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Trigeminal Nerve Injury ErbB3/ErbB2 Promotes Mechanical Hypersensitivity

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Abstract

BACKGROUND—Chronic constriction injury of trigeminal infraorbital nerve results in transient analgesia followed by whisker pad mechanical allodynia in rats. Neuregulin1 expressed on axonal membranes binds receptor tyrosine kinase ErbB promoting Schwann cell development and remyelination. This study investigated whether orofacial mechanical allodynia is signaled by ErbB3/ErbB2 heterodimers in injured nerves.

METHODS—Whisker pad mechanical allodynia (von Frey stimuli) was quantified in wild type rats and in transgenic rats with *Sleeping Beauty* transposon mutation for neuregulin1 transgene. Pain-related behavior was retested after intraperitoneal injection of ErbB2 inhibitor, Lapatinib, an agent shown by others to reduce breast cancer pain. Infraorbital nerve injury was evaluated histologically with myelin and neuronal biomarkers. ErbB3 changes over time were measured with western blots.

RESULTS—Whisker pad mechanical hypersensitivity began in week 2 in wild type rats (3.11 ± 5.93 *vs.* 18.72 ± 0.00g after sham surgery, n = 9, $p < 0.001$) indicating trigeminal neuropathic pain, but was not evident in transgenic rats (odds ratio: 1.12, 95% confidence interval: 0.38-3.35). Initiation of statistically significant mechano-hypersensitivity was delayed until week 6 after surgery in transgenic rats (3.44 ± 4.60g *vs.* 18.72 ± 0.00g, n = 4, $p < 0.001$). Mechanical allodynia which persisted 8 weeks in wild type rats was alleviated by Lapatinib (15 ± 3.89g *vs.* 2.45 ± 1.13g, n = 6, $p < 0.001$). Infraorbital nerve damage was verified histologically. Statistically significant ErbB3 increases (weeks 5 and 10) in wild type and transgenic rats (week 10) coincided with time points when mechanical hypersensitivity was present.

CONCLUSION—Neuregulin1/ErbB3/ErbB2 is a causal mechanism in nerve injury induced trigeminal neuropathic pain. Understanding peripheral glial mechanisms after nerve injury will improve neuropathic pain treatment.

Introduction

Trigeminal neuropathic pain is one of three main neuropathic pain-related diagnoses. The orofacial pain condition is characterized by chronic aching and burning pain as well as sudden excruciating, electric-like shooting pain caused by unintentional injury to the

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trigeminal system. There are few investigations on nociception mechanisms and analgesic trials in the trigeminal region. Thus, new analgesic strategies need to be explored before chronic trigeminal neuropathic pain can be successfully relieved.

Deterioration of trigeminal nerve myelin sheaths is one causal mechanism for trigeminal neuropathic pain. Mechanical hypersensitivity accompanying trigeminal neuropathic pain involves spontaneous and low-threshold activity in injured myelinated fibers [1]. These pathological changes cause ectopic discharge or impulse generation from sites along axons where damage has occurred, rather than just at sensory nerve endings [2]. Sciatic nerve constrictive injury induces demyelination and is a source of pathological ectopic firing leading to mechanical allodynia and heat hyperalgesia [3]. Neuregulin 1 (Nrg1) is a peptide ligand signaling via the receptor tyrosine kinase ErbB3/ErbB2 heterodimer [4]. The Nrg1/ErbB3/ErbB2 signaling complex plays a key role in axon-Schwann cell interactions promoting Schwann cell development and re-myelination after nerve injury. Nrg1 binds catalytically inactive ErbB3 while ErbB2 contributes tyrosine kinase activity in Schwann cells [5,6]. Blockade of ErbB receptor-initiated cell signaling in glial cells wrapping either myelinated or non-myelinated nerves produces unique sensory dysfunctions [7]. Thermal and mechanical hypersensitivity as well as thin myelination of sciatic nerves are found in ErbB receptor mutant mice [8,9]. Upregulation of ErbB3 messenger RNA is maintained in different types of median nerve regenerating axons after upper limb injury while ErbB2 messenger RNA is unchanged [10]. Possible changes of ErbB after nerve injury or a role in generating trigeminal neuropathic pain have not been explored and are the focus of this study. The present experiments utilized Nrg1 transgenic rats (Nrg1Tg) to explore the behavioral changes in whisker pad and ErbB receptor levels in injured nerve after infraorbital nerve chronic construction injury (CCI-ION). Lapatinib (Tykerb), an ErbB2 inhibitor, was applied after CCI-ION to investigate its effect on the pain related behavior. Lapatinib is used in ErbB2-positive breast cancer treatment and reportedly has reduced pain in a Phase II clinical trial [11]. We further investigated the relationship of ErbB3 levels in injured nerves to the development of whisker pad mechanical allodynia following CCI-ION. We hypothesized that ErbB receptor level increase at infraorbital nerve injury sites promotes mechanical hypersensitivity in the whisker pad.

Materials and Methods

Animals

Adequate measures were taken to minimize pain or discomfort in these studies. Experiments were carried out in accordance with the Guidelines of the National Institute of Health regarding the care and use of animals for experimental procedures. Experiments were approved by the Institutional Animal Care and Use Committee at the University of Kentucky, Lexington, Kentucky. Male Fisher 344 wild type or Nrg1Tg rats were accommodated in ventilated animal housing with a reverse 12/12 light/dark cycle and weighed 200-250g at the beginning of the experiments. A breeding pair of Fisher 344 Nrg1Tg rats generated by introduction of a *Sleeping Beauty* transposon with a bidirectional splice acceptor gene trap[12,13] was provided by the Knock Out Rat Consortium. A nonsense mutational insertion into intron 1 of the sequence encoding for Nrg1 domain resulted in ablation of all Nrg1 isoforms. The Nrg1Tg rats used in this study were bred in our animal facility, and the $Nrg1^{Tn(sb\text{-}T2/Bart3)2.183Mcwi}$ deficient genotype was confirmed.

Genotyping

Rats were genotyped by polymerase chain reaction using the following primers: 5’-AGGAACCAAAAAAGTAGACTCAGTGTG-3’, 5’-GTGAGCTTTTCTGTAAGGTGGTAACTT-3’, and transposon primer 5’-

CTGACCTAAGACAGGGAATT-3'. Polymerase chain reaction was carried out at 94°C for 2 min, and then 94°C for 15 s, 60°C for 30 s, and 72°C for 90 s for 35 cycles, followed by 5 min extension at 72°C by PTC-100 programmable thermal controller (MJ Research Inc., Waltham, MA). The expected 1,041 bp product for wild-type allele, 1,041 and 440 bp products for heterozygous allele, and 440 bp product for the mutant allele were separated on a 1% agarose gel.

Surgery

Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal), and all surgeries were performed in sterile conditions under a surgical microscope. The ION on one side was exposed using the surgical procedure described by Vos [14]. The ION was dissected free at its most rostral extent in the orbital cavity and two chromic gut (5-0) ligatures were loosely tied around the ION (2 mm apart). Nerve exposure but no ligation was performed in the sham operation. To obtain the desired degree of constriction a criterion formulated by Bennett and Xie [15] was applied, *i.e.,* ligations reduced the nerve diameter just noticeably. The ligation retarded but did not totally occlude circulation through the superficial vasculature.

Assessment of Mechanical Allodynia of Rat Whisker Pad and Drug Administration

Mechanical sensitivity of vibrissal whisker pad, which is the ION receptive field, was measured with eight von Frey fibers (0.4, 0.6, 1, 2, 4, 6, 8, 15 g; Stoelting, Wood Dale, IL) by modified up-and-down method with a default maximal threshold at 18.72g [16]. Mechanical stimuli were applied within the ION territory, near the whisker pad centers, both ipsilateral and contralateral to the surgery site. Responses to von Frey filaments applied to the rat whisker pad determined the threshold required for 50% head withdrawals. Each filament was applied five times at intervals of a few seconds. If head withdrawal was observed at least three times after probing with a filament, the rat was considered responsive to that filament. Whenever a positive response to a stimulus occurred, the next smaller von Frey filament was applied. Otherwise, the next higher filament was applied. Behavioral changes to mechanical stimuli were tested once a week for 10 weeks after surgery. Intraperitoneal injection of tyrosine kinase ErbB2 inhibitor Lapatinib (0, 0.2, 1, 5 mg/kg, in 300 μl dimethyl sulfoxide, Selleckchem, Houston, TX) [17] was administered to CCI-ION rats after mechanical allodynia was confirmed. Behavioral responses were determined for whisker pads on both sides at 30 min, 1 h, 3 h, and 6 h after Lapatinib administration.

Morphological Analysis

Aldehyde Fixation. Rats were anesthetized with sodium pentobarbital (70 mg/kg, intraperitoneal) and perfused transcardially with heparinized saline followed by 4% ice-cold paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.4). **Paraffin Embedding.** Infraorbital nerves were dissected out and placed in the same fixative solution at 4°C overnight. Samples were switched to 70% ethanol, dehydrated through graded ethanol, and embedded in paraffin. Infraorbital nerve tissue sections were cut (5 μm), mounted onto glass slides (Superfrost Plus, VWR, Radnor, PA), deparaffinized (Citrisolv, Fisher Scientific, Pittsburg, PA), dehydrated with graded ethanol, and rinsed in ddH_2O. **Hematoxylin and Eosin Staining.** Slides were immersed in 0.1% hematoxylin, washed in tap water, immersed in 0.1% eosin and washed in distilled water. Sections were dehydrated in ethanol and coverslipped (Permount, Fisher Scientific). **Immunofluorescence.** After block of nonspecific antigen sites with 3% normal goat serum (30 min), sections were incubated overnight (4°C) with rabbit anti-myelin protein zero (1:1,000, Abcam, Cambridge, MA) and mouse anti-pan-axonal-neurofilament protein (1:1,000, Convance, Princeton, NJ) antibodies. Subsequently, sections were incubated with secondary antibodies, fluorescein isothiocyanate

donkey anti-mouse antibody and Texas Red donkey anti-rabbit antibody (1:1,000, 1 h, Santa Cruz, Santa Cruz, CA). Sections were coverslipped with glycerol based mounting media (Vector Laboratories, Burlingame, CA). Staining was visualized using a Nikon E1000 microscope (Nikon Instruments, Inc., Melville, NY) equipped with MetaVue and Act-1 Programs. Photomicrographs were taken under the same conditions. Fluorescent intensity was analyzed offline using an advanced image-analysis system MetaMorph (Molecular Devices, Sunnyvale, CA). The numbers of axons with intact myelin sheaths in the best five representative sections of three rats from each group were counted [18,19]. The person who counted the number of myelinated axons was blinded to experimental groups.

Western blot

Infraorbital nerve fragments from the ligation site were dissected and homogenized in radioimmunoprecipitation assay buffer. Total protein samples (50 μg) were loaded onto SDS-PAGE (Bio-Rad, Hercules, CA), transferred to polyvinylidene fluoride membrane, blocked overnight and probed with rabbit anti-ErbB3 (1:200) and mouse anti-β-actin (1:10,000, Santa Cruz, Santa Cruz, CA) (2 h). The membrane was incubated with anti-rabbit and anti-mouse peroxidase-conjugated secondary antibody (1:10,000; GE Healthcare, Piscataway, NJ) (1 h). Immunoreactive proteins were detected by enhanced chemiluminescence kit (Amersham Biosciences, Pittsburgh, PA). Bands recognized by the primary antibody were visualized by X-ray film exposure and analyzed with Image J (National Institutes of Health, Baltimore, MD). The intensities of the ErbB3 bands were normalized to β-actin intensities and the relative intensity of ErbB3 from each group were compared.

Statistics

All data were expressed as mean ± SD, analyzed using the Prism 4 statistical program (Graph Pad Software, Inc., La Jolla, CA). The behavioral changes after nerve injury among four groups (Nrg1Tg and wild type rats with/without nerve ligation) for ipsilateral or contralateral sides were analyzed on a weekly basis by *two-way ANOVA* followed by Bonferroni post-tests. The odds ratio for the effect in week 2 for wild type versus transgenic rats was calculated using McNemar's test with a 95% confidence interval. The mechanical threshold changes on both sides of four groups (Nrg1Tg and wild type rats with/without nerve ligation) over 10 weeks and the time-course data from 0.5, 1, 3, and 6 h after Lapatinib administration were analyzed by repeated-measures *one-way ANOVA* followed by Tukey's Multiple Comparison post hoc tests. For the dose-dependent responses and the western blot data from each group and groups compared to initial level, comparisons were executed by independent *t-test* with two-tailed p values. The same comparisons were done for the contralateral side. A $p \leq 0.05$ was considered significant. There were no missing data from any of the experimental results.

Results

Orofacial Hypersensitivity after CCI-ION in Wild Type Rats

Behavioral testing to determine mechanical threshold of the whisker pad to von Frey fiber stimuli was performed once a week for 10 weeks after CCI-ION in both Nrg1Tg and wild type rats. Behavioral alterations observed after nerve injury were indicative of severe sensory disturbances within the injured nerve receptive territory (*i.e.*, whisker pad). After constrictive nerve injury, rats exhibited responses to mechanical stimuli in two phases. 1) First phase: During the first 2 weeks after CCI-ION there is no change in responses to mechanical stimuli applied to whisker pad with any of the eight test filaments. 2) Second phase (mechanical allodynia): Two weeks or more after CCI-ION surgery, low intensity stimuli applied to the injured nerve territory evokes a sensitized response in wild type rats.

Wild type rats showed mechanical allodynia (3.11 ± 5.93g, n = 9) in week 2 after nerve injury that persisted at least 8 weeks, *i.e.*, a statistically significant decrease in mechanical threshold compared to responses of the sham group (18.72 ± 0.00g, n = 4) ($p < 0.001$) (fig. 1A).

Delayed Hypersensitivity after CCI-ION in Nrg1Tg Rats

There was no mechanical threshold difference between wild type and Nrg1Tg rats prior to surgery or week 1 after CCI-ION. In Nrg1Tg rats development of second phase hypersensitive responses (4.39 ± 3.51g, n = 8) (lowered threshold) occurred with a statistically significant delay (week 6) compared to wild type rats, *i.e.*, the first phase lasted for 5 weeks rather than 1 week. Thus, differences in mechanical thresholds on the whisker pads were statistically significant between wild type and Nrg1Tg rats in week 2-5 (3.44 ± 4.60g *vs.* 18.72 ± 0.00g, $p < 0.001$) and in week 6 (3.05 ± 4.73g *vs.* 9.42 ± 7.74g, $p < 0.01$) postsurgery. Second phase hypersensitivity to mechanical stimuli persisted for 5 weeks in Nrg1Tg rats, from week 6 to week 10 ($p < 0.01$ in week 6 and $p < 0.001$ in week 7-10 compared to sham group of Nrg1Tg rats, n = 4). Mechanical thresholds on the contralateral whisker pads were decreased compared to thresholds of the contralateral sides for both sham groups (wild type and Nrg1Tg rats) (11.54 ± 4.70g, 11.01 ± 2.49g, 10.46 ± 5.10g, $p < 0.05$ in week 5, 8, 9 in wild type rats and 11.47 ± 6.65g, 10.89 ± 6.87g, $p < 0.05$ in week 8, 10 in Nrg1Tg rats) (fig. 1B).

Reversal of Mechanical Allodynia by Functional ErbB3/ErbB2 Heterodimer Inhibitor

Effect of ErbB2 inhibitor Lapatinib on mechanical allodynia was tested at different doses comparing 0.2, 1 and 5 mg/kg to vehicle treatment. Lapatinib was injected intraperitoneally to wild type rats with CCI-ION in week 4 postsurgery. Mechanical allodynia was confirmed before drug administration. Mechanical thresholds to von Frey filaments were tested on whisker pads at 30 min, 1 h, 3 h, and 6 h after drug injection. Lapatinib dose-dependently alleviated mechanical allodynia in wild type rats with CCI-ION at 3 h after drug administration (fig. 2A). Mechanical threshold increased ipsilaterally at 30 min, 1 h, and 3 h in response to 5 mg/kg Lapatinib. The effect reached a peak at 3 h (15 ± 3.89g *vs.* 2.45 ± 1.13g, $p < 0.001$, n = 6) and diminished at 6 h (fig. 2B). There was no difference in mechanical threshold on the contralateral sides either before or after Lapatinib administration (dimethyl sulfoxide vehicle, 0.2, 1, and 5 mg/kg).

Infraorbital nerve injury by loose ligation

Hematoxylin and eosin staining was performed on cross sections obtained from infraorbital nerve injury sites and equivalent nerve segments from the sham group 5 weeks after surgery. Sections from the sham group showed the normal peripheral nerve structure with dense labeling of axons and paler staining of myelin sheaths (fig. 3A inset). In injured nerves, axons were swollen, myelin was diminished, and normal structure was disrupted (fig. 3B inset). However, myelin disruption was more evident with immunofluorescence identifying myelin protein zero (red, fig. 3A). CCI-ION damaged myelin sheaths were evident when stained for myelin protein zero and counter-staining of axons with pan-axonal-neurofilament antibody (green) (fig. 3B). Most myelinated axons were damaged in the injured nerves and few remained with intact myelin sheaths (fig. 3C, table 1). Large myelinated axons (5-10 μM) were absent.

Infraorbital Nerve ErbB3 Increases in Rats with CCI-ION

The ErbB3 receptor protein levels at the CCI-ION site were examined using western blots normalized to β-actin. The ErbB3 receptor levels in injured infraorbital nerves from wild type (in week 1) and Nrg1Tg rats (in week 1 and week 5) were similar to rats with sham

surgery. ErbB3 level increases were statistically significant in weeks 5 and 10 relative to sham wild type rats and rats with CCI-ION in week 1 ($p = 0.038$ in week 5 and $p = 0.013$ in week 10 respectively, $p = 0.041$, 0.014 *vs.* CCI group in week 1, n = 3) (fig. 4A, B). ErbB3 level increases were statistically significant in week 10 ($p = 0.03$ *vs.* sham group in week 10, the CCI group in week 1 $p = 0.034$ and 5 $p = 0.016$, n = 3) in Nrg1Tg rats but not in week 5 ($p = 0.26$ *vs.* sham group in week 5, $p = 0.59$ *vs.* the CCI group in week 1) (fig. 4C, D). Statistically significant increases in ErbB3 receptor levels were concurrent with the behavioral hypersensitivity, *i.e.*, ErbB3 was increased in week 2 after injury in wild type rats and in week 5 in Nrg1Tg rats. These differences were not observed in the contralateral nerves (fig. 5).

Discussion

The present study characterizes behavioral, structural, and neurochemical changes after CCI-ION in both wild type and Nrg1Tg rats. Mechanical allodynia persisting at least 8 weeks in wild type rats in our study is consistent with the 120 day CCI-ION model time course reported by Vos [14]. Myelin sheaths were heavily damaged or destroyed by ligation in both wild type and Nrg1Tg rats. Trigeminal root biopsies from neuropathic pain patients exhibit pathological changes such as axonal loss and demyelination [20,21]. Constrictive sciatic nerve injury induces demyelination as sources of pathological ectopic firing accompanying mechanical allodynia and heat hyperalgesia [3]. Nrg1 is involved in peripheral Schwann cell/ axon communication, growth, migration, and myelination [22]. Mice deficient in *Bace*1 which cleaves the extracellular domain of Nrg1 show myelin impairment and nociceptive hypersensitivity [23]. The present study provided data in support of a role for the Nrg1/ErbB3/ErbB2 signaling complex in axon-Schwann cell interactions promoting mechanical allodynia after nerve injury.

There was initially no difference in infraorbital nerve ErbB3 expression from baseline in Nrg1Tg and wild type CCI-ION rats during the first phase. While mechanical allodynia develops immediately after CCI injury in sciatic nerve [24], infraorbital nerve constrictive injury induced mechanical hypersensitivity is typically evident several days later [14]. Although the first phase is speculated to be a residual effect of anesthetics or increases of endogenous opioids [25], differences in the regions affected (orofacial *vs.* somatic) and the mechanisms involved might be relevant. The increases in mechanical allodynia and ErbB3 were both statistically significant in week 5 after infraorbital nerve injury in wild type rats. However, development of mechanical allodynia was delayed in Nrg1Tg rats after CCI-ION. Our results showed that Nrg1Tg rats have a prolonged first phase during which there is no ErbB3 expression increase and no mechanical allodynia. Thus, increases in ErbB3 receptor levels after nerve injury were concurrent with whisker mechanical allodynia suggesting ErbB3 receptor activation is integrally involved in mechanical sensitization. Increases in ErbB3 phosphorylation and expression levels have been reported in distal nerves after transection of sciatic nerves [26,27].

ErbB3 activation is a crucial time-dependent event among nerve injury signaling in wild type and Nrg1Tg rats suggesting Nrg1 involvement in pain-related behavioral changes following injury. Nrg1 has been shown to increase ErbB3 and olfactory glial proliferation in culture [28]. Nrg1 delivered by virus in transected nerves promoted axonal and Schwann cell growth and increased responses to nociceptive stimuli after sciatic nerve axotomy [29]. Low Nrg1 in Nrg1Tg rats likely provides minimal ErbB3 activation after nerve injury. It is still unclear what compensatory mechanisms promote increased ErbB3 and sensitized behavior in week 10 in Ngr1Tg rats. It is possible that the Nrg1/ErbB response is slowed even more in the mutant Nrg1Tg rats during recovery from nerve trauma.

ErbB3 signals through its active tyrosine kinase heterodimer ErbB2 to trigger downstream cascades. However, in a study by ErbB2 mutant mice with sciatic nerve transection, ErbB2 was found to be nonessential for Schwann cell proliferation and survival [30]. Other potential ErbB mechanisms that might be involved include nerve ligation-induced microglial proliferation and activation initiated by Nrg1-ErbB and ERK1/2/p38 mediation of downstream signaling pathways [31,32].

Lapatinib is used in ErbB2 (HER2)-positive breast cancer therapy and in a phase 2 clinical trial reportedly improved patient functioning, symptom relief, and pain [11]. In the present study, the tyrosine kinase inhibitor, Lapatinib, provides significant alleviation of mechanical allodynia indicating ErbB3/2 involvement in whisker pad mechanical allodynia. Blocking ErbB3 downstream signaling with Lapatinib dose dependently ameliorated the behavioral allodynia in wild type rats. These results also suggest a relationship between whisker pad mechanical allodynia and infraorbital nerve injury-induced peripheral glial activation through ErbB3/ErbB2. The blunting of the behavioral pain-related behavior only on the ipsilateral side suggests a direct effect of the Lapatinib on the injured nerve firing and /or peripheral glial activation, while having no effect on ongoing central sensitization events at the time points tested. On the contralateral side which was uninjured, there was no difference in infraorbital nerve ErbB3 receptor expression comparing wild type and Nrg1Tg rats in week 1, 5, and 10 after the nerve injury. This finding serves as an internal control confirming that the ErbB3 increases in response to the injury.

One important factor contributing to the pathogenesis of trigeminal neuropathic pain is an abnormal compression of the trigeminal nerve by a vein or artery leading to deterioration of myelin sheaths [20]. The investigation of peripheral glial ErbB3/ErbB2 mechanisms in the current study using the constrictive nerve injury model provides implications for potential clinical therapies to alleviate the leading cause of trigeminal neuropathic pain. Reduction of the behavioral hypersensitivity by the ErbB2 inhibitor and the increase in ErbB3 protein after nerve injury suggest enhanced ErbB3/ErbB2 complex signaling following infraorbital nerve injury mediates mechanical allodynia in rat whisker pad.

Interventions addressing peripheral ErbB3/ErbB2 mechanisms could potentially be better choices as nonsurgical therapy instead of microvascular depression surgery, which is sometimes an effective treatment for trigeminal neuropathic pain. Similarly, other nerve constrictive injuries may also be alleviated by an ErbB2 inhibitor.

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MS #201110014 Final box summary

What we already know about this topic

- Peripheral nerve injury results in demyelination and activation of Schwann and other support cells around peripheral nerves
- The role of this process in neuropathic pain remains obscure

What this article tells us that is new

- In rats, genetic disruption of neuregulin-1, which signals proliferation and activation of Schwann cells, delayed downstream receptor signaling and hypersensitivity to touch on the whisker pad after infraorbital nerve injury
- Lapatinab, an experimental drug in Phase II trials the treatment of breast cancer and which targets this downstream receptor signaling pathway, reduced hypersensitivity in rats with nerve injury

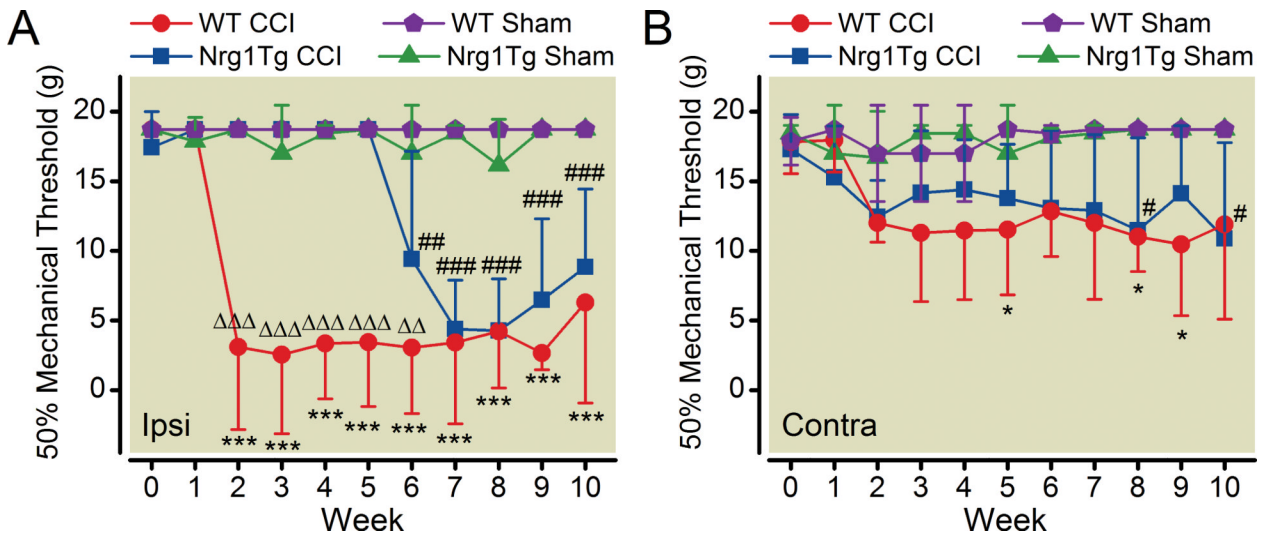

Fig. 1.

(A) Timecourse for development of mechanical allodynia. Mechanical allodynia develops with different time courses in the vibrassal pads of wild type (WT) and neuregulin1 transgenic (Nrg1Tg) rats after chronic constriction injury of infraorbital nerve (CCI-ION). Mechanical sensation threshold on the vibrassal pad in response to von Frey fiber stimulation was tested in both types of rats after CCI-ION. Mechanical allodynia was evident on the ipsilateral side of the nerve injury. Rats exhibit behavioral changes developing in two phases: 1) First hypo-responsive phase: After nerve injury, initially there was no change from baseline recorded in response to mechanical stimuli applied to the whisker pad with any strength test filament. The first phase persisted 5 weeks in Nrg1Tg rats versus 1 week in wild type rats. 2) Second hyper-responsive phase: Mechanical thresholds were markedly decreased during this phase for all groups except the surgical shams. Mechanical allodynia was present by week 2 in wild type rats. Development of allodynic responses was delayed until week 7 in Nrg1Tg rats. There was no difference between wild type and Nrg1Tg rats in weeks 7-10 of the hyper-responsive phase. $p<0.001$ *vs.* sham group of wild type rats; ##$p<0.01$, ###$p<0.001$ in week 7-10 *vs.* sham group of Nrg1Tg rats; ΔΔ$p<0.01$, ΔΔΔ$p<0.001$ *vs.* CCI group of Nrg1Tg rats. (mean±S.D)

(B) Contralateral whisker pad hypersensitivity after surgery. Increased responses (lowered mechanical threshold) were noted on the contralateral whisker pads after CCI in both wild type and Nrg1Tg rats. *$p<0.05$ *vs.* sham group in wild type rats; #$p<0.05$ *vs.* sham group in Nrg1Tg rats. (mean±S.D)

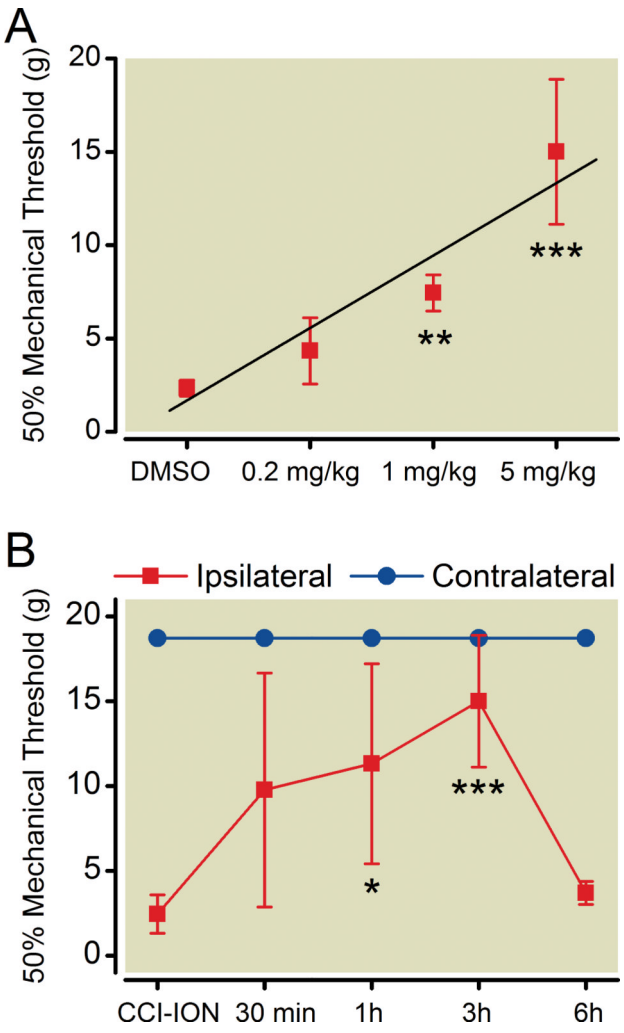

Fig. 2. ErbB2 tyrosine kinase inhibitor reduces mechanical allodynia

Whisker pad mechanical allodynia is alleviated by an active ErbB2 tyrosine kinase inhibitor, Lapatinib, in wild type (WT) rats with chronic constriction injury of infraorbital nerve (CCI-ION). In week 4 post CCI-ION, mechanical threshold in wild type rats was tested to confirm mechanical allodynia to von Frey filaments. Lapatinib was administered by intraperitoneal injection (vehicle, 0.2, 1 and 5mg/kg) after the pre-drug threshold was determined on both whisker pads. Bilateral behavioral responses to mechanical stimuli were monitored 30min, 1h, 3h and 6h post Lapatinib administration. **(A)** Lapatinib dose-dependently (Dimethyl sulfoxide (DMSO), 0.2, 1 and 5mg/kg) decreased mechanical allodynia in wild type rats with CCI-ION at 3h after drug administration. **p<0.01 at 1mg/kg, ***p<0.001 at 5mg/kg *vs.* DMSO. (mean±S.D) **(B)** Mechanical thresholds were increased by Lapatinib administration on the nerve injured side at 30min, 1h and 3h, with a maximal effect found at 3h post. *p<0.05 at 1h, ***p<0.001 at 3h *vs.* pre-drug. There was no change in mechanical threshold on the contralateral side after intraperitoneal injection of Lapatinib. (mean±S.D)

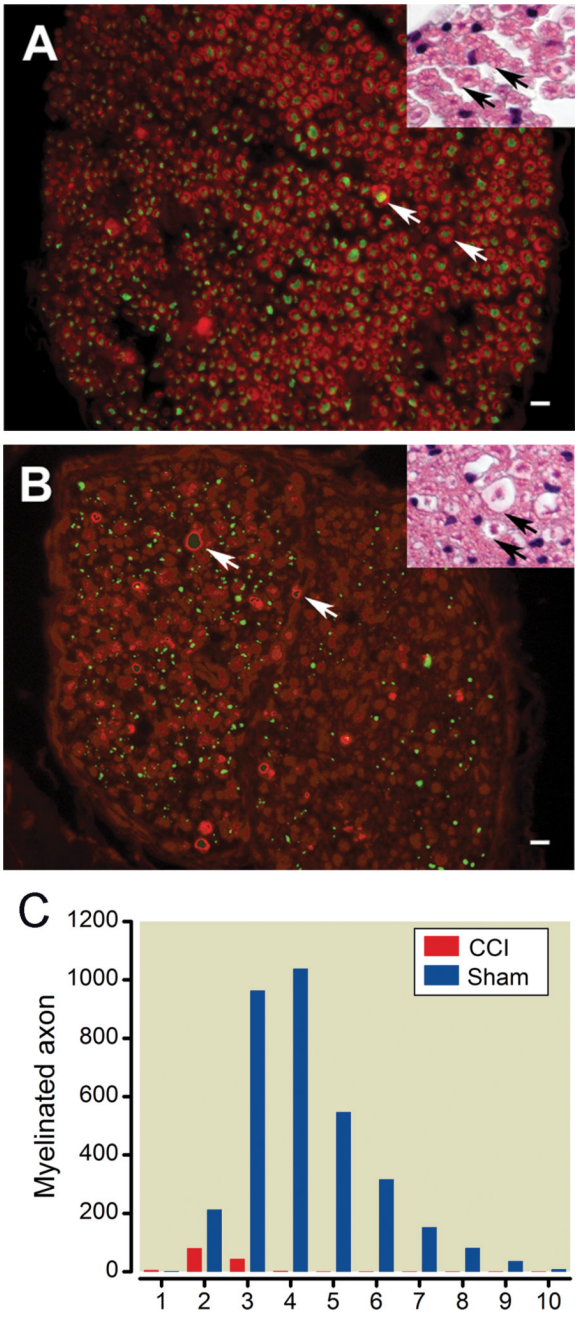

Fig. 3. Morphological alterations produced by chronic constriction injury

(A) Morphology of the sham control infraorbital nerve (ION): The cytoarchitecture of the ION in the surgical sham control group is shown in nerve cross sections. Axons stained for neurofilament 200 by immunofluorescence (green) are surrounded by myelin sheaths (white arrow) stained for myelin protein zero (red). Inset: Hematoxylin and eosin (H&E) staining shows the normal structure of a fascicle of the infraorbital nerve fiber. Each myelinated nerve fiber axon is densely stained and encircled by the lighter Schwann cell myelin sheath ring (black arrows). (B) Morphology after ION chronic constriction injury (CCI): Most MPZ stained myelin sheaths have lost their circular morphology and axonal staining in the center of their structure, i.e. the immunopositive structures (red) wrapping around the axons (green) have collapsed. Axons are seen scattered in the endoneurium without myelin sheaths or with

a few wraps of a damaged myelin sheath (white arrows). Inset: Myelin sheath in infraorbital nerve disrupted by ligation shown 5 weeks after surgery (black arrows). Bars: 10 μm **(C) ION size distribution:** The histogram shows the infraorbital nerve myelinated axon fiber size distribution and changes after constriction injury.

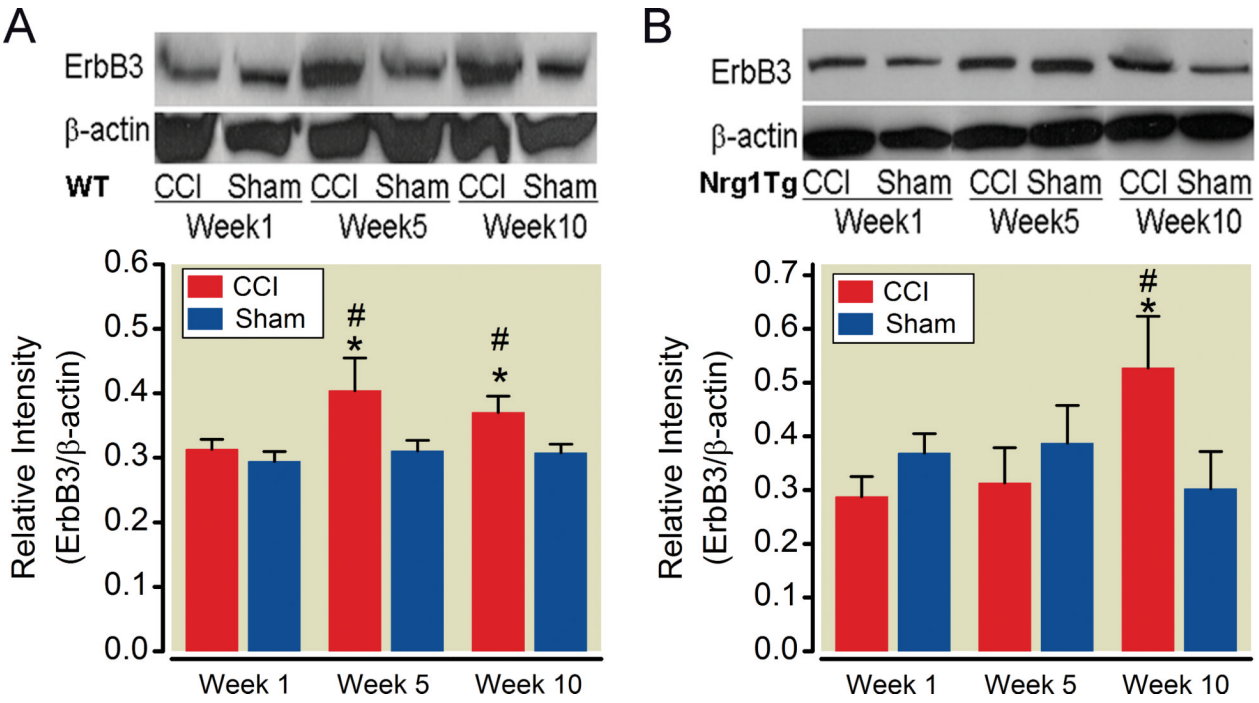

Fig. 4. Infraorbital nerve ErbB3 receptor level increases after nerve injury are delayed in neuregulin1 transgenic (Nrg1Tg) rats

Infraorbital nerve ErbB3 receptor level was determined by western blot analysis at different time points relevant to behavioral changes in rats with both phenotypes. **(A) ErbB3 in wild type rats.** ErbB3 receptor levels are shown for wild type (WT) animals with chronic constriction injury (CCI) and the surgical sham groups. **(B)** For wild type rats, there was no difference in infraorbital nerve ErbB3 receptor expression for the sham and surgery groups in week 1. Statistically significant increases in ErbB3 receptors were observed in week 5 and week 10 after injury in wild type rats with CCI compared to the sham group. **(C) ErbB3 in Nrg1Tg rats.** ErbB3 receptor levels in Nrg1Tg rats are shown for the CCI and the surgical sham groups. **(D)** The increase in ErbB3 receptor level in Nrg1Tg rats was not statistically significant compared to the sham group until week 10 after ligation. The increases in infraorbital nerve ErbB3 receptor levels were concurrent with the mechanical threshold changes in response to von Frey fiber stimuli. *p<0.05 *vs.* CCI group on week 1 in wild type and Nrg1 Tg rats. #p<0.05 *vs.* sham group in both week 5 and 10 in wild type rats, and week 10 in Nrg1 Tg rats. (mean±S.D)

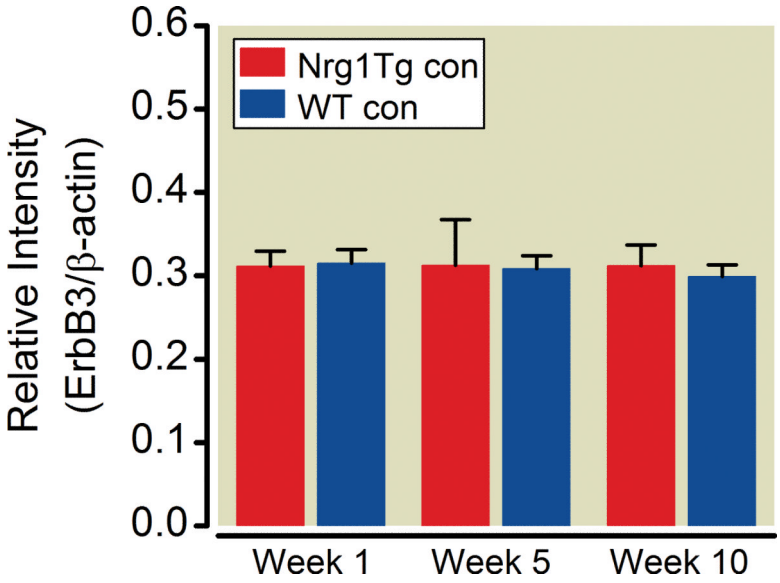

Fig. 5. No contralateral changes in ErbB3 receptor levels

No contralateral (CON) changes were evident in infraorbital nerve ErbB3 receptor levels in the wild type (WT) or neuregulin1 transgenic (Nrg1Tg) rats. Infraorbital nerve ErbB3 receptor level on the contralateral side was determined by western blot analysis at different time points relevant to the behavioral changes in rats with both phenotypes. There was no difference in infraorbital nerve ErbB3 receptor expression comparing wild type (WT) and Nrg1Tg rats in week 1, 5 and 10 after nerve injury on the contralateral side. (mean±S.D)

Table 1

Myelinated Axon Distribution in Infraorbital Nerve after Nerve Injury

	Axon Diameter (μm)									
Myelinated Axons	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sham	1	212	963	1038	546	316	152	81	36	8
CCI-ION	5	80	43	2	0	0	0	0	0	0

CCI-ION = chronic constriction injury of infraorbital nerve