Acute inflammation reveals GABA<sub>A</sub> receptor-mediated nociception in mouse dorsal root ganglion neurons via PGE<sub>2</sub> receptor 4 signaling

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Abstract
Gamma-aminobutyric acid (GABA) depolarizes dorsal root ganglia (DRG) primary afferent neurons through activation of CI<sup>-</sup> permeable GABA<sub>A</sub> receptors but the physiologic role of GABA<sub>A</sub> receptors in the peripheral terminals of DRG neurons remains unclear. In this study, we investigated the role of peripheral GABA<sub>A</sub> receptors in nociception using a mouse model of acute inflammation. In vivo, peripheral administration of the selective GABA<sub>A</sub> receptor agonist muscimol evoked spontaneous licking behavior, as well as spinal wide dynamic range (WDR) neuron firing, after pre-conditioning with formalin but had no effect in saline-treated mice. GABA<sub>A</sub> receptor-mediated pain behavior after acute formalin treatment was abolished by the GABA<sub>A</sub> receptor blocker picrotoxin and cyclooxygenase inhibitor indomethacin. In addition, treatment with prostaglandin E2 (PGE<sub>2</sub>) was sufficient to reveal muscimol-induced licking behavior. In vitro, GABA induced sub-threshold depolarization in DRG neurons through GABA<sub>A</sub> receptor activation. Both formalin and PGE<sub>2</sub> potentiated GABA-induced Ca<sup>2+</sup> transients and membrane depolarization in capsaicin-sensitive nociceptive DRG neurons; these effects were blocked by the prostaglandin E2 receptor 4 (EP4) antagonist AH23848 (10 µmol/L). Furthermore, potentiation of GABA responses by PGE<sub>2</sub> was prevented by the selective Na<sub>A</sub>1.8 antagonist A887826 (100 nmol/L). Although the function of the Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> co-transporter NKCC1 was required to maintain the Cl<sup>-</sup> ion gradient in isolated DRG neurons, NKCC1 was not required for GABA<sub>A</sub> receptor-mediated nociceptive behavior after acute inflammation. Taken together, these results demonstrate that GABA<sub>A</sub> receptors may contribute to the excitation of peripheral sensory neurons in inflammation through a combined effect involving PGE<sub>2</sub>-EP4 signaling and Na<sup>+</sup> channel sensitization.

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Introduction

Doral root ganglion (DRG) neurons are primary afferent neurons which conduct sensory information from the environment to the spinal cord. DRG neurons express the Cl⁻ permeable gamma-aminobutyric acid (GABA)A receptor (Morris et al. 1983; Farrant and Nusser 2005; Zeilhofer et al. 2012) which display membrane depolarization in response to GABA stimulation. It is suggested that GABAergic receptor-mediated tonic primary afferent depolarization (PAD) may serve to inhibit primary afferent signaling through the inactivation of voltage-gated channels such as Na⁺ and thus reduce neurotransmitter release from the afferent terminals to second-order neurons (Willis 1999; Kullmann et al. 2005; Guo and Hu 2014). In addition, GABA may itself exert an inhibitory effect via activation of metabotropic GABAB receptors expressed on DRG neurons (Hanack et al. 2015).

It has previously been demonstrated that co-application of a low dose (2 μmol/L, 30 μL) of the GABA_A receptor agonist muscimol with formalin into the mouse hind paw reduced formalin-induced biphasic nocifensive behavior (Carlton et al. 1999), a result consistent with the inhibition associated with PAD. However, a high dose of muscimol (1 mmol/L) was in fact found to increase formalin-induced biphasic nocifensive behavior (Carlton et al. 1999; Bravo-Hernandez et al. 2014), a phenomenon that was blocked by pre-treatment with the GABA_A receptor antagonist bicuculline (Bravo-Hernandez et al. 2014). These findings suggest that near-maximally activated GABA_A receptors may participate in nociceptive sensory transduction in pathological conditions. However, the molecular mechanisms underlying the contribution of peripheral GABA_A receptors to inflammatory pain remain unclear.

In the present study, we sought to investigate the mechanism behind this apparent shift in the role of peripheral GABA_A receptors in acute inflammation. We found that peripheral GABA_A receptor activation induces de novo pain behavior after formalin and prostaglandin E2 (PGE₂) pre-conditioning through a signaling pathway involving EP4 receptor activation. We also found that functional upregulation of tetrodotoxin-resistant voltage-gated sodium channels in the presence of PGE₂ allows GABA signaling to evoke robust neuronal activity under inflammatory conditions. These findings may open new avenues of research into the contribution of GABA_A receptors to sensory physiology.

Materials and Methods

Ethical approval

All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees of Seoul National University (SNU-120710-5-1) and the National Institute of Physiological Sciences (NIPS), Japan. Experiments were carried out and are reported here in accordance with the ARRIVE guidelines (Kilkenny et al. 2010) and the principles and best practice of The Journal of Physiology (Grundy 2015).

Animals

Adult C57BL/6J (wild type) male mice (6–8 weeks) were purchased from Daehan Biolink (Korea) and Japan SLC (Hamamatsu, Japan). Adult male and female Na⁺-K⁺-2Cl⁻ co-transporter 1 knockout (NKCC1⁻⁻⁻) mice were from a stock originally created by Dr. Gary Shull of the University of Cincinnati (Flagella et al. 1999) and maintained at the Department of Physiology, Korea University, Seoul. Animals were housed four to five mice per cage in a conventional facility with a 12 h light cycle (lights on 8.00 AM) and ad libitum access to water and chow. Mice were acclimatized for at least 1 week before experiments. All behavioral experiments were carried out between 12.00 PM and 6.00 PM. Drug treatments were assigned...
randomly to mice of the same litter by an independent observer. Mice were euthanized at the end of behavior experiments by rising concentration of CO₂, or by overdose of urethane followed by cervical dislocation at the end of in vivo recording, in accordance with the Schedule 1 of the UK Home Office Animals (Scientific Procedures) Act 1986.

**Formalin pre-conditioning and spontaneous pain behavior**

Mice were placed in an observation chamber (60 × 100 × 60 mm) and allowed to habituate for at least 30 min before drug administration. A mirror was positioned behind the observation chamber to provide an unobstructed view. After habituation, one experimenter restrained the mouse while another experimenter performed a ‘pre-conditioning’ injection of formalin (0.8%) or saline vehicle (20 μL) subcutaneously into the dorsum of the right hind paw using a 0.3 mL insulin syringe fitted with a 31 gauge needle. After subsidence of formalin-induced nocifensive behaviors (70 min after first injection) a second ‘stimulating’ injection of muscimol (1 mmol/L) or saline vehicle (20 μL) was given subcutaneously to the same dorsum area of the hind paw. For inhibition of GABAₐ receptors in vivo, picrotoxin was added at the indicated concentration to both the initial formalin solution and muscimol stimulation solution before paw injection. Care was taken with both injections to avoid leakage of the solutions from the paw. Spontaneous pain behaviors were assessed by measuring the time each animal spent licking the affected hind paw. The cumulative time spent licking was recorded during the 5 min immediately before drug administration and up to 110 min after the first drug administration. All behavior tests were video recorded and analyzed offline by an investigator who was blind to the treatment and genetic background of the mice.

**In vivo spinal cord extracellular recording**

The methods used for the present study were modifications of those used in preceding studies (Sugiyama et al. 2012; Funai et al. 2014). Adult C57BL/6J male mice were anesthetized with urethane (1.5 g/kg, i.p.) and monitored for loss of hind paw pinch reflex with supplemental injections of urethane (0.2 g/kg, i.p.) given if necessary during the experiment. A laminectomy was performed to expose the lumbar enlargement of the spinal cord. The mouse was placed in a stereotaxic apparatus (Model STS-A, Narishige, Tokyo, Japan). After the dura mater was opened, the pia-arachnoid membrane was cut to make a window to allow a tungsten electrode to enter into the spinal cord. The surface of the spinal cord was irrigated with 95% O₂ and 5% CO₂ equilibrated Krebs solution (in mmol/L: 117 NaCl, 3.6 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 11 glucose, and 25 NaHCO₃) at a flow rate of 10–15 mL/min at 38°C ± 1°C. The tungsten electrode (impedance, 1 MX, A-M systems, Sequim, WA) was advanced into the spinal cord using a micromanipulator (Model SM-11, Narishige). The tungsten electrode was placed into the spinal cord dorsal horn and action potentials in spinal cord neurons were extracellularly recorded with an AC differential amplifier (DAM 80, World Precision Instruments, Sarasota, FL). Wide dynamic range (WDR) neurons with a receptive field covering the ipsilateral hind paw were identified during electrode advancement by consistent spike responses to touch, brush and pinch stimuli applied to the hind paw. The firing rate of spinal cord neurons was analyzed with Offline Sorter software (version 3, Plexon, Dallas, TX). Injections were made into the dorsal surface of the ipsilateral hind paw in the same manner as for the behavioral experiments.

**DRG preparation**

DRG neurons were isolated from 6 to 8-week-old mice. Animals were killed in accordance with the Schedule 1 of the UK Home Office Animals (Scientific Procedures) Act 1986 by inhalation of a lethal, natural concentration of isoflurane (Hana Pharm. Co. Ltd., Korea) followed by decapitation. Bilateral DRG were rapidly removed under aseptic conditions and placed in ice-cold HBSS containing 20 mM HEPES. DRGs were digested in 1 mg/ml collagenase A (Roche) and 2.4 U/ml dispase II (Roche) in HBSS for 60 min, respectively, followed by 5 min in 0.25% trypsin (Sigma), all at 37°C. The DRGs were then washed in DMEM (Gibco) and resuspended in DMEM medium supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin (Sigma). DRGs were then mechanically dissociated using fire-polished glass pipettes, centrifuged (200 g, 5 min), resuspended in Neurobasal media (Gibco) with B27 supplement (Invitrogen), L-glutamine and 1% penicillin/streptomycin (Invitrogen), and plated on 0.5 mg/ml poly-D-lysine (Sigma)-coated glass coverslips. Cells were maintained at 37°C in a 5% CO₂ incubator. All experiments using DRG neurons were performed 12–36 h after plating.

**Patch-clamp electrophysiology**

Electrophysiologic responses were recorded using the patch-clamp recording technique with EPC-10 amplifier and Pulse 8.30 software (both from HEKA). For perforated-patch recordings in DRG neurons, we used an external bath solution of the following composition (in
mmol/L): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. Patch pipettes with resistances of 3–5 MΩ were made from borosilicate glass capillaries. Pipette solution contained (in mM): 140 K-gluconate, 1 CaCl₂, 2 MgCl₂, 10 EGTA, 5 K₂ATP, 10 HEPES, adjusted to pH 7.4 with KOH. A 50 mmol/L stock solution of gramicidin (Calbiochem, La Jolla, CA) was prepared in dimethylsulfoxide (DMSO; Sigma). Gramicidin was diluted into the pipette solution to a final concentration of 100 μg/mL and vortexed thoroughly before use. All drug solutions were applied to cells by local perfusion through a capillary tube (1.1 mm inner diameter) positioned near the cell of interest. After the formation of a tight seal, the progress of gramicidin perfusion was evaluated by monitoring the capacitive current transient produced by a 10 msec hyperpolarizing voltage step (−5 mV) from a holding potential of −60 mV. Cells were accepted for recording if the access resistance dropped to 20 MΩ within 20 min after seal formation. The solution flow was driven by gravity (flow rate, 3–5 ml/min) and controlled by miniature solenoid valves (The Lee Company). Perforated-patch recordings of membrane potential (V_m) were corrected offline using the following formula: V_{m} = V_p + V_{pf}(LJP), where V_p is the recorded potential, V_{pf} is the perforated-patch potential and LJP is the liquid junction potential between intracellular pipette and extracellular bath solutions. For whole-cell patch-clamp recordings of sodium currents the pipette solution contained (in mmol/L): 130 CsCl, 1 NaCl, 1 MgCl₂, 10 EGTA, 10 HEPES, adjusted to pH 7.4 with CsOH. The external solution was composed of (in mM): 131 NaCl, 10 TEACl, 10 CsCl, 1 CaCl₂, 2 MgCl₂, 0.1 CdCl₂, 3 4-aminopyridine, 10 HEPES, 10 glucose adjusted to pH 7.4 with NaOH. Tetrodotoxin (TTX)-resistant currents were recorded from DRG neurons in the presence of TTX (300 nmol/L). For quantification of activation of Nav1.8-mediated sodium currents, a 500 msec pre-pulse step at −50 mV, designed to inactivate TTX-resistant Na₈,1.9 channels (Berta et al. 2008), was followed by a series of voltage steps increments of +5 mV at a frequency of 1 Hz. A liquid junction potential of +6.0 mV was corrected offline for calculation of the conductance-voltage relationship. Data were fit with a Boltzmann function. The shift in voltage dependence of Na₈,1.8 activation was calculated from the difference in V_{1/2}.

Ca²⁺ imaging

We performed fura-2 AM-based (Molecular Probes) Ca²⁺ imaging experiments. Briefly, DRG neurons prepared as above were loaded with fura-2 AM (2 μmol/L) in DMEM for 50 min at 37°C in a 5% CO₂ incubator. The cells were then rinsed with DMEM and incubated for an additional 20 min to de-esterify the dye. Cells on slides were placed onto an inverted microscope and illuminated with a 175 W xenon arc lamp; excitation wavelengths (340/380 nm) were selected by a monochromatic wavelength changer. Intracellular calcium concentrations ([Ca²⁺]_i) were measured by digital video microfluorometry with an intensified charge-coupled-device camera (CasCade, Roper Scientific) coupled to the microscope and a computer with Metafluor software (Universal Imaging). All drugs were applied via bath perfusion at a flow rate of 3–5 mL/min. For Cl⁻ gradient depletion experiments, NaCl was substituted for equimolar Na-gluconate in the bath solution. The left axis of all scale bars for Ca²⁺ imaging traces represents the F_{340/380} ratio.

Drugs

Capsaicin, formalin, prostaglandin E₂, thapsigargin, picROTOXIN, lidocaine, γ-aminobutyric acid (GABA), muscimol (3-hydroxy-5-aminomethyl-isoxazole), HC-030031 (1,2,3,6-tetrahydro-1,3-dimethyl-N-4-(1-methylethyl)phenyl)-2,6-dioxo-7H-purine-7-acetamide), bumetanide (3-(aminosulfonyl)-5-(butylamino)-4-phenoxy benzoic acid), AH23848 ((4Z)-7-[[rel-1S,2S,5R)-5-[(1,1-dimethylethyl)phenyl]-4-yl)methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl)-4-heptenoic acid hemicalcium salt), AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid) and A887826 (5-(4-butoxy-3-chlorophenyl)-N-[2-(4-morpholinyl)-3-pyridiniyl]-2,6-dioxo-7H-purine-7-acetamide), were purchased from Sigma. For in vivo experiments, formalin and muscimol were dissolved in 0.9% saline with sonication. For in vivo experiments, formalin and muscimol were dissolved in 0.9% saline with sonication. For in vivo experiments, AH6809, A887826, bumetanide, and capsaicin were dissolved in 100% DMSO as stocks at 10 mmol/L and AH23848 was dissolved in saline +2% DMSO; all drugs were further diluted in bath solution immediately before experiments.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) unless otherwise indicated. In in vitro experiments, ’n’ refers to the number of cells recorded; in Ca²⁺ imaging experiments, the number of coverslips and number of mice used (i.e. repeat experiments) are also indicated. In behavior testing experiments, ’n’ indicates the number of animals tested. Sample sizes were based on the previous

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Peripheral GABAA receptors are nociceptive in acute inflammation

We investigated the modulatory effects of GABAA receptors on inflammatory pain by injecting the GABAA agonist muscimol (1 mmol/L, 20 μL) into the hind paw after cessation of 0.8% formalin-induced pain behavior in adult mice. This formalin pre-conditioning protocol revealed a novel peripheral GABAA receptor-mediated nocifensive behavior (Fig. 2A, B). In contrast, mice injected with muscimol after saline pre-injection showed no pain-like behavior in the 30 min after muscimol injection (Fig. 2A, B). These results suggest that GABAA receptors elicit nocifensive behavior in the presence of acute inflammation, but not in otherwise naïve animals.

We next investigated whether muscimol-induced nocifensive signaling can be transmitted to the spinal cord after formalin inflammation by performing extracellular recording of WDR neurons in adult mice in vivo. After a period (20 min) of baseline recording, muscimol (1 mmol/L, 20 μL) was injected subcutaneously into the hind paw. The spike frequency of all units did not differ compared with baseline in the 20 min after the first muscimol injection (Fig. 2C). Units representing the action potential discharge of 12 individual WDR neurons were identified based on spike profile (Fig. 2D). Subsequent formalin (0.8%) injection augmented spike frequency in 8 out of 12 WDR neurons (Representative units ‘a’ and ‘b’, Fig. 2C). After the activity of formalin-responsive neurons returned to baseline (approximately 60 min) a second muscimol injection facilitated action potential firing in 10 out of 12 WDR neurons. Units identified at a low frequency during the first muscimol injection were observed firing at higher frequency during the second muscimol injection after formalin conditioning (Fig. 2D, E). The overall number of action potentials recorded during the 20 min after the second muscimol injection was also significantly increased (Fig. 2F). Representative unit ‘c’, which did not respond to the formalin injection, was not affected by the second muscimol injection (Fig. 2C). These results indicate that activation of GABAA receptors in peripheral nociceptive neurons after acute inflammation evokes nocifensive signal transmission to second-order neurons in the spinal cord. In addition, we

Results

GABA induces Ca2+ transients by GABAA receptor activation in DRG neurons

We first employed ratiometric Ca2+ imaging to study the physiologic role of GABAA receptors in acutely dissociated adult mouse DRG neurons without disrupting the intracellular ionic milieu. GABA (300 μmol/L, 10 sec) applied via the bath perfusion induced Ca2+ transients (Fig. 1A) in a subpopulation of DRG neurons (52.8%; n = 507/960) (Fig. 4D). These transients were reproducible and stable across sequential applications of GABA (Fig. 1B). The amplitude of GABA-induced Ca2+ transients was concentration-dependent (Fig. 1C, D) with 300 μmol/L GABA representing a supramaximal response in our Ca2+ assay (Fig. 1D).

The GABAA receptor selectively conducts anions through its pore; therefore, we next sought the origin of Ca2+ transients induced by GABA. The GABA-induced Ca2+ transients were blocked by CdCl2 (100 μmol/L), a non-selective blocker of calcium channels (data not shown) suggesting that GABAA receptor activation leads to voltage-gated Ca2+ channel (VGCC) activation. We verified that GABA-induced Ca2+ transients were abolished by the removal of extracellular Ca2+ (Fig. 1E, F) but not by the depletion of intracellular Ca2+ stores (Fig. 1G, H). We confirmed that Ca2+ transients induced by supramaximal GABA (300 μmol/L) were blocked by the non-competitive GABAA receptor antagonist picrotoxin (Fig. I) in a concentration-dependent manner (Fig. 1I). Ca2+ responses could also be induced by the GABAA receptor selective agonist muscimol (10 μmol/L), which were again blocked by picrotoxin (100 μmol/L) (data not shown).

Gramacidin is an antibiotic agent which diffuses into the cell membrane forming small CI impermeable perforations, thereby giving electrical access without disruption of the intracellular CI concentration (Ebihara et al. 1995; Kyrozis and Reichling 1995). Using gramacidin-perforated patch-clamp technique we observed that GABA (300 μmol/L) evoked a fast, rapidly decaying depolarization of the membrane potential in small-sized (12–20 μm diameter) DRG neurons (Fig. 1K). The average membrane potential was recorded as −53.7 ± 0.8 mV at rest and a peak of −35.3 ± 0.6 mV during GABA (300 μmol/L) application (Fig. 1L); at this concentration of GABA action potentials were observed in 1/13 neurons (data not shown). Thus, GABA elicits membrane depolarization via activation of GABAA receptors, resulting in VGCC-mediated Ca2+ influx in DRG neurons.
confirmed that muscimol-induced licking behavior after formalin pre-conditioning was dose-dependently inhibited by the non-competitive GABA<sub>A</sub> receptor antagonist picrotoxin (Fig. 2G, H). Interestingly, co-injection of picrotoxin with formalin also dose-dependently inhibited the second phase of the formalin-induced behavior.

Formalin potentiates GABA response independent of TRPA1

We first examined the effect of formalin on GABA-induced Ca<sup>2+</sup> transients in nociceptive DRG neurons. GABA-induced Ca<sup>2+</sup> transient amplitudes were increased
Figure 2. Activation of peripheral GABA<sub>A</sub> receptors induces pain-like behavior after acute formalin inflammation but not in naïve mice. (A) Time course of hind paw licking behaviors during pre-conditioning with formalin (0.8%, 20 μL) followed by injection of muscimol (1 mmol/L, 20 μL) (open circles, n = 5 mice) or 0.9% saline (black circles, n = 4 mice) (‘stimulation’); pre-conditioning with 0.9% saline vehicle followed by injection of muscimol (open squares, n = 5 mice) or 0.9% saline (black squares, n = 5 mice) into the same dorsum hind paw area. (B) GABA<sub>A</sub> receptor agonist muscimol significantly increased hind paw licking behavior in formalin pre-conditioning group. Bar graph represents accumulative licking time during 30 min stimulation phase (**P < 0.01, one-way ANOVA with Bonferroni post-test compared with muscimol in saline pre-conditioning group; (*) < 0.05, one-way ANOVA with Bonferroni post-test compared with saline in formalin pre-conditioning group). (C) Extracellular unit recording of spinal WDR neurons showing the pattern of action potential (AP) discharge frequency of three units (a, b, and c) in response to sequential injections of muscimol (1 mmol/L, 20 μL) and formalin (0.8%, 20 μL) into the hind paw. (D–F) Pre-injection of formalin (0.8%) increases frequency of action potential (AP) discharge in spinal WDR neurons by injection of muscimol (1 mmol/L, 20 μL) into the hind paw. (D) Representative traces showing the AP discharge of two units induced by muscimol before and after formalin injection. (E) Frequency histogram of muscimol-induced AP discharge (1 min bins). (F) Mean frequency of total muscimol-induced AP discharge per min for 20 min, n = 12 units recorded from 6 animals, **P < 0.01, Wilcoxon signed-rank test. (G) Effect of GABA<sub>A</sub> antagonist picrotoxin (PTx) on time course of hind paw licking behaviors during pre-conditioning with formalin (0.8%, 20 μL) followed by injection of muscimol (1 mmol/L, 20 μL) into the same dorsum hind paw area. Picrotoxin was given at 1 mmol/L (gray triangle, n = 5 mice), 5 mmol/L (black squares, n = 6 mice) or vehicle (0.9% saline, open circles, n = 12 mice). (H) Picrotoxin significantly inhibited the second phase of the formalin response (*P < 0.05, ***P < 0.001, one-way ANOVA with Bonferroni post-test) as well as muscimol-induced pain behavior (**P < 0.01, P < 0.001, one-way ANOVA with Bonferroni post-test). WDR, wide dynamic range.
after pre-treatment of formalin (0.001%, 2 min) (Fig. 3A, B). Formalin is known to directly activate nociceptive neurons through TRPA1 (McNamara et al. 2007); however, formalin potentiation of GABA-induced Ca\(^{2+}\) transients occurred in neurons which had no response to formalin (0.001%) alone (Fig. 3A) and furthermore was not affected by the TRPA1 selective antagonist, HC030031 (30 μmol/L) (Fig. 3C, D).

**Prostaglandin E2 reveals GABA\(_A\) receptor-mediated nociception**

We next sought to determine the mechanism underlying peripheral GABA\(_A\) receptor-induced nociception in inflammation. Formalin-induced pain behavior can be attenuated by pre-treatment with the cyclooxygenase (COX) inhibitor indomethacin (Hunskaar et al. 1986), which reduces inflammation by blocking prostanoid synthesis. We found that systemic pre-treatment with indomethacin (40 mg/kg, i.p.) 30 min before formalin injection completely abolished the subsequent muscimol-induced licking behavior in adult mice (Fig. 4A, B). The prostaglandin E2 (PGE\(_2\)) derivative is a potent inflammatory mediator produced at the site of inflammation (Fulton et al. 2006). We observed that PGE\(_2\) (500 μmol/L, 20 μL)-induced licking behavior was also enhanced by co-injection of muscimol (Fig. 4C), confirming that PGE\(_2\) is sufficient for GABA\(_A\) receptor-mediated nocifensive behavior.

Next, we examined the effect of PGE\(_2\) on GABA-induced Ca\(^{2+}\) transients in cultured DRG neurons. Nociceptive DRG neurons were identified by their response to a 10 sec application of capsaicin (1 μmol/L) at the end of each experiment and made up 42.3% (406/960 neurons) of the total population, of which 17.6% (169/960 neurons) were also responsive to GABA (Fig. 4D). We found that PGE\(_2\) (10 μmol/L) potentiated GABA-induced Ca\(^{2+}\) transients (Fig. 4E, F) in a large subpopulation of capsaicin-responsive nociceptive DRG neurons (40.1%; n = 69/169 neurons) (Fig. 4G) but only a small number of capsaicin-insensitive DRG (1.8%; n = 6/338 neurons) (Fig. 4H). In addition, PGE\(_2\) (10 μmol/L) facilitated GABA-induced membrane depolarization resulting in action potential firing in a subpopulation of small-sized DRG neurons as recorded by gramicidin perforated patch (Fig. 4I–K). Collectively, our results suggest that PGE\(_2\) is one of the possible pro-inflammatory mediators that may contribute to GABA\(_A\) receptor-mediated nocifensive behavior during acute inflammation via action in a subpopulation of nociceptive DRG neurons.

**Activation of EP4 receptors increases GABA-induced nociceptive neuron activity**

PGE\(_2\) signals through a family of EP receptors (EP1-4) of which subtypes EP1, EP2, and EP4 are excitatory and promote inflammation (Fulton et al. 2006). PGE\(_2\)-potentiated GABA responses were inhibited by EP4 receptor antagonist, AH23848 (10 μmol/L) (St-Jacques and Ma 2011) (Fig. 5A, B) but not by EP1-2 receptor antagonist, AH6809 (50 μmol/L) (Lin et al. 2006) (Fig. 5A, B). GABA-induced action potential firing in the presence of PGE\(_2\) was also abolished by AH23848 (Fig. 5E, F). Furthermore, potentiation of GABA-induced Ca\(^{2+}\) transients by formalin was also blocked by AH23848 (10 μmol/L) (Fig. 5G, H). Together, these data suggest that formalin and PGE\(_2\) may potentiate GABA-induced responses through an EP4 receptor signaling mechanism in nociceptive DRG neurons.

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**Figure 3.** Formalin potentiates GABA-induced Ca\(^{2+}\) transients in a TRPA1 independent manner. (A, B) Formalin potentiates GABA-induced Ca\(^{2+}\) transients in nociceptive neurons. (A) Representative traces showing potentiated GABA (300 μmol/L)-induced Ca\(^{2+}\) by formalin pre-treatment (0.001%, 120 sec) in a capsaicin-sensitive DRG neuron. (B) Mean amplitude of GABA-induced Ca\(^{2+}\) transients (n = 18 cells, 6 coverslips from 3 mice; ***P < 0.001, one-way ANOVA). (C) Potentiated GABA-induced Ca\(^{2+}\) transients by formalin (0.001%) are not blocked by TRPA1 receptor selective antagonist HC030031 (30 μmol/L). (D) Quantification of GABA-induced Ca\(^{2+}\) transients in capsaicin-sensitive DRG neurons (n = 16 cells, 3 coverslips from 2 mice; ***P < 0.001, one-way ANOVA with Bonferroni post-test). DRG, dorsal root ganglia.
**Figure 4.** Prostaglandin E2 contributes to GABA\(_{\text{A}}\) receptor-mediated pain and neuronal excitability. (A) Effect of intraperitoneal injection of indomethacin (40 mg/kg) (open squares) or PBS vehicle (black circles) on muscimol (1 mmol/L, 20 μL)-induced hind paw licking behavior in formalin (0.8%, 20 μL) pre-conditioned mice. (B) Cyclooxygenase inhibitor indomethacin abolishes muscimol-induced hind paw licking behavior after formalin pre-conditioning (n = 6 mice per group, ***P < 0.05, Student’s t-test). (C) PGE\(_2\) potentiates muscimol-induced hind paw licking behavior. Quantification represents total duration of hind paw licking behavior during 30 min after PGE\(_2\) (10 nmol, 20 μL) or saline vehicle with or without muscimol (1 mmol/L, 20 μL) (n = 5–6 mice per group, *P < 0.05, ***P < 0.001, one-way ANOVA with Bonferroni post-test). (D) Venn diagram of GABA (300 μmol/L) and capsaicin (CAP; 1 μmol/L) sensitivity among KCl-responsive neuronal cells in acute (<24 h) cultures of adult mouse DRG (n = 960 cells, 19 coverslips from 3 mice). (E, F) PGE\(_2\) potentiates GABA-induced Ca\(^{2+}\) transients in nociceptive neurons. (E) Representative trace showing potentiated GABA (300 μmol/L) compared to control (n = 24), (F) Mean amplitude of GABA-induced Ca\(^{2+}\) transients (n = 34 cells, 11 coverslips from 7 mice; ***P < 0.001, one-way ANOVA). (G, H) The effect of PGE\(_2\) on the amplitude of GABA-induced Ca\(^{2+}\) transients. GABA-induced Ca\(^{2+}\) transients in capsaicin-sensitive (G) and capsaicin-insensitive (H) GABA-responsive neurons were classified according to whether the amplitude (change in F\(_{340/380}\) ratio) was increased (>0.03 ‘potentiated’), decreased (>0.02, or unchanged (–0.02 to +0.03) during PGE\(_2\) (10 μmol/L) compared to control responses (*P < 0.05, ***P < 0.001, paired two-tailed t-test). (I, J, K) PGE\(_2\) increases GABA-induced neuronal excitability. (I) Representative traces of GABA (100 μmol/L)-induced depolarization before and after pre-incubation with PGE\(_2\) (10 μmol/L, 180 sec). (J) Quantification of GABA-induced changes in membrane potentials and (K) number of action potentials by GABA before and after pre-incubation with PGE\(_2\) (10 μmol/L) (n = 21 cells). DRG, dorsal root ganglia.
Voltage-gated Na⁺ channels may contribute to GABA-induced neuronal excitability during PGE₂ sensitization

PGE₂ is an important mediator in the development of peripheral inflammation and peripheral sensitization (Vane 1971; Julius and Basbaum 2001). For example, PGE₂ can directly potentiate the TTX-resistant Na⁺ channels Nav1.8 (England et al. 1996; Gold et al. 1998) and Naᵥ1.9 (Rush and Waxman 2004) in peripheral nociceptive neurons. We therefore conducted experiments to examine whether modulation of Naᵥ channels contributes to GABA-induced responses during PGE₂ application.

The archetypal Naᵥ channel blocker lidocaine (Sheets et al. 2008) was applied to cultured DRG neurons during PGE₂-induced potentiation of the GABA response (Fig. 6A). Lidocaine (300 μmol/L) inhibited the potentiation of GABA-induced Ca²⁺ responses by PGE₂ (Fig. 6A, B) but had no effect on GABA-induced Ca²⁺ transients in control conditions (Fig. 6C, D). We confirmed that PGE₂ acutely potentiates TTX-resistant Naᵥ channel current (Fig 6E, F), as well as produce a leftward shift (−6.08 ± 1.8 mV, n = 6 cells) in the voltage dependence of Naᵥ channel activation.
Figure 6. PGE2 potentiates GABA–induced neuronal excitability through Na\textsubscript{1.8} channel modulation. (A) Potentiation of GABA (300 μmol/L)-induced Ca\textsuperscript{2+} transients by PGE2 (10 μmol/L) is inhibited by voltage-gated sodium channel blocker lidocaine (300 μmol/L) in capsaicin-sensitive DRG neurons. (B) Quantification of GABA-induced Ca\textsuperscript{2+} transients in the presence and absence of lidocaine relative to peak amplitude of 1st GABA response (n = 12 cells, 9 coverslips from 4 mice; ***P < 0.001, repeat-measures one-way ANOVA with Bonferroni post-test). (C) GABA-induced Ca\textsuperscript{2+} transients are unaffected by lidocaine (300 μmol/L, 360 sec). (D) Quantification of GABA-induced Ca\textsuperscript{2+} transients amplitudes in the presence of lidocaine (n = 17 cells, 4 coverslips from 3 mice; one-way ANOVA with Bonferroni post-test). (E) PGE2 (10 μmol/L, 180 sec) potentiates TTX-resistant sodium currents in small-sized DRG neurons. (F) Quantification of normalized TTX-resistant sodium currents amplitude (n = 5 cells, ***P < 0.001, paired Student’s t-test). (G) Normalized conductance-voltage relationship of Na\textsubscript{1.8} currents in the presence and absence of PGE2 (10 μmol/L) (n = 6 cells) fit with a Boltzmann function. Test pulses were preceded by a 500-msec step to -50 mV to inactivate TTX-resistant Na\textsubscript{1.9}. (H) A887826 (100 nmol/L) inhibits the membrane depolarization caused by PGE2 (10 μmol/L) application (n = 12–20 cells, *P < 0.05, unpaired Student’s t-test). (I) Potentiation of GABA (300 μmol/L)-induced Ca\textsuperscript{2+} transients by PGE2 (10 μmol/L) is abolished by Na\textsubscript{1.8} channel blocker A887826 (100 nmol/L) in DRG neurons (n = 9 cells). (J) The potentiating effect of PGE2 on GABA-induced changes in membrane potentials (K) and generating action potentials (L) in small-sized DRG neurons is blocked by Na\textsubscript{1.8} channel blocker A887826 (100 nmol/L) (n = 9 cells). DRG, dorsal root ganglia.
of Na+,l.8 activation (Fig 6G). PGE2 additionally induced a small depolarization from the resting membrane potential (Fig. 6H). Pre-incubation with the Na+,l.8 channel antagonist A887826 (100 nmol/L) (Zhang et al. 2010) not only blocked PGE2-induced depolarization (Fig. 6H) but also the PGE2 potentiation of GABA-induced Ca²⁺ responses (Fig. 6I, J) as well as GABA-induced action potential firing in the presence of PGE2 (Fig. 6K, L). Together, these results suggest that the activation of voltage-sensitive TTX-resistant Na⁺ channels may contribute to the nociceptive role of GABAA receptors during PGE2-mediated inflammation.

**NKCC1 is not required for potentiated GABA responses by pro-inflammatory mediators**

The Na⁺-K⁺-Cl⁻-co-transporter (NKCC1) is thought to be responsible for maintaining intracellular Cl⁻ levels in DRG neurons (Sung et al. 2000). Furthermore, NKCC1 activity is dynamically regulated by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) phosphorylation (Smith et al. 2008; Flemmer et al. 2010). We therefore hypothesized that NKCC1 may be a downstream target of EP4 receptor signaling (via PKA) during formalin conditioning in mice lacking NKCC1 (Fig. 7I, J). These results suggest that NKCC1 activity is not required for peripheral GABAA-mediated nociception during acute inflammation.

**Discussion**

Peripheral GABAA receptor nociception in vivo

GABA has long been observed to depolarize peripheral sensory nerves in rodents (Feltz and Rasminsky 1974; Deschenes et al. 1976; Sung et al. 2000) and humans (Carr et al. 2010); however, activation of peripheral GABAA receptors elicits neither spontaneous pain behavior nor mechanical sensitivity in naive animals. One proposal to this apparent paradox is the phenomenon of primary afferent depolarization (Rudomin and Schmidt 1999; Willis 1999) whereby sub-threshold depolarization results in inactivation of voltage-gated channels. Nevertheless, previous reports have suggested that the activation of peripheral GABAA receptors can facilitate inflammation-induced...
painless behavior (Carlton et al. 1999; Bravo-Hernandez et al. 2014). The formalin test is a well-characterized test of acute-inflammatory pain (Hunskaar and Hole 1987) which produces biphasic pain-like behavior that disappears 60 min after the original treatment. By a simple modification of the protocol - injection of muscimol after the return of formalin behavior to baseline - we were able to reveal a novel GABA_A receptor-mediated nociceptive behavior that could be used for further mechanistic study.

The phenomenon of GABA_A-mediated nociception after acute inflammation appears to contrast with observations that either application of GABA agonists directly to the DRG (Naik et al. 2008), or systemic administration of GABA_A allosteric modulators (Munro et al. 2008), can have an alleviating effect in certain models of chronic inflammatory or neuropathic pain, although the contribution of spinal GABA_A receptors cannot be completely excluded in these studies.

**Ca^2+** imaging as an indirect measure of GABA_A receptor function in vitro

Unlike whole-cell techniques, the **Ca**^2+ imaging technique allows the quantitative study of cell activity without disrupting the cell membrane. Thus, cells may be studied in a relatively undisturbed state with endogenous concentrations of intracellular ions and signaling factors. Application of GABA to DRG neurons induced consistent and concentration-dependent **Ca**^2+ transients that were mediated by GABA_A receptors specifically and blocked by CdCl_2, a non-selective voltage-gated **Ca**^2+ channel blocker. Moreover, manipulation of the **Cl**^- concentration gradient had a proportional effect on GABA-induced **Ca**^2+ transient amplitude. These results suggested that GABA elicited **Ca**^2+ influx through VGCC activation in DRG neurons of an amplitude proportional to the degree of membrane depolarization.

The high concentration of muscimol (>1 mmol/L) required to potentiate the formalin nociceptive response in vivo in this study and reported by others (Carlton et al. 1999) suggests that near-maximal activation of GABA_A receptor is required for conversion to a nociceptive role. Correspondingly, we used a relatively high concentration of GABA (300 μmol/L) for the majority of our in vitro studies, which represented a supramaximal response in our **Ca**^2+ assay. The physiologic concentration of GABA has been reported at >500 μmol/L at GABAergic synapses in the brain (Maconochie et al. 1994) with a peak concentration of 1.5–3 mmol/L GABA (Mozrzymas et al. 2003). Further work will be required to establish whether such concentrations are reached at peripheral GABA_A receptors in vivo.

**Formalin and PGE_2 potentiation of GABA responses are mediated by EP4 receptor**

The effect of formalin on DRG neurons in our in vitro culture system appeared to mimic the in vivo observation by potentiating the GABA-induced **Ca**^2+ response. Formalin-induced pain behavior is characterized by two phases: an acute first phase mediated by peripheral nociceptive transmission and a second phase thought to be driven by a combination of central sensitization (McNamara et al. 2007) and biphasic nociceptor activity (McCall et al. 1996). In addition, formalin is known to directly activate TRPA1 in nociceptive sensory neurons. However, a supramaximal concentration of the TRPA1 selective antagonist, HC030031, had no effect on the potentiation of the GABA-induced **Ca**^2+ responses by acute low concentration (0.001%) formalin suggesting a mechanism independent of TRPA1.

PGE_2 is a potent inflammatory mediator produced during formalin-induced inflammation (Malmberg and Yaksh 1995). PGE_2 sensitizes peripheral nociceptive neurons through EP receptors present on the peripheral terminals of high-threshold sensory neurons (Omote et al. 2002). In our experiments, PGE_2 potentiated GABA-induced **Ca**^2+ transients almost exclusively in capsaicin-sensitive DRG neurons suggesting that the effect is restricted to a population of nociceptive sensory neurons. PGE_2 also revealed additional muscimol-induced pain-like licking behaviors in vivo.

There are four subtypes of PGE_2 receptor known as EP receptors (EP1-4), all of which are G-protein-coupled receptors: EP1 coupled to G_αq/G_11, EP2 and EP4 coupled to G_αi and EP3 coupled to G_s and G_i. In particular, the EP4 receptor is highly expressed in primary sensory neurons and EP4 levels are known to increase in the DRG after peripheral inflammation (Lin et al. 2006). We found that GABA-induced **Ca**^2+ transients potentiated by PGE_2 were blocked by the EP4 receptor antagonist, AH23848, but not by EP1-2 receptor antagonist, AH6809. Furthermore, formalin-induced facilitation of GABA-induced **Ca**^2+ transients was also abolished by AH23848. These observations suggest that potentiation of the GABA response by formalin and PGE_2 may share the same downstream pathway through the EP4 receptor, although in the case of formalin we cannot exclude the possible contribution from other inflammatory mediators. Formalin application to DRG neurons may act indirectly on EP4 receptors via PGE_2 release. The source of PGE_2 released in our DRG cultures by formalin application in vitro is currently unknown but could include non-neuronal cells carried over during DRG dissociation and plating.
Sensitization of Na⁺ channels may increase the gain of GABA-induced responses in DRG

Previous studies have shown that the PGE₂ signaling pathway can modulate TTX-resistant Na⁺ channels to sensitize peripheral sensory neurons (England et al. 1996). We found that lidocaine blocked PGE₂-induced potentiation of GABA-induced Ca²⁺ transients, but did not affect the basal GABA-induced Ca²⁺ response, suggesting that simultaneous Na⁺ and Cl⁻ conductance are necessary for the conversion to a nociceptive state. To confirm the molecular link between PGE₂-induced sensitization and GABA signaling in primary sensory neurons, we applied the TTX-resistant Naᵥ₈.₈ channel blocker A887826 during the PGE₂ treatment, which successfully prevented the potentiation of GABA signaling by PGE₂. In some recordings (for example Fig. 4E), the potentiating effect of PGE₂ on GABA-induced Ca²⁺-transients appeared to last after washout until the following GABA application. However, on average GABA-induced Ca²⁺ transients returned to near their pre-PGE₂ control amplitude (Fig. 4F). This is consistent with the earlier work on PGE₂ sensitization of TTX-resistant Na⁺ channels where currents returned to their pre-exposure amplitudes within a few minutes of PGE₂ washout (Gold et al. 1996).

Our perforated patch-clamp experiments show that sub-threshold GABA-induced depolarizations in control conditions were enhanced during PGE₂ treatment and commonly resulted in action potentials. It has long been known that PGE₂ causes a leftward shift in the activation curve of TTX-resistant Na⁺ currents (England et al. 1996; Gold et al. 1996). In small-sized DRG neurons GABA_A receptor activation depolarized the membrane potential to approximately −35 mV (Fig. 1N), which just lies within the activation range of PGE₂-sensitized Naᵥ₁.₈ (See Fig. 6G); we speculate that this may lead ultimately to the action potential firing that we observe during application of GABA in the presence of PGE₂. These effects were completely prevented by selective EP4 receptor and Naᵥ₁.₈ channel antagonists, corroborating the pharmacology suggested by our Ca²⁺ imaging results. The concentration of A887826 used in this study (100 nmol/L) is consistent with the selective blockade of Naᵥ₁.₈ in a heterologous expression system as well as rodent DRG (Zhang et al. 2010), although the contribution from the TTX-resistant channel Naᵥ₁.₉, which is also sensitized by PGE₂ (Rush and Waxman 2004), cannot be ruled out. Further work however will be needed to elucidate the relationship between PGE₂-induced sensitization of voltage-gated Na⁺ channels and GABA_A receptor-mediated depolarization in vivo.

An increase in the responsiveness of isolated DRG neurons to GABA has previously been observed with Ca²⁺ imaging after chronic (3 days complete Freund’s adjuvant) inflammation in adult rats, and occurs concurrent with a decrease in low threshold K⁺ current density leading to membrane depolarization (Zhu et al. 2012b). Interestingly, this and another study by the same authors (Zhu et al. 2012a) report an increase in GABA current density with chronic inflammation, something that we did not observe in the presence of acute PGE₂ application. Notwithstanding the differences in acute and chronic inflammation we cannot therefore exclude the contribution of other ion conductances in the mechanism(s) of GABA-mediated nociception.

NKCC1 is not required for GABA_A nociception in inflammation

As we show in our in vitro characterization, GABA induces cell membrane depolarization in peripheral sensory neurons because of the high intracellular Cl⁻ concentration of DRG neurons. We further confirm that the GABA response in isolated DRG neurons is maintained by the constitutive activity of the Na⁺-K⁺-2Cl⁻ co-transporter NKCC1. A previous study reported that intracellular Cl⁻ concentration was increased in DRG neurons 1 h after treatment with a soup of inflammatory mediators (Funk et al. 2008). This has led to the suggestion that pathological upregulation of NKCC1 in inflammation could initiate the transition of peripheral GABA_A receptors to a nociceptive role (Morales-Aza et al. 2004). NKCC1 also represents a potential downstream target of EP4 signaling via PKA-mediated phosphorylation (Flemmer et al. 2010). Despite the evidence in favor of a role for NKCC1 in GABA_A-mediated nociception, our data surprisingly show the maintenance of muscimol-induced spontaneous licking behavior after formalin in mice lacking NKCC1, as well as PGE₂-induced potentiation of GABA-induced Ca²⁺ transients in NKCC1⁻/⁻ DRG neurons. Although intracellular Cl⁻ is reduced in the DRG of NKCC1⁻/⁻ mice it is not completely eliminated (Sung et al. 2000), suggesting that compensation by another Cl⁻ regulatory mechanism allows depolarization by GABA in these mice. Our results also show that an acute application of PGE₂ is sufficient to potentiate the GABA response, although we cannot exclude the possibility of an effect on intracellular Cl⁻ concentration via NKCC1 modulation with more chronic exposure to PGE₂.

Endogenous peripheral GABA_A receptor activation

As well as blocking muscimol-induced behavior, high dose picrotoxin in the hind paw also inhibited the second phase of the formalin response. This surprising result is
consistent with a previous study in which systemic tetrodotoxin treatment reduced formalin behavior (Heidari et al. 1996) and suggests that endogenous activators of the GABA A receptor may indeed play a role in the behavioral response to acute formalin inflammation.

One important remaining question is the endogenous source of GABA A receptor activation in DRG neurons. Expression of functional GABA A receptors at the central terminals of DRG (Labrakakis et al. 2003) creates the potential to receive spinal GABAergic signaling. Expression of GABA, or the GABA-synthesizing enzyme glutamine decarboxylase (GAD), has also been reported in various peripheral tissues, including keratinocytes (Ito et al. 2007) and macrophage (Tannahill et al. 2013). Whether these tissues are capable of synthesizing and more importantly releasing GABA remains to be explored.

In conclusion, our report suggests a nociceptive role of peripheral GABA A receptors in acute-inflammatory pain and presents a working model of the mechanism of GABA A receptor-mediated nociception.

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Conflict of Interests

All the authors have no competing interest in this study.

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