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Potential mechanisms for hypoalgesia induced by anti-nerve growth factor immunoglobulin are identified using autoimmune nerve growth factor deprivation

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Abstract

Nerve growth factor (NGF) antagonism has long been proposed as a chronic pain treatment. In 2010, the FDA suspended clinical trials using tanezumab, a humanized monoclonal anti-NGF antibody, to treat osteoarthritis due to worsening joint damage in 16 patients. Increased physical activity in the absence of acute pain which normally prevents self harm was purported as a potential cause. Such an adverse effect is consistent with an extension of tanezumab's primary mechanism of action by decreasing pain sensitivity below baseline levels. In animal inflammatory pain models, NGF antagonism decreases intraepidermal nerve fiber (IENF) density and attenuates increases in expression of nociception related proteins, such as calcitonin gene-related peptide (CGRP) and substance P (SP). Little is known of the effects of NGF antagonism in noninflamed animals and the hypoalgesia that ensues. In the current study, we immunized rats with NGF or cytochrome C (cytC) and examined 1) nocifensive behaviors with thermal latencies, mechanical thresholds, the hot plate test, and the tail flick test, 2) IENF density, and 3) expression of CGRP, SP, voltage-gated sodium channel 1.8 (Nav1.8), and glutaminase in subpopulations of dorsal root ganglion (DRG) neurons separated by size and isolectin B4 (IB4) labeling. Rats with high anti-NGF titers had delayed responses on the hot plate test but no other behavioral abnormalities. Delayed hot plate responses correlated with lower IENF density. CGRP and SP expression was decreased principally in medium (400-800 μm^2) and small neurons (<400 μm^2), respectively, regardless of IB4 labeling. Expression of Nav1.8 was only decreased in small and medium IB4 negative neurons. NGF immunization appears to result in a more profound antagonism of NGF than tanezumab therapy, but we hypothesize that decreases in IENF density and nociception related protein expression are potential mechanisms for tanezumab induced hypoalgesia.

Keywords

tanezumab; intraepidermal nerve fibers; calcitonin gene-related peptide; substance P; Nav1.8; glutaminase

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Nerve growth factor (NGF) is produced by peripheral tissues and influences the function of sensory and autonomic nerve fibers innervating these tissues. Inflammation increases production of NGF in human disease (Halliday et al., 1998, Friess et al., 1999, Miller et al., 2002) and animal pain models (Woolf et al., 1994, Sivilia et al., 2008), which sensitizes nociceptors (Pezet and McMahon, 2006) – the primary sensory neurons responsive to tissue damaging stimuli. Therefore, antagonizing NGF has become a proposed treatment for inflammatory pain (Hefti et al., 2006, Watson et al., 2008, Burgess and Williams, 2010, Cattaneo, 2010). Although small molecule NGF receptor antagonists have been developed (Owolabi et al., 1999, Colquhoun et al., 2004), antibodies against NGF remain the most effective means of blocking NGF-induced changes in nociceptors (Koltzenburg et al., 1999, Covaceuszach et al., 2005, Sevcik et al., 2005, Hefti et al., 2006, Abdiche et al., 2008). Passive immunization with anti-NGF immunoglobulin attenuates nociceptor sensitization and provides analgesia in animal inflammatory pain models (McMahon, 1996, Ma and Woolf, 1997, Bennett et al., 1998, Gould et al., 2000, Djouhri et al., 2001).

Tanezumab, a humanized IgG2 monoclonal anti-NGF antibody, is effective in relieving osteoarthritis pain in humans (Lane et al., 2010, Schnitzer et al., 2011). However, in 2010, the Food and Drug Administration (FDA) put clinical trials using this medication on hold. The phase III osteoarthritis clinical program and two phase II studies for diabetic peripheral neuropathy and chronic low back pain were temporarily suspended due to cases of worsening arthritis. Similar to individuals with congenital insensitivity to pain with anhidrosis (CIPA) who inadvertently hurt themselves by not sensing tissue damage, some patients treated with tanezumab have excessive joint wear in the absence of pain that would normally temper their physical activity (Wood, 2010). Sixteen patients enrolled in a phase III clinical trial for tanezumab treatment of osteoarthritis of the hip or knee exhibited worsening osteoarthritis with radiographic evidence of bone necrosis and subsequently required joint replacement. In addition, hypoesthesia was the most commonly reported adverse event of abnormal peripheral sensation in another study (Schnitzer et al., 2011).

NGF autoimmunization of adult animals leads to sympathectomy (Gorin and Johnson, 1979, Otten et al., 1979) and hypoalgesia (Chudler et al., 1997), which are symptoms similar to those of humans affected by CIPA where the high affinity NGF receptor, TrkA, is mutated (Indo, 2001, 2010). Studying sensory neurons from NGF immunized rats may help explain the mechanism behind tanezumab-induced hypoalgesia. Previous studies have examined the acute effects of local and systemic passive immunization against NGF. Autoimmunization against NGF is an effective experimental model for studying the effects of chronic NGF deprivation on peripheral neurons over a longer period of time.

NGF plays a role in regulating sensitivity of nociceptors (McMahon et al., 1995, McMahon, 1996, Bennett et al., 1998), which still express TrkA in adulthood (McMahon et al., 1994, Phillips and Armanini, 1996). The sensitizing effects of elevated NGF during inflammation are mediated by both post-translational and expression dependent changes of proteins important for nociception (Woolf and Ma, 2007), including calcitonin gene related peptide (CGRP), substance P (SP), and voltage-gated sodium channel (Nav) 1.8. Nav1.8 was the only Nav examined in this study, but Navs 1.3, 1.7, and 1.9 are also important mediators of pathological pain (Dib-Hajj et al., 2010) and would make excellent targets for study. In addition to SP and CGRP, nociceptors release glutamate at their peripheral (Omote et al., 1998, deGroot et al., 2000, Carlton, 2001, Jin et al., 2006, Brumovsky et al., 2007) and central terminals (Merighi et al., 1991, Zahn et al., 2002). Glutaminase (GLS) is the neuronal enzyme that converts glutamine into glutamate, and all dorsal root ganglion (DRG) neurons express this enzyme (Miller et al., 1993, Li et al., 1996, Hoffman et al., 2010, Miller et al., 2011). Peripheral inhibition of GLS decreases dorsal horn neuron activation (Hoffman and Miller, 2010) and attenuates hyperalgesia (Miller et al., 2010) during rat hind paw

inflammation, indicating that this enzyme plays a important role in nociceptor function. Basal GLS enzyme activity is decreased in DRG from rats exposed to anti-NGF antibodies *in utero* (McDougal et al., 1981). If basal GLS expression in mature DRG neurons is also dependent on peripheral NGF, then both peripheral and central glutamatergic transmission may be affected in chronic NGF deprivation, which could lead to decreased fidelity of nociceptive transmission.

Glutamate, CGRP, and SP are released by vesicular exocytosis from intraepidermal nerve fibers (IENF), which autostimulate and mediate nociception (Alvarez et al., 1988, Brumovsky et al., 2007, Miller et al., 2011). Sensitivity to painful stimuli is associated with intraepidermal nerve fiber (IENF) density (Casanova-Molla et al., 2011), which is another factor influenced by peripheral NGF levels (Albers et al., 1994, Bennett et al., 1998). Elevated NGF content in the skin of human with allergic contact eczema correlates with higher intraepidermal innervation density (Kinkelin et al., 2000). Biopsy confirms that many forms of neuropathy in humans have diminished IENF density (Kennedy and Wendelschafer-Crabb, 2005) and a similar decrease may contribute to the hypoalgesia seen in autoimmune NGF deprivation and tanezumab therapy.

The aim of the current study was to examine morphological and biochemical alterations in primary sensory neurons that could contribute to hypoalgesia resembling that seen in patients treated with tanezumab. A rat model of autoimmune NGF deprivation was employed to mimic repeated administration of the anti-NGF antibody. Immunization effectiveness was evaluated by measuring serum anti-NGF titers with enzyme-linked immunosorbent assay (ELISA). Deprivation of NGF was confirmed by observing superior cervical ganglion (SCG) neuron morphology. Nociceptive thresholds were assessed by hot plate responses, thermal latencies, mechanical thresholds, and the tail flick test. Changes in intraepidermal nerve fiber density and expression profiles of proteins important for nociception (Nav1.8, CGRP, SP, and GLS) were evaluated by immunofluorescence microscopy.

1. Experimental Procedures

1.1 Animals

Male and female Sprague-Dawley rats (n = 31) bred on site were housed on a 12 hour light: 12 hour dark cycle and given free access to food and water. Procedures were conducted according to guidelines from the International Association for the Study of Pain (Zimmermann, 1983) and were approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee. All appropriate efforts were made to minimize the number of animals used in this study.

1.2 Immunizations

To determine 1) the effect of immunization on behavioral responses, 2) the proportion of rats expected to have high antibody titer, and 3) the duration of antibody production, rats (n=12; 8 female, 4 male) were immunized against cytochrome C (cytC), and their behavioral responses and antibody titers were compared to naïve rats (n=3; all male). To determine the effects of NGF deprivation, rats in the second experiment were immunized with either cytC (n=6; 3 female, 3 male) or NGF (n=10; 5 female, 5 male). At six weeks of age, rats in both experiments were immunized with the appropriate antigen: cytC from horse heart (Sigma; St. Louis, MO) or 2.5S NGF from mouse submandibular gland (Affinity BioReagents; Golden, CO). Each rat was anesthetized with isoflurane prior to being given four dorsal paraspinal subcutaneous injections (~25 µL each) of antigen in complete Freund's adjuvant (CFA; Sigma); the total initial immunization dose for each rat was 20 µg in 100 µL CFA. At

ten weeks of age, rats were given a booster immunization with the appropriate antigen using the same injection method used for the initial immunization; the total booster immunization dose for each rat was 50 µg in 100 µL CFA.

1.3 Detection of antibodies

Antibody titer measurements were obtained from serum and CSF samples using an ELISA. For serum collection, rats were anesthetized with isoflurane and placed on a heating pad to aid in vasodilatation. A 26G needle attached to a syringe with the plunger removed was used to collect ~150 µL of blood from one of the lateral tail veins (Brown, 2006). Whole blood was incubated at 37°C for one hour to aid clotting and then stored overnight at 4°C. The sample was centrifuged at $10,000 \times g$ for 15 minutes at 4°C to dislodge the clot. Serum was collected from the top of the supernatant. The following dilutions of serum were made in PBS-Tween (100 mM sodium phosphate, 150 mM sodium chloride, 0.05% (v/v) Tween-20, pH 7.2): 1:2000, 1:4000, 1:8000, 1:16000, 1:32000, 1:48000, 1:64000, 1:96000. Multi-well plates were coated with 2 µg/mL of appropriate antigen (cytC or NGF) diluted in 50 mM sodium carbonate buffer, pH 9.6 overnight at 4°C. Wells were rinsed three times for five minutes with PBS-Tween. Wells were blocked with 5% (v/v) normal goat serum in PBS-Tween for one hour at room temperature. Dilutions of sera were applied and incubated for one hour at 37°C. Wells were rinsed three times for five minutes with PBS-Tween and incubated for one hour at 37° in horseradish peroxidase-conjugated goat anti-rat IgG (Sigma) diluted 1:1000 in PBS-Tween. Wells were rinsed three more times with PBS-Tween before adding 100 µL 0.04% (w/v) *o*-phenylenediamine in 50 mM phosphate-citrate buffer, pH 5.0 containing 0.012% (v/v) hydrogen peroxide. The reaction was stopped after incubating for 30 minutes at room temperature by adding 50 µL 2.5 N sulfuric acid. The reaction product was measured by determining the absorption at 492 nm. Titer was determined as the most dilute sera that gave an absorbance reading at least twice that of pre-immune serum samples from each rat taken at 5 weeks of age. Sera of rats in the first experiment (n=15) were measured at 8, 11, 12, 13, and 14 weeks of age. Sera of rats in the second experiment (n=16) were measured at 11, 12, and 14 weeks of age. Cerebrospinal fluid (CSF) titers were measured only at 14 weeks of age from cytC and high titer NGF rats. CSF was sampled immediately prior to tissue collection by surgically revealing the dura mater over cisterna magna in anesthetized rats and piercing it with the collection needle to avoid contamination with blood. Additional controls performed with each ELISA include: minus antigen control, minus primary control, and minus secondary control.

1.4 Sensory testing

Behavioral studies were performed to determine if sensory thresholds were affected by immunization with cytC or NGF as previously reported (Chudler et al., 1997). Four different tests were used: thermal latency, mechanical threshold, hot plate, and tail flick. Thermal latencies and mechanical thresholds were assessed at 5, 9, 11, 12, and 14 weeks of age by measuring each hind paw three times with at least 10 minutes between measurements. Thermal latencies were measured with the Plantar Test apparatus (Ugo Basile; Comerio, Italy) set at 55 mW/cm² with a cutoff of 32 seconds. Mechanical thresholds were measured with the Dynamic Plantar Aesthesiometer apparatus (Ugo Basile) set to apply a maximum force of 50 g at a ramp rate of 5 g/sec. The hot plate test was performed at 11, 12, 13, and 14 weeks of age by measuring the response time to lick a hind paw with at least 10 minutes between the three measurements from each rat. The Model 39D Analgesia Meter hot plate (IITC; Woodland Hills, CA) was set at 52°C, and a cutoff of 30 seconds was used. The tail flick test was performed only at 14 weeks. Tails were blackened with ink and tested three times with a Tail Flick Analgesia Meter apparatus (IITC), with a limit of 30 seconds.

1.5 Immunofluorescence

At 14 weeks of age, rats from the second experiment were anesthetized with Avertin and xylazine and transcardially perfused with 75 mL calcium-free Tyrode's solution followed by 300 mL 1.0% (w/v) formaldehyde, 0.8% (w/v) picric acid in 0.1 M sodium phosphate buffer. SCG, fifth lumbar (L5) DRG, and glabrous skin from the plantar surface of the hind paw were removed and post-fixed in the same fixative overnight at 4°C. Tissues were cryopreserved in antifreeze solution (Hoffman and Le, 2004) and stored at -20°C. Prior to sectioning, they were rinsed for 24 hours in 10% (w/v) sucrose in 0.01 M phosphate buffered 0.9% (w/v) saline (PBS). Sections of skin and ganglia were cut at 14 µm and 10 µm, respectively. Dried sections were rinsed three times with PBS. Skin sections were rinsed three times in PBS and then immediately incubated for four days at 4°C in anti-PGP9.5 (see Table 1) diluted in PBS-T with 0.5% (w/v) polyvinylpyrrolidone and 0.5% (w/v) bovine serum albumin. Ganglia sections were blocked in 10% (v/v) normal goat serum, 2% (w/v) bovine serum albumin, 2% (w/v) polyvinylpyrrolidone in PBS with 0.3% (v/v) Triton X-100 (PBS-T) and incubated for four days at 4°C in one of the following primary antibodies diluted in PBS-T: CGRP, SP, Nav1.8, or GLS (see Table 1). Sections of SCG were incubated with anti-tyrosine hydroxylase (TH). After incubation in primary antibody, sections were rinsed three times in PBS before incubating in 0.5 µg/mL Cy3 goat anti-rabbit IgG (Jackson ImmunoResearch; West Grove, PA), 1.33 µg/mL AlexaFluor 555 donkey anti-mouse IgG (Invitrogen; Carlsbad, CA), or 1 µg/mL biotinylated goat anti-rabbit IgG (Vector Laboratories; Burlingame, CA) diluted in PBS-T for one hour at room temperature. After three rinses, ganglia sections were incubated in biotinylated IB4 (Vector Laboratories) and 300 nM 4',6-diamidino-2-phenylindole (DAPI) diluted in PBS-T with 1 mM CaCl₂. Sections were then rinsed twice in PBS and once in sodium carbonate buffered saline, pH 9.6 before incubating for one hour in 1.0 µg/mL avidin-fluorescein isothiocyanate (FITC; Vector Laboratories) diluted in sodium carbonate buffered saline. Slides were cover slipped as above after three more PBS rinses.

1.6 Image analysis

Images were acquired with a 20X objective on a BX51 epifluorescence microscope (Olympus; Center Valley, PA) using a SPOT RT740 camera (Diagnostic Instruments; Sterling Heights, MI). For semi-quantitative analysis of DRG neurons, an exposure and gain combination was determined empirically for each antigen in which the dimmest regions of tissue could be discerned visually for tracing, but the brightest regions were not oversaturated. Three random fields of view were captured from at least three sections of each DRG separated by a minimum of 80 µm using filters for FITC (green), Cy3/AlexaFluor 555 (red), and DAPI (blue). All nucleated cells not touching the edge of the image were analyzed in ImageJ (National Institutes of Health, Bethesda, MD) by using a previously described method (Hoffman et al., 2010) to identify the cytoplasm of each neuron as regions of interest (ROI). Once all ROI for a given image were selected and added to the ROI manager, measurements for each cytoplasmic profile from the green and red images were exported to a spreadsheet for statistical analysis.

DRG neuron profiles were separated into small (<400 µm²), medium (400-800 µm²) and large (>800 µm²) cell size populations, which correspond approximately to C, Aδ, and Aα/β fiber neurons, respectively (Fang et al., 2006, Hoffman et al., 2010). The mean intensity of immunoreactivity (ir) or IB4 labeling for each cytoplasmic profile was used to determine if it would be considered positive (+ve) or negative (-ve) for a specific antigen or IB4 by setting a mean intensity threshold. This threshold was chosen by qualitative assessment of images and verified by a trough in the mean intensity frequency distribution (data not shown).

Ten random fields of view were photographed by a blinded investigator at 20X from three skin sections of each rat. The area comprising the vital layers of epidermis (i.e., excluding the strata lucidum and corneum) for each image was traced as an ROI in a similar manner to the DRG neurons as described above. Then, all IENFs were traced with the freehand line tool as individual ROIs. For each image, a total length of IENFs was obtained, and the epidermal area was multiplied by the section thickness (14 μm) to obtain a volume of epidermis sampled for that image. The IENF density, expressed in $\mu\text{m}/\text{mm}^3$, was calculated for each image and then average IENF densities were calculated for each of 16 rats.

Brightness, contrast, and color balance adjustments for all representative images used in figures were done with ImageJ. Alterations were applied uniformly to all images for a given comparison (IENF density, CGRP, SP, Nav1.8, GLS). Cropping and resizing of the original images was also performed with ImageJ. Original aspect ratios were constrained and bilinear interpolation was used to average when downsizing.

1.7 Statistical analysis

Prism version 5.01 (GraphPad Software, Inc.; LaJolla, CA) was used to make graphs and perform statistical tests. P values less than 0.05 were considered significant. Percentages of neurons in text are written as the mean $\pm$ the standard error. Means and SEM are represented in graphs. Kolmogorov-Smirnov tests were used to determine if data were normally distributed. The specific statistical test used is indicated in the figure legends.

2. Results

2.1 Autoimmunization with NGF

The first experiment provided needed information about the duration and magnitude of immunologic response to the immunization protocol used. Results were used to plan the number of rats to immunize against NGF in the second immunization experiment. Of the 12 rats immunized against cytC in the first experiment, four were put in a low titer group because their titers were zero at one week after the booster immunization (data not shown). Their titers eventually started to rise at 12 weeks, but were never higher than 16000. Three rats were put in a medium titer group because they had positive serum titers at week 11, but had not reached 96000 by week 12. The remaining five rats were considered high titer, because they all reached a titer of 96000 by week twelve and maintained this titer through week 14. Naïve rats never showed a positive titer. This heterogeneity of immunologic response has been previously observed (Chudler et al., 1997). No significant differences in weights or behavioral responses were noted between rats of different groups (data not shown). Since cytC and 2.5S NGF are similar in molecular weight, we predicted a comparable immune response to NGF. Power analysis of behavioral data from the first experiment indicated that at least four high anti-NGF titer rats would be needed for 80% power ($\beta = 0.2$) to detect a 50% difference. In order to have enough rats with high anti-NGF titer, ten rats were immunized against NGF. We decided to measure immunofluorescence changes in DRG and skin after allowing the rats to survive until they were 14 weeks old, based on results of the first cytC immunization experiment.

The responses to NGF immunization were split into high and low titer groups (Fig. 1). Five rats were considered high titer, because they had titers of 96000 by 12 weeks and maintained the high titer to 14 weeks. The remaining five rats were put into the low titer group. All of the low titer rats had titers of 32000 or less at 14 weeks. The CSF of four of the NGF immunized rats with high titer and three of the cytC immunized rats was tested for presence of anti-NGF and anti-cytC respectively. Antibody titers of CSF were zero in all cases as

previously reported (Chudler et al., 1997). In both immunization experiments, gender did not affect immunological response as measured by serum titer levels.

2.2 Verifying NGF deprivation by examination of sympathetic neurons

The sizes of SCG neurons were evaluated by examining TH-positive neuron profiles. The SCG neurons of rats immunized against cytC had a similar morphology to those of naïve rats (Fig. 2A, B). Qualitatively, SCG neurons from all rats immunized against NGF (both low and high titer) except one low titer animal showed atrophy with decreased neuronal cytoplasmic area, increased number of heterochromatic nuclei (non neuronal), and neuronal cell loss (Fig. 2C, D). Since these findings have already been documented repeatedly (Levi-Montalcini and Booker, 1960, Otten et al., 1979, Gorin and Johnson, 1980), our evaluation was limited to a qualitative assessment to verify effective NGF deprivation among NGF immunized rats.

2.3 Behavioral effects of NGF deprivation

As the rats matured, the thermal latencies and mechanical thresholds increased significantly between the pre- and post-immunization periods in all rats; however, the presence of anti-NGF had no effect on thermal latency or mechanical threshold at any of the time points tested when compared to cytC immunized rats (Fig. 3A, B). Rats in the high NGF titer group had significantly longer response times for the hot plate test at 11, 12, and 14 weeks, approximately twice as long as cytC immunized rats (Fig. 3C). Hot plate responses of low NGF titer rats were not significantly different from cytC rats, but there were three rats at 11 weeks and 2 rats at 12 weeks with low anti-NGF titers that had longer hot plate responses. It should be noted that there were also rats with high anti-NGF titers that had near normal hot plate responses at 12 and 14 weeks. There were no differences among the three groups for the tail flick test at 14 weeks (Fig. 3D).

2.4 Effect of NGF deprivation on intraepidermal nerve fibers

Individual nerve fibers in the epidermis and dermal nerve bundles were visualized in hind paw skin from all rats in the study using the pan-neuronal marker, PGP9.5 (Fig. 4A, B). The median IENF density in rats immunized against cytC was $0.486 \mu\text{m}/\text{mm}^3$, while rats with low and high anti-NGF titers had median IENF densities of 0.443 and $0.438 \mu\text{m}/\text{mm}^3$, respectively (data not shown). Although there was a trend for lower IENF density in NGF immunized rats, differences between these groups were not statistically significant. There was, however, a significant correlation between IENF density and hot plate responses ($p = 0.0297$) in which rats with longer hot plate response times had lower IENF densities (Fig. 4C).

2.5 Effect of NGF deprivation on DRG neurons

A total of 19,456 cytoplasmic profiles were included in the image analysis: 4,822 for CGRP, 4,693 for SP, 5,604 for Nav1.8, and 4,337 for GLS. The mean number of profiles analyzed per rat was 1297 and per rat per antibody was 347. Small and medium cells made up the majority of profiles, accounting for 10,866 and 5,793, respectively (Fig. 5). The frequency distributions of neuronal sizes were comparable for cytC and NGF immunized groups (Fig. 6A). IB4 labeling was restricted primarily to small and medium neurons and accounted for 60% of all profiles analyzed. Since there were only 82 (0.4%) large IB4+ve neurons in the entire study and several rats had none, large neurons were not subdivided into IB4+ve and IB4-ve groups.

2.5.1 Effects of NGF deprivation on neuropeptide-ir—Immunization with NGF significantly decreased the percentage of CGRP+ve DRG neurons from $48 \pm 2\%$ in the cytC

group to $32.5 \pm 2\%$ and $33 \pm 1\%$ in the low NGF and high NGF groups, respectively (Fig. 5A, 5E, 6B). Similarly, the mean percentage of SP+ve DRG neurons in cytC immunized rats was $28 \pm 2\%$ while the low NGF and high NGF groups were lower at $13 \pm 2\%$ and $11 \pm 1\%$, respectively (Fig. 5A, 5G, 6B). CGRP-ir was primarily limited to the small and medium neurons (Fig. 5A, E), whereas SP-ir was primarily limited to small neurons (Fig. 5C, G). The percentages of small and medium CGRP+ve neurons were decreased significantly in the low NGF and high NGF groups compared to the cytC immunized group, but there were not significant differences between the low NGF and high NGF groups (Fig. 5E, 6C). The percentages of small SP+ve were significantly lower in both low NGF and high NGF groups as compared to the cytC group, whereas only the high NGF group had a significantly decreased percentage of medium SP+ve neurons (Fig. 5G, 6D). There were no differences between the low NGF and high NGF groups.

Dividing small neurons by IB4 labeling showed that only small IB4+ve neurons had decreased percentages of CGRP+ve neurons, and this decrease was only observed in the high NGF group (Fig. 5F, 7A). Fewer medium IB4+ve and IB4-ve neurons were CGRP+ve in both the low NGF and high NGF groups as compared to the cytC group, but there were no differences between the low and high NGF groups (Fig. 5F, 7A). The percentages of SP+ve neurons were significantly decreased in small IB4+ve and IB4-ve DRG neurons from the low NGF and high NGF groups, but were not affected in IB4+ve or IB4-ve medium neurons (Fig. 5H, 7B).

2.5.2 Effects of NGF deprivation on Nav1.8-ir—In rats immunized against cytC, $66 \pm 5\%$ of all neurons were Nav1.8+ve, whereas $51 \pm 3\%$ and $53 \pm 4\%$ were Nav1.8+ve in the low NGF and high NGF groups, respectively (Fig. 5I, 5M, 6B). These differences were not significant, indicating that NGF deprivation had no significant effect on the total percentage of Nav1.8+ve DRG neurons. When DRG neurons were separated by size, there were significantly fewer small Nav1.8+ve neurons in the low NGF group, but not in the high NGF group (Fig. 5N, 6E). There were no significant changes in the percentages of Nav1.8+ve medium or large neurons. Further subdivision of small and medium neurons by IB4 labeling revealed that there were significantly fewer Nav1.8+ve small IB4-ve neurons in both the low NGF and high NGF groups, but there were no differences among small IB4+ve neurons (Fig. 5N, 7C). There was also a significant decrease in the percentage of Nav1.8+ve medium IB4-ve neurons in the high NGF group. This population in the low NGF group was not significantly affected. There were no significant differences for Nav1.8 among any of the medium IB4+ve neurons.

2.5.3 Effects of NGF deprivation on GLS-ir—Characterization of GLS immunoreactivity in the DRG could not be carried out as it was for CGRP, SP, and Nav1.8 by assessing the percentage of +ve cells because all DRG neurons have GLS-ir and are considered GLS+ve (Hoffman et al., 2010). There was no threshold set to delineate GLS+ve from GLS-ve neurons; instead, the mean intensity of GLS-ir itself was used for the analysis. All size classes of DRG neurons had similar levels of GLS-ir, and there were no differences between rats immunized with cytC and NGF (Fig. 5K, 5O, 8A). Additionally, dividing small and medium neurons by IB4 labeling revealed no significant changes in GLS-ir after NGF immunization among any subpopulation of DRG neurons (Fig. 5L, 5P, 8B).

3. Discussion

3.1 NGF autoimmunization as a model for anti-NGF pain therapy

NGF autoimmunization caused sustained elevated titers of anti-NGF for at least 28 days after the booster immunization (Section 2.1). Pharmacokinetic properties for tanezumab

have been described in cynomolgus monkeys (Zorbas et al., 2010). One fifth of the monkeys developed anti-tanezumab responses to the humanized monoclonal antibody, which decreased their exposure to the drug. In addition, the highest repeated doses had a half-life of 14 days took 100 days to achieve a steady state. NGF autoimmunization may represent a quicker and more profound antagonism of NGF than tanezumab treatment.

NGF autoimmunization of adult rats and rabbits produces sympathectomy as seen by sympathetic ganglion atrophy (Otten et al., 1979, Gorin and Johnson, 1980, Johnson et al., 1982). SCG neurons from rats immunized against NGF in the current study demonstrated atrophic morphology, confirming NGF deprivation (Section 2.2). In contrast, there were no morphological changes in the SCG neurons of monkeys given up to a 30 mg/kg weekly infusion of tanezumab for 26 weeks followed by an eight week recovery period (Zorbas et al., 2010). Such differences may again suggest that the NGF autoimmunization model is a more exaggerated version of repeated tanezumab treatment.

Thermal hypoalgesia as measured by the hot plate test has been documented previously (Chudler et al., 1997). We again demonstrated thermal hypoalgesia in some rats with high titers of anti-NGF, and a few with low anti-NGF titers, using the hot plate test (Section 2.3). The heterogeneity of hot plate responses mimics the pattern seen with humans treated with tanezumab in that only certain individuals developed a profound hypoalgesia resulting in joint damage. Thermal latencies, mechanical thresholds, and tail flick responses were not, however, altered by NGF immunization, regardless of anti-NGF titer. Considering the observed changes in the DRG of rats with low NGF titers (Section 2.5), it was discordant to see no change in behavioral responses from these rats. It should be noted however that there were some low anti-NGF titer rats with elevated hot plate response times. One reason we may not have been able to detect a significant difference for the whole group could have been a non-uniform behavioral response to NGF autoimmunization. Another reason may be the hot plate temperature used (52°C). It has been shown that lowering the hot plate temperature from 55°C to 50°C improved the sensitivity of the test to detect hypoalgesic effects (Plone et al., 1996). It was also unexpected to see no change in thermal latencies or tail flick responses since all of these test thermal stimuli. It has been shown that quickly heating the skin (6.5°C/s) activates A δ fibers, whereas slow heating (1°C/s) activates C fibers (Yeomans and Proudfoot, 1996). The thermal latency stimulus intensity used in the current study (55 mW/cm²) corresponds to heating at less than 1 °C/s. Since we observed changes in changes in the DRG and IENFs, we anticipated altered thermal latencies indicative of hypoalgesia. While slow heating with the thermal latency test can detect hypoalgesia (Lu et al., 1997), the test was designed and is most suitable for measuring hyperalgesic phenomena (Hargreaves et al., 1988, Le Bars et al., 2001) and may be why it did not detect hypoalgesia in the current study. A similar rationale applies to the lack of differences in the tail flick test.

Worsening osteoarthritis was presumed to be caused by hypoalgesia in the humans treated with tanezumab (Wood, 2010). Even though the current study did not show any evidence of hypoesthesia (as measured by mechanical thresholds), hypoalgesia was observed in some rats immunized against NGF. We hypothesize that similar changes may occur in humans, and purport that there is merit for using NGF autoimmunization as a model for anti-NGF pain therapy.

3.2 NGF deprivation decreases skin innervation

Quantitative sensory testing for pain thresholds in humans correlates well with IENF density (Selim et al., 2010). Patients experiencing chronic pain after burn injuries have elevated IENF densities (Hamed et al., 2011), whereas diabetic patients (Pittenger et al., 2004) and diabetic mice (Beiswenger et al., 2008) have decreased IENF density that follows after

symptoms. The effects of tanezumab therapy on innervation density are not known, but a clinical trial designed to describe these effects has been completed, and results are pending (clinicaltrials.gov identifier NCT01030640). Development of hypoalgesia among NGF immunized rats in the current study was not uniform (Section 2.3), but there was a significant correlation between IENF densities and hot plate responses of individual rats, indicating that a reduction in skin innervation may be responsible for thermal hypoalgesia as measured by the hot plate test. Results in the current study did not show a significant difference in IENF density based on anti-NGF titer levels showing that anti-NGF titer level was not necessarily the best predictor of hot plate response time. The possibility that PGP9.5 +ve Langerhan's cells could be contributing to the IENF counts cannot be excluded, although we did not encounter any PGP9.5 +ve Langerhans' cell bodies. Furthermore, these results suggest a lower epidermal innervation density as a potential mechanism in tanezumab induced hypoalgesia.

3.3 NGF deprivation diminishes neuropeptide content in specific DRG neuron subpopulations

The estimated percentages of CGRP+ve and SP+ve DRG neurons in rats immunized against cytC in this study were approximately 48% and 28%, respectively. These estimates are similar if not higher than those by other studies (Lawson et al., 1996, Kashiba et al., 1997, Schicho and Donnerer, 1999, Price and Flores, 2007), indicating that cytC does not decrease the neuropeptide content of DRG neurons. An inverse relationship between formaldehyde concentration and the percentage of DRG neurons considered positive for an antigen has been demonstrated previously (Hoffman et al., 2010). Previous studies have used formaldehyde at 3.7-4% (w/v) in the fixative, whereas, the current study used it at 1% (w/v), which could account for the higher and potentially more accurate estimations of CGRP and SP distribution.

NGF antagonism can prevent the increased percentage of CGRP+ve DRG neurons after CFA-induced inflammation, sciatic nerve crush injury, and intervertebral disc injury (Fukui et al., 2010, Iwakura et al., 2010, Orita et al., 2010). Administering anti-NGF antibody prevented the increase in SP+ve DRG neurons in a diabetic neuropathic pain model (Cheng et al., 2009). These studies evaluated the percentage of total DRG neurons positive for CGRP or SP, whereas subpopulations of DRG neurons divided by size and IB4 labeling were evaluated in the current study. The populations with decreased neuropeptide content were the small and medium classes, which correspond with the cell bodies of C and A δ fibers, respectively (Fang et al., 2006). Some classification schemes separate nociceptive DRG neurons into peptidergic and non-peptidergic neurons (Woolf and Ma, 2007). The peptidergic neurons are generally thought to be IB4-ve and TrkA+ve (Averill et al., 1995). However, both IB4+ve and IB4-ve medium neurons in the current study had decreased CGRP while small neurons irrespective of IB4 labeling had decreased SP. It might be expected that only IB4-ve neurons would be affected by decreased NGF levels, but a study has shown that 86% of TrkA mRNA+ve DRG neurons label with IB4 (Kashiba et al., 2001). Therefore, IB4 labeling status may not be predictive of responsiveness to NGF.

The neuropeptides CGRP and SP can directly stimulate peripheral nociceptor terminals as well as sensitize them to nociceptive stimuli (Nakamura-Craig and Gill, 1991, Carlton et al., 1996). They are also responsible for the efferent functions of nociceptors during neurogenic inflammation (Pedersen-Bjergaard et al., 1991, Sann and Pierau, 1998). CGRP and SP are also released from central nociceptors terminals onto second order sensory neurons in the spinal cord dorsal horn (Kuraishi et al., 1988, Wiesenfeld-Hallin et al., 1990). Decreased CGRP and SP contents in the cell bodies are likely to correlate with decreased neuropeptide content in both peripheral and central terminals. Peripheral treatment with vinpocetine, a retrograde transport blocker, inhibits endosomal retrograde NGF signaling and results in

decreased CGRP- and SP-ir in laminae I and II of the spinal cord dorsal horn (Knyihar-Csillik et al., 2007). When CGRP is neutralized by intrathecal administration of anti-CGRP antiserum, hyperalgesic responses of adjuvant- and carrageenan-injected rats are normalized (Kawamura et al., 1989).

The theory behind anti-NGF therapies is that nociceptor sensitization can be blocked, and the maladaptive chronic pain can be relieved. This is thought to be mediated in part by attenuating the increased CGRP and SP release from peripheral nociceptor terminals. If this primary mechanism of action is overextended, nociceptor sensitivity can actually be decreased such that acute pain, which is beneficial in preventing self-harm, is not perceived. If the nociceptors in patients treated with tanezumab also have decreased CGRP and SP available for release, they may have decreased nociceptor sensitivity and neurotransmission to spinal neurons.

3.4 NGF deprivation decreases Nav1.8 expression in IB4 negative DRG neurons

The estimated percentage of Nav1.8+ve DRG neurons in cytC immunized rats was 66%, which is similar to previous estimates (Fukuoka et al., 2008, Hoffman et al., 2010), demonstrating that cytC immunization did not decrease the percentage of Nav1.8+ve neurons.

NGF deprivation had no effect on the percentage of total Nav1.8+ve neurons, but significant changes were apparent once the neurons were divided into more homogeneous subpopulations. In contrast to the neuropeptides examined, Nav1.8 expression appeared to decrease in only small and medium IB4-ve neurons. These results conform to the notion that IB4-ve neurons are synonymous with NGF responsiveness. Taking into account the finding of TrkA mRNA+ve/IB4+ neurons (Kashiba et al., 2001), NGF responsiveness via the TrkA receptor is not likely to be the only explanation for observing Nav1.8 expression changes in IB4-ve neurons. These results also agree with a previous study where NGF autoimmunization led to decreased Nav1.8 *in situ* hybridization signals and Nav1.8 current density in IB4-ve DRG neurons (Fjell et al., 1999).

Nav1.8 is only expressed in nociceptive primary afferent neurons (Akopian et al., 1996), and is presumed to be important in acute pain (Akopian et al., 1999) as well as the hyperalgesia that develops with chronic pain (Jarvis et al., 2007, Abrahamsen et al., 2008). Despite the fact that Nav1.8 knockout mice and mice with Nav1.8-expressing neurons ablated have no change in responses to the hot plate test, they do have altered responses to other painful stimuli (Akopian et al., 1999, Abrahamsen et al., 2008). It was hypothesized that compensation by upregulation of other sodium channels was likely to explain the normal hotplate responses (Akopian et al., 1999). In contrast, decreased Nav1.8 expression in DRG neurons of aging rats has been suggested as a potential mechanism for decreased thermal sensitivity (Wang et al., 2006). It remains unclear as to whether the suppression of Nav1.8 expression is responsible for the thermal hypoalgesia seen on the hot plate test in the current study. However, a decrease in Nav1.8 expression could still play a role in decreased sensitivity to other noxious stimuli and could be part of the mechanism of tanezumab induced hypoalgesia.

3.5 Basal GLS expression is not dependent on peripheral NGF levels

Since all DRG neurons express GLS for synthesizing glutamate for release at both the peripheral and central terminals, a threshold intensity that determines positive and negative neurons could not be established. Instead, the mean GLS-ir for the cytoplasmic profile of all DRG neurons examined was used to assess any potential changes in GLS expression levels among specific subpopulations of DRG neurons.

When rats were exposed *in utero* to anti-NGF antibodies and then allowed to mature, the GLS enzyme activity of whole DRG was decreased, indicating a dependence on NGF during development for normal expression (McDougal et al., 1981). There were never any significant changes in GLS-ir after depriving mature DRG neurons of NGF in the current study. This may indicate that the supply of glutamate for release from peripheral and central terminals is not affected by neutralizing NGF. It is unlikely that decreased GLS expression plays a role in the NGF autoimmunization induced hypoalgesia or tanezumab induced hypoalgesia. Despite an absence of a role for GLS expression in anti-NGF induced hypoalgesia, peripheral GLS does appear to play a role in developing hyperalgesia during inflammation (Miller et al., 2006, Hoffman and Miller, 2010, Miller et al., 2011).

Conclusion

Autoimmunization of rats with NGF produces anti-NGF antibodies that neutralize NGF, cause sympathetic neuron atrophy, and can result in thermal hypoalgesia on the hot plate test. The current study suggests that this model produces an exaggerated deprivation of NGF as compared to tanezumab therapy, but we maintain that there is value in using this model to represent anti-NGF pain therapy in animals without the concern for anti-drug antibody responses. Reduced skin innervation density could be a key mechanism of anti-NGF and tanezumab induced hypoalgesia. Although they are not likely to be the only nociception related proteins whose expression is decreased by NGF antagonism, reduced expression of CGRP, SP, and Nav1.8 in specific populations of medium and small DRG neurons may also be responsible for hypoalgesia during NGF neutralization. In general, we found that examining more homogeneous populations of DRG neurons instead of all DRG neurons led to finding more expression changes that were significant. This likely reflects the heterogeneity of modality and receptor expression among DRG neurons. We hypothesize that morphological and biochemical alterations of nociceptive primary sensory neurons are potential mechanisms for tanezumab induced hypoalgesia.

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Abbreviations

CGRP	calcitonin gene-related peptide
CFA	complete Freund's adjuvant
CIPA	congenital insensitivity to pain with anhidrosis
CSF	cerebrospinal fluid
cytC	cytochrome C
DAPI	4',6-diamidino-2-phenylindole
DRG	dorsal root ganglion
ELISA	enzyme-linked immunosorbant assay

FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
GLS	glutaminase
IB4	isolectin B4
IENF	intraepidermal nerve fiber
ir	immunoreactivity
Nav	voltage-gated sodium channel
NGF	nerve growth factor
PBS	phosphate buffered saline
ROI	region of interest
SCG	superior cervical ganglion
SP	substance P
TH	tyrosine hydroxylase
+ve	positive
-ve	negative

Highlights

- In this study, we used a model of NGF autoimmunization to deprive rats of NGF.
- Thermal hypoalgesia developed in rats with high anti-NGF titers.
- NGF deprivation decreased intraepidermal nerve fiber density.
- CGRP, SP, and Nav1.8 expression in DRG neurons decreases in NGF immunized rats.
- NGF autoimmunization may be a suitable animal model for tanezumab pain therapy.

Figure 2.

Superior cervical ganglion (SCG) neurons from rats immunized against cytochrome C (cytC) or nerve growth factor (NGF). (A, B) Representative section of SCG from a cytC-immunized rat shows normal neuronal morphology and satellite glial cell number. (C, D) Representative section of SCG from a rat with a high anti-NGF titer shows marked neuronal atrophy (diminished TH+ve cytoplasmic area) and reactive gliosis (abundant glial nuclei). (green = tyrosine hydroxylase, blue = DAPI)

Figure 3.

Effect of nerve growth factor (NGF) deprivation on nocifensive behaviors. (A) Thermal latencies and (B) mechanical thresholds were not altered by NGF neutralization. (C) Hot plate (52°C) responses were significantly elevated at 11, 12, and 14 weeks in rats with high anti-NGF titers. (D) Tail flick responses were not altered by NGF neutralization. Individual data points are shown and mean values are indicated by horizontal bars. Data were analyzed by two-way ANOVA with Bonferroni post-tests.

Figure 4.

Effect of nerve growth factor (NGF) deprivation on intraepidermal nerve fiber (IENF) density. (A) A representative skin section from a cytochrome C (cytC) immunized rat shows IENFs extending to stratum granulosum. (B) In contrast, skin from NGF immunized rats had IENF that were generally much shorter and with less frequent despite no apparent change in the dermal nerve plexus density. The micrometer bar in panel A applies panel B. Solid arrows=IENFs; open arrowheads=dermal nerve plexus. (C) Graph plotting IENF densities versus average hot plate response times showed rats with longer hot plate response times had lower IENF densities. Generally NGF immunized rats had lower IENF densities and longer hot plate response times. Data were analyzed with Pearson correlation. The best fit

line (solid) and 95% confidence bands (dashed) of the best fit line are shown. Open circles = cytC, X's = low anti-NGF titer, solid boxes = high anti-NGF titer.

Figure 5.

Qualitative effects of nerve growth factor (NGF) deprivation on immunoreactivity for dorsal root ganglia (DRG) proteins important for nociceptive neurotransmission. (A, C) Calcitonin gene-related peptide (CGRP) is normally expressed primarily in small and medium DRG neurons, but the percentage of CGRP+ve medium DRG neurons irrespective of isolectin B4 (IB4) labeling and small IB4+ve neurons is reduced after NGF deprivation (E, F). (C, D) Substance P (SP) is normally expressed small and medium DRG neurons. (G, H) NGF deprivation decreases the percentage of SP+ve neurons in small IB4+ve and IB4-ve populations. (I, J) Voltage-gated sodium channel 1.8 is expressed primarily in small and medium neurons. (M, N) There are fewer small and medium IB4-ve neurons in NGF immunized rats. The micrometer bar in panel A applies to all panels. (solid arrowheads=small neurons: $<400\mu\text{m}^2$; open arrowheads=medium neurons: $400\text{--}800\mu\text{m}^2$; *=large neurons: $>800\mu\text{m}^2$; blue = DAPI) Data were analyzed with Kruskal-Wallis test with Dunn's post-test.

Figure 6.

Quantitative effects of nerve growth factor (NGF) deprivation on immunoreactivity for dorsal root ganglia proteins important for nociceptive neurotransmission. (A) Frequency distribution of neuronal size shows that similar populations of neurons were sampled, and NGF deprivation did not alter DRG neuron size. (B) There were decreased percentages of all calcitonin gene-related peptide (CGRP)+ve DRG neurons in rats with low and high anti-NGF titers and for substance P (SP)+ve DRG neurons only in rats with high anti-NGF titers. There were no changes in the percentage of all voltage-gated sodium channel 1.8 (Nav1.8)+ve neurons. (C) There were fewer CGRP+ve small and medium neurons in rats with low and high anti-NGF titers. (D) There were fewer SP+ve small DRG neurons in rats

with low and high anti-NGF titers, but only rats with high anti-NGF titers had fewer medium SP+ve neurons. (E) The only significant reduction in Nav1.8+ve neurons was in the small size group in rats with low anti-NGF titers. Data were analyzed with Kruskal-Wallis test with Dunn's post-test.

Figure 7.

Percentages of isolectin B4 (IB4) subpopulations of dorsal root ganglion (DRG) neurons with immunoreactivity for (A) calcitonin gene-related peptide (CGRP), (B) substance P (SP), and (C) voltage-gated sodium channel 1.8 (Nav1.8). There were fewer CGRP+ve small IB4+ve neurons in rats with high NGF titers, but all NGF immunized rats had fewer medium CGRP+ve neurons, regardless of IB4 labeling. The only populations to have decreased SP+ve neurons were the small IB4+ve and IB4-ve populations in rats with high and low anti-NGF titers. All NGF immunized rats had fewer small Nav1.8+ve/IB4-ve neurons, whereas only rats with high anti-NGF titers had decreased medium Nav1.8+ve/IB4-ve neurons. Data were analyzed with Kruskal-Wallis test with Dunn's post-test.

Figure 8.

Effect of nerve growth factor (NGF) deprivation on mean glutaminase (GLS) immunoreactivity (ir) levels in dorsal root ganglion (DRG) neurons. Mean GLS-ir in small, medium, and large DRG neurons (A) and neurons separated by isolectin B4 labeling (B) were not significantly different, irrespective of anti-NGF titer. Data were analyzed with Kruskal-Wallis test with Dunn's post-test.

Table 1

Primary antibodies used for immunofluorescence characterization of superior cervical ganglia, skin, and dorsal root ganglia.

Antibody	Immunogen	Species and clonality	Supplier (catalog #)	Dilution factor
tyrosine hydroxylase (TH)	denatured full length rat TH	rabbit polyclonal	Chemicon (AB152)	1:1,000
protein gene product 9.5 (PGP9.5)	full length human PGP9.5	rabbit polyclonal	Cedarlane (CL95101)	1:20,000
calcitonin gene-related peptide (CGRP)	full length rat CGRP	mouse monoclonal	Santa Cruz Biotechnology (4901)	1:8,000
substance P (SP)	full length mammalian SP	mouse monoclonal	R&D Systems (MAB4375)	1:2,000
voltage-gated sodium channel 1.8 (Nav1.8)	C-terminus of rat Nav1.8 (aa1724-1956)	mouse monoclonal	Neuromab	1:16,000
glutaminase (GLS)	full length GLS from rat kidney	rabbit polyclonal	N. Curthoys, Colorado State University	1:10,000