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Swedish Nerve Growth Factor Mutation (NGF^{R100W}) Defines a Role for TrkA and p75^{NTR} in Nociception

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Nerve growth factor (NGF) exerts multiple functions on target neurons throughout development. The recent discovery of a point mutation leading to a change from arginine to tryptophan at residue 100 in the mature NGFβ sequence (NGF^{R100W}) in patients with hereditary sensory and autonomic neuropathy type V (HSAN V) made it possible to distinguish the signaling mechanisms that lead to two functionally different outcomes of NGF: trophic versus nociceptive. We performed extensive biochemical, cellular, and live-imaging experiments to examine the binding and signaling properties of NGF^{R100W}. Our results show that, similar to the wild-type NGF (wtNGF), the naturally occurring NGF^{R100W} mutant was capable of binding to and activating the TrkA receptor and its downstream signaling pathways to support neuronal survival and differentiation. However, NGF^{R100W} failed to bind and stimulate the 75 kDa neurotrophic factor receptor (p75^{NTR})-mediated signaling cascades (i.e., the RhoA-Cofilin pathway). Intraplantar injection of NGF^{R100W} into adult rats induced neither TrkA-mediated thermal nor mechanical acute hyperalgesia, but retained the ability to induce chronic hyperalgesia based on agonism for TrkA signaling. Together, our studies provide evidence that NGF^{R100W} retains trophic support capability through TrkA and one aspect of its nociceptive signaling, but fails to engage p75^{NTR} signaling pathways. Our findings suggest that wtNGF acts via TrkA to regulate the delayed priming of nociceptive responses. The integration of both TrkA and p75^{NTR} signaling thus appears to regulate neuroplastic effects of NGF in peripheral nociception.

Key words. NGF; nociception; p75; sensory neuron; TrkA; trophic

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Significance Statement

In the present study, we characterized the naturally occurring nerve growth factor NGF^{R100W} mutant that is associated with hereditary sensory and autonomic neuropathy type V. We have demonstrated for the first time that NGF^{R100W} retains trophic support capability through TrkA, but fails to engage p75^{NTR} signaling pathways. Furthermore, after intraplantar injection into adult rats, NGF^{R100W} induced neither thermal nor mechanical acute hyperalgesia, but retained the ability to induce chronic hyperalgesia. We have also provided evidence that the integration of both TrkA- and p75^{NTR}-mediated signaling appears to regulate neuroplastic effects of NGF in peripheral nociception. Our study with NGF^{R100W} suggests that it is possible to uncouple trophic effect from nociceptive function, both induced by wild-type NGF.

Introduction

Nerve growth factor (NGF), discovered as a result of potent trophic actions on sensory and sympathetic neurons of the PNS in the 1950s (Levi-Montalcini and Hamburger, 1951), also regulates the trophic status of striatal and basal forebrain cholinergic neurons (BFCNs) of the CNS (Levi-Montalcini and Hamburger, 1951; Svendsen et al., 1994; Li and Jope, 1995; Kew et al., 1996; Conover and Yancopoulos, 1997; Lehmann et al., 1999). With the

discovery of brain-derived neurotrophic factor (BDNF), Neurotrophin 3 (NT-3) and Neurotrophin 4 (NT-4), NGF is now known as a member of the neurotrophin family (Chao and Hempstead, 1995; Huang and Reichardt, 2001; Chao, 2003). NGF acts via two known receptors, the 140 kDa tyrosine receptor kinase A (TrkA) and the 75 kDa neurotrophin receptor (p75^{NTR}), to transmit signals to the cytoplasm and nucleus of responsive neurons (Bothwell, 1995; Chao and Hempstead, 1995; Kaplan and Miller, 1997). NGF signaling through TrkA elicits many of the classical neurotrophic actions ascribed to NGF (Loeb and Greene, 1993). TrkB and TrkC mediate the signaling of NT-3 and NT-4, respectively (Huang and Reichardt, 2001; Chao, 2003). NGF and all members of the family also signal through p75^{NTR} (Huang and Reichardt, 2001; Chao, 2003). p75^{NTR} contributes to sphingomyelin-ceramide metabolism (Dobrowsky et al., 1994, 1995) and modulates RhoA activity to regulate axonal growth (Yamashita et al., 1999; Gehler et al., 2004). In addition, p75^{NTR} has been shown to activate the NF-κB, Akt, and JNK pathways (Harrington et al., 2002; Roux and Barker, 2002) to either induce apoptosis or to promote cell survival and differentiation (Chao and Hempstead, 1995; Casaccia-Bonnefil et al., 1998; Salehi et al., 2000; Roux and Barker, 2002; Nykjaer et al., 2005).

Given its robust trophic effects, NGF has been investigated for therapeutic properties in neurodegenerative disorders (Apfel et al., 1994, 1998; Andreev et al., 1995; Apfel and Kessler, 1995, 1996; Blesch and Tuszynski, 1995; Anand et al., 1996; Apfel, 1999a,b, 2000, 2002; Aloe et al., 2012). In one example, NGF's robust trophic effects on BFCNs has suggested a role in treating Alzheimer's disease (AD) in which this population degenerates (Olson, 1993; Hefti, 1994; Scott and Crutcher, 1994; Blesch and Tuszynski, 1995; Knusel and Gao, 1996; Koliatsos, 1996; Eriksdotter Jönhagen et al., 1998; Williams et al., 2006; Mufson et al., 2008; Schindowski et al., 2008; Schulte-Herbrüggen et al., 2008; Cuello et al., 2010). Unfortunately, features of the biology of NGF have limited the extent to which it could be evaluated. Inability to cross the blood–brain barrier prevented systemic administration. Delivery via the ventricular system even at low doses resulted in pain and studies in primates demonstrated Schwann cell hyperplasia that served to compromise CSF flow (Winkler et al., 1997). A recent phase 2 trial in which NGF was delivered via virus to the basal forebrain demonstrated safety and was not associated with pain (Tuszynski et al., 2015). Marked sprouting of BFCN fibers was evidence of a potent trophic effect, but cognitive measures were unaffected (Rafii et al., 2014). Significant efforts were also invested to investigate NGF as a treatment for diabetic polyneuropathy (Apfel and Kessler, 1995, 1996; Anand et al., 1996; Tomlinson et al., 1996; Elias et al., 1998; Apfel, 1999b; Goss et al., 2002; Murakawa et al., 2002; Kanda, 2009). NGF treatment demonstrated some benefit in phase 2 trial at 0.1 and 0.3 μg/kg, but was associated with dose-dependent hyperalgesia at the injection site (Apfel, 1999a,b). A large-scale phase 3 trial with a dose of 0.1 μg/kg showed no beneficial effect (Apfel et al., 1998; Apfel, 2002).

NGF is not only a trophic factor, but also functions as one of the key molecules for mediating inflammatory pain and neuropathic pain in the PNS (Lewin and Mendell, 1993; Lewin et al.,

1993; Chuang et al., 2001; Watanabe et al., 2008). Therefore, clinical trials in which large doses of NGF were infused in patients with AD had to be terminated due to the extreme side effects of pain (Aloe et al., 2012). Other clinical trials using NGF in treating diabetic neuropathies and peripheral neuropathies in HIV were also discontinued after reports of serious side effects such as back pain, injection site hyperalgesia, myalgia, and weight loss (Hellweg and Hartung, 1990; Lein, 1995; Apfel et al., 1998; Unger et al., 1998; Rask, 1999; McArthur et al., 2000; Quasthoff and Hartung, 2001; Schifitto et al., 2001; Apfel, 2002; Pradat, 2003; Walwyn et al., 2006). Therefore, the adverse effect of significant pain caused by NGF has severely limited its therapeutic use in treating neurodegenerative disorders. To overcome these pain-causing side effects of NGF, it is of paramount importance to elucidate the role of NGF and its receptor signaling by TrkA and p75^{NTR} in nociception.

A large body of genetic and clinical evidence has pointed to both TrkA and p75^{NTR} as contributing factors to sensitization of inflammatory pain mediated by NGF. For example, recessive mutations in TrkA cause hereditary sensory and autonomic neuropathy type IV [hereditary sensory autonomic neuropathy type V (HSAN V) (Online Mendelian Inheritance in Man (OMIM) #256800], also known as congenital insensitivity to pain with anhidrosis (Indo, 2001, 2002). Strong evidence supports a role of TrkA in mediating the sensitization effect of NGF: attenuation of TrkA expression (Malik-Hall et al., 2005; Alvarez and Levine, 2014) and pharmacological inhibition of TrkA-mediated signaling pathways extracellular signal-related kinase (ERK), phosphatidylinositol 3-kinase (PI3K), and phospholipase Cγ (PLCγ) all reduced NGF-induced hyperalgesia (Fang et al., 2005; Malik-Hall et al., 2005; Summer et al., 2006; Mantyh et al., 2011; Alvarez and Levine, 2014; Ashraf et al., 2016). Furthermore, NGF still evoked hyperalgesia in mice lacking p75^{NTR}, pointing to the involvement of TrkA (Bergmann et al., 1998). Evidence that p75^{NTR} has a role in pain signaling pathways is largely indirect. In one example, injecting a neutralizing antibody to p75^{NTR} prevented NGF-induced pain behavior and NGF-mediated increases in action potentials in sensory neurons (Zhang and Nicol, 2004; Watanabe et al., 2008; Iwakura et al., 2010). Therefore, both TrkA and p75^{NTR} signals contribute to pain induced by NGF. However, how these two receptors interact to mediate pain is poorly defined.

Recently, patients in consanguineous Swedish families suffering from length-dependent loss of pain that often leads to bone fractures and joint destruction were shown to harbor a homozygous missense mutation in NGF (Einarsdottir et al., 2004; Carvalho et al., 2011). The disorder was labeled HSAN V, OMIM #608654. Genetic analysis of these HSAN V patients revealed a point mutation (661C>T) causing a substitution of tryptophan (W) for arginine (R) at position 211 in the proform of the NGF polypeptide (pro-NGF^{R221W}); this residue corresponds to the position 100 in the mature protein (NGF^{R100W}) (Einarsdottir et al., 2004). Unlike HSAN type IV, which results from mutations in TrkA, HSAN V patients appear to have normal cognitive function, suggesting that the mutant NGF may retain its trophic functions in the CNS (Einarsdottir et al., 2004). We and others reasoned that NGF^{R100W} provides a tool with which to decipher possible differences in the trophic and nociceptive actions of NGF (Covaceuszach et al., 2010; Capsoni et al., 2011, 2014).

Initial characterization of NGF^{R100W} revealed that the R100 mutation may disrupt the processing of pro-NGF to mature NGF in cultured cells, resulting in relatively higher percentage of NGF secreted as the pro-form (Larsson et al., 2009). Given the difficulties in expressing NGF^{R100W}, Capsoni et al. (2011) examined a different series of residues at position 100, including NGF^{R100E}

and NGF double mutant (NGF$^{P61S/R100E}$), using recombinant techniques. They discovered that these NGF^{R100} mutants bound normally to TrkA, but failed to bind to p75^{NTR} (Covaceuszach et al., 2010). This finding suggested that failure to activate p75^{NTR} signaling was sufficient to attenuate pain induced by NGF and that TrkA signaling had little or no effect in pain (Capsoni et al., 2011). The investigators speculated that NGF^{R100} mutant would allow for development of p75^{NTR} antagonists such as NGF^{R100W} as a "painless NGF" therapeutic agent (Malerba et al., 2015).

In the present study, we examined the binding and signaling properties of the mature form of the naturally occurring mutant NGF (NGF^{R100W}) in HSAN V and compared the effects with those of wtNGF and NGF mutants that selectively engage and signal through either TrkA or p75^{NTR}. We discovered that NGF^{R100W} retains binding and signaling through TrkA to induce trophic effects, but not binding or activation of p75^{NTR}. Our findings are evidence that NGF^{WT} acts via both p75^{NTR} and TrkA to cause pain. Our findings are consistent with a necessary role for TrkA in both acute sensitization and delayed priming of nociceptive responses. In contrast, signaling through p75 appears to be necessary for acute sensitization, but does contribute to priming. The integration of both TrkA and p75 signaling thus appears to regulate neuroplastic effects of NGF in peripheral nociception.

Materials and Methods

Ethics statement. All experiments involving the use of animals were approved by the Institutional Animal Care and Use Committee of University of California–San Diego and University of California–San Francisco (UCSF). Surgical and animal procedures were performed strictly following the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals.*

Chemicals, oligos, and reagents. Streptavidin-QD605, Streptavidin-QD-655 (Q10103MP, Q10123MP) conjugates were from Invitrogen; all other chemicals were from Sigma-Aldrich unless noted otherwise. Recombinant extracellular domains of p75^{NTR} were a generous gift from Dr. Sung Ok Yoon of Ohio State University. Prostaglandin E2 (PGE$_2$) was from Sigma-Aldrich (catalog #82475). NGF (wtNGF, NGF^{R100W}, KKE, Δ9/13) proteins were produced in our own laboratory (Sung et al., 2011).

Cloning. Mouse pro-NGF was amplified by PCR from a NGF-GFP plasmid (a generous gift from Professor Lessmann, Mainz, Germany). The forward primer sequence was: 5′-acgaattccaccatgtccatgttgttctacactctg atcactgcg-3′ and the reverse primer sequence was: 5′-gatggatccttcgtgcca ttcgattttctgagcctcgaagatgtcgttcagaccgccaccgacctccacggcggtggc-3′. The reverse primer contains a sequence coding for the 17 aa AviTag: GGG LNDIFEAQKIEWHE. The sequence was based on the #85 AviTag peptide sequence described previously (Schatz, 1993). One glutamic acid residue was added to the C-terminal AviTag based on a finding by Avidity (www.avidity.com) that it greatly enhanced the biotinylation rate of the AviTag (Beckett et al., 1999). Platinum pfx DNA polymerase (Invitrogen, catalog #11708021) was used following the manufacture's instructions. The 50 μl reaction was denatured at 94°C for 4 min, followed by 25 cycles of amplification (30 s at 94°C; 30 s at 50°C; 90 s at 68°C). An additional extension was performed at 68°C for 4 min. The PCR product was purified and digested with EcoRI (Fermentas, catalog #FD0274) and BamHI (Fermentas, catalog #FD0054) and was ligated in-frame into the pcDNA3.1-myc-His vector that was predigested with EcoRI/BamHI. The resulting construct was designated as pcDNA3.1-NGFavi. BirA was amplified by PCR from pET21a-BirA (Addgene, plasmid #20857) (Howarth et al., 2005) using a forward primer (forward primer: 5′-gtgaac atg gctagcatgact-3′) and a reverse primer (5′-ggtgctcgagtcatgcggccgcaagct-3′ (containing an XhoI site). PCR was performed using Pfx as described above. The PCR product was digested with XhoI (Fermentas, catalog #FD0694) and subcloned into pcDNA3.1 myc.his (+) vector (Invitrogen) that was precut with EcoRV (Fermentas, catalog #FD0303) and XhoI. The resulting plasmid was designated as pcDNA3.1-BirA. All

primers were from Elim Biopharmaceuticals. All constructs were verified by sequencing (Elim Biopharmaceuticals). NGF^{R100W} was cloned using the same method, but with a point mutation at 661 (C>T). The KKE and Δ9/13 mutant constructs were obtained from Dr. K. Neet of Rosalind Franklin University (Hughes et al., 2001; Mahapatra et al., 2009) and subcloned to the pcDNA3.1-myc-His vector with an Avitag (Sung et al., 2011).

Protein purification. HEK293FT cells were grown in 15 cm plates to 70% confluency. Cells were changed to 25 ml of DMEM-high glucose, serum-free medium that was supplemented with 50 μM D-biotin (Sigma-Aldrich, catalog #B4639). Then, 15–21 μg f pcDNA3.1-NGFavi, NGF^{R100W}avi, KKEavi, and Δ9/13avi plasmids DNA plus 15–21 μg of pcDNA3.1-BirA plasmid DNA were mixed with 1 ml of DMEM-high glucose medium and 60 μl of Turbofect (Fermentas, catalog #R0531). The mixture was incubated at room temperature for 15 min and then added into the medium by the dropwise method. Transfected HEK293FT cells were incubated at 37°C, 5% CO$_2$. Seventy-two hours after transfection, media were collected for protein purification.

Media were harvested and adjusted to 30 mM phosphate buffer, pH 8.0, 500 mM NaCl, 20 mM imidazole, and a mixture of protease inhibitors (1 mM PMSF from Sigma-Aldrich, catalog #P7626, and 1 μl/ml aprotinin from Sigma-Aldrich, catalog #A6279). After incubation on ice for 15 min, media were cleared by centrifugation at 18,000 rpm for 30 min using a Beckman JA-20 rotor. Ni-NTA resins (Qiagen, catalog #30250) were rinsed with the washing buffer (30 mM phosphate buffer, pH 8.0, 500 mM NaCl, 20 mM imidazole, and a mixture of protease inhibitors from Sigma-Aldrich, catalog #S8820). Ni-NTA resins were added to the media at a concentration of 0.3 ml Ni-NTA/100 ml of media and incubated overnight with rotation at 4°C. The media/Ni-NTA slurry was loaded onto a column and the captured Ni-NTA resins were washed with 10 ml of wash buffer and eluted with 5 ml of elution buffer (30 mM phosphate buffer, pH 8.0, 500 mM NaCl, 300 mM imidazole, protease inhibitors). Every 500 μl volume of elution was collected. The purity and concentration of NGF was assessed by SDS-PAGE using a silver staining kit (Fast Silver, G-Biosciences, catalog #786–30). Known quantities of NGF purified from mouse submaxillary glands were used as standards. The first two eluted fraction normally contained most of purified proteins.

Cell culture and transfection. PC12 cells or a subclone of PC12 cells, PC12M, PC12^{nnr5} cells were cultured as described previously (Wu et al., 2001, 2007). NIH3T3-TrkA, NIH3T3-p75^{NTR} cells were as described previously (Huang et al., 1999). HEK293FT cells (Invitrogen, catalog #R70007) cells were cultured in DMEM-high glucose medium (4.5 g/L glucose, Mediatech, catalog #10-013-CV), 10% FBS, and 1% penicillin/ streptomycin.

Administration of wtNGF, NGF^{R100W}, prostaglandin E2 (PGE$_2$), and inhibitors to adult rats. Experiments were performed on adult male Sprague Dawley rats (220–240 g, Charles River Laboratories; RRID: RGD_737891). All experiments were performed following protocols that have been approved by the University of California–San Francisco Committee on Animal Research and conformed to the National Institutes of Health's *Guidelines for the Care and Use of Laboratory Animals.* Either 200 ng of wtNGF or NGF^{R100W} was injected intradermally on the dorsum of one hindpaw of adult rats. Nociceptive thresholds in the injected paws were then tested over time.

For studies using K252a (Sigma-Aldrich catalog #K1639) and GW4869 (Sigma-Aldrich catalog #D1692), both inhibitors were dissolved in DMSO (2 μg/μl) and then diluted to a concentration of 0.2 μg/μl in saline at the time of the experiments. Five minutes before injection of NGF, 5 μl (1 μg) of inhibitors was administered intradermally on the dorsum of the hindpaw at the same site where NGF was injected. The experimental design is illustrated in Figure 7A.

Randall–Selitto mechanical test and Hargreaves thermal test. Mechanical nociceptive threshold was measured using the Randall–Selitto paw pressure test (Randall and Selitto, 1957) with an Ugo Basile Algesymeter (Ferrari et al., 2010, 2013, 2015a,b). This device exerts a linear increase in force to the dorsum of the hindpaw of the rats. Before the test, rats were kept in individual restrainers for 20 min to acclimatize them to the experimental environment. The restrainers had openings that allowed rats

to extend hindpaws during the test. Mechanical thresholds were calculated as the average of three readings.

Thermal threshold was measured using the Hargreaves test, which applies heat stimuli by an adjustable high-intensity movable halogen projector lamp (Malmberg and Yaksh, 1993). Baseline responses were first measured and then averaged from at least two readings. Then, 600 ng of wtNGF or NGF^R100W was injected in the plantar surface of one hindpaw under isoflurane anesthesia. After recovery from the anesthesia, in less than 1 min, rats were placed on a glass plate and acclimatized for 20 min. Thermal nociceptive thresholds were evaluated within 1 h after the injections.

Binding and internalization assays of NGF-QD. At 50% confluence of NIH3T3-TrkA and NIH3T3-p75^NTR, cells were starved in serum free DMEM-high glucose medium for 4 h at 37°C. NGF or NGF mutants were conjugated with QD 655-streptavidin (Invitrogen, catalog #Q10121MP) on ice 4°C for 30 min. Then, 0.2 nM conjugate was applied to cells. Cells were incubated at 20°C for 20–30 min, washed with serum free DMEM-high glucose medium, and then surface binding was quantified. For the internalization assay, cells were incubated with 1 nM conjugates for 30 min or 2 h, for 3T3-TrkA and 3T3-p75^NTR, respectively, at 37°C. Cells were washed and then subjected to imaging.

Pull-down assay. The recombinant protein, Fc-75^NTR extracellular domain (ECD) was a kind gift from Dr. Sung Ok Yoon, the Ohio State University. Five microliters of supernant from insect lysate expressing Fc-p75^NTR was incubated with a range of either wtNGF or NGF^R100W (~0–10 ng) overnight at 4°C. Avidin–agarose beads (20 μl) were added/incubated for 2 h at 4°C. Beads were washed, boiled with protein sample buffer, and subjected to SDS-PAGE. Western blotting was performed using the function blocking antibodies against the extracellular domain of p75^NTR (REX) (Mischel et al., 2001).

Dorsal root ranglion (DRG) culture, live-cell imaging, and data analysis. Embryonic DRGs at embryonic day 15 (E15) to E16 were isolated from Sprague Dawley rats as described previously (Cui et al., 2007; Wu et al., 2007; Sung et al., 2011) with some minor modifications. Cells were maintained with alternation between growth media (MEM media containing 10% heat inactivated FBS and 100 ng/ml of NGF) and selection media (MEM media containing 0.5–1 μM cytosine β-D-arabinofuranoside (Sigma-Aldrich catalog #C1768 and 100 ng/ml NGF) every 2 d.

For survival analysis, only the cells with round and transparent cell bodies were counted as DRG neurons to exclude possible fibroblast populations. In phase contrast images, the cell bodies of DRGs look whitish and transparent, whereas fibroblasts have black and flattened cell bodies.

For live cell imaging, dissociated DRGs were cultured in microfluidic chambers for 7–10 d (Cui et al., 2007). The microfluidic chambers, manufactured in-house, were plated onto 24 mm × 48 mm glass coverslips that were precoated with poly-L-lysine (Sigma-Aldrich catalog P8920) as described previously (Taylor et al., 2006). Dissociated DRG neurons were plated into the cell body chamber. The growth/selection scheme outlined above was repeated. Axons from the DRG neurons started to cross the microgrooves after 3 d and reached the axonal chamber in another 7–8 d. Before live imaging of axonal transport of NGF, all compartments (cell body and axonal chambers) of the DRG neurons were thoroughly rinsed and depleted of NGF in NGF-free, serum-free MEM for 3 h. NGF-QD605 was prepared following the protocol described above. NGF-QD605 was added to a final concentration of 0.2 nM to the axonal chambers for 2 h at 37°C. Live cell imaging of NGF-QD605 transport with the axons was performed using a modified inverted microscope (Nikon TE300) for pseudo-TIRF illumination (Zhang et al., 2010). The microscope stage was equipped to maintain a constant temperature (37°C). CO_2 level (5%) was maintained using CO_2-independent medium (Invitrogen catalog #18045-088) during live imaging. The laser beam of 532 nm was used and penetrated ~1 μm into aqueous solution at an incident angle. Fluorescence emission was filtered with QD605/20 emission filter (Chroma Technology). Time-lapse images were acquired at the speed of 10 frames/s and were captured using an EMCCD camera (Cascade 512B, Photometric). All data were processed and analyzed using a MATLAB software pipeline (RRID:SCR_001622).

Single-cell patch-clamp recording. All recordings were obtained from small- to medium-diameter cells from cultured DRGs at room temperature. One-electrode whole-cell voltage-clamp recording was performed using Axopatch-1D amplifiers (Molecular Devices). A 2–5 MW sized patch electrode and puffing pipette were used. All cells were clamped at −60 mV holding potential to measure low pH-evoked current.

Standard external solution contained the following (in mM): 145 NaCl, 5 KCl, 2 CaCl_2, 1 MgCl_2, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH, and was used to incubate DRG neurons. To block possible synaptic transmission via activation of ionotropic receptors, 1 mM TTX, 10 mM CNQX, 50 mM AP5, and 50 mM picrotoxin were added to the bath. Internal recording solution contained the following (in mM): 130 K-gluconate, 10 HEPES, 0.6 EGTA, 5 KCl, and 2.5 Mg-ATP, adjusted to pH 7.3 with KOH. Low pH puffing solution was made from external solution and was adjusted to pH 5.5 by adding 1 N HCl.

Cells were starved for 2 h with MEM before recording. To evoke a low pH response, pH 5.5 external solution was applied briefly through an additional glass pipette placed 50 mm away from the recorded cell and by a Picospritzer (5–10 psi, for 50–100 ms, Picospritzer II, General Valve). The responses seen at this configuration were not evoked by mechanical stimulation due to air puffing as when neutral pH solution was used, so no response was observed. wtNGF or NGF^R100W at a concentration of 50 ng/ml was applied directly to the bath and incubated for 10 min, followed by low pH puffing. Data analysis was done in pClamp software (Molecular Devices, RRID:SCR_011323) by calculating the charge transfer for 100 ms and normalized by computing the ratio of response after NGF/response before NGF.

Antibodies and SDS-PAGE/blotting. Standard protocols were followed for SDS-PAGE and blotting. Rabbit anti-pro-NGF IgGs were a generous gift of Dr. B.L. Hempstead of Cornell University. Rabbit anti-NGF IgGs were from Santa Cruz Biotechnology (catalog #sc-549; RRID:AB_632012). Rabbit IgGs against Trk-pTyr490, pErk1/2, total Erk1/2, and p-Cofilin were from Cell Signaling Technology (catalog #9141; RRID:AB_2298805, 9101; RRID:AB_331646, 9102; RRID:AB_330744, 3311; and RRID:AB_330239 respectively). Mouse IgGs against pJNK and mouse IgGs against total PLC-γ were from Santa Cruz Biotechnology (sc-6254; RRID:AB_628232, sc-7290; RRID:AB_628119, respectively) and rabbit IgGs against pPLC-γ were from GeneTex (GTX61714; RRID:AB_10621534). Rabbit functional blocking antibodies against the extracellular domain of p75^NTR (REX) (Mischel et al., 2001) was a generous gift of Dr. L. Reichardt of UCSF. Rabbit monoclonal antibodies against pAkt were from Epitomics (catalog #2214-1; RRID:AB_1266979). Rabbit anti-AviTag IgGs were from Genescript (catalog #A00674; RRID:AB_915553).

Immunostaining. Immunostaining was performed according to published protocols (Weissmiller et al., 2015). Briefly, PC12^nnr5 cells (RRID:CVCL_C128) were cultured on coverslips that were precoated with Matrix gel (BD Biosciences). Aafter serum starvation for 2 h, cells were treated with either 50 ng/ml wtNGF or 50 ng/ml NGF^R100W. Cells were then fixed for 10 min with 4% paraformaldehyde at 37°C and permeabilized for 15°C at room temperature with 0.1% Triton X-100; 3% BSA and 5% goat serum in PBS were used for blocking. The cells were then incubated overnight at 4°C with blocking solution containing 1/100 diluted primary antibody, active RhoA from New East Biosciences (catalog #26904; RRID:AB_1961799). After washing 3× primary antibody with PBS and rocking fir 5 min, the cells were incubated with a 1/800 dilution of Alexa Fluor 488-goat anti-mouse IgG (Invitrogen catalog #A1100) for 1 h at room temperature with rocking covered with foil. After 3 washes with PBS, nuclei were labeled with 1 μg/ml Hoechst 33342 (bisBenzimide H 33342 trihydrochloride, Sigma-Aldrich catalog #B2261) for 5 min at room temperature. Cells were rinsed, air-dried, mounted, and examined with a Leica microscopy using a 100× oil objective lens.

Statistical analysis. All experiments were repeated at least three times independently. Statistical analyses of results and calculation of *p*-values were performed using Prism 5 software (GraphPad Software; RRID:SCR_015807). For un-pairwise comparisons, the Student's *t* test was used. For multiple comparisons, the Tukey one-way ANOVA (RRID:SCR_002427) test was used. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Results

NGF^{R100W} does not elicit acute thermal or mechanical hyperalgesia *in vivo*

Previous studies suggested that the NGF mutation associated with HSAN V disrupted either the processing of the proform (i.e., NGF^{R221W}) or secretion of the mature form (NGF^{R100W}), resulting in preferential secretion of the NGF^{R221W} (Larsson et al., 2009; Covaceuszach et al., 2010; Carvalho et al., 2011; Capsoni, 2014). However, these findings and interpretations are perplexing because HSAN V patients report no autonomic symptoms (Capsoni, 2014), which would argues against increased levels of pro-NGF, a p75 ligand that induces death of sympathetic neurons (Roux and Barker, 2002; Nykjaer et al., 2005; Khodorova et al., 2013). Furthermore, these patients have intact mental abilities, arguing against clinically meaningful CNS neuronal loss and dysfunction. To further explore the binding, signaling, and actions of NGF^{R100W}, we produced and characterized the mature form of the protein in HEK293 cells.

Although superficial sensation in patients homozygous for NGF^{R100W} is normal, it is unknown whether this mutant NGF can induce nociceptor sensitization or transition to chronic pain. Therefore, we measured the behavioral response of adult rats to mechanical stimuli or noxious thermal stimulus after a single injection of either wtNGF or NGF^{R100W}. Proteins were administered intradermally to the hindpaws of adult male Sprague Dawley rats following published protocols (Taiwo et al., 1991).

We used the Randall–Sellito method to measure mechanical threshold (Randall and Selitto, 1957). For all experiments, baseline values were measured before the injection of NGF. Previous reports showed that 10 ng to 1 μg of injected wtNGF induced significant mechanical hyperalgesia (Andreev et al., 1995; Malik-Hall et al., 2005). When 200 ng of wtNGF (Fig. 1A–C, blue) was injected to the dorsum of the rat's hindpaw, the mechanical nociceptive threshold was reduced by ~25% compared with baseline ($p < 0.0001$, unpaired t test, 0 m vs 60 m on the first day; Fig. 1B,C, blue). Mechanical hyperalgesia was evident as soon as 15 min and reached the maximum by 1 h, an effect that lasted for ≥24 h. Hyperalgesia was diminished to ~15% by 5 d (Fig. 1C). In marked contrast to wtNGF, injection of 200 ng of NGF^{R100W} had no effect on mechanical nociceptive threshold (Fig. 1B,C, red).

We then measured thermal hyperalgesia using the Hargreaves method (Hargreaves et al., 1988). Intraplantar injection of 600 ng of wtNGF to the hindpaw of rats induced thermal hyperalgesia, as demonstrated by as much as a 34.8% decrease in nociceptive threshold ($p = 0.0186$, 0 vs 45 m, unpaired t test; Fig. 1D–F, blue). Acute thermal hypergelsia was first observed 20–30 min after injection, with the maximal effect at 45 min, followed by a return to baseline (Fig. 1E,F, blue). In contrast, injection of 600 ng of NGF^{R100W} did not induce acute thermal hyperalgesia (Fig. 1E,F, red). We conclude that, unlike wtNGF, NGF^{R100W} does not induce acute thermal or mechanical hyperalgesia.

NGF^{R100W} induces hyperalgesic priming

To test whether NGF^{R100W} could contribute to chronic pain, we took advantage of the hyperalegesic priming paradigm with the injection of prostaglandin E2 (PGE$_2$) (Reichling and Levine, 2009). Because wtNGF was shown to induce hyperalgesic priming in rats (Ferrari et al., 2010), we used this model to examine responses to wtNGF and NGF^{R100W}. Seven days after injection of 200 ng of either wtNGF or NGF^{R100W}, PGE$_2$ (100 ng/5 μl) was injected into the same injection site into adult rats (Fig. 1G). If a priming effect for chronic pain is absent, then injection of PGE$_2$ only induces acute hyperalgesia that disappears by 4 h (Ferrari et al., 2010, 2013, 2015b). However, in the presence of priming, the same dose of PGE$_2$ induces a much prolonged hyperalgesia to mechanical stimuli (Aley and Levine, 1999; Reichling and Levine, 2009).

As reported previously (Ferrari et al., 2010), intradermal injection of wtNGF (200 ng) induced acute mechanical hyperalgesia that lasted through day 7 (Fig 1H,I). As before, NGF^{R100W} failed to induce acute mechanical hyperalgesia (Fig. 1H,I). On the seventh day after measuring the threshold baseline, PGE$_2$ was injected into the same site as for NGF and the mechanical threshold was measured at 30 min and 4 h thereafter. Consistent with previous findings (Aley and Levine, 1999; Ferrari et al., 2010), in animals treated with wtNGF, PGE$_2$ induced prolonged hyperalgesia unattenuated at least for 4 h (Fig. 1H,I, blue), whereas in naive subjects, PGE$_2$ induced only acute hyperalgesia at 30 min and the hyperalgesic effect was largely dissipated at 4 h (Fig. 1H,I, black, naive). Remarkably, NGF^{R100W} was also as potent as wt-NGF in inducing priming with prolonged PGE$_2$ hyperalgesia, lasting at least 4 h (Fig. 1H,I, red, NGF^{R100W}). We conclude that NGF^{R100W} retains the binding and signaling necessary to induce hyperalgesic priming (Fig. 1H,I) despite its inability to induce acute hyperalgesia (Fig. 1B,C,E,F). These findings suggest that studies of NGF^{R100W} may provide insights into mechanisms underlying the nociceptive and trophic functions of NGF in sensory neurons.

NGF^{R100W} does not potentiate low H$^+$-evoked response in sensory neurons *in vitro*

Among the known mechanisms of pain transduction, NGF produces acute hypersensitivity by potentiating nociceptive ion channels such as the capsaicin receptor (also known as TRPV1) and acid-sensing ion channels (ASICs) (Szallasi and Blumberg, 1999; Qiu et al., 2012; McCleskey and Gold, 1999; Chuang et al., 2001; Julius and Basbaum, 2001; Mamet et al., 2003; Yen et al., 2009). ASICs and TRPV1 are proton-gated nonselective cation channels that mediate acid-evoked pain in peripheral sensory neurons (Caterina et al., 2000; Davis et al., 2000; Chen et al., 2002; Hellwig et al., 2004). We next performed patch-clamp recordings to test whether NGF^{R100W} no longer elicited nociceptive response at the cellular level in cultured rat E15.5 DRG sensory neurons. At 8 d in culture (DIC8), neurons were starved for NGF for 2 h. We used a moderate acidic solution, pH 5.5, to activate ASICs and TRPV1 by puffing the patch-clamped cell body (Fig. 2A). Proton-evoked currents were measured before and after a 10 min application of either wtNGF or NGF^{R100W} (Fig. 2B). The responses were normalized by calculating the ratio of the "after NGF" value to the "before NGF" value. Consistent with previous studies (Koplas et al., 1997; Shu and Mendell, 1999a,b), 10 min of wtNGF treatment produced an acute sensitization, as displayed by a 1.5-fold increase in proton evoked current (Fig. 2C). However, NGF^{R100W} did not induce hypersensitization (Fig. 2C; wtNGF vs NGF^{R100W}; $p < 0.01$; paired t test).

NGF^{R100W} induces differentiation and supports survival of rat E15.5 DRG neurons

HSAN V patients show reduced responses to painful stimuli but retain normal cognitive function (Einarsdottir et al., 2004). We thus speculated that NGF^{R100W} would sustain trophic signaling, resulting in the differentiation and survival of DRG sensory neurons (Winter et al., 1988; Wu et al., 2007). To compare the bioactivity of NGF^{R100W} with wtNGF, we performed a dose–response survival assay of DRG neurons for both wtNGF and NGF^{R100W} (0, 10, 50, and 100 ng/ml). We used E15.5 DRG and
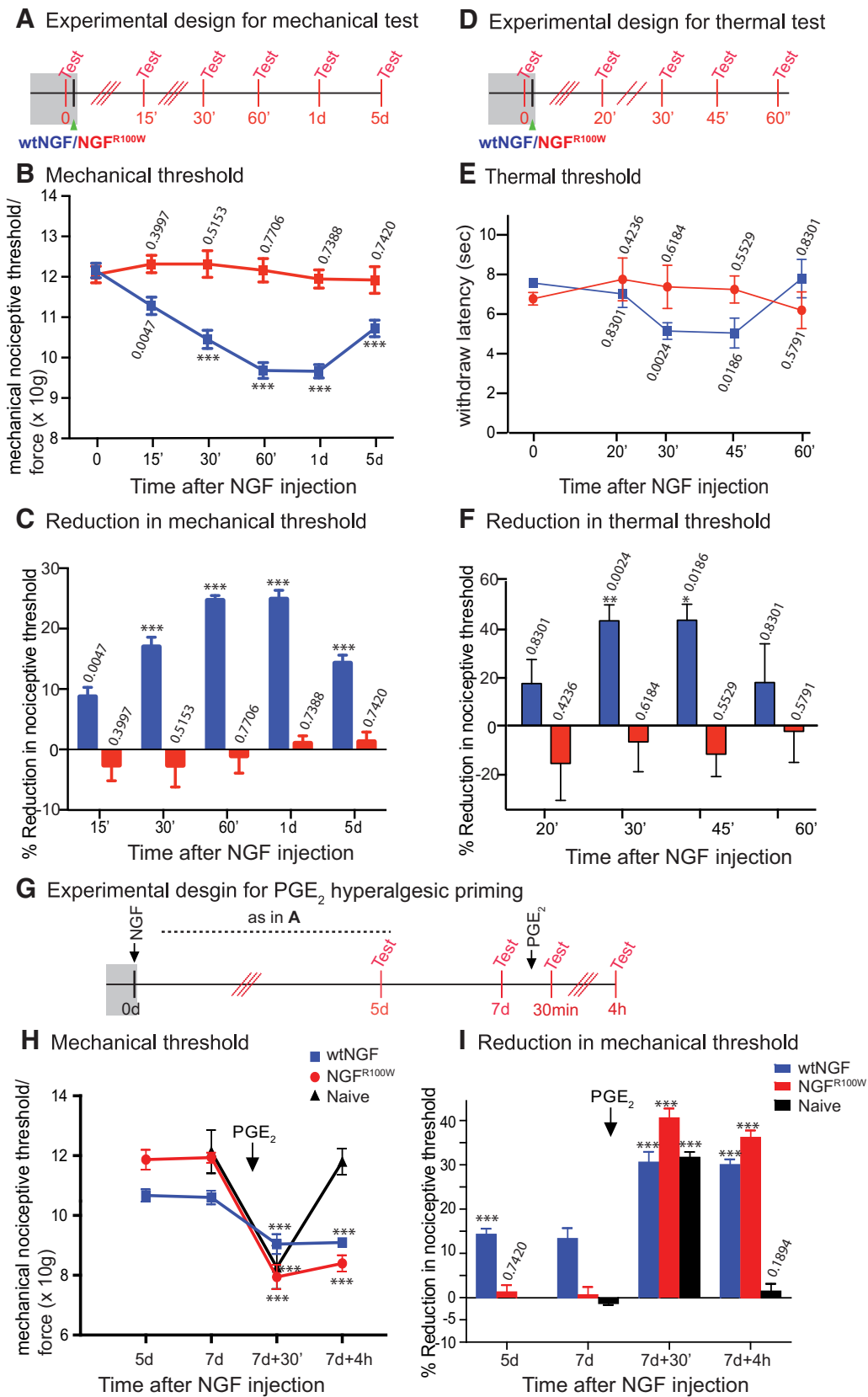
Figure 1. NGF^{R100W} does not induce acute sensitization in adult rats. **A–C**, Mechanical hyperalgesia. The Randall–Selitto method was used to measure mechanical hyperalgesia in adult rats. After intradermal injection of 200 ng of wtNGF ($n = 6$) or NGF^{R100W} ($n = 6$) into the rat's hindpaws, mechanical threshold was measured at the indicated times. A significant decrease in mechanical nociceptive threshold in rats injected with wtNGF was seen within 15 min, reached a maximum by 1 h, and lasted at least 5 d. In contrast, NGF^{R100W} did not produce a significant decrease in the threshold during the 5 d period. **D–F**, Thermal hyperalgesia. The Hargreaves test was used to measure thermal hyperalgesia. Baseline response was first measured for each test animals before NGF injection. Hindpaws were injected with 600 ng of either wtNGF or NGF^{R100W}. Thermal threshold was measured at the indicated times. Animals that were injected with wtNGF showed a significant decrease at 30 min. The decrease was dissipated after 1 h. NGF^{R100W} failed to reduce thermal nociceptive threshold during the entire 1 h test period. Six rats for (*Figure legend continues.*)

A

C

B
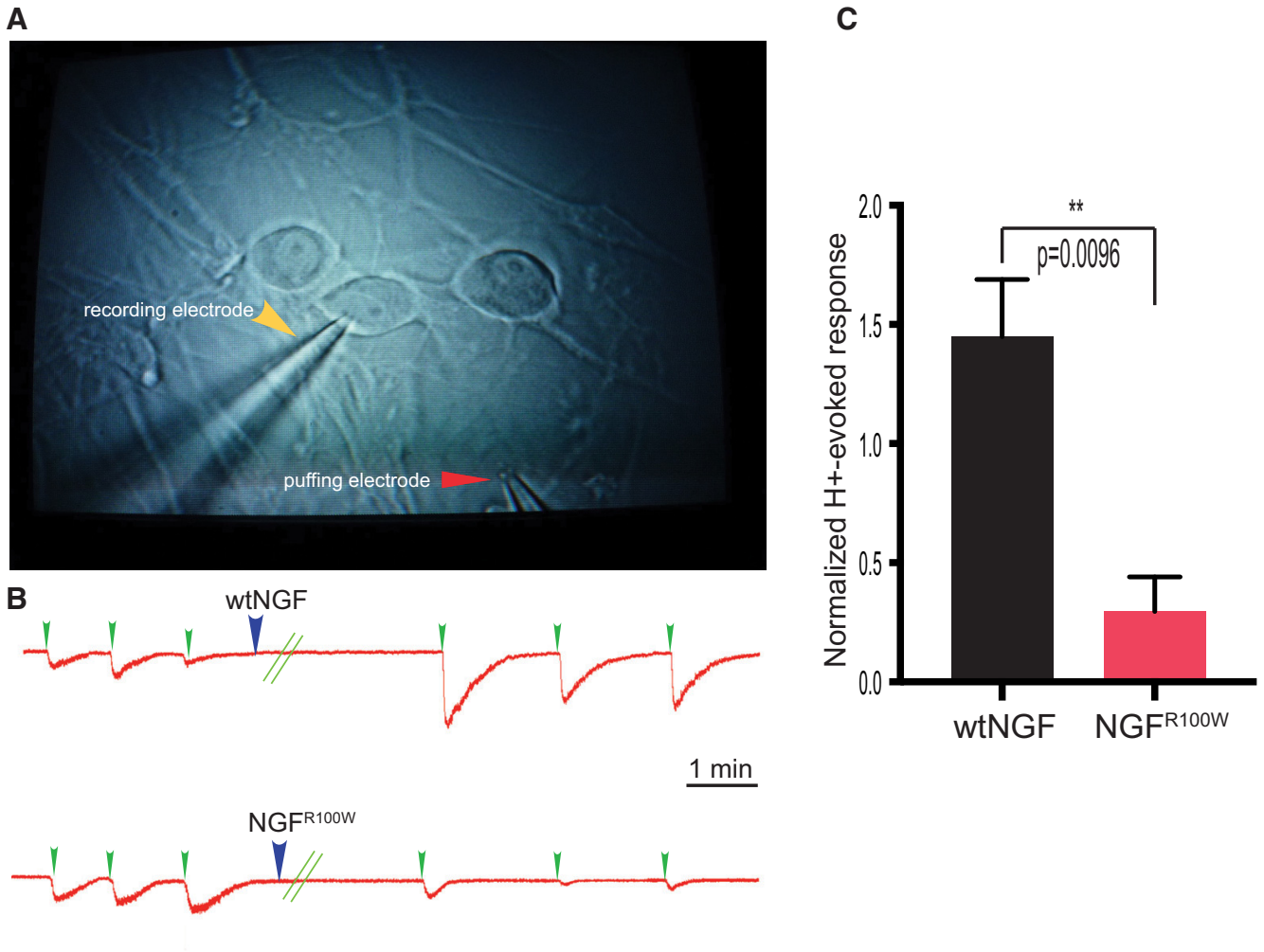
Figure 2. Low H$^+$-evoked response by single-cell patch clamping. Rat E15.5 DRG neurons were cultured as described in the Materials and Methods. At DIC5, DRG neurons were deprived of NGF for 2 h. **A**, Phase contrast image of DRG neurons (under 60× magnification) showing the experimental setup. The patch pipette approached from the left and another glass pipette that applied brief puffs of pH 5.5 solution to the nearby cell body was placed at a distance. This pipette did not induce mechanical responses. **B**, Whole-cell patch-clamp recording was performed at a holding potential of −60 mV in DRG. The proton-evoked response was measured after a brief application of moderate acidic solution of pH 5.5 (blue arrow) onto the cell body to induce inward current (designated as "before NGF response"). Then, 50 ng/ml of wtNGF or NGF^{R100W} (green arrow) was applied to the bath solution for 10 min and three additional puffs were applied to record the after NGF response. **C**, The data were normalized by calculating the ratio of the after NGF response to the before NGF response. Bar graphs represent mean ± SEM. wtNGF sensitized the inward current by 1.45-fold, but not NGF^{R100W} ($p = 0.0096$; wtNGF vs NGF^{R100W}; unpaired t test). **$p < 0.01$.

measured the number of healthy DRGs at DIC8 after treating with either wtNGF or NGF^{R100W} (Fig. 3A). At DIC8, phase contrast images of DRG cultures were taken and survival was quantitated (Fig. 3B). Unpaired t test was performed to compare the number of healthy DRGs treated with wtNGF or NGF^{R100W}. At

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(*Figure legend continued.*) each group ($n = 6$) were used in the test. Unpaired t tests were performed against the baselines within each NGF injection group to produce p-values. Comparisons were done between the threshold before injection with either wtNGF or NGF^{R100W} to produce p-values that were noted in the figure. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **G–I**, PGE$_2$-hyperalgesic priming effect. On d 7 after NGF administration, PGE$_2$ was injected intradermally (100 ng/5 μl) to test hyperalgesic priming. Naive rats (nontreated with NGF) are shown as a control. Naive animals showed acute hyperalgesia within 30 min, but not at 4 h, after PGE$_2$ injection. Rats pretreated with intradermal injection of wtNGF showed a decrease in the mechanical nociceptive threshold after PGE$_2$ injection at 30 min, like the controls, but unlike controls, it lasted longer than 4 h. Rats pretreated with NGF^{R100W} showed significant decrease in mechanical nociception after PGE$_2$ injection, which lasted at least 4 h and was comparable to wtNGF. Data are presented as mean ± SEM. Unpaired t tests were performed versus values before PGE$_2$ injection. ***$p < 0.001$ Six paws ($n = 6$) were used for wtNGF or NGF^{R100W}.

any concentration of treatment, NGF^{R100W} exerted a trophic effect to support the survival of DRGs comparably to wtNGF. As a control, cultures with no addition of NGF showed significant death (Fig. 3A). We thus conclude that NGF^{R100W} supports the survival of rat E15.5 DRG neurons and that it does so as effectively as wtNGF.
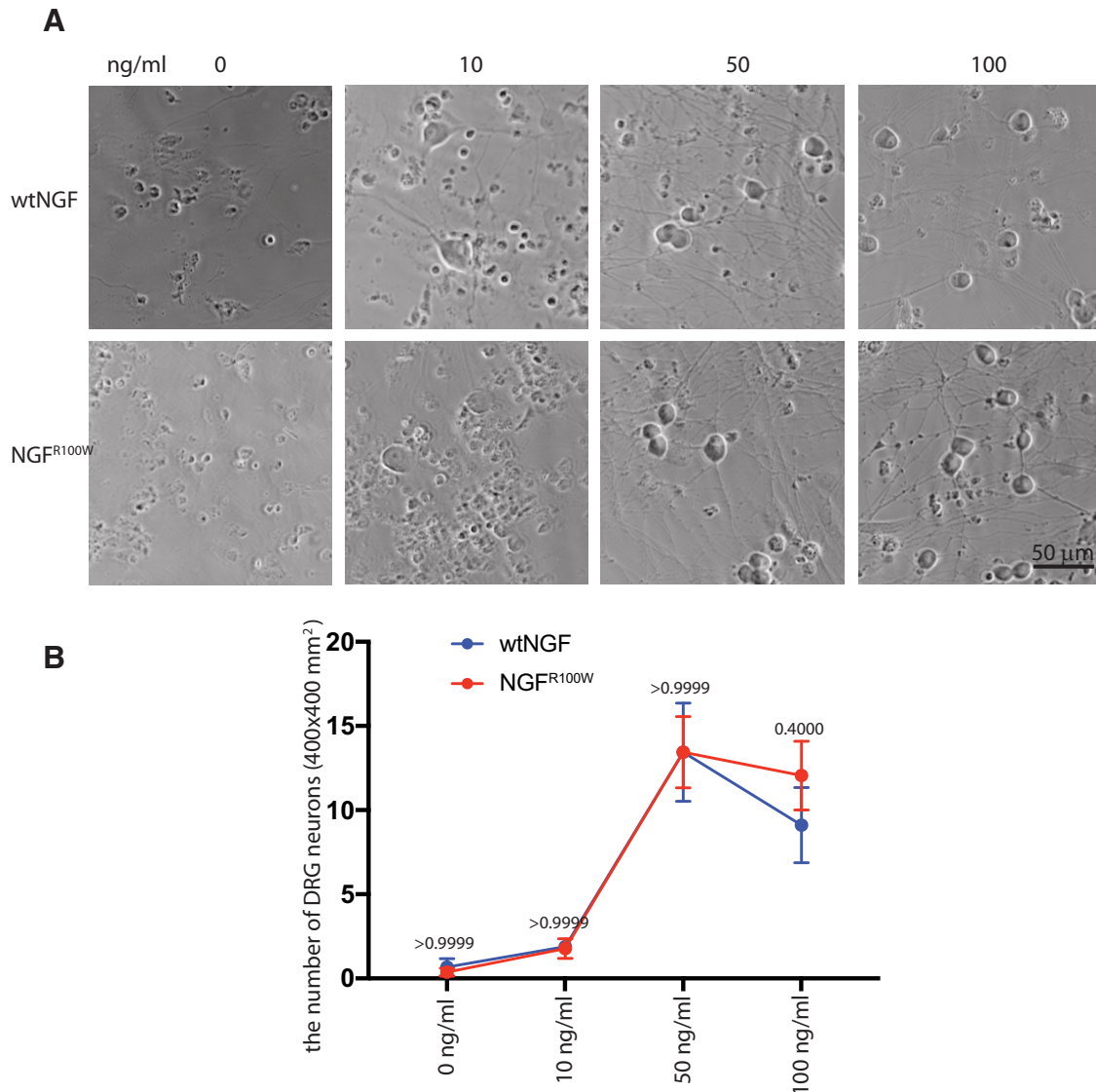
NGF^{R100W} binds to and is internalized through TrkA, but not p75^{NTR}

NGF binds and signals through TrkA and/or p75^{NTR} receptors to effect neuronal function (Frade and Barde, 1998; Yoon et al., 1998; Sofroniew et al., 2001; Chao, 2003). We then explored whether NGF^{R100W} differed from wtNGF in binding and internalization through TrkA and p75^{NTR}. We used a NIH3T3 cell line that stably expresses either TrkA or p75^{NTR} (Hempstead et al., 1991; Kaplan et al., 1991; Zhou et al., 1994; Huang et al., 1999) to perform in-cell binding assays. NGF^{R100W} and wtNGF were each labeled with Quantum Dots 655 (QD655) before incubating with either NIH3T3-TrkA-, or NIH3T3-p75^{NTR} cells. Saturable binding was demonstrated by using a range of NGF concentrations

Figure 3. DRG survival assays. Rat E15.5 DRG neurons were cultured as described in the Materials and Methods. Parallel cultures were supplied with either wtNGF or NGF^{R100W} at a range from 0 to 100 ng/ml. **A**, Phase contrast images of DRG neurons at 8 d *in vitro* were captured and representative images are shown. Negative control (no NGF, 0 ng/ml) failed to maintain the survival of DRGs. NGF^{R100W} maintained the survival as potently as wtNGF. **B**, The survival rate of DRG neurons (i.e., cell counts) by wtNGF or NGF^{R100W} was not significantly different. **E**, Unpaired *t* test was performed on wtNGF versus NGF^{R100W} ($p > 0.9999$ at 0, 10, and 50 ng/ml, $p = 0.4000$ at 100 ng/ml). Data are presented as mean ± SEM.

(Fig. 4 *A*, *B*). Binding data were fit to a classical hyperbolic binding curve (one-site binding) and nonlinear regression analysis using Prism 6 software. The results showed that NGF^{R100W} binding to TrkA ($K_d = 2.79$ nM) was essentially indistinguishable from that for wtNGF ($K_d = 2.27$ nM; Fig. 4*A*). In contrast, NGF^{R100W} showed minimal binding to p75^{NTR} even at concentrations at which wtNGF binding was essentially saturated (Fig. 4*B*).

To further confirm that NGF^{R100W} binding to p75^{NTR} was markedly reduced, we performed *in vitro* binding assays for p75^{NTR} using the extracellular domain (p75^{NTR}-ECD). Either wtNGF or NGF^{R100W} in their biotinylated forms at a final concentration ranging from 0 to 0.25 nM was incubated with recombinant p75^{NTR}-ECD; streptavidin–agarose beads were used to pull down p75^{NTR}-ECD that bound to biotinylated wtNGF or NGF^{R100W}. The levels of p75^{NTR}-ECD were assayed by immunoblotting using the anti-p75^{NTR} antibody, REX. Figure 4, *C* and *E*, shows that increasing concentrations of wtNGF resulted in increasing amounts of p75^{NTR}-ECD in the pull-down complex. In

contrast, NGF^{R100W} failed to pull down detectable p75-ECD even at the highest concentration (Fig. 4*D*). We conclude that the binding affinity of NGF^{R100W} for p75^{NTR} is markedly reduced with respect to that for wtNGF; therefore, NGF^{R100W} fails to bind p75^{NTR}.

To extend these analyses, we then assayed whether wtNGF and NGF^{R100W} differed with respect to TrkA- or p75^{NTR}-receptor-mediated internalization. Because NGF^{R100W} showed reduced or absent binding to p75^{NTR}, NGF^{R100W} would also fail to be internalized via p75^{NTR}. We performed live cell imaging using NIH3T3-TrkA- or NIH3T3-p75^{NTR}-expressing cells (Huang et al., 1999). In addition to wtNGF and NGF^{R100W}, we also took advantage of two well characterized NGF mutants: the KKE mutant, which shows a significant decrease in binding affinity for p75^{NTR} but unaltered binding affinity for TrkA (Ibáñez et al., 1992; Mahapatra et al., 2009), and the Δ9/13 mutant, which poorly binds to TrkA while maintaining normal binding affinity for p75^{NTR} (Hughes et al., 2001). Accordingly, KKE and Δ9/13 NGF served as
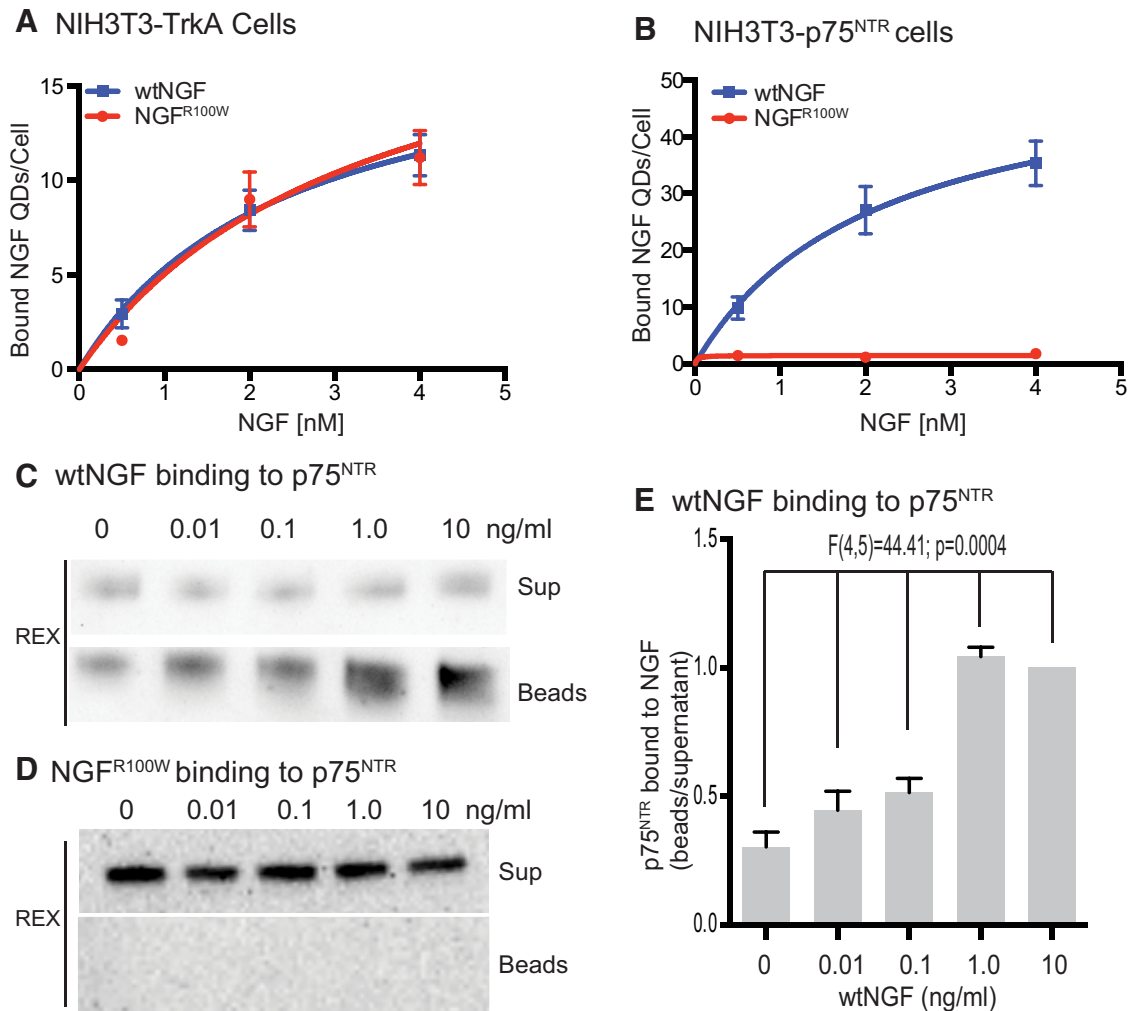
Figure 4. Classical hyperbolic saturation curves of wtNGF and NGF^{R100W} bound to TrkA or p75^{NTR}-expressing NIH3T3 cells. *A*, *B*, Either NIH3T3 TrkA or p75^{NTR} cells were exposed to wtNGF-QD655/or NGF^{R100W}-QD655 for 20 –30 min at 20°C with a range of different NGF-QD655 concentrations. Nonlinear regression analysis using observed data were performed to examine the binding of NGF to the TrkA receptor (*A*) or p75^{NTR} (*B*). Blue squares and red circles represent surface-bound wtNGF and NGF^{R100W}, respectively. *C*, *D*, Biotinylated wtNGF (*C*) or NGF^{R100W} (*D*) was incubated with recombinant extracellular domain of p75^{NTR} at 4°C for 2 h. The complex that contained biotinylated NGF was pulled down using streptavidin–agarose (SA) beads at 4°C for 2 h. The beads were washed and boiled in SDS sample buffer. Both an aliquot of the supernatant (control) and the bead-bound samples were analyzed by Western blotting using a specific antibody against p75^{NTR} (REX). Representative blot shows p75^{NTR} bound to wtNGF-SA beads (*C*) and p75^{NTR} bound to NGF^{R100W}-SA beads (*D*). Relative REX signal from pulled-down beads was significantly increased with increased amount of wtNGF, as shown in *C*. *E*, ANOVA analysis was performed with different amounts of wtNGF, suggesting that pulled-down p75 ECDs were significantly increased according to the increased amount of wtNGF ($F_{(4,5)}$ = 44.41, p = 0.0004). *D*, In contrast, pulled-down p75 ECD was not detectable even with 10 ngf of NGF^{R100W} and only reached the level of baseline Rex signal intensity. Data are presented as mean ± SEM.

positive controls for binding to TrkA and p75^{NTR}, respectively. We produced monobiotinylated forms of KKE and Δ9/13 NGF along with wtNGF and NGF^{R100W} to facilitate conjugation to QD655. These QD-655 labeled forms of NGF were incubated with cells at a final concentration of 0.2 nM at 37°C (30 min for NIH3T3-TrkA cells and 2 h for NIH3T3-p75^{NTR} cells) to allow for internalization. Incubations were followed by extensive washing at 4°C in PBS (3×). QD655 signals were captured by live imaging and defined as internal if the QD signal was found within the perimeter of the cell and at the same focal level as the nucleus. As with wtNGF and the KKE mutant, NGF^{R100W} was internalized by NIH3T3-TrkA cells (Fig. 5A). The process was receptor mediated because premixing with 100× wtNGF (i.e., not conjugated to QD 655) eliminated NGF^{R100W}-QD655 internalization. The Δ9/13 mutant was not internalized in NIH3T3-TrkA cells (Fig. 5A,C). As predicted, NGF^{R100W} and the KKE mutant failed to be internalized into NIH3T3-p75^{NTR} cells; with both wtNGF and the Δ9/13 mutant, bright QD signals were detected inside

NIH3T3-p75^{NTR} cells (Fig. 5B,C). As a control, nonconjugated QDs were not detected in either cell line. These results are further evidence that NGF^{R100W} is similar to wtNGF in that it binds to TrkA, but differs in not binding to p75^{NTR}.

NGF^{R100W} activates TrkA-mediated signaling pathways, but fails to stimulate a p75^{NTR} downstream effector

Based on the binding and internalization results, we predicted that NGF^{R100W} activated the TrkA-mediated, but not the p75^{NTR}-mediated signaling, pathway. We next determined whether wt-NGF and NGF^{R100W} activated two main effectors of TrkA downstream signaling cascades: Erk1/2 and Akt using PC12 cells (Fig. 6A). For this purpose, PC12 was stimulated with 50 ng/ml of either wtNGF or NGF^{R100W}. Cells then were lysed and analyzed using immunoblotting. Figure 6H, shows that NGF^{R100W} induced phosphorylation of TrkA, Erk1/2, and Akt to the extent comparable to wtNGF. We also performed semiquantitative measurement of pTrkA activated by wtNGF or NGF^{R100W}. The signals
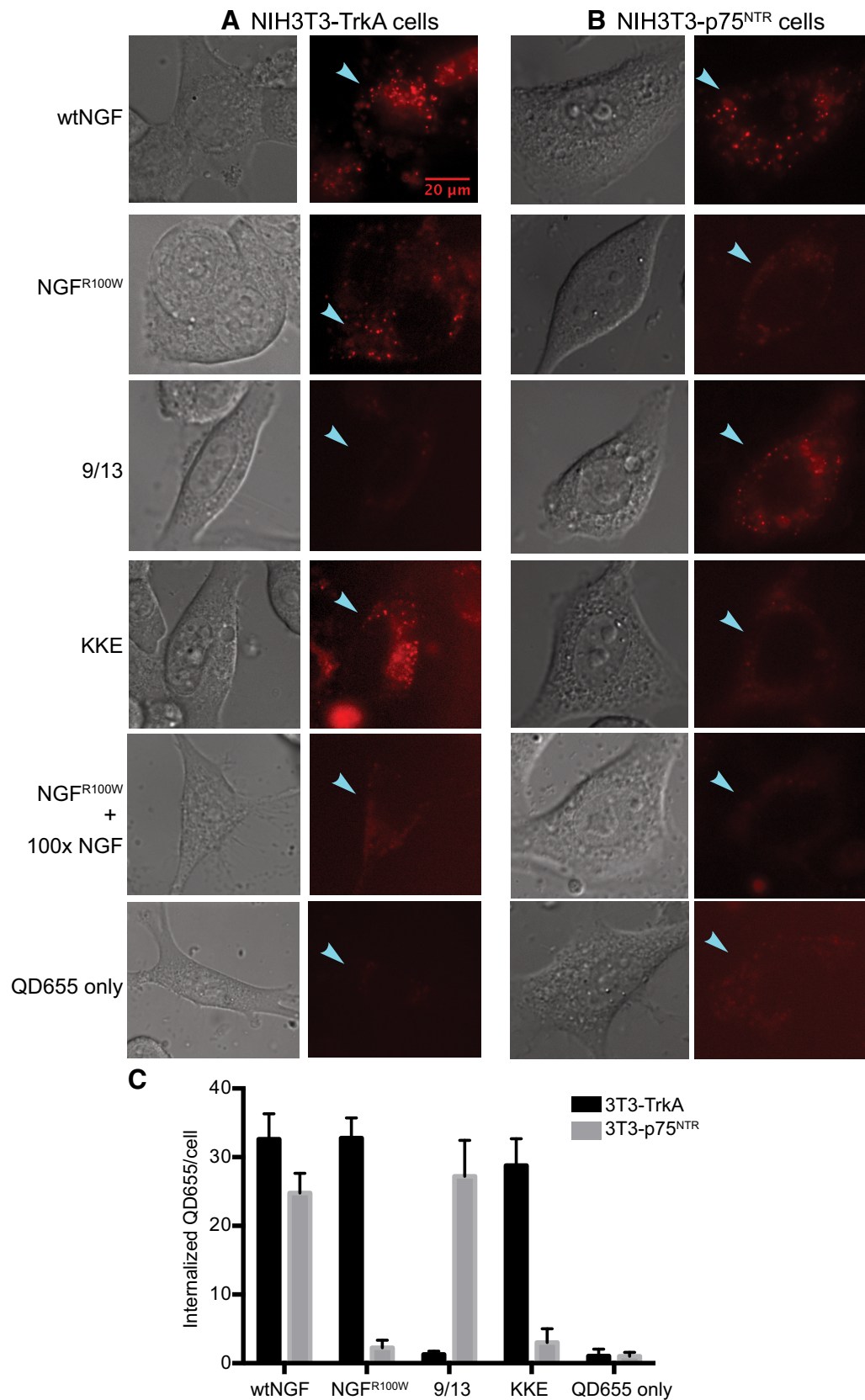
Figure 5. Imaging analysis of binding and internalization into NIH3T3-TrkA- or -p75[NTR]-expressing cells. *A*, NIH3T3-TrkA cells were cultured on coverslips that were precoated with poly-L-lysine. Cells were rinsed and serum-starved for 2 h. Cells were then incubated with 0.2 nM of wtNGF, or NGF[R100W], KKE, or delta9/13-QD655 conjugates or with QD655 alone for 30 min at 37°C. After extensive rinses, QD655 signals were captured by live cell imaging. Representative images are shown. wtNGF, NGF[R100W], and the KKE mutant that were used as positive controls for the TrkA receptor showed bright QD655 signals inside the cells. To ensure that internalization of NGF[R100W] was receptor mediated, we premixed NGF[R100W] with 100-fold unlabeled NGF before live imaging. The results show little internalization of NGF[R100W], indicating the internalization of NGF[R100W] into NIH3T3-TrkA cells was TrkA specific. *B*, Similarly, NIH3T3-p75[NTR] cells were (*Figure legend continues.*)

for pTrkA were normalized against GAPDH (Fig. 6-1, available at: https://doi.org/10.1523/JNEUROSCI.1686-17.2018.f6-1). We did not detect a significant difference in pTrkA (Fig. 6-1, available at: https://doi.org/10.1523/JNEUROSCI.1686-17.2018.f6-1). These findings suggest that NGF^{R100W} maintains its ability to activate TrkA, Erk 1/2, and Akt signaling pathways, which are primarily involved in providing trophic support for neuronal survival and differentiation.

We then assessed whether both wtNGF and NGF^{R100W} stimulated two signaling pathways downstream of p75^{NTR} receptors. We investigated whether RhoA and Cofilin, two effectors downstream to p75^{NTR} (Yamashita and Tohyama, 2003; Vardouli et al., 2005), were phosphorylated or activated by wtNGF or NGF^{R100W}. Using PC12^{nnr5} cells that express p75^{NTR} with little or no TrkA (Loeb and Greene, 1993), we tested whether treatment of these cells with either wtNGF or NGF^{R100W} induced phosphorylation of Cofilin (i.e., p-Cofilin) by immunoblotting using an antibody that specifically recognizes the phosphorylated form of Cofilin. Our results show that Cofilin was activated when cells were treated by wtNGF. However, the level of phosphorylated Cofilin was significantly less in cells treated by NGF^{R100W} (Fig. 6D,E). We then assayed for activation of RhoA, a signaling molecule upstream of Cofilin in the p75^{NTR} signaling cascades (Vardouli et al., 2005). As demonstrated by immunostaining with specific antibody to activated RhoA (i.e., RhoA^{GTP}), RhoA^{GTP} exhibited marked activation by wtNGF, as evident by strong cytosolic staining (Fig. 6F). In contrast, NGF^{R100W} resulted in much less activation of RhoA^{GTP}, with only sparse speckles of signals in the cytoplasm (Fig. 6F). These data further confirm that NGF^{R100W} is ineffective in activating signaling cascades downstream of p75^{NTR}.

Interestingly, we observed that NGF^{R100W} was unable to fully induce phosphorylation of PLC-γ (Fig. 6G). Therefore, we investigated whether failure of activation of the p75^{NTR} signaling by NGF^{R100W} was responsible for the inability to fully phosphorylate PLC-γ. Indeed, there has been evidence suggesting that activated p75^{NTR} downstream effectors positively affect downstream of TrkA signaling (Ruiz-Argüello et al., 1996; Basáñez et al., 1997; Ruiz-Argüello et al., 1998; Cremesti et al., 2002). For example, others have shown that ceramide-induced changes in membrane microenvironments facilitate PLC signaling. We therefore speculated that treating cells with wtNGF under conditions in which p75^{NTR} was inhibited would cause a reduction in PLC-γ activation, as was the case for NGF^{R100W} (Fig. 6H). We used PC12 cells to determine whether p75^{NTR} inhibition affected PLC-γ by possible crosstalk between p75^{NTR} and TrkA. Our results showed that phosphorylation of PLC-γ was decreased when PC12 was pretreated with a p75^{NTR} inhibitor, TAT-pep5 (Head et al., 2009), followed by stimulation with wtNGF (Hasegawa et al., 2004), as shown in Figure 6F. These results suggest that failure of p75^{NTR} signaling by NGF^{R100W} leads to failure of PLC-γ signaling by as yet unknown mechanisms, even though NGF^{R100W} stimulates other downstream signaling such as Erk1/2 and Akt under TrkA. NGF^{R100W} activates most TrkA downstream signaling events, but not those mediated by p75.

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(*Figure legend continued.*) used to investigate internalization of the different forms of NGF proteins and representative images are shown after incubating with NGF-QD655 for 2 h at 37℃. The results show that both wtNGF and Δ9/13, which are known to bind to the p75^{NTR} receptor, were internalized into NIH3T3-p75^{NTR} cells and the QD655 signals were mostly concentrated around the peripheries of the cell. No signals were observed in the NIH3T3-p75^{NTR} cells when treated with either the KKE mutant or NGF^{R100W}. *C*, Internalized QD655 within the cells were quantitated. Data are presented as mean ± SEM.

NGF^{R100W} is retrogradely transported in a fashion similar to wtNGF

Axons feature prominently in facilitating trafficking and signaling of NGF. A possibility existed that the loss of pain signaling in NGF^{R100W} was due to its inability to be effectively transported retrogradely to the cell soma in sensory neurons. The ability of R100W to induce priming suggested that it was effectively transported retrograde in axons to engage the cell body responses that support priming. To test this possibility, we established microfluidic cultures of dissociated rat E15.5 DRG neurons. To visualize trafficking of NGF, we conjugated biotinylated wtNGF or NGF^{R100W} with streptavidin-QD605 at a 1 NGF dimer to 1 QD605 ratio. This experimental paradigm was used to track axonal movement of a single NGF dimer by live imaging to produce highly quantitative results (Sung et al., 2011). wtNGF was retrogradely transported with an average speed of 1.5 μm/s (Fig. 7A), which is consistent with previous results (Cui et al., 2007; Sung et al., 2011). Based on the analysis of >100 endosomes/condition, the average moving speed of NGF^{R100W} revealed no marked difference from that of wtNGF (Fig. 7B).

NGF is known to exhibit a "go and stop" behavior during transport (Cui et al., 2007; Sung et al., 2011). Therefore, we tested whether the moving speed during the "go" period differed significantly between wtNGF and NGF^{R100W}. Our results demonstrated that this was not the case (Fig. 7D). By superimposing the kymographs of NGF^{R100W} onto those of wtNGF (Fig. 7C), our results confirmed that NGF^{R100W} behaved exactly like wtNGF during retrograde transit from the axonal terminal to the cell body. We thus conclude that the critical function in retrograde axonal transport of NGF in sensory neurons is preserved in NGF^{R100W}.

Study of contribution of TrkA or p75^{NTR} to NGF-induced sensitization effect *in vivo*
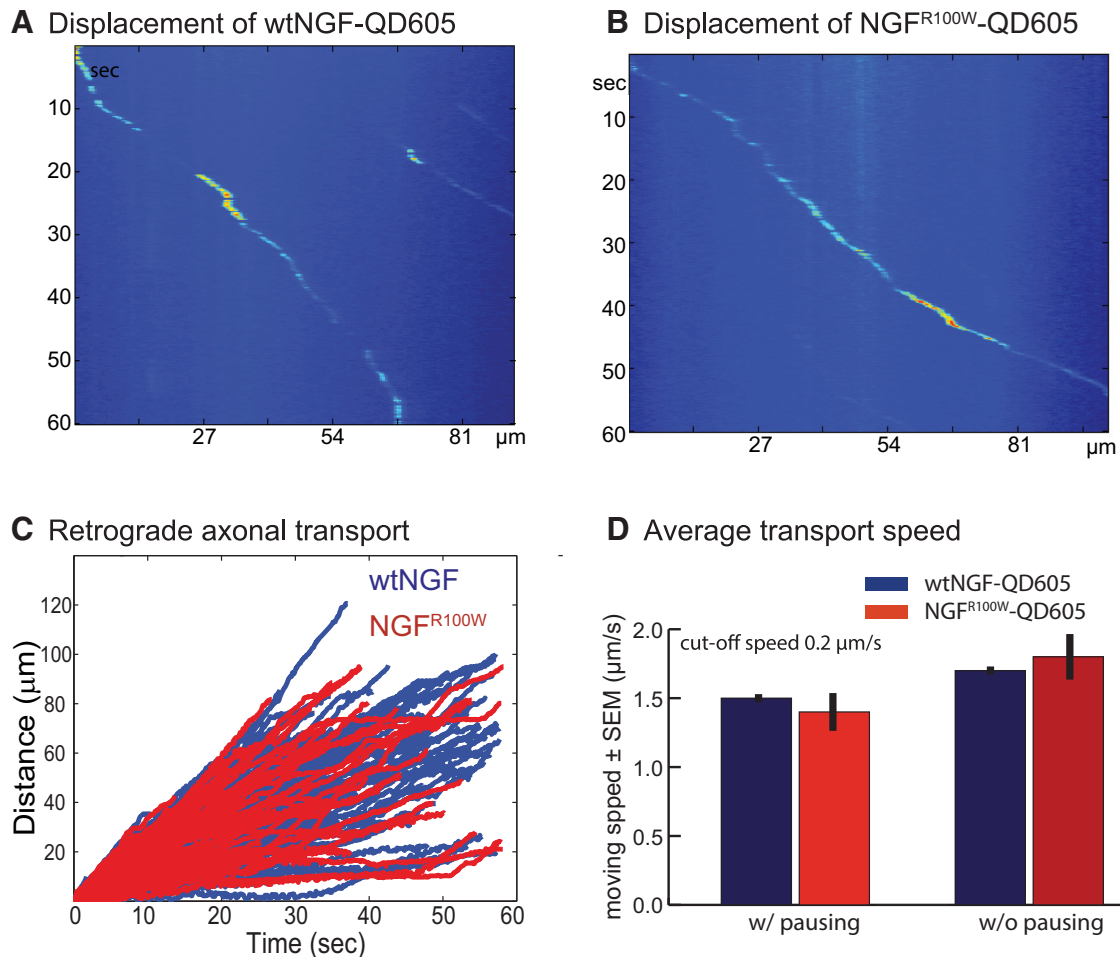
Our findings suggest that NGF^{R100W} retained its ability to bind to and activate TrkA while failing to engage p75^{NTR}. However, when injected into adult rats, NGF^{R100W} still induced hyperalgesic priming without causing acute sensitization to mechanical stimuli. These results raise the possibility that TrkA and p75^{NTR} may play a distinct role(s) in NGF-induced hyperalgesia and hyperalgesic priming. We used pharmacological reagents to selectively inhibit signaling downstream of TrkA and/or p75^{NTR}: K252a for blocking TrkA activation and GW4869 for inhibiting neutral sphingomyelinase downstream of p75^{NTR}.

Vehicles or inhibitors (K252a, GW4869, K252a + GW4869) were injected only once 5 min before NGF injection (Fig. 8A). We then measured the mechanical nociceptive threshold at 1 h and 7 d after NGF injection, as outlined in the experimental design (Fig. 8A). K252a produced a robust reduction ($\sim$30%) in NGF hyperalgesia ($p = 0.0001$); GW4869 had a small but insignificant reduction ($\sim$7%, $p = 0.0557$); combination of K252a with GW4869 caused a $\sim$25% reduction ($p < 0.0001$). Consistent with results presented in Figure 1, NGF-induced hyperalgesia had returned to baseline nociceptive threshold at day 7 after NGF treatment regardless of inhibitors (Fig. 8A).

We then measured the impact of these inhibitor treatments on priming after injection of PEG$_2$, as in Figure 1. Treatment with K252a, GW4869, or both did not change acute sensitization significantly 30 min after PEG$_2$ injection (Fig. 8A). However, at 4 h after PEG$_2$ injection, K252a and GW4869 each alone produced a moderate reduction in PGE$_2$ hyperalgesia ($p = 0.0076$ for K252a; p0.0359 for GW4869); Strikingly, combination of K252a with GW4869 induced complete elimination of PGE$_2$ hyperalgesia
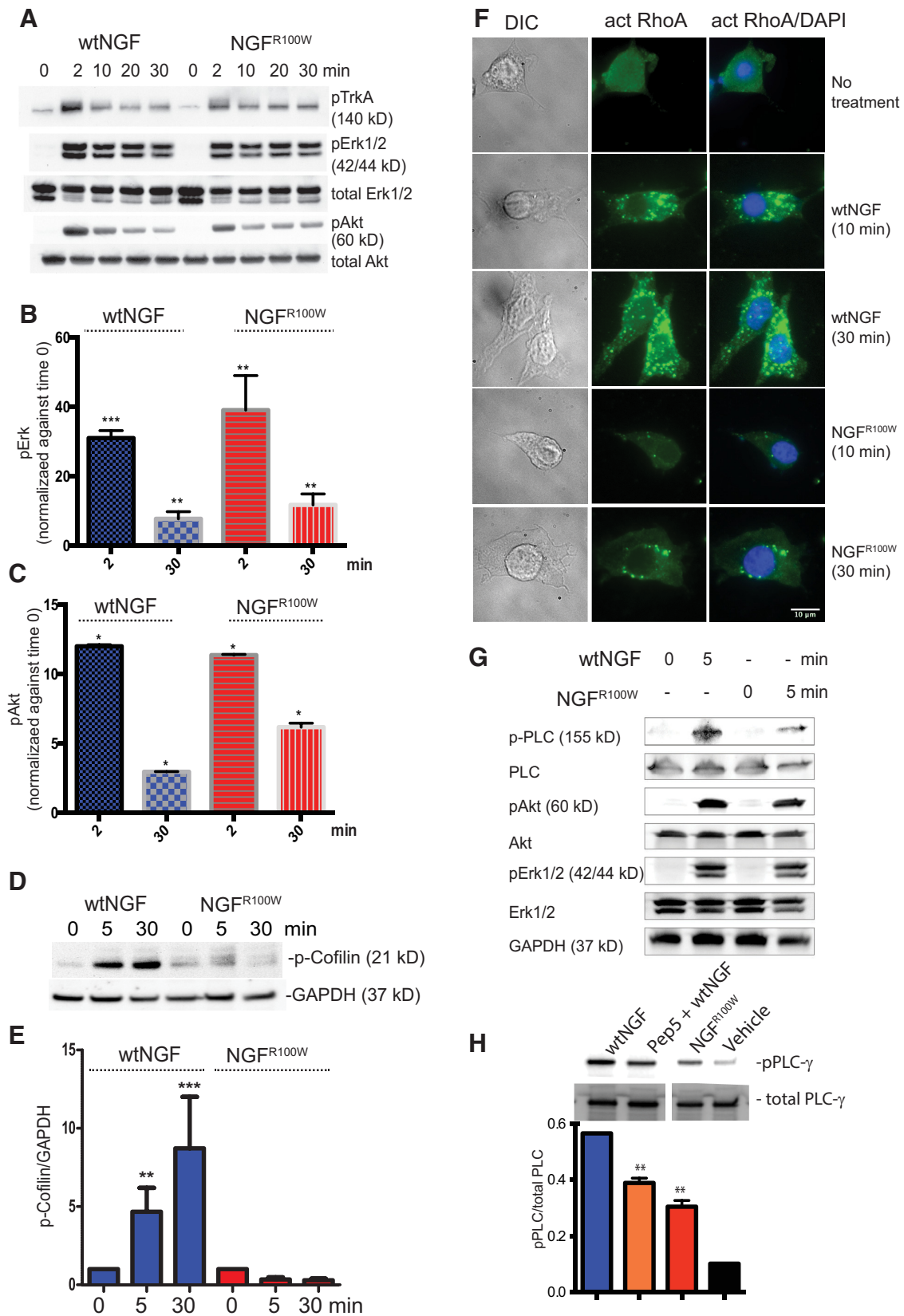
Figure 6. Analysis of TrkA- and p75^{NTR}-mediated signaling pathways. **A–C**, PC12 cells were serum-starved and treated with 50 ng/ml of either wtNGF or NGF^{R100W} for the indicated time intervals. Cell lysates were analyzed by SDS-PAGE and immunoblotting with specific antibodies as indicated. Treatment with either wtNGF or NGF^{R100W} induced phosphorylation; i.e., activation of TrkA, Erk 1/2, and Akt. The blots were reprobed for total Akt or Erk1/2 as loading controls. We also measured the signals for pTrkA activated by wtNGF or NGF^{R100W}. The signals for pTrkA were normalized against GAPDH (Fig. 6-1, available at: https://doi.org/10.1523/JNEUROSCI.1686-17.2018.f6-1). **D**, **E**, Analysis of downstream signaling of p75^{NTR} in PC12 nnr5 by immunoblotting. The cells were prepared, treated, and cell lysates analyzed by SDS-PAGE/immunoblotting with specific antibodies as in **A**. There was a significant reduction in phosphorylation of Cofilin by NGF^{R100W} compared with wtNGF. **F**, Immunostaining of RhoA-GTP in PC12 nnr5. PC12 nnr5 were plated on the coverslip coated by poly-L-lys, starved for 2 h, treated with (*Figure legend continues.*)

A Displacement of wtNGF-QD605

B Displacement of NGF^{R100W}-QD605

C Retrograde axonal transport

D Average transport speed

Figure 7. Analysis of retrograde axonal transport by live imaging. **A**, **B**, Rat E15.5 DRG neurons were cultured in microfluidic chamber. Kymographs of axonal movement of wtNGF and NGF^{R100W} based on real-time imaging series of axonal transport assays are shown. The graphs represent spatial position of QD605 signals (in micrometers) over time (seconds). The results for both wtNGF and NGF^{R100W} showed similar slopes, suggesting that NGF^{R100W} moves at a speed within the axon similar to that of wtNGF. **C**, Overlayed kymographs of displacement of axonal QD605 signals for wtNGF and NGF^{R100W}. The 100 –150 QD605 signals for either wtNGF (blue) or NGF^{R100W} (red) were analyzed and superimposed. The results demonstrate that axonal movement of wtNGF and NGF^{R100W} behaves in a strikingly similar fashion. **D**, Total average transport speeds including pausing for wtNGF and NGF^{R100W} were calculated to be 1.5 and 1.4 mm/s, respectively. The moving velocity without pausing, that is, during the "go" motion period, was 1.7 mm/s for both wtNGF and NGF^{R100W}.

($p = 0.0006$; Fig. 8A). These results further support an interaction between TrkA and p75^{NTR} in mediating the priming effect by NGF.
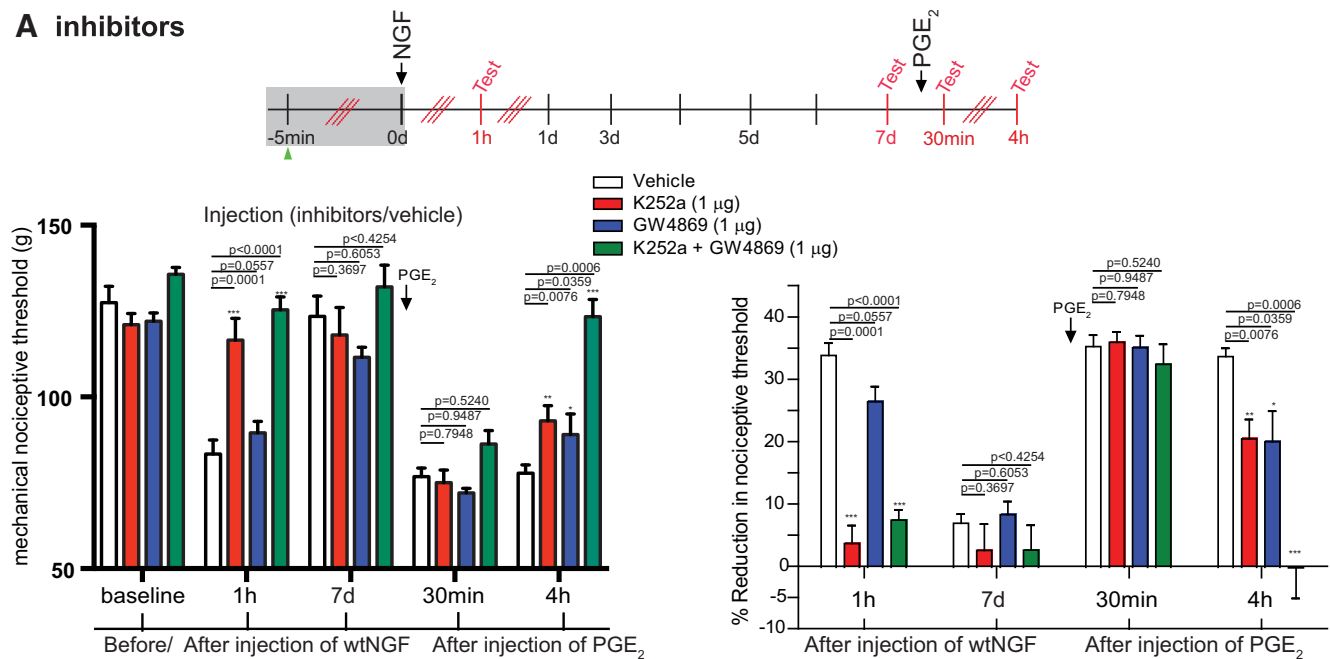
Because there is discrepancy between NGF^{R100W} injection studies (Fig. 1 B,C) versus GW4869 treatment in inducing acute hyper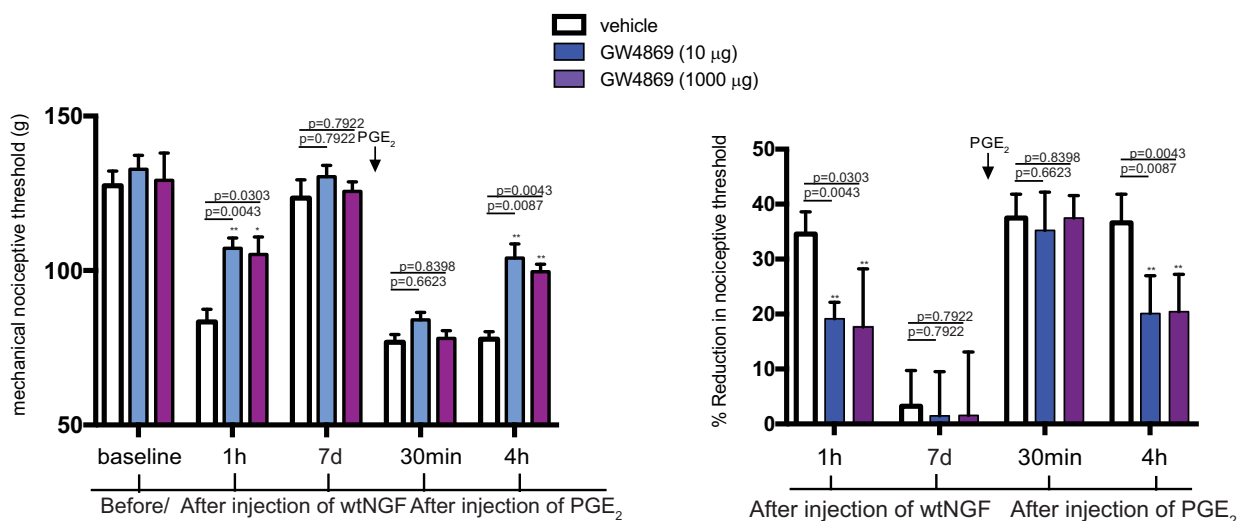sensitivity (Fig. 8A), we repeated the Randall–Selitto experiment with higher dosages of GW 4869. We chose two different dosages, 10 μg and 1000 μg. Previous studies have demonstrated that 11.55 μg/injection of GW 4869 was effective in blocking acute sensitization by NGF (Khodorova et al., 2013, 2017). The 1000 μg dose was to ensure that a maximal effect was achieved. As demonstrated in Figure 8B, 10 μg/injection appeared to yield a maximal effect; both dosages, 10 μg and 1000 μg, successfully prevented NGF-induced acute hyperalgesia at 1 h after NGF injection, which is consistent with published studies (Khodorova et al., 2013, 2017), but also from our own studies presented in Figure 1, B and C. At 1 h after NGF injection, vehicle injection decreased the mechanical pain threshold ~34.57% and 10 μg and 1000 μg of GW4869 decreased the threshold ~19.14% ($p = 0.0043$) and ~17.64% ($p = 0.0303$), respectively. (Fig. 8B). We then measured priming after injection of PGE$_2$ with these two high dosages of GW4869. Both dosages also blocked priming significantly compared with vehicle ($p = 0.0087$ and $p = 0.0043$; Fig. 8B). These data further confirmed our result pointing to the loss of p75^{NTR} downstream signaling in NGF^{R100W} as a mechanism underlying loss of pain perception. Together, these data suggest that both TrkA and p75^{NTR} are responsible for mediating acute hypersensitivity and priming.

←

(*Figure legend continued.*) either wtNGF or NGF^{R100W}, and the preparations were fixed and permeabilized, followed by the protocol. Differential interference contrast imaged cells show single staining for active form of RhoA (RhoA-GTP) or double staining for active RhoA and nucleus. RhoA-GTP staining revealed that RhoA activation was stronger in the cell treated with wtNGF than in the cell treated with NGF^{R100W}. **G**, **H**, Analysis of PLC-γ signaling in PC12 cells by immunoblotting. **G** shows that PLC-γ stimulated by NGFR100W differs significantly from the one by wtNGF. In contrast, the same lysate showed similar amount of activation of Erk1/2 and Akt. PC12 cells were pretreated with the p75^{NTR} inhibitor Pep15, followed by treatment with 50 ng/ml NGF. In parallel samples, cells were treated with vehicle, 50 ng/ml NGF, or 50 ng/ml NGF^{R100W}. Cell lysates were analyzed by SDS-PAGE/immunoblotting with specific antibodies as indicated. The data show that activation of PLC-γ was markedly suppressed by NGF^{R100W} compared with wtNGF. wtNGF failed to fully activate PLC-γ when p75^{NTR} was functionally inhibited, similar to partially activated PLC-γ when treated by NGF^{R100W}. Data are presented as mean $\pm$ SEM. p values were calculated using student unpaired t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

A inhibitors

B dosage response of GW4869

Figure 8. Role of TrkA and p75^{NTR} in nociceptive response and hyperalgesic priming. ***A***, Five microliters (1 μg) of K252a, GW4869, or vehicle (10% DMSO) was administered intradermally on the dorsum of the hindpaw of adult rats at the same site where NGF was going to be injected. After NGF injection (200 ng of wtNGF), the Randall–Selitto method was used to measure mechanical hyperalgesia as in ***A***. $n = 4$ for vehicle, K252a, GW4869; $n = 6$ for K252a + GW4869. Data are presented as mean ± SEM. p-values were calculated using unpaired t test. ***$p < 0.001$. ***B***, Higher dosages of GW4869, 10 μg ($n = 4$) and 1000 μg ($n = 4$), were injected intradermally on the dorsum of the hindpaw of adult rats following by NGF injection as in ***A***. The same method was applied to measure mechanical hyperalgesia used as in ***A***. Data are presented as mean ± SEM. p-values were calculated using unpaired t test. *$p < 0.05$, **$p < 0.01$.

Discussion

Studies on a naturally occurring mutation in HSAN V patients, NGF^{R100W}, have provided new insights into NGF-mediation of nociceptor function. Using molecular, cellular, and biochemical techniques, we demonstrated that NGF^{R100W} retained the ability to bind and signal through TrkA receptors and robustly support the trophic status of DRGs. In contrast, NGF^{R100W} failed to bind or activate of p75^{NTR}. Our *in vivo* studies have demonstrated that inhibition of TrkA and p75^{NTR} signaling, as well as delivery of antisense reagents targeting these receptors, provided evidence for a role for both receptors in nociceptor regulation. Whereas TrkA activation mediated acute sensitization and priming, p75^{NTR}

signaling appeared to contribute only to priming, thereby augmenting the TrkA response. Remarkably, and in contrast to its ability to induce trophic responses via TrkA, NGF^{R100W} induced nociceptor priming, but differed from wtNGF by not causing acute sensitization. These observations are evidence for distinct TrkA signaling pathways mediating trophic effects and nociceptor function and provide insights into the biology of HSAN V.

Consistent with previous results (Covaceuszach et al., 2010; Capsoni et al., 2011), we confirmed that, whereas NGF^{R100W} binding and activation of TrkA is robust, binding and signaling through p75^{NTR} is essentially absent. Important to its ability to prime nociceptors, NGF^{R100W} was internalized at axonal tips of

DRG sensory neurons and traveled toward the cell body at the speed and with the velocity characteristic of wtNGF. Given properties in common with wtNGF, it is unclear why NGF^{R100W} failed to induce acute sensitization. Possibilities include differences with respect to wtNGF in the structure of the NGF^{R100W}/TrkA complex, the structure of the complex in signaling endosomes, or the downstream partners recruited to these complexes. Furthermore, it is hard to exclude the possibility that TrkA downstream signaling events implicated in pain could be positively affected by crosstalk between p75^{NTR} and TrkA, as suggested by only partial PLC activation by NGF^{R100W}. This suggests the possibility that TrkA downstream events that support trophic functions are retained in NGF^{R100W}, whereas p75^{NTR} downstream and some TrkA downstream events that affect pain threshold are reduced in NGF^{R100W}. In any case, it is apparent that differences must exist for the signaling events that subserve TrkA trophic functions and its acute effects on nociceptor sensitization. Further studies to decipher the basis for these differences will benefit from the ability to compare the signaling properties of NGF^{R100W}/TrkA with wtNGF/TrkA.

Our data help to explain the clinical manifestations of NGF^{R100W} mutation. Studies of affected families point to considerable interpatient variability. Both homozygous and heterozygous individuals demonstrate orthopedic manifestations, the most frequent of which is multiple painless fractures, typically in the legs and feet (Einarsdottir et al., 2004; Minde et al., 2004; Larsson et al., 2009; Capsoni, 2014). Homozygous patients show decreased pain sensation, mainly at the forearms and legs. In contrast, they respond to truncal pain and register visceral pain. Distal testing of temperature thresholds showed increases in some homozygous and heterozygous patients. Sensitivity to soft touch, joint position, vibration sensation, and visceral pain are normal. Sural nerve biopsy reveals loss of C and A delta sensory fibers in both homozygotes and heterozygotes (Minde et al., 2004, 2009; Minde, 2006; Sagafos et al., 2016). All patients show reduced sensory innervation of skin and reduced sympathetic innervation of sweat glands, with more marked changes in homozygotes (Axelsson et al., 2009). Although a decrease in pain is consistent with the inability of NGF^{R100W} to induce acute nociceptor sensitization, the clinical picture is that of a length-dependent sensory and sympathetic neuropathy. Indeed, this explains best the painless fractures and decreased pain sensation in distal lower limbs, increased distal thresholds for cold and heat perception, loss of sensory and sympathetic innervation of skin, and preservation of truncal pain. In view of the preservation of TrkA signaling by NGF^{R100W}, the question arises as to how NGF^{R100W} causes the syndrome. The likely cause is failure to secrete the protein in sufficient amounts to support the distal axons of sensory and sympathetic neurons. NGF is critical for the survival and maintenance of sympathetic and sensory neurons (Ibáñez et al., 1992; Kew et al., 1996; Casaccia-Bonnefil et al., 1998; Sofroniew et al., 2001; Chao, 2003). Decreased secretion of NGF^{R100W}, as demonstrated in other studies, must be confirmed with human cells expressing the mutant protein (Larsson et al., 2009; Covaceuszach et al., 2010).

A causal link between NGF deficiency in innervated targets and neuronal dysfunction and degeneration was established during the earliest studies on NGF (Levi-Montalcini and Hamburger, 1951; Cohen and Levi-Montalcini, 1956; Levi-Montalcini and Angeletti, 1961, 1963; Levi-Montalcini, 1964) and has been shown in studies of the developing and mature nervous system. Attempts to increase NGF availability in the hopes of reducing neurodegeneration (Wilcox and Johnson,

1988; Apfel et al., 1994; Apfel and Kessler, 1995, 1996; Apfel, 1999a,b, 2002; McArthur et al., 2000; Cattaneo et al., 2008) have failed in part due to NGF dose-limiting pain (Eriksdotter Jönhagen et al., 1998; Apfel, 2000, 2002). That NGF^{R100W} maintains trophic functions without acutely sensitizing nociceptors has suggested that this isoform of NGF might be used to provide trophic support without causing pain (Capsoni et al., 2011, 2014). That NGF^{R100W} maintains the ability of wt NGF to prime nociceptors raises the caution that NGF^{R100W} treatment may not fully avoid the pain induced by wild-type NGF.

Increasing evidence supports that both TrkA- and p75^{NTR}-mediated signaling pathways are intimately involved in NGF-induced hyperalgesia (Nicol and Vasko, 2007). Extensive human genetic studies strongly support an essential role played by TrkA in pain sensation; mutations in TrkA that result in loss or reduced TrkA activity are associated with congenital insensitivity to pain with anhidrosis (Indo, 2001, 2002). In addition, inhibiting of TrkA-mediated signaling pathways such as Erk1/2 (Aley et al., 2001; Dai et al., 2002), PI3K/Akt (Zhuang et al., 2004), and PLC-γ (Chuang et al., 2001) has been shown to block NGF-induced sensitization both *in vivo* and *in vitro*. Consistent with these findings, we used specific antisense oligos to attenuate expression of TrkA *via* intrathecal administration or administered K252a to inhibit TrkA activation via acute intraplantar injection. Both approaches affirmed that TrkA is required for both the acute and chronic phase of sensitization induced by NGF.

Unlike TrkA, a role for p75^{NTR} in NGF-induced hyperalgesia has been implicated largely by indirect evidence: (1) intrathecal administration of anti-p75^{NTR} into animals reduced temperature hyperalgesia and mechanical allodynia after nerve injury (Obata et al., 2006); (2) direct application of an p75^{NTR} antibody to a crushed sciatic nerve suppressed mechanical allodynia (Fukui et al., 2010); (3) pretreatment with a p75^{NTR} antibody prevented the increase in the number of action potentials induced by NGF (Zhang and Nicol, 2004); (4) intraplantar injection of proNGF, which selectively activates p75^{NTR}, and not TrkA, induced hyperalgesia (Khodorova et al., 2013); and (5) NGF-induced sensitization was attenuated by inhibiting p75^{NTR}-mediated activation of the sphingomyelin-ceramide-sphingosine 1 phosphate and the c-JUN kinase pathway (Zhang et al., 2002, 2006; Doya et al., 2005; Obata et al., 2006; Khodorova et al., 2013).

We confirmed a role for p75^{NTR} in acute sensitization of mechanical nociceptors using GW4869. However, a discrepancy exists between priming studies of NGF^{R100W} in Figure 1 versus GW4869 in Figure 8A; NGF^{R100W} induced a priming effect comparably to wtNGF, whereas GW4869 failed to block the priming effect by wtNGF even at higher dosages (Fig. 8B). This could be explained by the lack of specificity of GW4869 in blocking neutral SMase2 (Canals et al., 2011). The GW4869 specificity issue was also raised in studies showing that GW4869 not only blocks SMase, but also PLC and PP2A (Luberto et al., 2002). Moreover, previous studies have shown that GW4869 abrogated NGF-mediated TrkA trophic effects on cell viability (Candalija et al., 2014), suggesting that block of nSMase2 could lead to partial block of TrkA-mediated trophic signaling such as pAkt (Gills et al., 2012). Therefore, the GW4869 effect could not be the same as loss of p75 downstream by NGF^{R100W}. This may explain the inconsistency in our data showing incapability of priming by GW4869 versus the priming capability by NGF^{R100W}.

We are also aware of the specificity issues associated with the use of K252a inhibitor to block TrkA. K252a selectivity has

been studied in many studies. For example, K252A is known to inhibit PKCs, which is also implicated in p75 downstream (Mizuno et al., 1993). Not only inhibiting TrkA, at certain range of concentration, K252a was also found to act as partial inhibitor of the PDGF receptor (Nye et al., 1992).

Given all the caveats associated with the use of inhibitors in our studies, our data suggest that p75^NTR plays an important role in NGF-induced pain function (Zhang and Nicol, 2004; Watanabe et al., 2008; Iwakura et al., 2010; Khodorova et al., 2013, 2017). When p75 inhibition is combined with TrkA suppression, both acute response and priming resulted in a greater reduction in NGF-mediated hyperalgesic priming. These results support a role for p75^NTR that is most evident in its ability to synergize with TrkA to mediate NGF-induced hyperalgesic priming. Therefore, the nociceptive functions of NGF include contributions from both TrkA and/or p75^NTR.

To avoid the pitfalls associated with the use of inhibitors for TrkA- or p75NTR-signaling pathways, future studies using novel genetic models are needed to further study the contributions of TrkA and p75^NTR to NGF-induced sensitization. For example, TrkA activity can be specifically inhibited by injection of nanomolar concentrations of derivatives of the general kinase inhibitor PP1 (1NMPP1 or 1NaPP1) to block NGF signaling in TrkA (F592A)-knock-in mice (Chen et al., 2005). Ablation of p75^NTR can be induced in conditional p75^NTR knock-out mice (Bogenmann et al., 2011; Wehner et al., 2016). Use of these models is expected to further clarify the contributions of TrkA and p75^NTR and suggest the possibility that their signaling pathways interact to effect NGF-induced sensitization.

References

Aley KO, Levine JD (1999) Role of protein kinase A in the maintenance of inflammatory pain. J Neurosci 19:2181–2186. Medline

Aley KO, Martin A, McMahon T, Mok J, Levine JD, Messing RO (2001) Nociceptor sensitization by extracellular signal-regulated kinases. J Neurosci 21:6933–6939. Medline

Aloe L, Rocco ML, Bianchi P, Manni L (2012) Nerve growth factor: from the early discoveries to the potential clinical use. J Transl Med 10:239. CrossRef Medline

Alvarez P, Levine JD (2014) Screening the role of pronociceptive molecules in a rodent model of endometriosis pain. J Pain 15:726–733. CrossRef Medline

Anand P, Terenghi G, Warner G, Kopelman P, Williams-Chestnut RE, Sinicropi DV (1996) The role of endogenous nerve growth factor in human diabetic neuropathy. Nat Med 2:703–707. CrossRef Medline

Andreev NYu, Dimitrieva N, Koltzenburg M, McMahon SB (1995) Peripheral administration of nerve growth factor in the adult rat produces a thermal hyperalgesia that requires the presence of sympathetic postganglionic neurones. Pain 63:109–115. CrossRef Medline

Apfel SC (1999a) Neurotrophic factors in the therapy of diabetic neuropathy. Am J Med 107:34S–42S. Medline

Apfel SC (1999b) Neurotrophic factors and diabetic peripheral neuropathy. Eur Neurol 41:27–34. CrossRef Medline

Apfel SC (2000) Neurotrophic factors and pain. Clin J Pain 16:S7–S11. CrossRef Medline

Apfel SC (2002) Nerve growth factor for the treatment of diabetic neuropathy: what went wrong, what went right, and what does the future hold? Int Rev Neurobiol 50:393–413. CrossRef Medline

Apfel SC, Kessler JA (1995) Neurotrophic factors in the therapy of peripheral neuropathy. Baillieres Clin Neurol 4:593–606. Medline

Apfel SC, Kessler JA (1996) Neurotrophic factors in the treatment of peripheral neuropathy. Ciba Found Symp 196:98–108; discussion 108–112. Medline

Apfel SC, Arezzo JC, Brownlee M, Federoff H, Kessler JA (1994) Nerve growth factor administration protects against experimental diabetic sensory neuropathy. Brain Res 634:7–12. CrossRef Medline

Apfel SC, Kessler JA, Adornato BT, Litchy WJ, Sanders C, Rask CA (1998) Recombinant human nerve growth factor in the treatment of diabetic polyneuropathy. NGF study group. Neurology 51:695–702. CrossRef Medline

Ashraf S, Bouhana KS, Pheneger J, Andrews SW, Walsh DA (2016) Selective inhibition of tropomyosin-receptor-kinase A (TrkA) reduces pain and joint damage in two rat models of inflammatory arthritis. Arthritis Res Ther 18:97. CrossRef Medline

Axelsson HE, Minde JK, Sonesson A, Toolanen G, Högestätt ED, Zygmunt PM (2009) Transient receptor potential vanilloid 1, vanilloid 2 and melastatin 8 immunoreactive nerve fibers in human skin from individuals with and without norrbottnian congenital insensitivity to pain. Neuroscience 162:1322–1332. CrossRef Medline

Barrett GL, Naim T, Trieu J, Huang M (2016) In vivo knockdown of basal forebrain p75 neurotrophin receptor stimulates choline acetyltransferase activity in the mature hippocampus. J Neurosci Res 94:389–400. CrossRef Medline

Basáñez G, Ruiz-Argüello MB, Alonso A, Goñi FM, Karlsson G, Edwards K (1997) Morphological changes induced by phospholipase C and by sphingomyelinase on large unilamellar vesicles: a cryo-transmission electron microscopy study of liposome fusion. Biophys J 72:2630–2637. CrossRef Medline

Beckett D, Kovaleva E, Schatz PJ (1999) A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. Protein Sci 8:921–929. CrossRef Medline

Bergmann I, Reiter R, Toyka KV, Koltzenburg M (1998) Nerve growth factor evokes hyperalgesia in mice lacking the low-affinity neurotrophin receptor p75. Neurosci Lett 255:87–90. CrossRef Medline

Blesch A, Tuszynski M (1995) Ex vivo gene therapy for Alzheimer's disease and spinal cord injury. Clin Neurosci 3:268–274. Medline

Bogenmann E, Thomas PS, Li Q, Kim J, Yang LT, Pierchala B, Kaartinen V (2011) Generation of mice with a conditional allele for the p75 (NTR) neurotrophin receptor gene. Genesis 49:862–869. CrossRef Medline

Bothwell M (1995) Functional interactions of neurotrophins and neurotrophin receptors. Annu Rev Neurosci 18:223–253. CrossRef Medline

Canals D, Perry DM, Jenkins RW, Hannun YA (2011) Drug targeting of sphingolipid metabolism: sphingomyelinases and ceramidases. Br J Pharmacol 163:694–712. CrossRef Medline

Candalija A, Cubí R, Ortega A, Aguilera J, Gil C (2014) Trk receptors need neutral sphingomyelinase activity to promote cell viability. FEBS Lett 588:167–174. CrossRef Medline

Capsoni S (2014) From genes to pain: nerve growth factor and hereditary sensory and autonomic neuropathy type V. Eur J Neurosci 39:392–400. CrossRef Medline

Capsoni S, Covaceuszach S, Marinelli S, Ceci M, Bernardo A, Minghetti L, Ugolini G, Pavone F, Cattaneo A (2011) Taking pain out of NGF: a "painless" NGF mutant, linked to hereditary sensory autonomic neuropathy type V, with full neurotrophic activity. PLoS One 6:e17321. CrossRef Medline

Carvalho OP, Thornton GK, Hertecant J, Houlden H, Nicholas AK, Cox JJ, Rielly M, Al-Gazali L, Woods CG (2011) A novel NGF mutation clarifies the molecular mechanism and extends the phenotypic spectrum of the HSAN5 neuropathy. J Med Genet 48:131–135. CrossRef Medline

Casaccia-Bonnefil P, Kong H, Chao MV (1998) Neurotrophins: the biological paradox of survival factors eliciting apoptosis. Cell Death Differ 5:357–364. CrossRef Medline

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306–313. CrossRef Medline

Cattaneo A, Capsoni S, Paoletti F (2008) Towards non invasive nerve growth factor therapies for Alzheimer's disease. J Alzheimers Dis 15:255–283. CrossRef Medline

Chao MV (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 4:299–309. CrossRef Medline

Chao MV, Hempstead BL (1995) p75 and trk: a two-receptor system. Trends Neurosci 18:321–326. CrossRef Medline

Chen CC, Zimmer A, Sun WH, Hall J, Brownstein MJ, Zimmer A (2002) A role for ASIC3 in the modulation of high-intensity pain stimuli. Proc Natl Acad Sci U S A 99:8992–8997. CrossRef Medline

Chen X, Ye H, Kuruvilla R, Ramanan N, Scangos KW, Zhang C, Johnson NM,

England PM, Shokat KM, Ginty DD (2005) A chemical-genetic approach to studying neurotrophin signaling. Neuron 46:13–21. CrossRef Medline

Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns (4,5)P2-mediated inhibition. Nature 411:957–962. CrossRef Medline

Cohen S, Levi-Montalcini R (1956) A nerve growth-stimulating factor isolated from snake venom. Proc Natl Acad Sci U S A 42:571–574. CrossRef Medline

Conover JC, Yancopoulos GD (1997) Neurotrophin regulation of the developing nervous system: analyses of knockout mice. Rev Neurosci 8:13–27. Medline

Covaceuszach S, Capsoni S, Marinelli S, Pavone F, Ceci M, Ugolini G, Vignone D, Amato G, Paoletti F, Lamba D, Cattaneo A (2010) In vitro receptor binding properties of a "painless" NGF mutein, linked to hereditary sensory autonomic neuropathy type V. Biochem Biophys Res Commun 391:824–829. CrossRef Medline

Cremesti AE, Goni FM, Kolesnick R (2002) Role of sphingomyelinase and ceramide in modulating rafts: do biophysical properties determine biologic outcome? FEBS Lett 531:47–53. CrossRef Medline

Cuello AC, Bruno MA, Allard S, Leon W, Iulita MF (2010) Cholinergic involvement in Alzheimer's disease: a link with NGF maturation and degradation. J Mol Neurosci 40:230–235. CrossRef Medline

Cui B, Wu C, Chen L, Ramirez A, Bearer EL, Li WP, Mobley WC, Chu S (2007) One at a time, live tracking of NGF axonal transport using quantum dots. Proc Natl Acad Sci U S A 104:13666–13671. CrossRef Medline

Dai Y, Iwata K, Fukuoka T, Kondo E, Tokunaga A, Yamanaka H, Tachibana T, Liu Y, Noguchi K (2002) Phosphorylation of extracellular signal-regulated kinase in primary afferent neurons by noxious stimuli and its involvement in peripheral sensitization. J Neurosci 22:7737–7745. Medline

Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 405:183–187. CrossRef Medline

Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265:1596–1599. CrossRef Medline

Dobrowsky RT, Jenkins GM, Hannun YA (1995) Neurotrophins induce sphingomyelin hydrolysis: modulation by co-expression of p75NTR with trk receptors. J Biol Chem 270:22135–22142. CrossRef Medline

Doya H, Ohtori S, Fujitani M, Saito T, Hata K, Ino H, Takahashi K, Moriya H, Yamashita T (2005) c-jun N-terminal kinase activation in dorsal root ganglion contributes to pain hypersensitivity. Biochem Biophys Res Commun 335:132–138. CrossRef Medline

Einarsdottir E, Carlsson A, Minde J, Toolanen G, Svensson O, Solders G, Holmgren G, Holmberg D, Holmberg M (2004) A mutation in the nerve growth factor beta gene (NGFB) causes loss of pain perception. Hum Mol Genet 13:799–805. CrossRef Medline

Elias KA, Cronin MJ, Stewart TA, Carlsen RC (1998) Peripheral neuropathy in transgenic diabetic mice: restoration of C-fiber function with human recombinant nerve growth factor. Diabetes 47:1637–1642. CrossRef Medline

Eriksdotter Jönhagen M, Nordberg A, Amberla K, Bäckman L, Ebendal T, Meyerson B, Olson L, Seiger, Shigeta M, Theodorsson E, Viitanen M, Winblad B, Wahlund LO (1998) Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease. Dement Geriatr Cogn Disord 9:246–257. CrossRef Medline

Fang X, Djouhri L, McMullan S, Berry C, Okuse K, Waxman SG, Lawson SN (2005) trkA is expressed in nociceptive neurons and influences electrophysiological properties via Nav1.8 expression in rapidly conducting nociceptors. J Neurosci 25:4868–4878. CrossRef Medline

Ferrari LF, Bogen O, Levine JD (2010) Nociceptor subpopulations involved in hyperalgesic priming. Neuroscience 165:896–901. CrossRef Medline

Ferrari LF, Levine E, Levine JD (2013) Role of a novel nociceptor autocrine mechanism in chronic pain. Eur J Neurosci 37:1705–1713. CrossRef Medline

Ferrari LF, Araldi D, Levine JD (2015a) Distinct terminal and cell body mechanisms in the nociceptor mediate hyperalgesic priming. J Neurosci 35:6107–6116. CrossRef Medline

Ferrari LF, Bogen O, Reichling DB, Levine JD (2015b) Accounting for the delay in the transition from acute to chronic pain: axonal and nuclear mechanisms. J Neurosci 35:495–507. CrossRef Medline

Frade JM, Barde YA (1998) Nerve growth factor: two receptors, multiple functions. Bioessays 20:137–145. CrossRef Medline

Fukui Y, Ohtori S, Yamashita M, Yamauchi K, Inoue G, Suzuki M, Orita S, Eguchi Y, Ochiai N, Kishida S, Takaso M, Wakai K, Hayashi Y, Aoki Y, Takahashi K (2010) Low affinity NGF receptor (p75 neurotrophin receptor) inhibitory antibody reduces pain behavior and CGRP expression in DRG in the mouse sciatic nerve crush model. J Orthop Res 28:279–283. CrossRef Medline

Gehler S, Gallo G, Veien E, Letourneau PC (2004) p75 neurotrophin receptor signaling regulates growth cone filopodial dynamics through modulating RhoA activity. J Neurosci 24:4363–4372. CrossRef Medline

Gills JJ, Zhang C, Abu-Asab MS, Castillo SS, Marceau C, LoPiccolo J, Kozikowski AP, Tsokos M, Goldkorn T, Dennis PA (2012) Ceramide mediates nanovesicle shedding and cell death in response to phosphatidylinositol ether lipid analogs and perifosine. Cell Death Dis 3:e340. CrossRef Medline

Goss JR, Goins WF, Lacomis D, Mata M, Glorioso JC, Fink DJ (2002) Herpes simplex-mediated gene transfer of nerve growth factor protects against peripheral neuropathy in streptozotocin-induced diabetes in the mouse. Diabetes 51:2227–2232. CrossRef Medline

Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32:77–88. CrossRef Medline

Harrington AW, Kim JY, Yoon SO (2002) Activation of rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis. J Neurosci 22:156–166. Medline

Hasegawa Y, Yamagishi S, Fujitani M, Yamashita T (2004) p75 neurotrophin receptor signaling in the nervous system. Biotechnol Annu Rev 10:123–149. CrossRef Medline

Head BP, Patel HH, Niesman IR, Drummond JC, Roth DM, Patel PM (2009) Inhibition of p75 neurotrophin receptor attenuates isoflurane-mediated neuronal apoptosis in the neonatal central nervous system. Anesthesiology 110:813–825. CrossRef Medline

Hefti F (1994) Development of effective therapy for Alzheimer's disease based on neurotrophic factors. Neurobiol Aging 15:S193–S194. Medline

Hellweg R, Hartung HD (1990) Endogenous levels of nerve growth factor (NGF) are altered in experimental diabetes mellitus: a possible role for NGF in the pathogenesis of diabetic neuropathy. J Neurosci Res 26:258–267. CrossRef Medline

Hellwig N, Plant TD, Janson W, Schäfer M, Schultz G, Schaefer M (2004) TRPV1 acts as proton channel to induce acidification in nociceptive neurons. J Biol Chem 279:34553–34561. CrossRef Medline

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV (1991) High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature 350:678–683. CrossRef Medline

Howarth M, Takao K, Hayashi Y, Ting AY (2005) Targeting quantum dots to surface proteins in living cells with biotin ligase. Proc Natl Acad Sci U S A 102:7583–7588. CrossRef Medline

Huang CS, Zhou J, Feng AK, Lynch CC, Klumperman J, DeArmond SJ, Mobley WC (1999) Nerve growth factor signaling in caveolae-like domains at the plasma membrane. J Biol Chem 274:36707–36714. CrossRef Medline

Huang EJ, Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 24:677–736. CrossRef Medline

Hughes AL, Messineo-Jones D, Lad SP, Neet KE (2001) Distinction between differentiation, cell cycle, and apoptosis signals in PC12 cells by the nerve growth factor mutant delta9/13, which is selective for the p75 neurotrophin receptor. J Neurosci Res 63:10–19. CrossRef Medline

Ibáñez CF, Ebendal T, Barbany G, Murray-Rust J, Blundell TL, Persson H (1992) Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the trk gene product. Cell 69:329–341. CrossRef Medline

Indo Y (2001) Molecular basis of congenital insensitivity to pain with anhidrosis (CIPA): mutations and polymorphisms in TRKA (NTRK1) gene encoding the receptor tyrosine kinase for nerve growth factor. Hum Mutat 18:462–471. CrossRef Medline

Indo Y (2002) Genetics of congenital insensitivity to pain with anhidrosis (CIPA) or hereditary sensory and autonomic neuropathy type IV. clinical,

biological and molecular aspects of mutations in TRKA (NTRK1) gene encoding the receptor tyrosine kinase for nerve growth factor. Clin Auton Res 12:I20–I32. CrossRef Medline

Iwakura N, Ohtori S, Orita S, Yamashita M, Takahashi K, Kuniyoshi K (2010) Role of low-affinity nerve growth factor receptor inhibitory antibody in reducing pain behavior and calcitonin gene-related peptide expression in a rat model of wrist joint inflammatory pain. J Hand Surg Am 35:267–273. CrossRef Medline

Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. Nature 413:203–210. CrossRef Medline

Kanda T (2009) Peripheral neuropathy and blood-nerve barrier [Article in Japanese]. Rinsho Shinkeigaku 49:959–962. CrossRef Medline

Kaplan DR, Miller FD (1997) Signal transduction by the neurotrophin receptors. Curr Opin Cell Biol 9:213–221. CrossRef Medline

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 252:554–558. CrossRef Medline

Kew JN, Smith DW, Sofroniew MV (1996) Nerve growth factor withdrawal induces the apoptotic death of developing septal cholinergic neurons in vitro: protection by cyclic AMP analogue and high potassium. Neuroscience 70:329–339. CrossRef Medline

Khodorova A, Nicol GD, Strichartz G (2013) The p75NTR signaling cascade mediates mechanical hyperalgesia induced by nerve growth factor injected into the rat hindpaw. Neuroscience 254:312–323. CrossRef Medline

Khodorova A, Nicol GD, Strichartz G (2017) The TrkA receptor mediates experimental thermal hyperalgesia produced by nerve growth factor: modulation by the p75 neurotrophin receptor. Neuroscience 340:384–397. CrossRef Medline

Knusel B, Gao H (1996) Neurotrophins and Alzheimer's disease: beyond the cholinergic neurons. Life Sci 58:2019–2027. CrossRef Medline

Koliatsos VE (1996) Biological therapies for Alzheimer's disease: focus on trophic factors. Crit Rev Neurobiol 10:205–238. CrossRef Medline

Koplas PA, Rosenberg RL, Oxford GS (1997) The role of calcium in the desensitization of capsaicin responses in rat dorsal root ganglion neurons. J Neurosci 17:3525–3537. Medline

Larsson E, Kuma R, Norberg A, Minde J, Holmberg M (2009) Nerve growth factor R221W responsible for insensitivity to pain is defectively processed and accumulates as proNGF. Neurobiol Dis 33:221–228. CrossRef Medline

Lehmann M, Fournier A, Selles-Navarro I, Dergham P, Sebok A, Leclerc N, Tigyi G, McKerracher L (1999) Inactivation of rho signaling pathway promotes CNS axon regeneration. J Neurosci 19:7537–7547. Medline

Lein B (1995) Potential therapy for painful neuropathy. PI Perspect 16:11. Medline

Levi-Montalcini R (1964) Growth control of nerve cells by a protein factor and its antiserum: discovery of this factor may provide new leads to understanding of some neurogenetic processes. Science 143:105–110. CrossRef Medline

Levi-Montalcini R, Angeletti PU (1961) Growth control of the sympathetic system by a specific protein factor. Q Rev Biol 36:99–108. CrossRef Medline

Levi-Montalcini R, Angeletti PU (1963) Essential role of the nerve growth factor in the survival and maintenance of dissociated sensory and sympathetic embryonic nerve cells in vitro. Dev Biol 6:653–659. Medline

Levi-Montalcini R, Hamburger V (1951) Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J Exp Zool 116:321–361. CrossRef Medline

Lewin GR, Mendell LM (1993) Nerve growth factor and nociception. Trends Neurosci 16:353–359. CrossRef Medline

Lewin GR, Ritter AM, Mendell LM (1993) Nerve growth factor-induced hyperalgesia in the neonatal and adult rat. J Neurosci 13:2136–2148. Medline

Li X, Jope RS (1995) Selective inhibition of the expression of signal transduction proteins by lithium in nerve growth factor-differentiated PC12 cells. J Neurochem 65:2500–2508. Medline

Loeb DM, Greene LA (1993) Transfection with trk restores "slow" NGF binding, efficient NGF uptake, and multiple NGF responses to NGF-nonresponsive PC12 cell mutants. J Neurosci 13:2919–2929. Medline

Luberto C, Hassler DF, Signorelli P, Okamoto Y, Sawai H, Boros E, Hazen-Martin DJ, Obeid LM, Hannun YA, Smith GK (2002) Inhibition of tumor necrosis factor-induced cell death in MCF7 by a novel inhibitor of neutral sphingomyelinase. J Biol Chem 277:41128–41139. CrossRef Medline

Mahapatra S, Mehta H, Woo SB, Neet KE (2009) Identification of critical residues within the conserved and specificity patches of nerve growth factor leading to survival or differentiation. J Biol Chem 284:33600–33613. CrossRef Medline

Malerba F, Paoletti F, Bruni Ercole B, Materazzi S, Nassini R, Coppi E, Patacchini R, Capsoni S, Lamba D, Cattaneo A (2015) Functional characterization of human ProNGF and NGF mutants: identification of NGF P61SR100E as a "painless" lead investigational candidate for therapeutic applications. PLoS One 10:e0136425. CrossRef Medline

Malik-Hall M, Dina OA, Levine JD (2005) Primary afferent nociceptor mechanisms mediating NGF-induced mechanical hyperalgesia. Eur J Neurosci 21:3387–3394. CrossRef Medline

Malmberg AB, Yaksh TL (1993) Spinal nitric oxide synthesis inhibition blocks NMDA-induced thermal hyperalgesia and produces antinociception in the formalin test in rats. Pain 54:291–300. CrossRef Medline

Mamet J, Lazdunski M, Voilley N (2003) How nerve growth factor drives physiological and inflammatory expressions of acid-sensing ion channel 3 in sensory neurons. J Biol Chem 278:48907–48913. CrossRef Medline

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

McArthur JC, Yiannoutsos C, Simpson DM, Adornato BT, Singer EJ, Hollander H, Marra C, Rubin M, Cohen BA, Tucker T, Navia BA, Schifitto G, Katzenstein D, Rask C, Zaborski L, Smith ME, Shriver S, Millar L, Clifford DB, Karalnik IJ (2000) A phase II trial of nerve growth factor for sensory neuropathy associated with HIV infection. AIDS clinical trials group team 291. Neurology 54:1080–1088. CrossRef Medline

McCleskey EW, Gold MS (1999) Ion channels of nociception. Annu Rev Physiol 61:835–856. CrossRef Medline

Minde JK (2006) Norrbottnian congenital insensitivity to pain. Acta Orthop Suppl 77:2–32. Medline

Minde J, Toolanen G, Andersson T, Nennesmo I, Remahl IN, Svensson O, Solders G (2004) Familial insensitivity to pain (HSAN V) and a mutation in the NGFB gene: a neurophysiological and pathological study. Muscle Nerve 30:752–760. CrossRef Medline

Minde J, Andersson T, Fulford M, Aguirre M, Nennesmo I, Remahl IN, Svensson O, Holmberg M, Toolanen G, Solders G (2009) A novel NGFB point mutation: a phenotype study of heterozygous patients. J Neurol Neurosurg Psychiatry 80:188–195. CrossRef Medline

Mischel PS, Smith SG, Vining ER, Valletta JS, Mobley WC, Reichardt LF (2001) The extracellular domain of p75NTR is necessary to inhibit neurotrophin-3 signaling through TrkA. J Biol Chem 276:11294–11301. CrossRef Medline

Mizuno K, Saido TC, Ohno S, Tamaoki T, Suzuki K (1993) Staurosporine-related compounds, K252a and UCN-01, inhibit both cPKC and nPKC. FEBS Lett 330:114–116. CrossRef Medline

Mufson EJ, Counts SE, Perez SE, Ginsberg SD (2008) Cholinergic system during the progression of Alzheimer's disease: therapeutic implications. Expert Rev Neurother 8:1703–1718. CrossRef Medline

Murakawa Y, Zhang W, Pierson CR, Brismar T, Ostenson CG, Efendic S, Sima AA (2002) Impaired glucose tolerance and insulinopenia in the GK-rat causes peripheral neuropathy. Diabetes Metab Res Rev 18:473–483. CrossRef Medline

Nicol GD, Vasko MR (2007) Unraveling the story of NGF-mediated sensitization of nociceptive sensory neurons: ON or OFF the trks? Mol Interv 7:26–41. CrossRef Medline

Nye SH, Squinto SP, Glass DJ, Stitt TN, Hantzopoulos P, Macchi MJ, Lindsay NS, Ip NY, Yancopoulos GD (1992) K-252a and staurosporine selectively block autophosphorylation of neurotrophin receptors and neurotrophin-mediated responses. Mol Biol Cell 3:677–686. CrossRef Medline

Nykjaer A, Willnow TE, Petersen CM (2005) p75NTR: live or let die. Curr Opin Neurobiol 15:49–57. CrossRef Medline

Obata K, Katsura H, Sakurai J, Kobayashi K, Yamanaka H, Dai Y, Fukuoka T, Noguchi K (2006) Suppression of the p75 neurotrophin receptor in uninjured sensory neurons reduces neuropathic pain after nerve injury. J Neurosci 26:11974–11986. CrossRef Medline

Olson L (1993) NGF and the treatment of Alzheimer's disease. Exp Neurol 124:5–15. CrossRef Medline

Pradat PF (2003) Treatment of peripheral neuropathies with neutrotrophic

factors: animal models and clinical trials [Article in French]. Rev Neurol (Paris) 159:147–161. Medline

Qiu CY, Liu YQ, Qiu F, Wu J, Zhou QY, Hu WP (2012) Prokineticin 2 potentiates acid-sensing ion channel activity in rat dorsal root ganglion neurons. J Neuroinflammation 9:108. CrossRef Medline

Quasthoff S, Hartung HP (2001) Nerve growth factor (NGF) in treatment of diabetic polyneuropathy. One hope less? [Article in German] Nervenarzt 72:456–459. CrossRef Medline

Rafii MS, Baumann TL, Bakay RA, Ostrove JM, Siffert J, Fleisher AS, Herzog CD, Barba D, Pay M, Salmon DP, Chu Y, Kordower JH, Bishop K, Keator D, Potkin S, Bartus RT (2014) A phase1 study of stereotactic gene delivery of AAV2-NGF for Alzheimer's disease. Alzheimers Dement 10:571–581. CrossRef Medline

Randall LO, Selitto JJ (1957) A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther 111:409–419. Medline

Rask CA (1999) Biological actions of nerve growth factor in the peripheral nervous system. Eur Neurol 41:14–19. CrossRef Medline

Reichling DB, Levine JD (2009) Critical role of nociceptor plasticity in chronic pain. Trends Neurosci 32:611–618. CrossRef Medline

Roux PP, Barker PA (2002) Neurotrophin signaling through the p75 neurotrophin receptor. Prog Neurobiol 67:203–233. CrossRef Medline

Ruiz-Argüello MB, Basáñez G, Goñi FM, Alonso A (1996) Different effects of enzyme-generated ceramides and diacylglycerols in phospholipid membrane fusion and leakage. J Biol Chem 271:26616–26621. CrossRef Medline

Ruiz-Argüello MB, Goñi FM, Alonso A (1998) Vesicle membrane fusion induced by the concerted activities of sphingomyelinase and phospholipase C. J Biol Chem 273:22977–22982. CrossRef Medline

Sagafos D, Kleggetveit IP, Helås T, Schmidt R, Minde J, Namer B, Schmelz M, Jørum E (2016) Single-fiber recordings of nociceptive fibers in patients with HSAN type V with congenital insensitivity to pain. Clin J Pain 32:636–642. CrossRef Medline

Salehi AH, Roux PP, Kubu CJ, Zeindler C, Bhakar A, Tannis LL, Verdi JM, Barker PA (2000) NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis. Neuron 27:279–288. CrossRef Medline

Schatz PJ (1993) Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in Escherichia coli. Biotechnology (N Y) 11:1138–1143. CrossRef Medline

Schifitto G, Yiannoutsos C, Simpson DM, Adornato BT, Singer EJ, Hollander H, Marra CM, Rubin M, Cohen BA, Tucker T, Koralnik IJ, Katzenstein D, Haidich B, Smith ME, Shriver S, Millar L, Clifford DB, McArthur JC; AIDS Clinical Trials Group Team 291 (2001) Long-term treatment with recombinant nerve growth factor for HIV-associated sensory neuropathy. Neurology 57:1313–1316. CrossRef Medline

Schindowski K, Belarbi K, Buée L (2008) Neurotrophic factors in Alzheimer's disease: role of axonal transport. Genes Brain Behav 7:43–56. CrossRef Medline

Schulte-Herbrüggen O, Jockers-Scherübl MC, Hellweg R (2008) Neurotrophins: from pathophysiology to treatment in Alzheimer's disease. Curr Alzheimer Res 5:38–44. CrossRef Medline

Scott SA, Crutcher KA (1994) Nerve growth factor and Alzheimer's disease. Rev Neurosci 5:179–211. Medline

Shu X, Mendell LM (1999a) Nerve growth factor acutely sensitizes the response of adult rat sensory neurons to capsaicin. Neurosci Lett 274:159–162. CrossRef Medline

Shu XQ, Mendell LM (1999b) Neurotrophins and hyperalgesia. Proc Natl Acad Sci U S A 96:7693–7696. CrossRef Medline

Sofroniew MV, Howe CL, Mobley WC (2001) Nerve growth factor signaling, neuroprotection, and neural repair. Annu Rev Neurosci 24:1217–1281. CrossRef Medline

Summer GJ, Puntillo KA, Miaskowski C, Dina OA, Green PG, Levine JD (2006) TrkA and PKC-epsilon in thermal burn-induced mechanical hyperalgesia in the rat. J Pain 7:884–891. CrossRef Medline

Sung K, Maloney MT, Yang J, Wu C (2011) A novel method for producing mono-biotinylated, biologically active neurotrophic factors: an essential reagent for single molecule study of axonal transport. J Neurosci Methods 200:121–128. CrossRef Medline

Svendsen CN, Kew JN, Staley K, Sofroniew MV (1994) Death of developing

septal cholinergic neurons following NGF withdrawal in vitro: protection by protein synthesis inhibition. J Neurosci 14:75–87. Medline

Szallasi A, Blumberg PM (1999) Vanilloid (capsaicin) receptors and mechanisms. Pharmacol Rev 51:159–212. Medline

Taiwo YO, Levine JD, Burch RM, Woo JE, Mobley WC (1991) Hyperalgesia induced in the rat by the amino-terminal octapeptide of nerve growth factor. Proc Natl Acad Sci U S A 88:5144–5148. CrossRef Medline

Taylor AM, Rhee SW, Jeon NL (2006) Microfluidic chambers for cell migration and neuroscience research. Methods Mol Biol 321:167–177. CrossRef Medline

Tomlinson DR, Fernyhough P, Diemel LT (1996) Neurotrophins and peripheral neuropathy. Philos Trans R Soc Lond B Biol Sci 351:455–462. CrossRef Medline

Tuszynski MH, Yang JH, Barba D, U HS, Bakay RA, Pay MM, Masliah E, Conner JM, Kobalka P, Roy S, Nagahara AH (2015) Nerve growth factor gene therapy: activation of neuronal responses in alzheimer disease. JAMA Neurol 72:1139–1147. CrossRef Medline

Unger JW, Klitzsch T, Pera S, Reiter R (1998) Nerve growth factor (NGF) and diabetic neuropathy in the rat: morphological investigations of the sural nerve, dorsal root ganglion, and spinal cord. Exp Neurol 153:23–34. CrossRef Medline

Vardouli L, Moustakas A, Stournaras C (2005) LIM-kinase 2 and Cofilin phosphorylation mediate actin cytoskeleton reorganization induced by transforming growth factor-beta. J Biol Chem 280:11448–11457. CrossRef Medline

Walwyn WM, Matsuka Y, Arai D, Bloom DC, Lam H, Tran C, Spigelman I, Maidment NT (2006) HSV-1-mediated NGF delivery delays nociceptive deficits in a genetic model of diabetic neuropathy. Exp Neurol 198:260–270. CrossRef Medline

Watanabe T, Ito T, Inoue G, Ohtori S, Kitajo K, Doya H, Takahashi K, Yamashita T (2008) The p75 receptor is associated with inflammatory thermal hypersensitivity. J Neurosci Res 86:3566–3574. CrossRef Medline

Wehner AB, Milen AM, Albin RL, Pierchala BA (2016) The p75 neurotrophin receptor augments survival signaling in the striatum of presymptomatic Q175 (WT/HD) mice. Neuroscience 324:297–306. CrossRef Medline

Weissmiller AM, Natera-Naranjo O, Reyna SM, Pearn ML, Zhao X, Nguyen P, Cheng S, Goldstein LS, Tanzi RE, Wagner SL, Mobley WC, Wu C (2015) A gamma-secretase inhibitor, but not a gamma-secretase modulator, induced defects in BDNF axonal trafficking and signaling: evidence for a role for APP. PLoS One 10:e0118379. CrossRef Medline

Wilcox CL, Johnson EM Jr (1988) Characterization of nerve growth factor-dependent herpes simplex virus latency in neurons in vitro. J Virol 62:393–399. Medline

Williams BJ, Eriksdotter-Jonhagen M, Granholm AC (2006) Nerve growth factor in treatment and pathogenesis of Alzheimer's disease. Prog Neurobiol 80:114–128. CrossRef Medline

Winkler J, Ramirez GA, Kuhn HG, Peterson DA, Day-Lollini PA, Stewart GR, Tuszynski MH, Gage FH, Thal LJ (1997) Reversible schwann cell hyperplasia and sprouting of sensory and sympathetic neurites after intraventricular administration of nerve growth factor. Ann Neurol 41:82–93. CrossRef Medline

Winter J, Forbes CA, Sternberg J, Lindsay RM (1988) Nerve growth factor (NGF) regulates adult rat cultured dorsal root ganglion neuron responses to the excitotoxin capsaicin. Neuron 1:973–981. CrossRef Medline

Wu C, Lai CF, Mobley WC (2001) Nerve growth factor activates persistent Rap1 signaling in endosomes. J Neurosci 21:5406–5416. Medline

Wu C, Ramirez A, Cui B, Ding J, Delcroix JD, Valletta JS, Liu JJ, Yang Y, Chu S, Mobley WC (2007) A functional dynein-microtubule network is required for NGF signaling through the Rap1/MAPK pathway. Traffic 8:1503–1520. CrossRef Medline

Yamashita T, Tohyama M (2003) The p75 receptor acts as a displacement factor that releases rho from rho-GDI. Nat Neurosci 6:461–467. CrossRef Medline

Yamashita T, Tucker KL, Barde YA (1999) Neurotrophin binding to the p75 receptor modulates rho activity and axonal outgrowth. Neuron 24:585–593. CrossRef Medline

Yen YT, Tu PH, Chen CJ, Lin YW, Hsieh ST, Chen CC (2009) Role of acid-sensing ion channel 3 in sub-acute-phase inflammation. Mol Pain 5:1. CrossRef Medline

Yoon SO, Casaccia-Bonnefil P, Carter B, Chao MV (1998) Competitive

signaling between TrkA and p75 nerve growth factor receptors determines cell survival. J Neurosci 18:3273–3281. Medline

Zhang K, Osakada Y, Vrljic M, Chen L, Mudrakola HV, Cui B (2010) Single-molecule imaging of NGF axonal transport in microfluidic devices. Lab Chip 10:2566–2573. CrossRef Medline

Zhang YH, Nicol GD (2004) NGF-mediated sensitization of the excitability of rat sensory neurons is prevented by a blocking antibody to the p75 neurotrophin receptor. Neurosci Lett 366:187–192. CrossRef Medline

Zhang YH, Vasko MR, Nicol GD (2002) Ceramide, a putative second messenger for nerve growth factor, modulates the TTX-resistant Na (+) current and delayed rectifier K (+) current in rat sensory neurons. J Physiol 544:385–402. CrossRef Medline

Zhang YH, Vasko MR, Nicol GD (2006) Intracellular sphingosine 1-phosphate mediates the increased excitability produced by nerve growth factor in rat sensory neurons. J Physiol 575:101–113. CrossRef Medline

Zhou J, Holtzman DM, Weiner RI, Mobley WC (1994) Expression of TrkA confers neuron-like responsiveness to nerve growth factor on an immortalized hypothalamic cell line. Proc Natl Acad Sci U S A 91:3824–3828. CrossRef Medline

Zhuang ZY, Xu H, Clapham DE, Ji RR (2004) Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization. J Neurosci 24:8300–8309. CrossRef Medline