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Reduced $GABA_A$ receptor α6 expression in the trigeminal ganglion alters inflammatory TMJ hypersensitivity

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Abstract

Trigeminal ganglia neurons express the $GABA_A$ receptor subunit alpha 6 (Gabrα6) but the role of this particular subunit in orofacial hypersensitivity is unknown. In this report the function of Gabrα6 was tested by reducing its expression in the trigeminal ganglia and measuring the effect of this reduction on inflammatory temporomandibular joint (TMJ) hypersensitivity. Gabrα6 expression was reduced by infusing the trigeminal ganglia of male Sprague Dawley rats with small interfering RNA (siRNA) having homology to either the Gabrα6 gene (Gabrα6 siRNA) or no known gene (control siRNA). Sixty hours after siRNA infusion the rats received a bilateral TMJ injection of complete Freund's adjuvant to induce an inflammatory response. Hypersensitivity was then quantitated by measuring meal duration, which lengthens when hypersensitivity increases. Neuronal activity in the trigeminal ganglia was also measured by quantitating the amount of phosphorylated ERK. Rats in a different group that did not have TMJ inflammation had an electrode placed in the spinal cord at the level of C1 sixty hours after siRNA infusion to record extracellular electrical activity of neurons that responded to TMJ stimulation. Our results show that Gabrα6 was expressed in both neurons and satellite glia of the trigeminal ganglia and that Gabrα6 positive neurons within the trigeminal ganglia have afferents in the TMJ. Gabrα6 siRNA infusion reduced Gabrα6 gene expression by 30% and significantly lengthened meal duration in rats with TMJ inflammation. Gabrα6 siRNA infusion also significantly increased p-ERK expression in the trigeminal ganglia of rats with TMJ inflammation and increased electrical activity in the spinal cord of rats without TMJ inflammation. These results suggest that maintaining Gabrα6 expression was necessary to inhibit primary sensory afferents in the trigeminal pathway and reduce inflammatory orofacial nociception.

Keywords

pain; nociception; trigeminal ganglia; temporomandibular joint; satellite glia; GABA

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1.1 Introduction

γ-aminobutyric acid (GABA) exerts its inhibitory effects by binding one of two receptor subtypes: $GABA_A$ or $GABA_B$. Activation of ionotropic $GABA_A$ receptors causes increased Cl^- ion conductance, whereas; activation of metabotropic $GABA_B$ receptors primarily activates a second messenger system and modulates G protein gated calcium and potassium channels (Usherwood and Grundfest, 1964; Gahwiler and Brown, 1985; Pfrieger et al., 1994), although direct activation of ion channels is possible upon $GABA_B$ activation (Pfrieger et al., 1994). Evidence suggests that activation of $GABA_A$ receptors outside the brain and spine will reduce pain (Limmroth et al., 1996; Naik et al., 2008). For example, blockade of $GABA_A$ receptors within the temporomandibular joint (TMJ) will increase orofacial hypersensitivity (Cairns et al., 1999; Cai et al., 2001)

The prevalence of TMJ disorders in the United States is estimated at 4.6% (Plesh et al., 2011) and these disorders are the leading cause of chronic orofacial pain (Dworkin et al., 1990). Although a large number of people suffer with TMJ disorders the mechanisms causing this pain are often unknown. A recent search for genes involved in TMJ disorders demonstrated that there was an association between the $GABA_A$ receptor subunit alpha 6 (Gabrα6) and the nociceptive response resulting from TMJ inflammation (Puri et al., 2011). The involvement of GABA in TMJ nociception is not new, previous work has shown neuronal activity in the medullary dorsal horn/spinal cord, the trigeminal ganglia and the TMJ can be affected by changes in GABA signaling (Ginestal and Matute, 1993; Kondo et al., 1995; Almond et al., 1996; Cairns et al., 1999; Cai et al., 2001; Hayasaki et al., 2006; Vit et al., 2009; Anderson et al., 2009). GABA in the trigeminal subnucleus caudalis region has the potential to bind $GABA_A$ receptors and this binding results in neuronal inhibition at both superficial and deep laminae (Ginestal and Matute, 1993; Kondo et al., 1995; Almond et al., 1996; Anderson et al., 2009). It has been found that GABA produced by glutamate decarboxylase (GAD) 65 in the trigeminal ganglia can and will bind to the $GABA_A$ receptor to induce a inhibitory neuronal chloride current (Hayasaki et al., 2006). Moreover, application of a $GABA_A$ receptor antagonist to the trigeminal ganglia will reverse the neuronal inhibition caused by GABA and increase inflammatory orofacial nociception (Vit et al., 2009).

From these previous studies cited above neuronal input from the TMJ can be reduced by $GABA_A$ receptor activation, but to date the contribution of individual $GABA_A$ receptor subunits on orofacial hypersensitivity is unknown. In our lab Gabrα6 was found to be expressed in the trigeminal ganglia and interestingly, mice with a Gabrα6 gene deletion, in addition to having no Gabrα6 expression, showed a reduced number of $GABA_A$ receptors (Nusser et al., 1999), consistent with the idea that the α6 subunit could modulate pain by reducing the number of $GABA_A$ receptors. Thus, we hypothesized that Gabrα6 had a role in modulating orofacial pain (Hayasaki et al., 2006; Puri et al., 2011). To address this hypothesis Gabrα6 expression was knocked down in the trigeminal ganglia, then orofacial hypersensitivity and neuronal activity was measured after the induction of TMJ inflammation.

1.2 Experimental Procedures

Experimental Design

Animal care and welfare—All animal experiments were approved by the Baylor College of Dentistry Institutional Animal Care and Use Committee in accordance with the guidelines of the USDA and National Research Council's "Guide for Care and Use of Laboratory Animals". Male Sprague-Dawley rats (250–300g) were purchased from Harlan Industries, Houston, TX. Female rats respond to pain differently at different stages of the estrous cycle

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and require that the effect of knockdown be analyzed at each estrous stage. A study in females would be a large independent experiment. Upon arrival the animals were housed individually in a temperature-controlled room (23°C) under a 12/12-hour light/dark cycle with lights on at 6:00 AM. The rats were given chow (Harlan Industries) and water ad libitum.

Retrograde labeling of TMJ afferents show Gabrα6 expression—The upper joint space of 3 naïve male rats was injected unilaterally (two rats were injected on the right side and one rat was injected on the left side) with 15 μl of green Lumafluor beads (Lumafluor Incorporated) and after two weeks the trigeminal ganglia were isolated. After sectioning the tissue was stained with Gabrα6 antibody as described below with the exception that the slides were mounted with a 25% sucrose solution instead of Fluormount-G medium due to potential quenching by the glycerol. Green beads were observed only in ganglia ipsilateral to the injected TMJ (data not shown).

Fluorescently labeled siRNA was infused to delineate its spread within the central nervous system—After infusion of 6-carboxyfluorescein (FAM) labeled control siRNA the rat head was embedded in plastic. Embedding included perfusing the rat with 5% paraformaldehyde 5 hours after siRNA infusion, soaking the head for 21 days in a series of solutions; 50% to 100% ethanol, acetone and methyl methacrylate. Soaking occurred for 3 days in each solution. Once the methyl methacrylate hardened the block was sectioned with an IsoMet 1000 saw (Buehler, Lake Bluff, IL) and the 0.5 mm sections were imaged.

Effect of Gabrα6 siRNA infusion on inflammatory TMJ hypersensitivity—Male rats were infused with either saline, control siRNA or Gabrα6 siRNA; sixty hours after infusion the rats received bilateral TMJ injections of saline or CFA. The infused and injected rats were divided into six groups; 1) trigeminal ganglia saline infusion/TMJ saline injection; 2) trigeminal ganglia saline infusion/TMJ CFA injection; 3) trigeminal ganglia control siRNA infusion/TMJ saline injection; 4) trigeminal ganglia control siRNA infusion/TMJ CFA injection; 5) trigeminal ganglia Gabrα6 siRNA infusion/TMJ saline injection; and 6) trigeminal ganglia Gabrα6 siRNA infusion/TMJ CFA injection. Meal duration was recorded before and after infusion and injection. Two days following TMJ injection the animals were sacrificed and the trigeminal ganglia were collected. A trigeminal ganglia from one side was sectioned and stained on alternate slides for phosphorylated extracellular signal-regulated kinase (p-ERK) and the neuronal marker NeuN or Gabrα6 and NeuN. Stained cells were then counted. The trigeminal ganglia from the opposite side were assayed by western blot. There were 9 rats in each treatment group.

Effect of Gabrα6 siRNA infusion on neuronal electrical activity—After bilateral cannulation of the trigeminal ganglia the rats were divided such that one group was infused with Gabrα6 siRNA and the second group was infused with control siRNA. Sixty hours later, electrodes were placed in the lamina II/III layer at the C1 level of the spinal cord and the neuronal activity was recorded at baseline and in response to three repeated adenosine triphosphate (ATP) injections into the TMJ (30 min intervals). A multiple injection paradigm allowed for confirmation that knocking down gene expression would modulate spike activity. It has been previously shown that TMJ nociceptive afferents terminate in the upper lamina at the level of C1 and are activated by injecting ATP into the TMJ (Tashiro et al., 2008; Tashiro et al., 2009b). Only a single recording was performed on each rat. 12 rats were included for each treatment group.

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General Methods

Guide cannula placement—Rats were anesthetized with a mixture of ketamine (75 mg/kg) and xylazine (0.5 mg/kg) injected intramuscularly (Bellinger and Tillberg, 1997). Guide cannulas (22 GA, Plastics One Inc, Roanoke, VA) were stereotaxically (David Kofp Instruments, Tujunga, California) placed in the trigeminal ganglia bilaterally using coordinates 4.3 mm posterior of bregma, 3.4 lateral of the midline at a depth of 10.3 mm (Paxinos and Watson, 2007). After surgery the animals were given 75,000 units of penicillin G prophylactically and were allowed to recover for a week. Stereotaxic coordinates were established in a preliminary trial by injecting India ink through the cannulas followed by a postmortem histological examination (data not shown). In all animals cannula placement was confirmed by x-ray (Fig. 1A, superior view and Fig 1B, lateral view).

Trigeminal ganglion infusions—Rat were anaesthetized with 5% isoflurane and infused bilaterally with 5 μl of control or siRNA solution over a 5 min period (Plastics One Inc, Roanoke, VA). Depending on the experiment the infusion solution was 0.9% saline (i.e., control) or a polyethyleneimine (PEI) solution containing 2.5 μg Gabrα6 siRNA or 2.5 μg control siRNA/ganglia. In one experiment the control siRNA was conjugated to FAM. Gabrα6 siRNA or control siRNA (Invitrogen, Carlsbad, CA) was mixed with linear PEI (In vivo JetPEI, Cat# 201-10, PolyPlus Transfection) to increase the transfection efficiency (Boussif et al., 1995). The ratio of cationic PEI amines (N) to nucleic acid phosphates (P) was N/P=6. This amount of siRNA and PEI was based on a previous study where siRNA knockdown in vivo had been optimized (Kramer et al., 2010b), although we do not exclude the possibility that further optimization would be possible. The control siRNA had no homology to any known gene and was identified as Silencer Negative Control #1 siRNA (5′-AGUACUGCUUACGAUACGGtt-3′, 5′-CCGUAUCGUAAGCAGUACUtt-3′) and the Gabrα6 siRNA sequence was sense (5′-GGAACGAUCCUGUACACCA tt-3′), anti-sense (3′-UGGUGUACAGGAUCGUUCCat-5′). Animals were alert and mobile less than 5 minutes after exposure to the isoflurane.

TMJ injections—Rats were infused as described above and 60 hours later the rats received TMJ injections performed under 5% isoflurane anesthesia. Bilateral TMJ injections included 15 μg of CFA or 0.9% saline (15 μl). The animals were then sacrificed two days after the TMJ injection and the trigeminal ganglia were harvested for immunocytochemistry and western blots.

TMJ hypersensitivity assay: Meal pattern analysis—Meal duration is a proven, dose dependent behavioral marker of TMJ hypersensitivity in undisturbed male and female rats (Kerins et al., 2005; Guan et al., 2005; Bellinger et al., 2007; Kramer and Bellinger, 2009; Kramer et al., 2010a). In these studies rats were housed individually in 32 sound-attenuated chambers equipped with computer-activated pellet feeders (Med Assoc. Inc., East Fairfield, VT). The rats were given 45 mg rodent chow pellets (Product No. FO 165, Bioserv, Frenchtown, NJ). When a rat removes a pellet from the feeder trough, a photobeam placed at the bottom of the trough is no longer blocked, signaling the computer to drop another pellet. The computer records the date and time that each pellet was removed, and keeps a running tally of the total daily food consumption. The computer record of pellets dropped over time establishes the meal duration, which is a continuous non-invasive biological marker of TMJ hypersensitivity in undisturbed male and female rats. Animals were placed in the feeders a week after trigeminal ganglia cannulation surgery and were allowed to acclimate to the feeders for 4 days before infusion of the siRNA.

Extracellular recording of electrical activity—The experimental design included a series of three 20 μl TMJ injections of the neurotransmitter ATP (1 mM, Sigma, St. Louis,

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MO) injected over a 4–7 sec time period with an inter-injection interval of 30 minutes. The physiological concentration of ATP used in this experiment does not cause inflammation (Morris et al., 1985; Green et al., 1993) and can be repeatedly injected into the TMJ without causing sensitization or tachyphylaxis of the evoked response (Tashiro et al., 2008).

Extracellular recordings were performed on animals after injection with sodium pentobarbital (50 mg/kg, i.p.). Anesthesia was maintained by continuous intravenous administration of sodium pentobarbital at a rate of 5 mg/hour. Proper depth of anesthesia was identified by the absence of pupil and hind limb withdrawal reflexes. The rats were monitored throughout the experiment and body temperature was maintained at 37 °C using a feedback controlled heating pad and thermal probe. Tracheotomy was performed and artificial respiration was administered. The cervical vertebrae C1 and C2 were removed to expose the caudal brainstem and spinal cord by laminectomy and the exposed area was bathed with 37°C mineral oil. A tungsten microelectrode (10–12 MΩ, FHC) was used for the electrophysiological recordings and this electrode was connected to a CED 1401Plus data acquisition system (Cambridge Electronic Design Ltd, UK). The spike activity was recorded using SPIKE2 computer software; SPIKE2 was also used to calculate the spike activity/second as well as the mean spike activity during a 5 minute period.

Neuronal activity in this study was acquired from cells in lamina II/III layer at the level of C1. These neurons were responsive to press of the skin and overlying muscle of the TMJ condyle with a 6.1 von Frey filament (i.e., 980 miliNewtons). When applied to the investigators skin the force of the press was near pain threshold. A brush of the skin with soft bristles was also completed on a region overlying the condyle. Previous studies further classified these neurons as either wide-dynamic range or nociceptive (Okamoto et al., 2003). Wide-dynamic range neurons were excited by brush and nociceptive neurons were activated by press of the 6.1 von Frey filament. The same number of wide-dynamic range and nociceptive neurons was analyzed for each treatment group. A minority of the cells that were responsive to only brushing of the skin were not included in this study. The recording electrode was placed on the left side of the midline at the C1 level for collecting data on electrical activity.

Sample collection, tissue processing and immunofluorescence—A trigeminal ganglia from each treated rat was removed and fixed in 5% paraformaldehyde and then stored in a 25% sucrose solution. Each trigeminal ganglia was sagittally cut at a thickness of 24 μm on a cryostat. The sections were stored in 25% sucrose until used. Immunohistochemistry was completed on floating sections by first quenching 30 minutes with 0.3% hydrogen peroxide followed by three 20 minute (phosphate buffered saline) PBS rinses and a 1 hour blocking step (PBS, 5% normal goat serum, 0.3% Triton X-100). Following three rinses in PBS, the sections were incubated overnight at 4 °C with the primary antibodies in a solution of PBS, 1% BSA and 0.3% Triton X-100. The primary antibodies included a mixture anti-neuronal marker NeuN (1:1000, Millipore Billerica, MA, monoclonal) and anti-Gabrα6 (1:1000, Millipore, rabbit polyclonal) or an antibody against p-ERK (1:500, Cell Signaling Technology, Danvers, MA, rabbit polyclonal). Alternate sections were incubated with primary antibodies against the satellite glial marker glutamine synthetase (Millipore, 1:300, monoclonal) and the anti-Gabrα6 antibody. To determine the specificity of the Gabrα6 antibody a control experiment was performed where 1.5 μg of Gabrα6 antibody was mixed with 3 μg of peptide (KLEDEGNFYSKNISR, Biomatik) in 1.5 ml of the above antibody solution. After an overnight incubation at 4 °C the pre-absorbed antibody was placed on tissue for another overnight incubation. After the overnight incubation with primary antibodies the slides were rinsed 3 times in PBS followed by a 2 hour incubation step with fluorescently tagged secondary antibodies (PBS, 1% BSA, 0.3% Triton X-100). The secondary antibodies included a goat anti-rabbit 488 or 568 and a goat

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anti-mouse 488 or 633 from Invitrogen. After incubation with the secondary antibodies the sections were rinsed again three times in PBS, placed on slides and the sections were mounted with fluoromount-G medium (Southern Biotech, Birmingham, AL). Occasionally the medium contained 4′-6-Diamidino-2-phenylindole (DAPI). Images were captured using a Nikon epifluorescent microscope and a Photometrics CoolSnap K4 CCD camera (Roper Scientific, Inc, Duluth, GA). Nikon Imaging Software-Elements (Melville, NY) controlled the camera. Controls in which the primary antibody was not included showed no signal (data not shown).

Cell counts—Cells that had a green (often cytoplasmic) and red (often nuclear) or yellow signal within a single cell, that cell was counted as a Gabra6/NeuN or a p-ERK/NeuN positive cell. Any red signal in the nuclei was counted as a NeuN positive cell. The percentage of Gabrα6/NeuN and p-ERK/NeuN positive cells was determined on every fifth section to obtain a representative sample through the entire trigeminal ganglia region (West and Slomanka, 2001). When calculating the percent of Gabrα6 neurons we used the formula = (# of cells co-expressing Gabrα6 and NeuN/total # cells expressing NeuN) × 100. When calculating the percent of p-ERK positive neurons we used the formula = (# of cells co-expressing p-ERK and NeuN/total # cells expressing NeuN) × 100.

Western blot analysis—A trigeminal ganglia from each rat was ground in 300 μl of T-Per tissue protein extraction reagent containing Halt Protease Inhibitor (Thermo Scientific, Rockford, IL). Total protein was determined in each sample using a BCA protein assay (Thermo Scientific) and 15 μg of total protein was loaded into each well of a 4–12% Bis-Tris acrylamide gel (Invitrogen, Carlsbad, CA). The gel was electrophoresed at 200 volts for 35 minutes and the proteins transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was rinsed in Tris-buffered saline containing 0.1% Tween-20 and then blocked for one hour in this buffer containing 5% milk. After three more rinses the membranes were placed in the block solution with either an antibody against 1) Gabrα6 (1:1,500) or 2) against p-ERK (1:2000) or 3) against β-actin (1:2000, rabbit polyclonal, Cell Signaling). The membranes were probed first with anti-Gabrα6, stripped (Re-blot Plus Mild, Millipore) and probed second with anti-p-ERK and then stripped again and probed third with anti-β-actin. For each antibody an overnight incubation at 4°C was completed and the membranes were rinsed three times and incubated with an HRP conjugated goat anti-rabbit antibody (1:500, Cell Signaling) at room temperature for 90 minutes. After incubation in this second antibody the membranes were rinsed three times and reacted with the ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire UK). After exposure of the membrane to the film and development of the film, the bands were quantitated with Scion Image software. An area and mean value were multiplied to obtain the optical density of the band. Values were reported as a ratio of the optical density of the protein band of interest (i.e., Gabrα6 or p-ERK) divided by the optical density of the β-actin band. Ratio values were multiplied by a factor of 10 to yield an integer value. Controls in which the primary antibody was not included showed no signal (data not shown).

Statistical analysis—Meal duration, spike activity and western data were analyzed by a two-way ANOVA (ABstat software, V1.94). The independent variables included the control siRNA/Gabrα6 siRNA infusion and TMJ injection treatment in addition to the day of treatment, when applicable. The dependent variable was meal duration, spike activity or the optical density ratio. Statistically significant data was further analyzed with a Bonferroni post hoc test. Statistical analysis on the cell count data was completed using a student's t-test (Graph Pad Prism version 5.0, Graph Pad, San Diego, CA). Data with $p < 0.05$ was considered significant. All values were presented as mean ± SEM.

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1.3 Results

$GABA_A$ receptor α6 subunit expression in trigeminal ganglia neurons

Gabrα6 expression was present in neurons (Fig. 2A and D, red cells) and satellite glial cells (Fig. 2A, yellow, arrows) but not all satellite glia in the trigeminal ganglia stained for Gabrα6 (Fig. 2D–F). Satellite glia (Fig. 2C and F) surround the trigeminal ganglia neurons (Fig. 2B and E) as expected. CFA injected animals showed a similar result as the non-injected rats (data not shown). Insert in panel 2E is a representative image of trigeminal ganglia sections incubated with pre-absorbed Gabrα6 antibody, no signal for Gabrα6 was observed on 5 trigeminal sections stained with pre-absorbed antibody.

Gabrα6 positive neurons innervate the TMJ

Retrograde tracer was injected into the TMJ two weeks prior to sacrifice and trigeminal ganglia cells that were loaded with tracer (Fig. 3A) were also positive for Gabrα6 (Fig. 3B and C, arrows).

Reduction of Gabrα6 expression by infusion of siRNA directly into the trigeminal ganglia

A fluorescent signal was observed in the trigeminal ganglia and cells lining the interior of the ventricle after infusion of FAM labeled siRNA (Fig. 4A, green). Infused FAM siRNA was not observed 9.0 mm posterior to bregma (data not shown). Panel 4B shows the anatomy of the coronal brain slice imaged in panel 4A. Panel 4C shows the location of the infusion cannula on a cartoon image of the trigeminal ganglia.

Gabrα6 expression was reduced in the trigeminal ganglia of Gabrα6 siRNA infused rats as compared to control siRNA infused rats (compare Fig. 4D and 4E). After infusion of control siRNA 86% of the small neurons (<30 μm), 74% of the medium size neurons (30–39 μm) and 74% of the large neurons (>40 μm) were Gabrα6 positive. A count of Gabrα6 positive neurons showed that the percentage of Gabrα6 positive neurons decreased approximately 30% after infusing Gabrα6 siRNA versus infusing control siRNA. Consistent with the cell count data a significant reduction in the amount of Gabrα6 protein on the western blot was observed in rats infused with Gabrα6 siRNA versus controls (Fig. 4F). CFA injection did not significantly alter Gabrα6 expression in either control siRNA or Gabrα6 siRNA infused animals (Fig. 4F).

Gabrα6 knock-down in the trigeminal ganglia enhances hypersensitivity

There was no change in the 24 hour meal duration after infusion of Gabrα6 siRNA or control siRNA (Fig. 5, 2 days (d) post-siRNA). Note: this is before TMJ injection of CFA. Meal duration was significantly longer 24 and 48 hours after TMJ CFA injection (Fig. 5, 1d post-CFA, 2d post-CFA). In rats with CFA induced TMJ arthritis Gabrα6 siRNA infusion resulted in significantly longer meal duration than was observed in saline or control siRNA infused rats (Fig. 5).

Knock-down of Gabrα6 increased p-ERK expression in the trigeminal ganglia

P-ERK staining was higher in the rats that received Gabrα6 siRNA as compared to control siRNA rats, whether the TMJ was injected with saline (compare Fig. 6A to 6C) or CFA (compare Fig. 6B to D). To measure changes in p-ERK expression, a western blot for p-ERK protein expression was performed (Fig. 6E). Quantitation of the bands from the western blot indicated that infusion of Gabrα6 siRNA significantly increased p-ERK protein expression in the trigeminal ganglia versus controls (Fig. 6F). Moreover, two-way ANOVA of this western data indicated a significant CFA effect ($P<0.01$) for p-ERK in the trigeminal ganglia, consistent with previous work (Liverman et al., 2009)..

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Increased neuronal activity after knock-down of the Gabrα6 subunit

Animals infused with Gabrα6 siRNA had a five-fold increase in the number spikes per second in comparison to rats infused with control siRNA (Fig. 7A, compare middle and top histogram). These observations were supported by quantitation of the data both before and after ATP injection where the mean spike activity increased as a result of Gabrα6 siRNA infusion (Fig. 7B). After Gabrα6 siRNA infusion the 1st and 2nd ATP injection increased spike activity as compared to the "before injection" group but the 3rd injection of ATP did not significantly increase activity (Fig. 7B).

1.4 Discussion

TMJ hypersensitivity, as measured by meal duration and p-ERK expression increased after Gabrα6 knock-down. Meal duration is a continuous non-invasive biological marker of TMJ nociception (surface and deep) in undisturbed male and female rats (Kerins et al., 2003; Kerins et al., 2005; Kramer and Bellinger, 2009). In this report meal duration increased after knock-down of Gabrα6 expression suggesting Gabrα6 inhibited nociception in a rat with an inflamed TMJ. Because p-ERK is widely used as a marker for neuronal activation in both TMJ and tooth pulp models (Shimizu et al., 2006; Suzuki et al., 2007; Liverman et al., 2009), the increase in p-ERK observed in this study suggests neuronal activity increased in the trigeminal ganglia after Gabrα6 siRNA infusion. In addition to hypersensitivity and neuronal activation, electrical activity was quantitated from regions of the spinal cord that were responsive to TMJ stimulation. After injecting the TMJ with green retrograde beads we estimate 9 out of 10 green cells were Gabrα6 positive. This preliminary result is consistent with the idea that a majority of the TMJ's primary afferents are Gabrα6 positive and that a majority of the recorded cells (responsive to TMJ stimulation) were Gabrα6 positive. If a majority of the electrical recordings were from TMJ responsive neurons containing Gabrα6 then Gabrα6 within these primary afferents likely inhibits inflammatory TMJ nociceptive responses.

How might a decrease in Gabrα6 expression reduce the inhibitory action GABA. Previous work has demonstrated that a reduction in specific $GABA_A$ receptor subunits can lead to a reduction in the amount of functional $GABA_A$ receptor and inhibit the activity of GABA (Yu et al., 1996; Nusser et al., 1999; Wei et al., 2003; Glykys et al., 2008). In the spinal cord, neuronal inhibition will occur after blockade or deletion of $GABA_A$ receptor subunits α2, α3 and α5 (Munro et al., 2008; Knabl et al., 2008; Knabl et al., 2009), and a recent study demonstrated presynaptic inhibition resulting from primary afferent depolarization (PAD) required the primary afferents to express α2 (Witschi et al., 2011). $GABA_A$ receptor α1, α5, β2, β3, γ1, γ2 and γ3 subunits have been observed in most trigeminal ganglia neurons but α3, α4, α6, and δ subunits are expressed to a lesser degree and no α2 expression can be detected (Hayasaki et al., 2006). From our recent work we did not see any changes in α1, β2 and γ2 transcript when TMJ hypersensitivity changed significantly but these were the only $GABA_A$ receptor subunits measured (Puri et al., 2011). In previous work deletion of Gabrα6 reduced the amount of $GABA_A$ β2, β3 and γ2 subunits by 50%, 20% and 40%, respectively resulting in half the number of $GABA_A$ receptors in the cerebellum (Nusser et al., 1999). Alternatively, the α1 and α5 subunits found in all the trigeminal ganglia neurons might compensate for depletion of the alpha6 subunits (Hayasaki et al., 2006) but previous work has shown that alternate subunits did not rescue a Gabrα6 deletion (Jones et al., 1997). These results are consistent with the idea that the increased hypersensitivity resulting from Gabrα6 knock-down is due to an overall reduction in functional $GABA_A$ receptors. Quantitation of other GABA receptor subunits along with receptor binding assays would reveal potential reductions in the amount of $GABA_A$ receptor after Gabrα6 knock-down.

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GABA, within the inhibitory pathway, has been shown to be predominantly located in the interneurons in the sensory trigeminal nuclei (Avendano et al., 2005). GABA immune-reactive neurons are present in the trigeminal subnucleus caudalis region (Ginestal and Matute, 1993; Kondo et al., 1995; Almond et al., 1996) and $GABA_A$ receptor-mediated inhibition occurs in the superficial and deep laminae of the spinal cord (Anderson et al., 2009). Studies have also demonstrated the role that activation of GABAergic neurons have in the trigeminal nucleus caudalis (Vc) and trigeminal nucleus interpolaris (Vi) following tooth pulp stimulation (Wu et al., 2010) and these neurons respond to $GABA_A$ receptor modulators muscimol and bicculline following stimulation (Takemura et al., 2000). GABA binding to its receptor can induce chloride channel opening and an outward chloride current causing PAD and presynaptic inhibition (varez-Leefmans et al., 1988; Sung et al., 2000). This presynaptic inhibition resulting from the chloride is dependent on active inward pumping of Cl^- ions potentially by the presence of the NKCC1 cotransporter or a lack of the KCC2 cotransporter in the primary afferents (Toyoda et al., 2005; Price et al., 2006). In addition to the trigeminal nuclei, GABA could have some effect within the ganglia. At a molecular level glutamate decarboxylase 65 (GAD65), an enzyme that synthesizes GABA from glutamate, is present in the sensory neurons of the trigeminal ganglia and GABA, once produced, accumulates in cells of the trigeminal ganglia (Szabat et al., 1992; Kuroda et al., 2000; Hayasaki et al., 2006). Measurement of GABA release from isolated trigeminal ganglia cells suggests that high extracellular K+ induces a Ca^{2+} dependent release of GABA (Hayasaki et al., 2006). Once released GABA binds to $GABA_A$ receptors within the trigeminal ganglia inducing an inhibitory inward Cl^- current (Kondo et al., 1994; Hayasaki et al., 2006; Vit et al., 2009), desensitizing voltage-sensitive channels, resulting in inhibited neuronal activity. Patch-clamp studies with trigeminal ganglia slices after treatment with Gabrα6 or control siRNA and modulators of ion pumps could address the role of this receptor subunit in modulating GABA action within the ganglia itself.

Interestingly, the NKCC1 cotransporter is expressed on satellite glia (Price et al., 2006) as well as neurons and it would be interesting study if Gabrα6 containing satellite glia have a role in neuronal inhibition. Gabrα6 was expressed on satellite glia in this study and may affect pain by modulating the physiology of satellite glia as well as neurons. Based on the western blot data siRNA infusion reduced Gabrα6 expression by 30% but an experiment counting glutamine synthase/Gabrα6 positive cells is needed to directly show siRNA knocked down Gabrα6 in satellite glia. In a previous study (Vit et al., 2009) $GABA_A$ receptors appeared to be present on satellite glia although the study did not utilize a glutamine synthase marker to verify localization. Using isolated, cultured satellite glia could determine the response of these cells to GABA after modulation of Gabrα6 expression.

In this study control rats had a maximum activity of 50–70 spikes per second after the first and second injection of ATP into the TMJ, which is within range of previous work in rats (Tashiro et al., 2009a; Tashiro et al., 2009b), but the reduction in spike activity on the third injection is unique and may result from cannulation of the ganglia. Alternatively, neurotransmitter stores in the neurons became depleted with subsequent ATP injections, thus causing a reduction in the spike activity. Alternate explanations could include either, “adaptation”- a decrease in sensitivity due to repetitive stimuli (Mumford, 1965; Ernst et al., 1986; Hummel et al., 1996) or desensitization of the ATP receptors (Mierlak and Farb, 1988). This reduction in neuronal spike activity could correlate to reduced pain perception, as has been reported through repeated stimulation in a given subject (Ernst et al., 1986).

TMJ inflammation did not significantly alter Gabrα6 expression in the trigeminal ganglia but previous work has shown increased expression of $GABA_A$ and $GABA_B$ receptors when peripheral inflammation is present (Castro-Lopes et al., 1992; Castro-Lopes et al., 1994; McCarson and Enna, 1999). GABA binding can increase in the presence of inflammation

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(Castro-Lopes et al., 1995) and nerve ligature will reduce $GABA_A$ receptor expression in the dorsal root ganglia (Fukuoka et al., 1998). But we saw no change in Gabrα6 expression after induction of TMJ inflammation. One explanation for this results could be that only a subset of neurons were altered by CFA injection, an experiment where localized changes in expression and receptor binding were measured in the trigeminal ganglia should address this question.

Conclusion

In summary, knock-down of Gabrα6 in the trigeminal ganglia increased TMJ hypersensitivity and neuronal activity in the trigeminal ganglia and induced spike activity in neurons receiving input from the TMJ, consistent with the idea that GABA inhibits nociceptive input from the TMJ by a mechanism dependent, in part, on Gabrα6.

Acknowledgments

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List of Abbreviations

GABA	γ-aminobutyric acid
Gabrα6	$GABA_A$ receptor subunit alpha 6
TMJ	temporomandibular joint
ERK	extracellular signal-regulated kinase
siRNA	small interfering RNA
CFA	complete Freund's adjuvant
FAM	6-carboxyfluorescein
GAD65	glutamate decarboxylase 65
PBS	phosphate buffered saline
DAPI	4′-6-Diamidino-2-phenylindole
PVDF	polyvinylidene fluoride
PAD	primary afferent depolarization
SEM	standard error of the mean

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Highlights

- GABA receptor subunit Gabrα6 is expressed in trigeminal ganglia.
- Gabrα6 is expressed in primary afferents of the temporomandibular joint
- siRNA in trigeminal ganglia reduced Gabrα6 expression.
- reduced Gabrα6 expression increased inflammatory hypersensitivity in the jaw joint.
- Gabrα6 part of pathway regulating inflammatory temporomandibular pain

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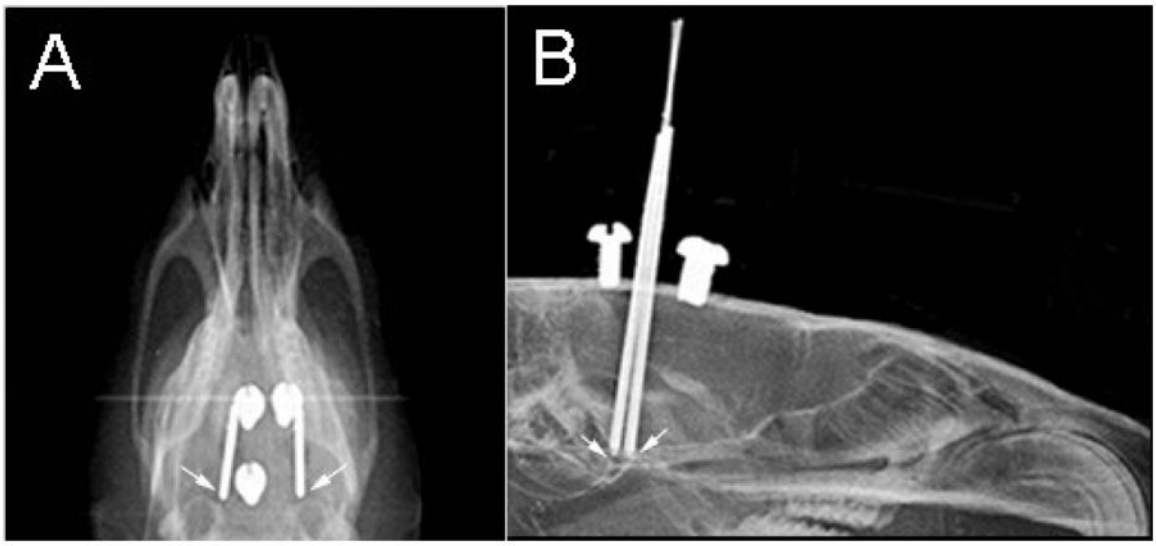

Figure 1. Radiograph of a rat head shows that the trigeminal ganglia guide cannula placement
The arrows point at the tip of the guide cannulas placed in the trigeminal ganglia V_3 region, a superior view (A). In a lateral view (B) an obturator extends 0.5 mm below the guide cannula (arrows).

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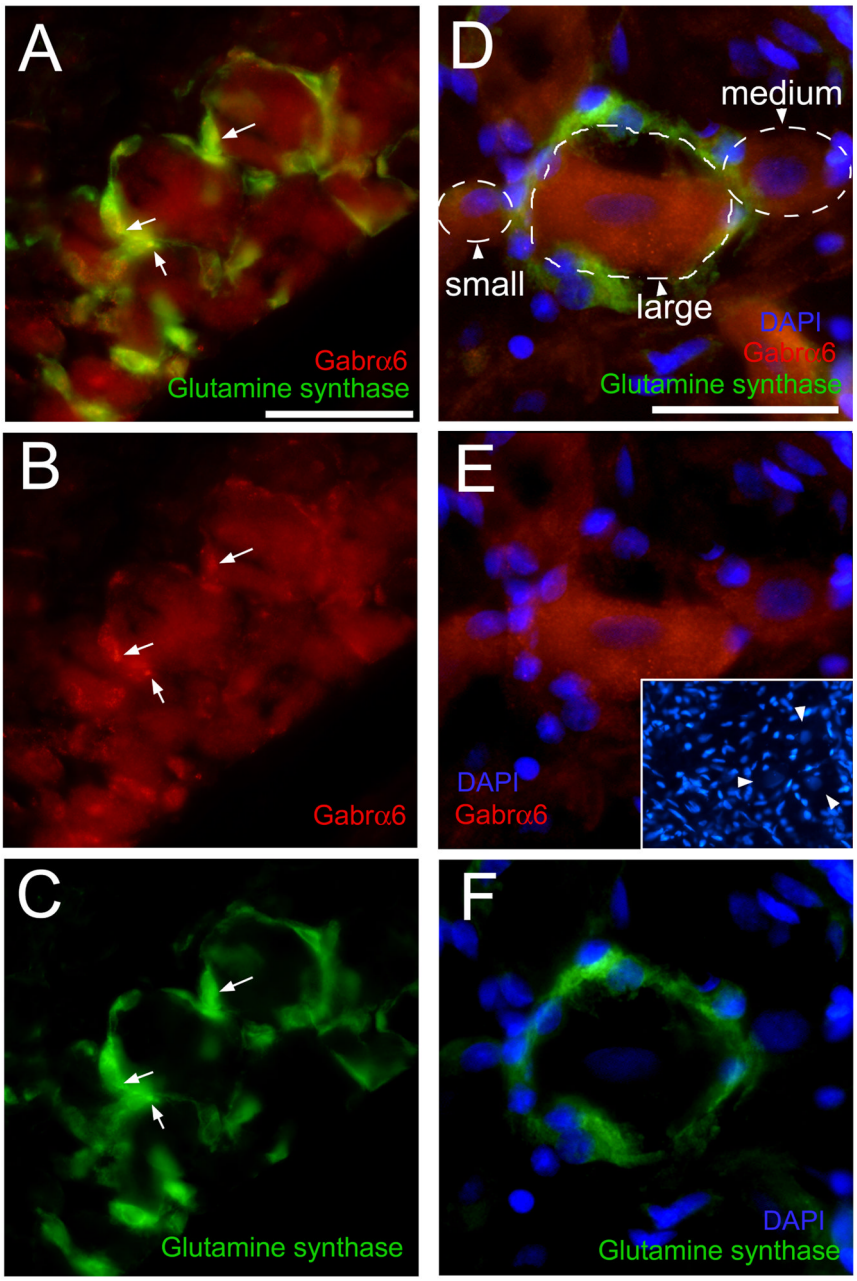

Figure 2. Expression of Gabrα6 in trigeminal ganglia neurons and satellite glia

(A) Gabrα6 (red) and glutamine synthase (green) are co-expressed in several cells (yellow, arrows) but in other cells (D) Gabrα6 expression did not co-localize with glutamine synthase. Cells were stained for Gabrα6 (panels B and E) or the satellite glial marker glutamine synthase (panels C and F). (D) Gabrα6 expression was observed in small (<30 μm), medium (30–39 μm) and large neurons (>40 μm); representative cells are indicated by white dashes and arrowheads. Insert in panel E shows trigeminal ganglia tissue after staining with a Gabrα6 antibody pre-absorbed with a Gabrα6 peptide (arrowheads point to large nuclei indicative of neurons). Nuclear DAPI stain is in blue. Size bar equals 50 μm.

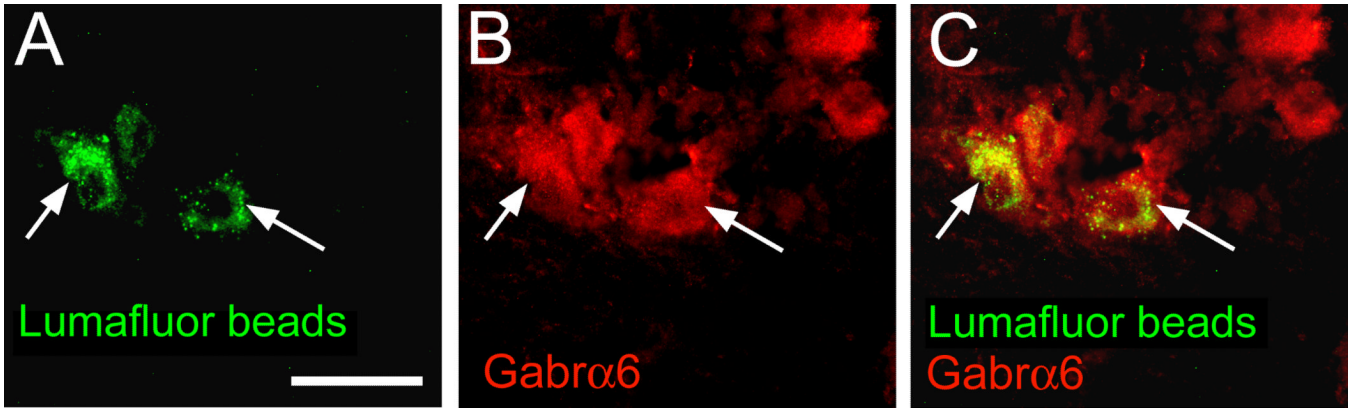

Figure 3. Trigeminal ganglia neurons projecting to the TMJ expressed Gabr α6

Lumafluor beads were injected into the TMJ and after two weeks the trigeminal ganglia were isolated, sectioned and stained for Gabrα6. (A) Green Lumafluor beads were present in trigeminal ganglia cells that stained for Gabrα6 (panel B, red). (C) Double labeled cells are yellow (arrows). Images are representative of three rats. Size bar equals 50 μm.

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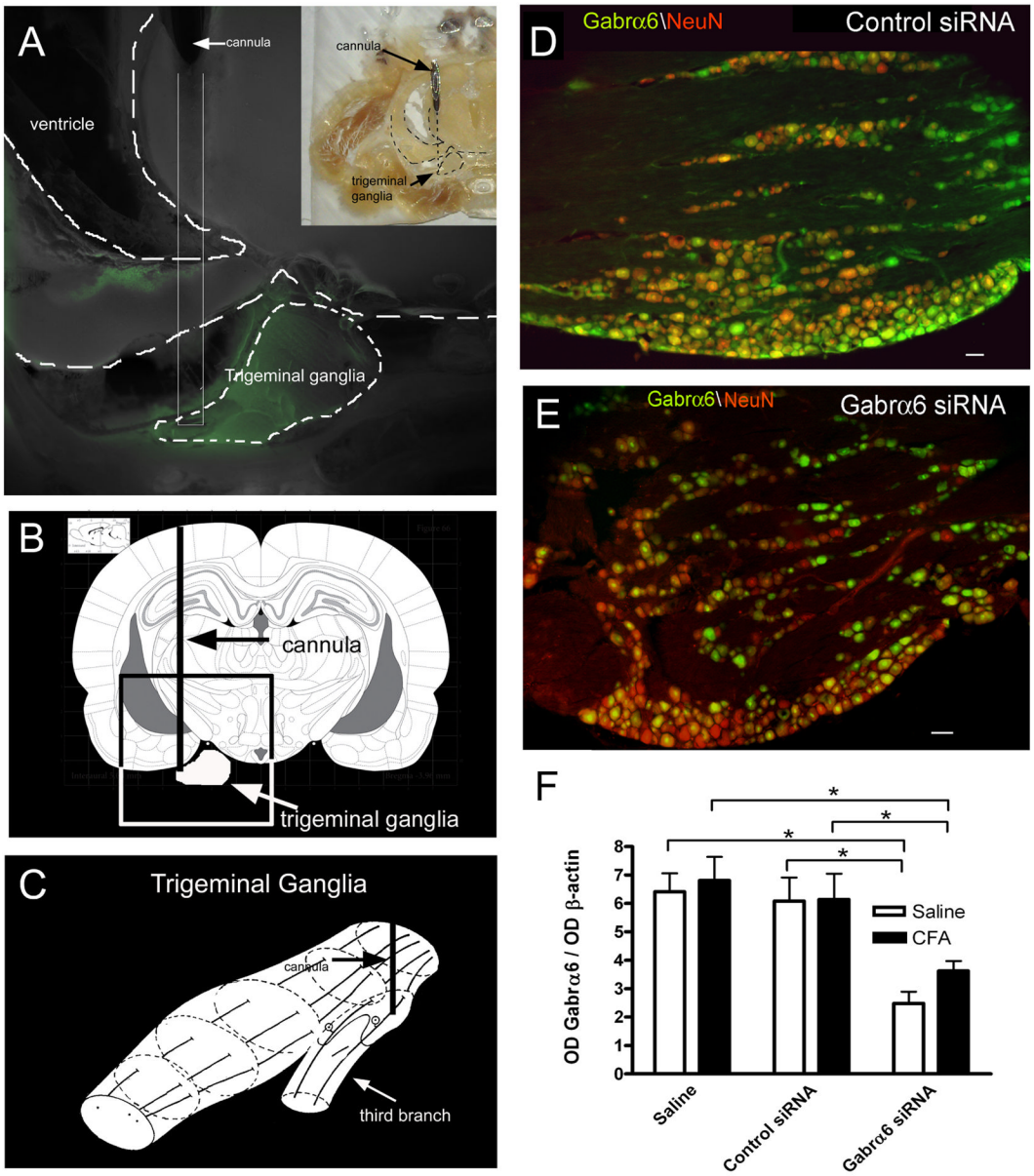

Figure 4. Gabrα6 siRNA infusion reduced Gabrα6 expression in the trigeminal ganglia

In a first experiment FAM labeled control siRNA was infused into the trigeminal ganglia of a rat head that was then embedded in methyl methacrylate and sectioned. (A) FAM fluorescence (green) was imaged on a whole head coronal section containing the trigeminal ganglia. The insert in panel A shows a picture of the head section with the trigeminal ganglia and stainless steel guide cannula and obturator labeled (arrow). Dashed outline in panel A delineates the bottom of the brain, the ventricle and the trigeminal ganglia. The boxed region in panel B outlines the region imaged in panel A (Paxinos and Watson, 2007). The cannula was placed near the junction of the third branch of the trigeminal ganglia, panel C. In a second experiment the trigeminal ganglia was bilaterally infused with either control siRNA (D) or siRNA with homology to the Gabrα6 gene (E); 60 hours later the TMJ was injected with saline; and 48 hours after the TMJ injection the rats were sacrificed for immunostaining. Cells in the trigeminal ganglia were stained for Gabrα6 (green) and the neuronal marker NeuN (red); double stained cells appear yellow. Bar=50 μm. F) In a third

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experiment the trigeminal ganglia was infused with saline, control siRNA or Gabrα6 siRNA and 60 hours after infusion the TMJ was injected with either 0.9% saline or CFA. A western blot was performed with trigeminal ganglia tissue harvested 48 hours after the TMJ injection. Optical density (OD) of the bands obtained after blotting for the Gabrα6 and β-actin protein was determined and a ratio of the optical density for the Gabrα6 and β-actin bands was calculated. $p<0.05$*. Values are mean ± SEM. There were 9 rats in each treatment group for panels C, D and E.

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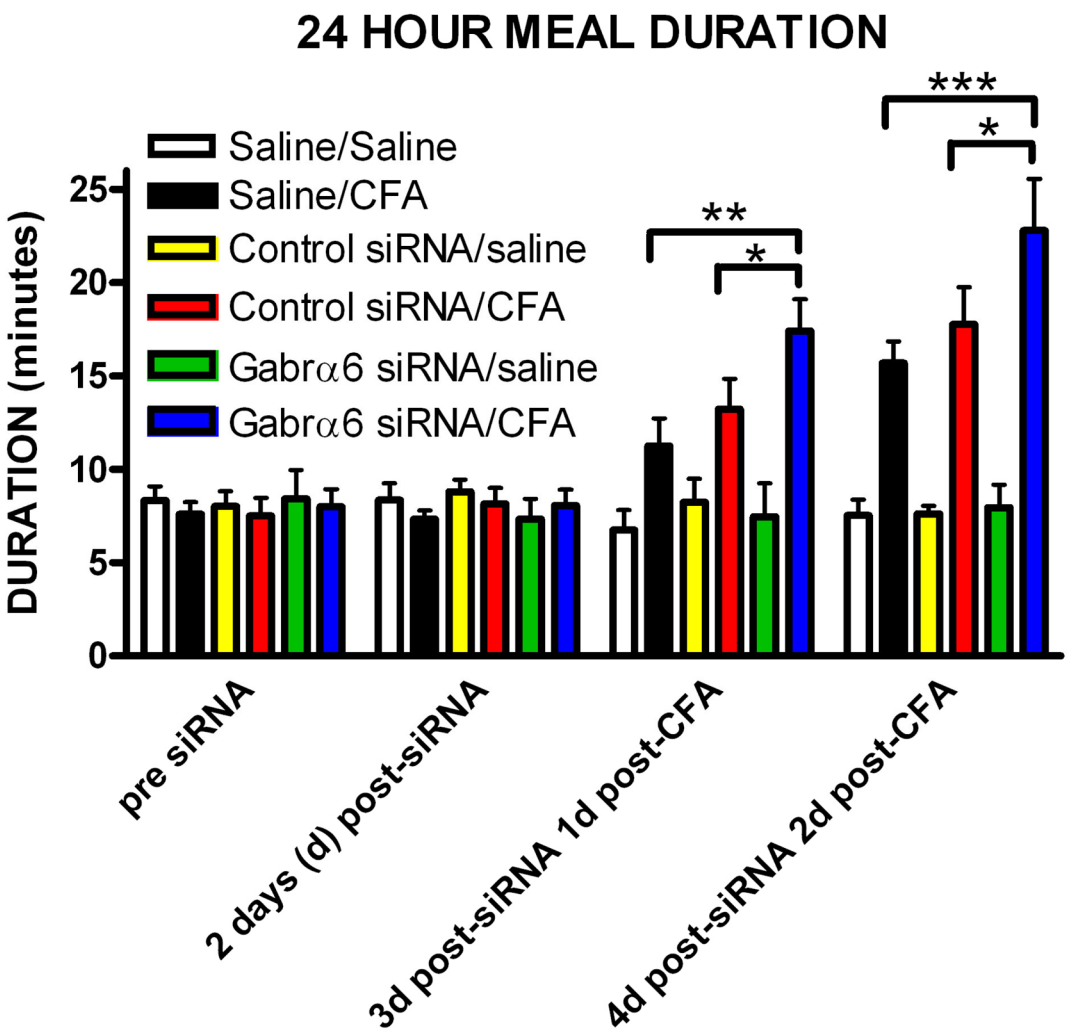

Figure 5. Hypersensitivity in a rat with TMJ arthritis after trigeminal ganglia infusion of siRNA

Daily meal duration (a behavioral correlate of hypersensitivity) was quantitated in rats before and after trigeminal ganglia infusion (saline or control siRNA or Gabrα6 siRNA) followed by a TMJ injection of 0.9% saline or CFA 60 hours later. Meal duration is shown before trigeminal ganglia infusion (pre siRNA); 48hours after siRNA infusion [2 days (d) post-siRNA]; 60 hours after siRNA infusion/24 hours after TMJ injection [3 days post-siRNA 1d post-CFA]; and 84 hours after siRNA infusion/48 hours after TMJ injection [4 days post-siRNA 2d post-CFA]., $p<0.05$=* $p < 0.01$=**, $p<0.001$***. Values are the mean ± SEM. Nine rats were in each treatment group.

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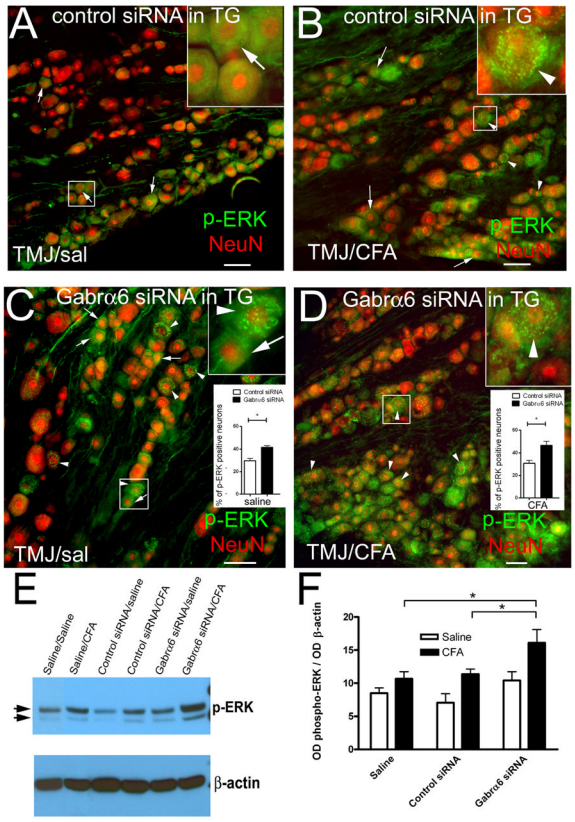

Figure 6. p-ERK expression was quantitated in the trigeminal ganglia after siRNA infusion

A–D) The trigeminal ganglia of male rats were infused with control siRNA or Gabrα6 siRNA and 60 hours later the rats TMJ were injected with saline (sal) or CFA. Fourty-eight hours after TMJ injection the trigeminal ganglia (from one side) were immunostained for p-ERK (green) and the neuronal marker, NeuN (red); double stained cells appear yellow. White boxes encompass individual cells or cell groups that are enlarged (upper right corners of each panel). Cells with diffuse p-ERK staining are marked with an arrow and cells with more punctuate staining are marked with an arrow head. The histogram inserted in panels C and D show the percent of p-ERK positive neurons present on the immunostained sections for that treatment group. E) Western blots were performed with the trigeminal ganglia from the opposite side of these treated rats using antibodies against p-ERK and β-actin (45 kDa). Arrows point to the phosphorylated p44 and p42 MAP kinase (Erk1 and Erk2). F) The histogram shows the optical density of the bands obtained after blotting for the p-ERK and β-actin; values are the ratio of the optical density (OD) for the p-ERK band divided by the OD of the β-actin band. $p<0.05$*. Bar=50 μm. Values are mean ± SEM. Nine rats were in each treatment group.

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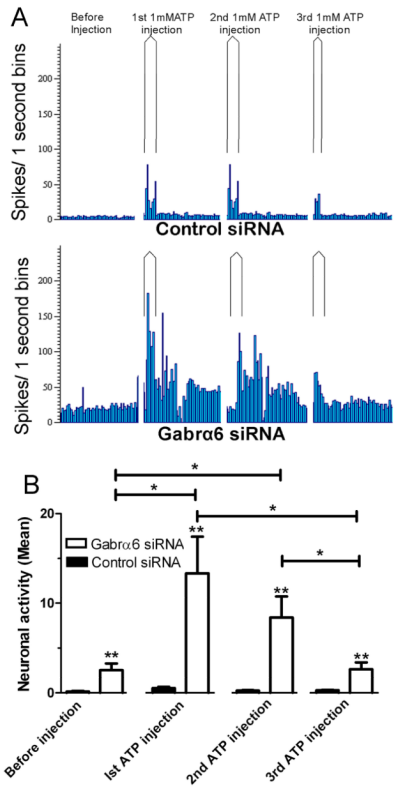

Figure 7. Greater spike activity was present in the C1 lamina II/III region after a series of TMJ ATP injections in rats with Gabrα6 knock-down

In panel A, two histograms show a representative rat for either the control siRNA (top histogram) or the Gabrα6 siRNA (middle histogram) infused treatment group. Extracellular recording data was represented as the number of spikes per second after injecting a series of three separate doses of 1mM ATP into the TMJ. The graph shows 1 second bins thus, 1 bar of the histogram equals the number of spikes in a second's worth of recording. Each injection took 4–7 seconds and about 30 seconds of data is shown before the TMJ injection (Before injection) and for 30 seconds after each of three separate TMJ injections (1st 1mM ATP injection, 2nd 1 mM ATP injection, 3rd 1mM ATP injection). The histogram in panel B shows the mean spike activity for a 5 minute period before injection and for 5 minutes after each of the three ATP injections. The ATP injections were given one after the other with a 30 minute period between each injection. A significant difference between the control siRNA and the Gabrα6 siRNA group before and after TMJ ATP injection was identified, $p<0.01$**. Values are the mean ± SEM. *=$p<0.05$. The data is representative of recordings from 12 animals per treatment group.