” NGF mutant described in HSANV patients, NGF<sup>R121W</sup>, which has been described to bind to TrkA receptors but not provoke nociceptive signaling.

2.2. Generation and characterization of NGF<sup>R121W-SNAP</sup>

A C-terminal fusion of NGF<sup>R121W</sup> and SNAP (NGF<sup>R121W-SNAP</sup>) was produced in Chinese hamster ovary cells at yields similar to wildtype NGF<sub>SNAP</sub> and could be readily labelled with BG549 indicating that the SNAP-tag was fully functional (Fig. 2A). To characterize the binding and signaling properties of NGF<sup>R121W-SNAP</sup>, we first assessed its capacity to label Hek293T cells transfected with neurotrophin receptor plasmids. NGF<sup>R121W-SNAP</sup> was coupled in vitro with BG-Surface549 and applied to cells. Similar to wildtype NGF<sub>SNAP</sub>, we observed robust membrane labelling of TrkA-/p75-expressing cells (Fig. 2B) but no signal in TrkB/p75- or TrkC/p75-transfected cells (Figs. 2C and D). We further analyzed downstream signaling provoked by wildtype NGF<sub>SNAP</sub> and NGF<sup>R121W-SNAP</sup> by assessing phosphorylation of MAPK and AKT upon treatment of PC12 cells with ligands. Wildtype NGF treatment produced a substantial increase in phosphorylation of MAPK and AKT, while levels of MAPK and AKT phosphorylation in cells treated with NGF<sup>R121W-SNAP</sup> were no different from untreated cells (Figs. 2E and F).

We next evaluated the neurotrophic activity of wildtype NGF and NGF<sup>R121W-SNAP</sup> by quantifying the number of differentiated PC12 cells (assessed by neurite outgrowth) after 6 days of incubation with ligands. A significantly higher proportion of differentiated PC12 cells were present in samples treated with NGF<sub>SNAP</sub>, while those treated with NGF<sup>R121W-SNAP</sup> were not significantly different from untreated controls (Figs. 2G–I). Finally, we considered the pronociceptive activity of NGF<sub>SNAP</sub> and NGF<sup>R121W-SNAP</sup> by injecting ligands into the hind paw of mice and evaluating mechanical and thermal hyperalgesia with the von Frey and hotplate tests. We found that wildtype NGF induced substantial sensitization to both mechanical and thermal stimuli, while injection of NGF<sup>R121W-SNAP</sup> did not change thresholds to either of these stimuli (Figs. 2K and L).

2.3. NGF<sup>R121W-SNAP</sup>-mediated photoablation and acute nociception

We next assessed the efficacy of NGF<sup>R121W-SNAP</sup> IR700-mediated photoablation in vitro in Hek293T cells transfected with TrkA/p75. Similar to NGF<sub>SNAP</sub>, the mutant ligand induced a robust and concentration-dependent increase in cell death 24 hours after application to cells and NIR illumination (Figs. 3A–C). By contrast, NGF<sup>R121W-SNAP</sup> IR700 and NIR illumination was without effect in mock-transfected cells not expressing TrkA/p75 (Fig. 3D).

To characterize NGF<sup>R121W-SNAP</sup>-mediated photoablation in vivo, we evaluated its efficacy in models of acute nociception. Wildtype C57BL/6J male mice were injected for 3 consecutive days in the hind paw with either NGF<sup>R121W-SNAP</sup>, with or without IR700, or with IR700 alone (some controls shown in Supplementary Fig. 2, available at http://links.lww.com/PAIN/A822) followed by NIR illumination. We first tested mice for sensitivity to mechanical stimuli using von Frey filaments. As shown in Figure 3E, 2 days after ablation, mice treated with NGF<sup>R121W-SNAP</sup> IR700 displayed significantly reduced sensitivity to these stimuli, requiring weights above 3 g to provoke a behavioral response, and this insensitivity persisted throughout the 8-day monitoring period. Similarly, responses to nociceptive pin-prick stimuli applied to the paw were also substantially reduced in photoablated compared with control mice (Fig. 3F).

We further assessed sensitivity to non-nociceptive dynamic brush stimuli and observed no effect of NGF<sup>R121W-SNAP</sup> IR700 (Fig. 3G) indicating that the photoablation is selective for nociceptors. We also tested thermal responsiveness using the hotplate test. Intriguingly, first-line reflex latencies were not different between photoablated and control animals; however, licking...
behavior evoked by sustained noxious thermal stimuli was reduced in treated animals at 46°C and 50°C (Figs. 3H and I). Thus, TrkA-positive neurons may mediate pain resulting from tissue damaging thermal stimuli, rather than reflexive defensive responses. Finally, we asked whether NGF<sup>R121W-SNAP</sup> IR700 can be applied topically to alleviate acute noceception. A microemulsion premixed with either NGF<sup>R121W-SNAP</sup> with or without IR700, or IR700 alone (Supplementary Fig. 2e, available at http://links.lww.com/PAIN/A822), was applied to the tail skin and illuminated with NIR light. One week later, a calibrated clip was applied to the base of the tail and responses monitored. We observed significantly reduced behavioral responses to the clip in NGF<sup>R121W-SNAP</sup> IR700 mice throughout the monitoring period, such that mice appeared essentially insensitive to this noxious stimulus (Fig. 3J; Supplementary Video 1, available at http://links.lww.com/PAIN/A823).

2.4. NGF<sup>R121W-SNAP</sup>-mediated photoablation in models of inflammatory, osteoarthritic, and neuropathic pain

We investigated the effects of NGF<sup>R121W-SNAP</sup>-mediated photoablation on persistent pain using models of inflammatory, osteoarthritic, and neuropathic pain.

To model inflammatory pain, mice were injected in the hind paw with complete Freund’s adjuvant (CFA). After 48 hours, mechanical and thermal hyperalgesia were evident, and mice were treated for 3 days with NGF<sup>R121W-SNAP</sup> with or without IR700 (Supplementary Fig. 2e, available at http://links.lww.com/PAIN/A822), or with NGF<sup>R121W-SNAP</sup> IR700, with or without near NIR illumination (Fig. 4A and B). Behavioral thresholds to mechanical and thermal stimuli were monitored for 12 days after CFA injection. As shown in Figure 4A, mice which received NGF<sup>R121W-SNAP</sup> and NIR inflammation displayed a complete reversal of mechanical hypersensitivity that lasted throughout the 12-day observation period (Fig. 4A). Of note, thermal hyperalgesia induced by CFA was not altered by photoablation (Fig. 4B).

To model OA-like pain, mice received an intra-articular injection into the knee of monosodium iodoacetate (MIA). Seven days after injection, significant fibrosis was evident in the joint (Supplementary Fig. 1a–f, available at http://links.lww.com/PAIN/A822), and mice developed referred mechanical hypersensitivity to von Frey filaments applied to the plantar surface of the paw (Supplementary Fig. 1g, available at http://links.lww.com/PAIN/A822). NGF<sup>R121W-SNAP</sup> IR700 was injected into the knee, and the area was illuminated with NIR light through the skin for 3 consecutive days. Control mice received NGF<sup>R121W-SNAP</sup> IR700 but no illumination, or IR700 alone with illumination (Supplementary Fig. 2f, available at http://links.lww.com/PAIN/A822). In treated mice, we observed a complete recovery of mechanical thresholds to preinjury levels, which persisted for 3 weeks before returning to the preablation state (Fig. 4C). Control animals displayed no recovery and remained mechanically hypersensitive throughout the 40-day monitoring period (Fig. 4C; Supplementary Fig. 2f, available at http://links.lww.com/PAIN/A822).

For neuropathic pain, we used the spared nerve injury (SNI) model. Seven days after nerve injury when all mice had developed mechanical hypersensitivity, NGF<sup>R121W-SNAP</sup> IR700 was injected into the paw, and the skin was illuminated with NIR light on 3 consecutive days. Control animals received NGF<sup>R121W-SNAP</sup> IR700 without illumination (Figs. 4D–F), IR700 with illumination, or NIR illumination.
alone (Supplementary Fig. 2g, available at http://links.lww.com/PAIN/A822). Mice were then assayed for hypersensitivity to mechanical punctate stimuli, dynamic brush stimuli, and evaporative cooling. In mice treated with NGF R121W-SNAP IR700 and NIR illumination, we again observed that thresholds to punctate mechanical stimuli recovered to preinjury levels for about 3 weeks, before returning to

Figure 2. Characterization of NGF R121W-SNAP. (A) NGF R121W-SNAP was coupled with BG549 in vitro, run on an SDS-Page, stained with Coomassie blue, and in-gel fluorescence visualized under a UV light. (B–D) Confocal images of Hek293T cells transiently transfected with TrkA, TrkB, or TrkC (B–D, respectively) and p75 stained with 100-nM NGF R121W-SNAP conjugated to surface BG-546. NGF R121W-SNAP selectively binds only to TrkA-/p75-transfected cells, with virtually no binding to TrkB- or TrkC-transfected cells. Scale bar 20 μm. (E) Representative Western blot of 3 independent experiments, showing the expression level of MAPK, phospho-MAPK, AKT, phospho-AKT, and actin (loading control) in untreated PC12 cells (lane 1), treated with NGF (lane 2) or NGF R121W-SNAP (lane 3). (F) Levels of each protein were expressed as ratio between the phosphorylated form and the total counterpart and then normalized to the NGF-treated sample. (G–J) Neuron differentiation in untreated (G), NGF-treated (H), and NGF R121W-SNAP-treated (I) PC12 cells, after 6 days of treatment. Arrows indicate differentiated cells. Scale bar 20 μm. (J) Quantitation of neuron-like differentiated PC12 cells, expressed as percentage (%); for the analysis, 241 untreated cells, 246 NGF-treated cells, and 285 NGF R121W-SNAP-treated cells were considered. Error bars indicate SEM. (K) von Frey thresholds after injection of NGF (shaded box) or NGF R121W-SNAP (open box) into the hind paw (ipsi) of mice (n = 4 animals). Box plots represent the force expressed in grams (G) required to trigger a 50% response. *P = 0.05 (two-tailed t test). (L) Hotplate test after injection of NGF (n = 6 animals, red box) and NGF R121W-SNAP (n = 5 animals, gray box) into the hind paw of the mice. Box plots represent the latency expressed in seconds (s) of the paw withdrawal in response to heat (52°C). *P = 0.009 (two-tailed t test). NGF, nerve growth factor.
a hypersensitive state (Fig. 4D). Intriguingly, responses to dynamic brushing mechanical stimuli were not altered by photoablation (Fig. 4E) nor were responses to evaporative cooling (Fig. 4F).

2.5. Mechanism of NGFR121W-SNAP–mediated photoablation

To understand how NGFR121W-SNAP–mediated photoablation underlies the loss and then recovery of mechanical sensitivity to noxious stimuli in treated mice, we performed a histological analysis of skin at 7 and 28 days after treatment.

We first investigated the degree of cell death of non-neuronal cells in the skin by performing TUNEL staining to detect apoptotic DNA fragmentation. Skin was harvested at 7 and 28 days after treatment in animals injected with NGFR121W-SNAP IR700 and illuminated with NIR light, or at 7 days after treatment in control animals receiving only IR700 and illumination. Sections were then stained with propidium iodide (PI) to detect apoptotic DNA fragmentation. The results showed a decrease in apoptotic cell death in the skin of treated mice compared to control animals.

Error bars indicate SEM, *P = 0.001 (two-way ANOVA followed by Bonferroni post hoc test).
processed for TUNEL staining and immunohistochemistry with an anti-K14 antibody to mark keratinocytes. As shown in Figures 5A–C, very few apoptotic cells could be detected in the skin in all conditions, and there was no difference in treated vs control samples (Figs. 5A–D). The number of keratinocytes was also constant across conditions and time points. Thus, at the concentrations and light intensities used here, there is no detectable effect of photoablation on non-neuronal cells in the skin.

To investigate the effects of NGFR121W-SNAP-guided photoablation on innervation of the skin, we performed immunohistochemistry using an anti-Pgp9.5 antibody to detect neuronal fibers. Again, skin samples were taken at 7 and 28 days after treatment in control animals (IR700 and illumination). We observed a significant reduction in the number of the intraepidermal free nerve endings in treated animals compared with control at 7 days after injection (Figs. 5E and F). Importantly, at 28 days after ablation, the number of fibers was restored to a value similar to the one in the control group (Fig. 5G). Thus, after photoablation, we observe a retraction of nociceptors from the skin that then reinnervate the epidermis with a similar time course to the loss and return of mechanical sensitivity seen in behavioral experiments.

3. Methods

3.1. Animals

Wildtype C57BL/6J and Balb/c mice were used at age of 8 to 10 weeks for all the experiments. All mice were bred and maintained at the EMBL Neurobiology and Epigenetic Unit, Rome, in accordance with Italian legislation (Art. 9, 27. January 1992, no 116). Experiments were performed under license from the Italian Ministry of Health and in compliance with the ARRIVE guidelines.

3.2. Production of recombinant NGF SNAP and NGFR121W-SNAP

cDNAs encoding for murine NGF SNAP and NGFR121W-SNAP including a C-terminal polyhistidine tag (His6) inserted for purification purposes were cloned into the pMBS-PB vector as a fusion protein. Protein expression was performed using Chinese hamster ovary cells (ATCC, mycoplasma negative) as described by Balasubramanian.2 Secreted NGF SNAP and NGFR121W-SNAP were purified from cell medium using a Ni-NTA resin (Qiagen, #30210) and eluted with an excess of imidazole. Eluted fractions were then pooled, concentrated, and stored for further analysis.

3.3. Synthesis of BG-IR700

A total of 3 mg of IRDye700DX N-hydroxysuccinimide ester fluorophore (LI-COR Biosciences GmbH, Bad Homburg, Germany) were dissolved in 150 μL of DMSO and treated with 1.5 mg of BG-PEG11-NH₂ and 5 μL of disopropylethylamine. After 1 hour, the product BG-PEG11-IRDye700DX was purified by HPLC using a Waters Sunfire Prep C18 OBD 5 μm; 19 × 150-mm column using 0.1 M of triethylammonium acetate (TEAA) (pH 7.0) and 0.1 M of TEAA in water/acetonitrile 3:7 (pH 7.0) as mobile phases A and B, respectively. A linear gradient from 100% A to 100% B within 30 minutes was used. The fractions containing the product were lyophilized. Stock solutions of BG-IR700 were...
stored at high concentration in DMSO. Before conjugation with proteins, BG-IR700 was diluted in physiological buffer.

3.4. In vitro experiments

Hek293T cells were transfected with TrkA, TrkB, TrkC, and p75 plasmid using Lipofectamine 2000 (Thermo). For the labelling, 1 mM NGFR121W-SNAP was coupled with 3 mM BG549 surface (NEB # S9112) for 1 hour at 37˚C in calcium imaging buffer (NaCl 140 mM; KCl 4 mM; CaCl2 2 mM; MgCl2 1 mM; NaOH; 4.55 mM; glucose 5 mM; and HEPES 10 mM; pH 7.4). The coupling reaction was filtered through a PD MiniTrap G-25 column (GE Healthcare #28-9180-07) to remove the excess of BG. Cells were incubated with the coupling reaction for 15 to 20 minutes at 37˚C, then washed 3 times in calcium imaging buffer.

To assess NGF signaling, PC12 cells were cultured on collagen IV–coated 6-well plate in DMEM/F12 medium containing 1% horse serum. Cells were treated for 2 minutes with PBS or 1 mM NGF or 1 mM NGFR121W-SNAP, followed by lysis in Ripa buffer.

For the neuronal differentiation assay, PC12 cells were daily exposed to 100-ng/mL NGF (Alomone labs, #N-100) or NGFR121W-SNAP for 6 days. The number of neuron-like cells was counted at day 6.

All images were visualized with a Leica SP5 confocal microscope.

3.5. SDS-Page and Western blot

To assess the coupling reaction, 1 mM NGFR121W-SNAP was coupled with 1.5 mM BG549 for 1 hour at 37˚C. The coupling reactions were analyzed by SDS-Page on a precast acrylamide gel (BioRad #456-9034), along with the same concentrations of NGF R121W-SNAP alone. The bands corresponding to the binding of NGF R121W-SNAP with BG549 were visualized by gel fluorescence. All the samples were visualized by Coomassie staining.

For NGF-mediated signaling, PC12 cells were collected and lysed in Ripa Buffer (Sigma, #R0278) with proteases inhibitor cocktail (Roche #11873580001). Protein lysates were quantified by Bradford assay. For all the experiments, 30-μg total lysate were separated on 10% SDS-Page gel and transferred to a nitrocellulose membrane (Protran #10600007). Membranes were incubated with the following antibodies: anti-MAPK (Cell Signaling #4695), anti-phospho MAPK (Thr202/Tyr204) (Cell Signaling #9106), anti-AKT (Cell Signaling #4691), anti-phospho AKT (Ser473) (Cell Signaling #9271), anti-Actin (Cell signaling #4970). Bands were visualized using the ECL detection system (Amersham #RPN2106); band density was calculated using ImageJ, and the levels of phosphorylated proteins were normalized to the total counterpart.

Figure 5. NGFR121W-SNAP-guided photoablation induces reversible nerve retraction without affecting keratinocytes in the skin. (A–D) Representative paw skin cryosections stained for keratin 14 (K14, in green) and TUNEL TMR (in red) with quantitation of keratinocytes cell death in mice (n = 3 animals) injected for 3 days with IR700 followed by NIR illumination (A), NGF R121W-SNAP + IR700 followed by NIR illumination (B and C). Skin was collected 7 days after (A and B) and 28 days after (C) the last day of injection. The number of TUNEL+ keratinocytes is expressed as percentage (D). (E–H) Representative paw skin cryosections stained for the neuronal marker Pgp9.5 (in green) of mice injected for 3 days with IR700 followed by NIR illumination (E), NGF R121W-SNAP + IR700 followed by NIR illumination (F and G). The skin was collected 7 days after (E and F) and 28 days after (G) the last day of injection. Arrows indicate free nerve endings. Quantitation of the epidermal free nerve endings for the indicated sample; 8 sections were analyzed (H). *P = 0.001 (two-tailed t test). NIR, near-infrared.
3.6. Immunofluorescence

Skin from the plantar region of the mice hind paw was dissected 7 days and 28 days after treatment of SN1 mice with IR700, with or without NGF R121W-SNAP, followed by near-IR illumination. Skin was fixed with 4% PFA overnight at 4°C, cryoprotected in 30% sucrose for 6 hours and embedded in Tissue-Tek O.C.T. compound. Twenty-five micrometer thick sections were cut using a cryostat (Leica, CM3050S), permeabilized with 0.3% Triton-X, blocked in 5% goat serum + 0.3% Triton-X, and stained for one of the antibodies listed below, prepared in PBS containing 5% goat serum + 0.3% Triton-X. We used K14 (1:200 dilution; Covance # PRB155P) antibody marking for keratinocytes; TUNEL TMR (In situ cell death detection kit—TMR red, Roche # 012156792910) for detecting apoptotic cells; Pgp9.5 (1:200 dilution; Dako # Z5116) marking for nerve fibers. DAPI (1 μg/mL, Invitrogen # D1306) was used to stain nuclei. Slides were mounted with Prolong gold antifade (Invitrogen # P36930), imaged with a Leica SP5 confocal microscope and analyzed with ImageJ. To quantify keratinocytes apoptosis, TUNEL + K14 + double positive cells were counted in the epidermal basal layer expressed as percentage. For Pgp9.5 + fibers quantitation, the region of dermis/epidermis junction was analyzed and only the intraepidermal free nerve endings per section were considered for counting.38

3.7. Knee histology

After intra-articular injection with saline or MIA, knees were dissected out after 7 and 28 days. The surrounding tissues were trimmed, and knees were postfixed overnight and then placed into a decalcifying solution for 72 hours. Decalcified knees were then washed and embedded in paraffin wax. Eight micrometer thick sections were cut and stained for hematoxylin and eosin and for trichrome staining.

3.8. Behavioral testing

All behavior experiments were performed on adult male mice (8-10 weeks old). Mice were placed on an elevated platform with a mesh floor and habituated for minimum 30 minutes, unless otherwise specified.

For the von Frey test, the plantar side of the hind paw was stimulated with calibrated von Frey filaments (North Coast medical, #NC12775-99). The 50% paw withdrawal threshold was calculated using the Up–Down method.14 After MIA injection, von Frey testing was confined to the posterior-heel region of the hind paw; post-SNI, von Frey testing was confined to the sural nerve innervating region of the paw.

To measure dynamic allodynia, the plantar hind paw was stimulated by stroking using a paint brush (heel-to-toe direction). The responses were scored as previously described18 as 0 = no response; 1 = brief paw withdrawal; 2 = prolonged paw withdrawal, and 3 = paw flicking or licking. Test was performed 5 times, with 1-minute interval between trials.

To measure thermal nociception, mice were placed on a hotplate (Ugo Basile, #35150) preset at 52°C and video recorded. The latency to response (flicking or licking the hind paw) was measured. To avoid tissue damage, a cutoff of 30 seconds was defined.

A hotplate was also used to assess the response to prolonged nocuous stimuli. The hotplate was set at 46°C and 50°C, and different cutoffs were set for each temperature: 3 minutes for 46°C, and 1 minute for 50°C, as previously described.25

Thermal nociception was also evaluated using Hargreaves test, in which a thermal heat stimulus (IR 35) was focused onto the plantar region of the hind paw. To avoid tissue damage, a cutoff of 20 seconds was set. The test was repeated 3 times, with 5-minute interval between trials.

To assess evaporative cold allodynia, the acetone drop test was performed.16 Briefly, a single drop of cold acetone was applied onto the plantar side of the hind paw of mice using a syringe without touching the paw of the mice. The responses were scored according to the following scheme: 0 = no response, 1 = paw withdrawal or a single flick, 2 = repeated flicking of the paw, and 3 = licking of the paw. The test was repeated 5 times with 1-minute interval between trials.

For the pin-prick test, the plantar side of the hind paw was gently touched using an insect pin attached to a 1 g von Frey filament, without penetrating the skin, and the yes/no responses were noted for 10 trials, with 1-minute interval between the trials.

For the clip tail test, an alligator clip covered with a rubber casing to reduce the potential tissue damage, was placed on the tail of the mouse, near the base of the tail; then, each mouse was placed on a plexiglass chamber and video recorded. A response was scored when the mice showed a response to the clip by biting, vocalization, grasping of tail, or jumping. A cutoff of 90 seconds was set, and data are plotted as the time to respond (latency) for each mouse.

3.9. Complete Freund’s Adjuvant model

Complete Freund’s Adjuvant (CFA, 20 μL, 2% in 1:1 saline) (Sigma-Aldrich, F5881) was injected into the hind paw of mice. Behavioral tests were performed at 24- and 48-hour after CFA injection to assess inflammation, followed by photoablation experiments.

3.10. Spared nerve injury model

Peripheral nerve injury was induced by SN1.46 Briefly, mice were anesthetized using 2.5% isoflurane. The sciatic nerve near the thigh region was exposed, and the peroneal and tibial branches were ligated and cut, leaving the sural nerve intact. Behavioral tests were performed starting from 1 week after injury, followed by photoablation experiments.

3.11. Osteoarthritis model

As previously described,45 10-μL monosodium iodoacetate (MIA, 1 mg) (Sigma-Aldrich, I2512) was injected intra-articular, through the infrapatellar ligament, into the knee. Sterile saline was injected in the control mice. Behavioral testing was performed 1 week after the injection.

3.12. In vivo photoablation

The hind paw, the knee, or the tail of the mice was treated for 3 consecutive days with NGF R121W-SNAP (5 μM) coupled to BG-IR700 (15 μM) (20 μL and 10 μL for paw and knee injections, respectively, and 20 μL for microemulsion application). Depending on the experiment, the coupling reaction was delivered by injection, or topically using a microemulsion, as described previously.44 Twenty minutes after delivery, NIR light at 690 nm was applied to the treated skin for 1 minute, at 120 to 150 J/cm² (PSU-III-FDA diode laser, CNI Laser, China). Behavioral tests were performed starting from 1 day after the last photoablation.
3.13. Statistical analysis

All statistical data are presented as SEM along with the number of samples analyzed (n). Student t test and/or analysis of variance followed by the appropriate post hoc test were used. Statistical significance was assumed at $P < 0.05$. Sample sizes were determined from the power of the statistical test performed. No animals were excluded, and all experiments were performed blinded with order of testing randomized.

4. Discussion

Here, we describe an approach based on ligand-targeted, light-activated delivery of a phototoxic agent to ablate nociceptors in the skin and reduce pain behavior in mice. To achieve selective nociceptor ablation in vivo, we engineered the “painless” HSANV NGF$^{R121W}$ mutant ligand to specifically deliver a photoactivatable photosensitizer locally to TrkA expressing nociceptors. Upon illumination with NIR light, we show that there is a highly effective reversal in pain behavior in mouse models of inflammatory, neuropathic, and osteoarthritic pain.

The ligand-mediated photoablation technology we describe here has been previously validated in mice using engineered TrkB- and IL31-receptor ligands to control mechanical pain hypersensitivity and inflammatory itch.18,44 Based on extensive evidence supporting a role for TrkA signaling in pain,1,7,16,27,31,34–36,39,56,62 we selected NGF as a delivery agent to target cutaneous nociceptors in vivo. After local illumination of the skin, we observed retraction of fibers innervating the epidermis, corresponding to a long lasting but reversible reduction in nociceptive behavior. When the pain behavior returned 3 weeks after photoablation, we also observed a concomitant regrowth of the cutaneous sensory fibers, suggesting that we were targeting nociceptors, the major transducers of pain. Importantly, no damage to other cellular types was detected under the conditions used, despite the fact that TrkA receptors are expressed on keratinocytes and other cells in the skin.7,36,55 This is in agreement with our previous study using interleukin-31 (IL-31) as a ligand to target prurceptors in the skin. Like NGF receptors, IL-31 receptors are highly expressed in keratinocytes; however, we found that IL-31-guided photoablation induced keratinocyte cell death only at very high concentrations, and at much higher light intensities than those used here.44 This would suggest that neurons are more sensitive to IR700-induced photoablation than other cells in the skin.

Several recent studies have described naturally occurring mutations in the NGF gene in patients affected by a rare form of congenital insensitivity to pain, HSANV. The variants pro-NGF$^{R251W}$ (corresponding to the mature NGF$^{R102W}$) and NGF$^{R121W}$ have been characterized at the protein level,13,54,57 and it has been demonstrated that both retain the binding to their receptors TrkA and p75, while downstream signaling (autophosphorylation of TrkA and phosphorylation of MAPK) is impaired, preventing the nociceptive activity typically exerted by NGF. The identification of these “painless” NGF forms opens up the possibility that these mutated ligands may be exploited as drug delivery agents that bind to nociceptors but do not activate them. We explored this possibility here by fusing a SNAP-tag to NGF$^{R121W}$, and using it to target the small molecule photosensitizer IR700 to nociceptors. Further development of this approach may also allow for the conjugation of other biologically active small molecules to NGF$^{R121W}$ to deliver them into nociceptors in vivo and directly interfere with neuronal activity.

Previous work has shown that selective antagonism of endogenous NGF is highly effective in animal models of many acute and chronic pain states.23,39,51 Among several agents developed to counteract NGF-mediated sensitization, particular attention has focused on monoclonal antibodies such as tanezumab and fasinumab, which have proven effective in the management of osteoarthritic pain.5,8 Although these anti-NGF antibodies showed greater efficacy in chronic pain compared with the nonsteroidal anti-inflammatory drugs, serious adverse effects, including osteonecrosis, neurogenic arthropathy, and morphologic changes in the sympathetic nervous system were reported,4 leading the US Food and Drug Administration (FDA) to place a hold from 2010 to 2015 on clinical studies involving this class of drugs. The reported adverse effects were similar across anti-NGF monoclonal antibodies, suggesting that they are “class-specific effects”.8 Although new evidence is generated in ongoing phase III clinical trials involving tanezumab and fasinumab in OA, the current evaluation of risk/benefit ratio of anti-NGF therapies remains challenging.5

Similar safety concerns may also be pertinent to NGF-mediated photoablation. However, there are a number of important differences in the photoablation approach compared with monoclonal anti-NGF–based strategies for treating pain. First, photoablation targets cells rather than one specific molecular mediator of pain, enabling the treatment to bypass the enormous molecular complexity that underlies pain, and to circumvent molecular redundancy, a major issue in developing new analgesic drugs. Second, the photoablation is local and can be applied on demand. Thus, dosage, light intensity, and frequency of application may constitute useful checkpoints for patient-personalized modulation. Compared with approaches based on monoclonal antibodies, which are administered systemically, this may reduce off-target effects on systems other than nociception. Third, our data indicate that a single treatment regime of photoablation is sufficient to obtain analgesia that lasts for several weeks, rather than hours or days, potentially reducing toxicity. Indeed, it may also be possible to increase the effect duration further by performing photoablation in the nerve innervating the injured area, or even to selectively ablate nociceptors permanently in the dorsal root ganglion. However, before these approaches are tested, it will be necessary to investigate whether ligand-receptor complexes are endocytosed and transported along the axon to the dorsal root ganglion cell body, and to determine whether the R121W mutation influences NGF trafficking. It will also be important to establish the safety and efficacy of repeated cycles of photoablation.

We found that photoablation using NGF$^{R121W}$-SNAP was most effective at reducing responses to punctate nociceptive mechanical stimuli. Thus, we observed substantially increased von Frey thresholds under basal, inflammatory, osteoarthritic, and neuropathic states and decreased sensitivity to painful clip and pinprick. Of note, behavioral responses to dynamic mechanical stimuli were not altered by photoablation. In previous work, we have demonstrated that these dynamic stimuli evoke nociceptive reflexes through TrkB-positive mechanoreceptors after nerve injury, and that photoablation using BDNF$^{SNAP}$ is an effective means of reducing these responses.18 As well as demonstrating that NGF$^{R121W}$-SNAP- and BDNF$^{SNAP}$-mediated photoablation is selective, these data highlight the complexity of sensory processing under different conditions and indicate that solely targeting nociceptors may not be an optimal strategy in certain pain states.

Given the prominent role of NGF in thermal hyperalgesia, we expected to see a reduction in thermal nociceptive withdrawal latencies in photoablation-treated mice. For example, using a 52°C stimulus, we observed similar latencies between ablated and
nonsensory nociceptors underlie reflexive responses that limit injury, while TRPV1-positive neurons underlie behavioral responses to prolonged thermal stimulation at 46°C and 50°C. It has recently been demonstrated that these 2 behaviors are driven by distinct inputs to the periphery, and it was shown that nonpeptidergic MRGPRD-positive nociceptors mediate thermal nociceptive withdrawal reflexes.

Although we saw no difference in withdrawal reflexes to heat, we did observe a significant reduction in nociceptive behavior upon prolonged thermal stimulation at 46°C and 50°C. This has been demonstrated that these 2 behaviors are driven by distinct inputs from the periphery and the spinal cord to the thalamus. Indeed, in a recent study, it has been shown that acute noxious inflammatory nerve growth factor, on the sympathetic nervous system in adult cynomolgus monkeys (Macaca fascicularis); a stereodenic: histopathologic, and cardiovascular assessment. Toxicol Sci 2017;158:319–33.

Conflict of interest statement
The authors have no conflicts of interest to declare.

Acknowledgements
The authors acknowledge the assistance of David Hacker, Laurence Durrer, and Soraya Quinche of the Protein Production and Structure Core Facility of the EPFL in generation of NGF SNAP and NGFαβ12W-SNAP. The authors also thank Violetta Paribeni and Valerio Rossi for the technical support provided with the animals. This work was funded by EMBL.

Appendix A. Supplemental digital content
Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A822.

Supplemental video content
Video content associated with this article can be found online at http://links.lww.com/PAIN/A823.

Article history:
Received 13 March 2019
Received in revised form 7 May 2019
Accepted 10 May 2019
Available online 26 June 2019

References
[1] Axelrod FB, Hilz MJ. Inherited autonomic neuropathies. Semin Neurol 2003;23:381–90.
[2] Balsubramanian S, Matasci M, Kadlecova Z, Baldi L, Hacker DL, Wurm FM. Rapid recombinant protein production from piggyBac transposon-mediated stable CHO cell pools. J Biotechnol 2015;200:61–9.
[3] Bannwarth B, Kostine M. Targeting nerve growth factor (NGF) for pain management: what does the future hold for NGF antagonists? Drugs 2014;74:619–26.
[4] Belanger P, Butler P, Butt M, Bhattacharya P, Fosset S, Shelton D, Evans M, Andrus R, Hurst S, Ökerberg C, Cummings T, Potter D, Steidl-Nichols J, Zorbas M. From the cover: evaluation of the effects of tanezumab, a monoclonal antibody against nerve growth factor, on the sympathetically evoked responses in adult cynomolgus monkeys (Macaca fascicularis): a stereologic, histopathologic, and cardiovascular assessment. Toxicol Sci 2017;158:319–33.
[5] Berenbaum F. Targeting nerve growth factor to relieve pain from osteoarthritis: what can we expect? Joint Bone Spine 2018;86:127–8.
[6] Bhangar KP, Kaye AD, Knezevic NN, Candido KD, Urman RD. An analysis of new approaches and drug formulations for treatment of chronic low back pain. Anesth Analg 2017;155:341–50.
[7] Botchkareva VA, Yaar M, Peters EM, Raychaudhuri SP, Botchkareva NV, Marconi A, Raychaudhuri SK, Paus R, Pincelli C. Neurotrophins in skin biology and pathology. J Invest Dermatol 2006;126:1719–27.
[8] Bramson C, Herrmann DN, Carey W, Keller D, Brown MT, West CR, Verburg KM, Dyck PJ. Exploring the role of tanezumab as a novel treatment for the relief of neuropathic pain. Pain Med 2015;16:1162–76.
[9] Busse JW, Wang L, Kamaleldin M, Craigie S, Riva JJ, Montoya L, Mulla SM, Lopes LC, Vogel N, Chen E, Kirmayr K, De Oliveira K, Olivier L, Kaushal A, Chaparro LE, O'Bryan J, Agarwal A, Couban R, Tosi L, Lam T, Vrandyk PO, Hsu S, Bala MM, Schandelmeier S, Scheidecker A, Ebrahim S, Ashoctor V, Rehman Y, Hsu PJ, Ross S, Johnston BC, Kurnz R, Sun X, Buckley N, Sessler DI, Guyatt GH. Opioids for chronic noncancer pain: a systematic review and meta-analysis. JAMA 2018;320:2448–60.
[10] Capsoni S, Covacevich S, Marinelli S, Cici M, Bernardi A, Minghetti L, Ugozil G, Pavone F, Cattaneo A. Taking pain out of NGF: a “painless” NGF mutant, linked to hereditary sensory autonomic neuropathy type V, with full neurotrophic activity. PLoS One 2011;6:e17321.
[11] Carroll SL, Silos-Santiago I, Freese SE, Ruit KG, Milbrandt J, Snider WD. Dorsal root ganglion neurons expressing trk are selectively sensitive to NGF deprivation in utero. Neuron 1992;9:779–88.
[12] Canvalho OP, Thornton GK, Hertecant J, Houlden H, Nicholas AK, Cox JJ, Reilly M, Al-Gazzali L, Woods CG. A novel NGF mutation clarifies the molecular mechanism and extends the phenotypic spectrum of the HSNS neuropathy. J Med Genet 2011;48:131–5.
[13] Chang DS, Hsu E, Hottinger DG, Cohen SP. Anti-nerve growth factor in pain management: current evidence. J Pain Res 2016;9:373–83.
[14] Chaplain SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994;53:55–63.
[15] Chi KR. Outlook for NGF inhibitor painkiller class brightens. Nat Biotechnol 2016;34:679–80.
[16] Choi Y, Yoon YW, Na HS, Kim SH, Chung JM. Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. PAIN 1994;59:369–76.
[17] Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. PAIN 2000;87:149–58.
[18] Dhandapani K, Arkarija CM, Taberner FJ, Pacifico P, Doshi B, Nocchi L, Carvalho OP, Thornton GK, Hertecant J, Houlden H, Nicholas AK, Cox JJ, Reilly M, Al-Gazzali L, Woods CG. A novel NGF mutation clarifies the molecular mechanism and extends the phenotypic spectrum of the HSNS neuropathy. J Med Genet 2011;48:131–5.
[19] Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. PAIN 2000;87:149–58.
[20] Dhandapani K, Arkarija CM, Taberner FJ, Pacifico P, Doshi B, Nocchi L, Carvalho OP, Thornton GK, Hertecant J, Houlden H, Nicholas AK, Cox JJ, Reilly M, Al-Gazzali L, Woods CG. A novel NGF mutation clarifies the molecular mechanism and extends the phenotypic spectrum of the HSNS neuropathy. J Med Genet 2011;48:131–5.
[21] Einarsdottir E, Carlsson A, Minde J, Toolanen G, Svensson O, Solders G, Holmgren G, Portulano C, Franciosa F, Maffei M, Hussain AF, de Castro Reis F, Raychaudhuri SK, Paus R, Pincelli C. Neurotrophins in skin biology and pathology. J Invest Dermatol 2006;126:1719–27.
[22] Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci U S A 1976;73:2424–8.
[23] Hefti FF, Rosenthal A, Walicke PA, Wyatt S, Vergara G, Shelton DL, Davies AM. Novel class of pain drugs based on antagonism of NGF. Trends Pharmacol Sci 2006;27:95–9.
[24] Hochberg MC. Serious joint-related adverse events in randomized controlled trials of anti-nerve growth factor monoclonal antibodies. Osteoarthr Cartilage 2015;23(suppl 1):S18–21.
Huang T, Lin SH, Malewicz NM, Zhang Y, Zhang Y, Goulding M, LaMotte RH, Ma Q. Identifying the pathways required for coping behaviours associated with sustained pain. Nature 2019;565:86–90.

Ikeda-Miyagawa Y, Kobayashi Y, Yamazaki H, Kurokawa K, Okubo M, Wang S, Dai Y, Yag H, Hirose M, Noguchi K. Peripheralised increased artemin is a key regulator of TRPA1/V1 expression in primary afferent neurons. Mol Pain 2015;11:8.

Indo Y, Tsuruta M, Hayashida Y, Karim MA, Onha K, Kawano T, Mitsuhashi H, Tonoki H, Awaysa Y, Matuda I. Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. Nat Genet 1996;13:485–8.

Johnson EM Jr, Gorin PD, Braderis LD, Pearson J. Dorsal root ganglion neurons are destroyed by exposure in utero to maternal antibody to nerve growth factor. Science 1980;210:916–18.

Kalso E, Aldington DJ, Moore RA. Drugs for neuropathic pain. BMJ 2013;347:f7339.

Larsen E, Kuma R, Norberg A, Minde J, Holmberg M. Nerve growth factor R221W responsible for insensitivity to pain is defectively processed and accumulates as proNGF. Neurobiol Dis 2000;33:221–8.

Lewin GR, Lechner SG, Smith ES. Nerve growth factor and nociception: from experimental embryology to new analgesic therapy. Handb Exp Pharmacol 2014;220:251–82.

Lewin GR, Mendell LM. Nerve growth factor and nociception. Trends Neurosci 1993;16:353–9.

Lewin GR, Ritter AM, Mendell LM. Nerve growth factor-induced hyperalgesia in the neonatal and adult rat. J Neurosci 1993;13:2136–48.

Lewin GR, Rueff A, Mendell LM. Peripheral and central mechanisms of NGF-induced hyperalgesia. Eur J Neurosci 1994;6:1903–12.

Lindsay RM, Harmar AJ. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 1989;343:362–4.

Mantyh PW, Koltenburg M, Mendell LM, Tive L, Shelton DL. Antagonism of nerve growth factor-TrkA signaling and the relief of pain. Anesthesiology 2011;115:189–204.

Matasci M, Baldi L, Hacker DL, Wurm FM. The PiggyBac transposon enhances the frequency of CHO stable cell line generation and yields recombinant lines with superior productivity and stability. Biotechnol Bioeng 2011;108:2141–50.

McArthur JC, Stocks EA, Hauer P, Comblath DR, Griffin JW. Epidermal nerve fiber density: normative reference range and diagnostic efficiency. Arch Neurol 1998;55:1513–20.

McArthur JC, Stocks EA, Hauer P, Comblath DR, Griffin JW. Epidermal nerve fiber density: normative reference range and diagnostic efficiency. Arch Neurol 1998;55:1513–20.

McArdle I, De Clercq K, Mulier M, Held K, Pinto S, Van Ranst N, Segal Vennekens R, De Clercq K, Mulier M, Held K, Pinto S, Van Ranst N, Segal

McArdle I, De Clercq K, Mulier M, Held K, Pinto S, Van Ranst N, Segal

McArdle I, De Clercq K, Mulier M, Held K, Pinto S, Van Ranst N, Segal

McArdle I, De Clercq K, Mulier M, Held K, Pinto S, Van Ranst N, Segal

Pezet S, McMahon SB. Neurotrophins: mediators and modulators of pain. Annu Rev Neurosci 2006;29:507–38.

Petrič M, Gosselin RD, Decosterd I. The spared nerve injury model of neuropathic pain. Methods Mol Biol 2012;851:205–12.

Petyt BG, Comblath DR, Adomato BT, Chaudhry V, Flexner C, Wachsmann M, Siniropi D, Burton LE, Peroutka SJ. The effect of systemically administered recombinant human nerve growth factor in healthy human subjects. Ann Neurol 1994;36:244–6.

Pezet S, McMahon SB. Neurotrophins: mediators and modulators of pain. Annu Rev Neurosci 2006;29:507–38.

Petrič M, Gosselin RD, Decosterd I. The spared nerve injury model of neuropathic pain. Methods Mol Biol 2012;851:205–12.

Petyt BG, Comblath DR, Adomato BT, Chaudhry V, Flexner C, Wachsmann M, Siniropi D, Burton LE, Peroutka SJ. The effect of systemically administered recombinant human nerve growth factor in healthy human subjects. Ann Neurol 1994;36:244–6.