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# Inhibition of G protein-coupled P2Y<sub>2</sub> receptor induced analgesia in a rat model of trigeminal neuropathic pain

Na Li<sup>1,2†</sup>, Zhan-ying Lu<sup>1†</sup>, Li-hua Yu<sup>1†</sup>, Geoffrey Burnstock<sup>3,4</sup>, Xiao-ming Deng<sup>2\*</sup> and Bei Ma<sup>1\*</sup>

## Abstract

**Backgrounds:** ATP and P2X receptors play important roles in the modulation of trigeminal neuropathic pain, while the role of G protein-coupled P2Y<sub>2</sub> receptors and the underlying mechanisms are less clear. The threshold and frequency of action potentials, fast inactivating transient K<sup>+</sup> channels (I<sub>A</sub>) are important regulators of membrane excitability in sensory neurons because of its vital role in the control of the spike onset. In this study, pain behavior tests, QT-RT-PCR, immunohistochemical staining, and patch-clamp recording, were used to investigate the role of P2Y<sub>2</sub> receptors in pain behaviour.

**Results:** In control rats: 1) UTP, an agonist of P2Y<sub>2</sub>/P2Y<sub>4</sub> receptors, caused a significant decrease in the mean threshold intensities for evoking action potentials and a striking increase in the mean number of spikes evoked by TG neurons. 2) UTP significantly inhibited I<sub>A</sub> and the expression of Kv1.4, Kv3.4 and Kv4.2 subunits in TG neurons, which could be reversed by the P2 receptor antagonist suramin and the ERK antagonist U0126. In ION-CCI (chronic constriction injury of infraorbital nerve) rats: 1) mRNA levels of Kv1.4, Kv3.4 and Kv4.2 subunits were significantly decreased, while the protein level of phosphorylated ERK was significantly increased. 2) When blocking P2Y<sub>2</sub> receptors by suramin or injection of P2Y<sub>2</sub>R antisense oligodeoxynucleotides both led to a time- and dose-dependent reverse of allodynia in ION-CCI rats. 3) Injection of P2Y<sub>2</sub> receptor antisense oligodeoxynucleotides induced a pronounced decrease in phosphorylated ERK expression and a significant increase in Kv1.4, Kv3.4 and Kv4.2 subunit expression in trigeminal ganglia.

**Conclusions:** Our data suggest that inhibition of P2Y<sub>2</sub> receptors leads to down-regulation of ERK-mediated phosphorylation and increase of the expression of I<sub>A</sub>-related Kv channels in trigeminal ganglion neurons, which might contribute to the clinical treatment of trigeminal neuropathic pain.

**Keywords:** Antisense oligodeoxynucleotides, ERK, Excitability, I<sub>A</sub> channels, P2Y<sub>2</sub> receptors, Trigeminal ganglion, Trigeminal neuropathic pain

## Introduction

Trigeminal neuropathic pain disorders, as typical, atypical, or post-therapeutic trigeminal neuralgias, are pain that is either spontaneous or can be elicited by harmless but crucial activities, such as eating and talking, or by light touch to facial skin [1]. The current treatments do not provide long-lasting relief for these frequently

treatment-refractory patients due to a limited understanding of their pathophysiology. Chronic constriction nerve injury (CCI) has characteristics of inflammation and nerve injury [2,3]. Previous studies using a chronic constriction nerve injury model of the infraorbital nerve (ION-CCI) have reported it to be a good model that mimics trigeminal neuralgia of humans [4-7]. The major pathologic changes for trigeminal neuralgia are axonal loss and demyelination in trigeminal root [8]. Constrictive infraorbital nerve injury like constrictive sciatic nerve injury induces demyelination as sources of pathological ectopic firing accompanying mechanical allodynia and heat hyperalgesia [4].

\* Correspondence: deng\_x@yahoo.com; mabei08@aliyun.com

†Equal contributors

<sup>2</sup>Department of Anesthesiology, Changhai hospital, Second Military Medical University, Shanghai 200433, P.R. China

<sup>1</sup>Department of Physiology, The Key Laboratory of Molecular Neurobiology, Ministry of Education, Second Military Medical University, Shanghai 200433, P.R. China

Full list of author information is available at the end of the article

Adenosine 5'-triphosphate (ATP) and uridine 5'-triphosphate (UTP) are released from cells as a consequence of tissue injury and mediate their bio-effects through binding to a large group of cell surface receptors of both P2X or P2Y receptor families [9]. There were early hints that ATP might be involved in pain, including the demonstration of pain produced by injection of ATP into human skin blisters [10,11]. In trigeminal ganglion (TG) neurons, the highly selective distribution of P2X<sub>3</sub> and P2X<sub>2/3</sub> receptors within the nociceptive system has suggested a potential role for ATP as a pain mediator [12,13]. Expression of P2Y<sub>1</sub>, 2, 4, and 6 receptors has also been reported in TG neurons [14]. P2Y<sub>2</sub> receptors are typically expressed on small, nociceptive neurons [15]. *In vitro* studies have demonstrated that co-activation of P2Y<sub>2</sub> receptors and TRPV channels by ATP could underlie ATP-induced pain [16]. UTP, a selective agonist for P2Y<sub>2</sub> and P2Y<sub>4</sub> receptors, activates cutaneous afferent fibers [17], mediates excitation of dorsal root ganglion (DRG) neurons [18] and sensitizes mouse bladder sensory neurons [19]. These results suggest that UTP may be an endogenous nociceptive messenger. However, *in vivo* studies have shown that UTP significantly alleviates mechanical allodynia in a neuropathic pain model [20,21]. However, the effect of activation of P2Y<sub>2</sub> receptors on neuropathic pain is not clear and requires further study.

Multiple types of voltage-gated ion channels are related to neuronal excitability, such as voltage-gated K<sup>+</sup> (Kv) channels, which are important regulators of membrane potentials and action potentials in nociceptive sensory neurons [22,23]. In rat small TG neurons, Kv currents have been divided into three types: slow inactivating transient K<sup>+</sup> current (I<sub>D</sub>), fast inactivating transient K<sup>+</sup> current (I<sub>A</sub>) and dominant sustained K<sup>+</sup> current (I<sub>K</sub>) [24]. I<sub>A</sub> is particularly important in the control of the spike onset, the threshold of the action potential firing, and the firing frequency [25]. Many studies have shown that the Kv1.4, Kv3.4, Kv4.2, and Kv4.3 subunits contribute to the I<sub>A</sub> channels in DRG neurons [26-28], which suggests that I<sub>A</sub> has the ability to regulate the neuronal activity of nociceptive neurons. After sciatic nerve injury, the expression of Kv1.4 was decreased in small-diameter DRG neurons [28]. Another study showed that activation with the GABA<sub>B</sub> receptor agonist baclofen inhibited the excitability of TG neurons, which was mediated by potentiation of both I<sub>A</sub> and I<sub>K</sub> in rat small-diameter TG neurons [29]. I<sub>A</sub>, I<sub>K</sub> and the total K<sup>+</sup> currents were significantly reduced in rats with inferior alveolar nerve transection and ION-CCI [3,30]. A recent report demonstrated that P2Y<sub>2</sub> receptors mediate an excitation of DRG neurons through inhibition of K<sub>v</sub>7 channels [18].

In this study, we hypothesize that activation of P2Y<sub>2</sub> receptors might mediate trigeminal neuropathic pain through regulating the expression and function of Kv1.4,

Kv3.4, Kv4.2, and Kv4.3 subunits. We have used pain behavior tests, quantitative reverse transcription-polymerase chain reaction analysis (QT-RT-PCR), immunohistochemical staining and patch-clamp recording to investigate the role of P2Y<sub>2</sub> receptors in pain behavior, excitability of TG neurons, and modulation of I<sub>A</sub> channels in rats.

## Materials and methods

### Animals

Experiments were performed on male Sprague-Dawley rats weighing 200–250 g. Rats were kept under standard laboratory conditions with food and water *ad libitum*. They were housed three per cage and maintained on a 12:12 h light: dark schedule at a constant ambient temperature (24 ± 1°C). All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Second Military Medical University.

### Drugs and drug administration

ATP, α,β-methylene-ATP (α,β-meATP), 2-methylthio ADP (2-MesADP), UTP, suramin, U0126 and TEA were purchased from Sigma-Aldrich (St. Louis, MO). Fluoro-Gold was purchased from Biotium (Hayward, CA). For electrophysiology and RT-PCR, ATP, UTP, α,β-meATP, 2-MesADP, suramin and U0126 were dissolved in distilled water to 10 mM, and then diluted to the final concentration (details see the Results). For animal behavioral tests, suramin was diluted in distilled water to 150 μg/50 μl and 15 μg/50 μl and injected only once per dilution, respectively. P2Y<sub>2</sub> receptor antisense oligodeoxynucleotides (AS-ODN) was dissolved in water to 15 μg/50 μl and usually injected every 12 h within a 48 h period.

For animal behavioral tests, we performed a peripheral target injection to the TG via the infraorbital foramen as described previously by Neubert [31]. Briefly, at day 9 after surgery, rats were anaesthetized with diethyl ether. A sterile stainless steel needle was inserted medial (1–2 mm) to the palpated portion of the zygomatic process through the infraorbital foramen. The needle was positioned at ~10° angle relative to the midline of the head. The tip of the needle was advanced approximately 20 mm along the infraorbital canal and subsequently through the foramen rotundum, then the corresponding drugs were injected. The mechanical pain threshold was then determined every 5 min or 12 h after injection.

### P2Y<sub>2</sub> receptor antisense oligodeoxynucleotides (AS-ODN)

P2Y<sub>2</sub> receptor AS-ODN was purchased from Invitrogen Company. Oligonucleotides to rats P2Y<sub>2</sub> were synthesized and purified by Integrated DNA Technologies (ADT). The sequence was as follow: antisense 5'-CCAGGAGTCCAGGCCTGCTGCCATTGCC-3'. The sequences were checked for uniqueness using the National Center for

Biotechnology Information's Local Alignment Searchtool (BLAST) based on a previous study [32].

### **Surgery and behavior test**

#### ***Chronic constriction injury of the infraorbital branch of trigeminal nerve (ION-CCI) and sham surgery***

Rats underwent CCI of the right ION as previously described [3]. Briefly, rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and a small incision (approximately 5 mm) was made at the juncture between the zygomatic arch and nasal bone, under the right eye. The muscle was dissected to exposure the infraorbital branch of the TG nerve until it was clearly visible through the incision and two ligatures (4–0 chromic catgut) were loosely tied (with about 2 mm spacing) around it. The loose ligature method was used according to the procedure developed by Imamura et al. [33]: the ligatures reduced the diameter of the nerve by a just noticeable amount and retarded, but did not interrupt the circulation through the superficial vasculature. The incision was sutured with 1.0 silk. Sham-operated rats were treated identically, but no ligatures were applied to the ION. All operations were performed aseptically. After surgery, all rats were maintained in a warm room until they recovered from anesthesia and no antibiotics were administered.

### **Behavior test**

Rats were allowed to acclimate for at least 3 days before use in experiments. All the experiments were carried out between 9:00 and 14:00 in the animal housing room. Before each testing session, animals were placed in individual plastic cages and left to adapt to the environment for at least 15 min. The mechanical pain threshold was tested one day before and every three days after surgery in the two groups. In accordance with our previous study, we injected drugs and performed behavior tests at day 9 after surgery when the mechanical pain threshold of rats was lowest. Each rat received drugs only once and was used in only one experiment. The mechanical pain threshold (PWT) was determined with a rigid von Frey filament coupled with a force transducer (Electro-vonfrey, model no: 2391, IITC Inc. Woodland Hills, CA), as previously described [34]. Stimuli were applied within the ION territory, around the center of the vibrissal pad, on the hairy skin surrounding the mystacial vibrissae, ten consecutive times with 2 s each time at 30-sec intervals on the nerve-injured side [35]. The PWT was considered as the lowest force of the filaments that produced a brisk head withdrawal, touching or scratching the facial regions upon mechanical stimulation [35]. The mechanical pain threshold were measured every 5 min or 12 h following drug administration. The

persons conducting the behavioral measurements were blind to the treatments.

### **Retrograde labeling of TG neurons innervating the facial skin**

TG neurons innervating the facial skin were identified for electrophysiology and immunohistochemistry using the fluorogold (FG)-labeling method [36]. A FG solution (2% in distilled water, 50  $\mu$ l) was injected into the facial skin in the bilateral region of the whisker pad using a 31-gauge microsyringe.

### **Cell culture**

Control male Sprague–Dawley rats were used for patch-clamp recording and RT-PCR analysis. Rats were decapitated and bilateral TG were rapidly removed, de-sheathed, cut and incubated in 2 ml  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free Hanks' balanced salt solution with 10 mM HEPES buffer (pH 7.4) (HBSS; Life Technologies) containing 1.5 mg/ml collagenase (Class II, Worthington Biochemical Corporation, UK) and 6 mg/ml bovine serum albumin (Sigma Chemical Co., Poole, UK) at 37°C in a shaking bath (170 rpm) for 30 min. This was followed by incubation in 2 ml HBSS containing 1 mg/ml trypsin (Sigma) at 37°C in a shaking bath (170 rpm) for 10 min. The solution was replaced with 1 ml growth medium comprising L-15 medium supplemented with 10% bovine serum, 50 ng/ml nerve growth factor, 0.2%  $\text{NaHCO}_3$ , 5.5 mg/ml glucose, 200 i.u./ml penicillin and 2 g/ml streptomycin. The ganglia were dissociated into single neurons by gentle mechanical trituration and plated onto 35 mm Petri dishes coated with 10  $\mu$ g/ml laminin (Sigma) [37]. Cells were maintained at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$ . All neurons were studied after 16 h removal from the animals. For RT-PCR, cultured TG neurons were incubated in growth medium without 10% bovine serum, in order to eliminate glia cells.

### **Real-time quantitative reverse transcription–polymerase chain reaction analysis**

#### ***RNA extraction and RT-PCR***

Total RNA was extracted using an RNeasy Mini Kit (QIAGEN; Clifton Hill, Australia). RNA purity was determined using a method of ultraviolet spectrophotometry at a wavelength of 260–280 nm. 2  $\mu$ g of total RNA was reversely transcribed to complementary DNA in a 20  $\mu$ l reaction mixture containing 1 $\times$  reverse transcriptase buffer (15 mM  $\text{MgCl}_2$ , 375 mM KCl, 50 mM DTT, 250 mM Tris–HCl, pH 8.3), 10 mM dNTP, 20 U RNase inhibitor, 200 U M-MLV reverse transcriptase, and 50 ng of oligo (deoxythymidine)<sub>15</sub> primer. Reaction time was at least 1 h at 42°C. The cDNA was stored at –20°C until real-time polymerase chain reaction (RT-PCR). All

reagents, with the exception of the RNeasy Mini Kit, were from Promega Corp. (Madison, WI).

#### Real-time RT-PCR

Quantitative RT-PCR amplification was performed with SYBRGreen (Applied Biosystems; Scoresby, Australia) using Roto-gene RG3000 (Australia) in a 20  $\mu$ l reaction mixture. The solution consisted of 1.0  $\mu$ l diluted RT-PCR product, 0.25  $\mu$ M of each of the paired primers, and 10  $\mu$ l real-time PCR SYBR Green Master Mix (QIAGEN, Clifton Hill, Australia). RNA levels were measured with specifically designed primers. That for Kv1.4 was: 5'-TTG TGA ACG CGT GGT AAT AAA TGT GT-3' (forward), 5'-GGC GGC CTC CTG ACT GGT AAT AAT A-3' (reverse); for Kv3.4: 5'-CCA CGG GGC AAT GAC CAC ACC-3' (forward), 5'-ACA CAG CGC ACC CAC CAG CAT TCC T-3' (reverse); for Kv4.2: 5'-GCC GCA GCG CCT AGT CGT TAC C-3' (forward), 5'-TGA TAG CCA TTG TGA GGG AAA AGA GCA-3' (reverse); and for Kv4.3: 5'-CTC CCT AAG CGG CGT CCT GGT CAT T-3' (forward), 5'-CTT CTG TGC CCT GCG TTT ATC TGC TCT C-3' (reverse) against the sequences downloaded from Genbank (accession no. X90651, 708–731 and 1126–1147). The PCR condition was 95°C for 2 min, followed by 40 cycles of 95°C, 20 s; 65°C, 25 s; 72°C, 25 s for Kv1.4, Kv3.4, Kv4.2, Kv4.3 and was 95°C for 2 min, followed by 40 cycles of 95°C, 20 s; 63°C, 25 s; 72°C. RT-PCR for the housekeeping gene  $\beta$ -actin was performed for each sample. The primer for amplification of  $\beta$ -actin was: ATGGTGGGTATGGGTCAGAAAGG (forward); TGGCTGGGGTGTGAAGGTC (reverse). The absolute mRNA level of target gene in each sample was calculated using a standard curve and then by the ratio to  $\beta$ -actin in each sample. The specificity of the primers was verified by examining the melting curve as well as sequencing of the QT-RT-PCR products. The melting curve of QT-RT-PCR showed a single sharp peak for Kv1.4, Kv3.4, Kv4.2, Kv4.3 and  $\beta$ -actin PCR products. The lengths of PCR products of Kv1.4, Kv3.4, Kv4.2, Kv4.3 and  $\beta$ -actin mRNA were 199, 134, 261, 108 bp and 265 bp, respectively.

#### Whole-cell patch clamp recording

Whole-cell patch-clamp recording was undertaken at room temperature with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Membrane potential held at -60 mV, signals were filtered at 2 kHz (-3 dB frequency, Bessel filter, 80 dB per decade), then digitized at 10–50 kHz (Digidata 1320A interface, Axon Instruments). The leak current was subtracted from the potassium currents using Clampfit programs. Patch electrodes had resistance of 2–5 M $\Omega$ . For voltage-recordings, the pipette solution contained the following (in mM): K gluconate 120, KCl 10, NaCl 5, MgCl<sub>2</sub>•6H<sub>2</sub>O 2, CaCl<sub>2</sub>•2H<sub>2</sub>O 1, HEPES 10, EGTA 11, Mg-ATP 2, Li-

GTP 1 (pH adjusted to 7.4 with KOH). The external solution contained (in mM): NaCl 145, KCl 3, CaCl<sub>2</sub>•2H<sub>2</sub>O 1, MgCl<sub>2</sub>•6H<sub>2</sub>O 2, HEPES 10, glucose 10, (pH adjusted to 7.4 with NaOH). Test solutions bathing the cytoplasmic face of the patch membrane contained (in mM): NMDG 145, TEA 25, KCl 3, MgCl<sub>2</sub>•6H<sub>2</sub>O 0.6, CdCl<sub>2</sub> 1, CaCl<sub>2</sub>•2H<sub>2</sub>O 2.5, HEPES 10, glucose 10 (pH adjusted to 7.4 with tris-base and 300 mOsM). CdCl<sub>2</sub> was included to block voltage-gated calcium channels. NMDG and TEA were included to reduce currents from voltage-gated sodium channels, I<sub>K</sub> currents, hyperpolarization-activated cation channels, and capsaicin-induced inward currents [27,38]. A protocol was used as previous described [39], briefly, a pre-pulse (-120 mV, 100 ms) was followed by test pulses (400 ms) from -60 to +60 mV with 10 mV increments, and only those cells that exhibited minimal outward currents during the pre-pulse were analyzed. For current-recordings, action potentials were recorded under current-recordings. During a 400-ms injection of a positive current (ranging from -40 to 450 pA), a single action potential could be evoked, depending on the type of neuron (A $\beta$ -, A $\delta$ - and C-units) [3], for example A $\delta$ -units were frequently encountered at a later period after ION-CCl. Cultured TG neurons with soma diameters ranging from 18 to 39  $\mu$ m were used for action potential recording, for they are consistent with nociceptive A $\delta$ - and C-neurons [39]. Those neurons with retrograde labelling were used for I<sub>A</sub> recording. The amplitude of the I<sub>A</sub> was measured at the peak. Whole-cell current-voltage (I-V) curves for individual neurons were generated by calculating the peak outward current at each testing potential and normalizing to the cell capacitance.

#### Western blotting analysis

TG were harvested and homogenized in cold lysis buffer (20 mM Hepes buffer, pH 7.4, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 5 mg/mL pepstatin A, 10 mg/mL leupeptin and 10 mg/mL aprotinin) using a Dounce homogenizer. Protein concentration was determined with a bicinchoninic acid (BCA) assay kit using bovine serum albumin as a standard (Pierce Biotechnology, Inc., Rockford, IL) and then heated to 95°C. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% Tris-HCl gels (BioRad, Hercules, CA) and electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, USA) at 120 V for one and half an hour in Towbin buffer, pH 8.3, to which 20% (V/V) methanol had been added. After transfer, the membranes were blocked with 5% (mass/vol) non-fat dried milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 hour, then incubated with the primary antibodies:



P2Y<sub>2</sub> (rabbit anti-rat polyclonal, IgG 1:500, Santa Cruz Biotechnology, Santa Cruz, CA) or ERK (rabbit anti-rat polyclonal, IgG 1:1000, Cell Signaling) and  $\beta$ -actin (mouse monoclonal, IgG 1:8000, Sigma, USA). After three washes with TBST, the membranes were incubated with the secondary antibody (goat anti-rabbit polyclonal, IgG 1:8000, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were rinsed at room temperature in Tris-buffered saline containing Tween 20 (TBST) followed by TBS 3 times and visualized using an Odyssey Infrared Imaging System (LICOR, Lincoln, NE). Densitometric quantification of the P2Y<sub>2</sub>, ERK and  $\beta$ -actin protein bands of the Western blot were determined using Odyssey software version 1.0 (LI-COR, Lincoln, NE) and expressed as a relative ratio of P2Y<sub>2</sub>/ $\beta$ -actin and ERK/ $\beta$ -actin.

#### Immunofluorescence histochemistry

Rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused transcardially with 0.1 M phosphate buffer solution (PBS), pH 7.4 and subsequently with fresh 4% paraformaldehyde phosphate buffer (PB) solution. After the perfusion, TGs were harvested and fixed in 4% paraformaldehyde for 4 hours. They were then transferred into 20% sucrose for at least 3 days. Series frozen transverse sections (10  $\mu$ m thick) were made through the TG with a cryostat (Leica, CM1850, Germany), collected and then washed 3  $\times$  5 min in cold PBS. The preparations were then preincubated in antiserum solution 1 (10% normal bovine serum, 0.2% Triton X-100, 0.4% sodium azide in 0.01 mol/l PBS pH 7.2) for 30 min. For double-immunostaining of P2Y<sub>2</sub> and Kv1.4 or Kv3.4 or Kv4.2 or Kv4.3, sections were incubated in a mixture of rabbit polyclonal P2Y<sub>2</sub> (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal Kv1.4 (1:200 dilution, Abcam, HongKong, China) or goat polyclonal KCNC4 (Kv3.4) (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat polyclonal Kv4.2 (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat polyclonal Kv4.3 (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The sections were subsequently incubated with FITC-conjugated affini-pure donkey anti-rabbit IgG (1:200 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h to visualize P2Y<sub>2</sub> receptors, fluorophore-labeled donkey anti-mouse IgG (1:1500 dilution, Invitrogen life technologies, Grand Island, NY, USA) for 1 h to visualize Kv1.4, Cy3-conjugated affini-pure donkey anti-goat IgG (1:200 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h to visualize Kv3.4, Cy3-conjugated affini-pure donkey anti-goat IgG (1:300 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h to

visualize Kv4.2 or Kv4.3, respectively. All staining procedures were carried out at room temperature and all the incubations were separated by three washes in PBS, 5 min each. The immunoreactivity was visualized by fluorescence microscopy.

#### Statistical analysis

All data are presented as means  $\pm$  SEM. The electrophysiological data were analyzed using the clampfit 9.0 and origin 7.0. For current-clamp recording, differences between the means of action potentials were tested for significance using unpaired Student's t-tests. For voltage-clamp recording and animal behavior test, differences between the means were tested for significance using repeated measures ANOVA followed by Dunnett's analysis. For RT-PCR results, differences among groups were tested for significance using two way ANOVA followed by Dunnett's analysis. For Western-blot and immunofluorescence histochemical results, differences among groups were tested for one-way ANOVA followed by Tukey's HSD and unpaired Student's t-tests. Differences were considered as statistically significant when the p value was lower than 0.05 ( $p < 0.05$ ).

#### Results

##### Effects of P2Y<sub>2</sub> receptors on Kv1.4, Kv3.4, Kv4.2 and Kv4.3 in control rat TG neurons

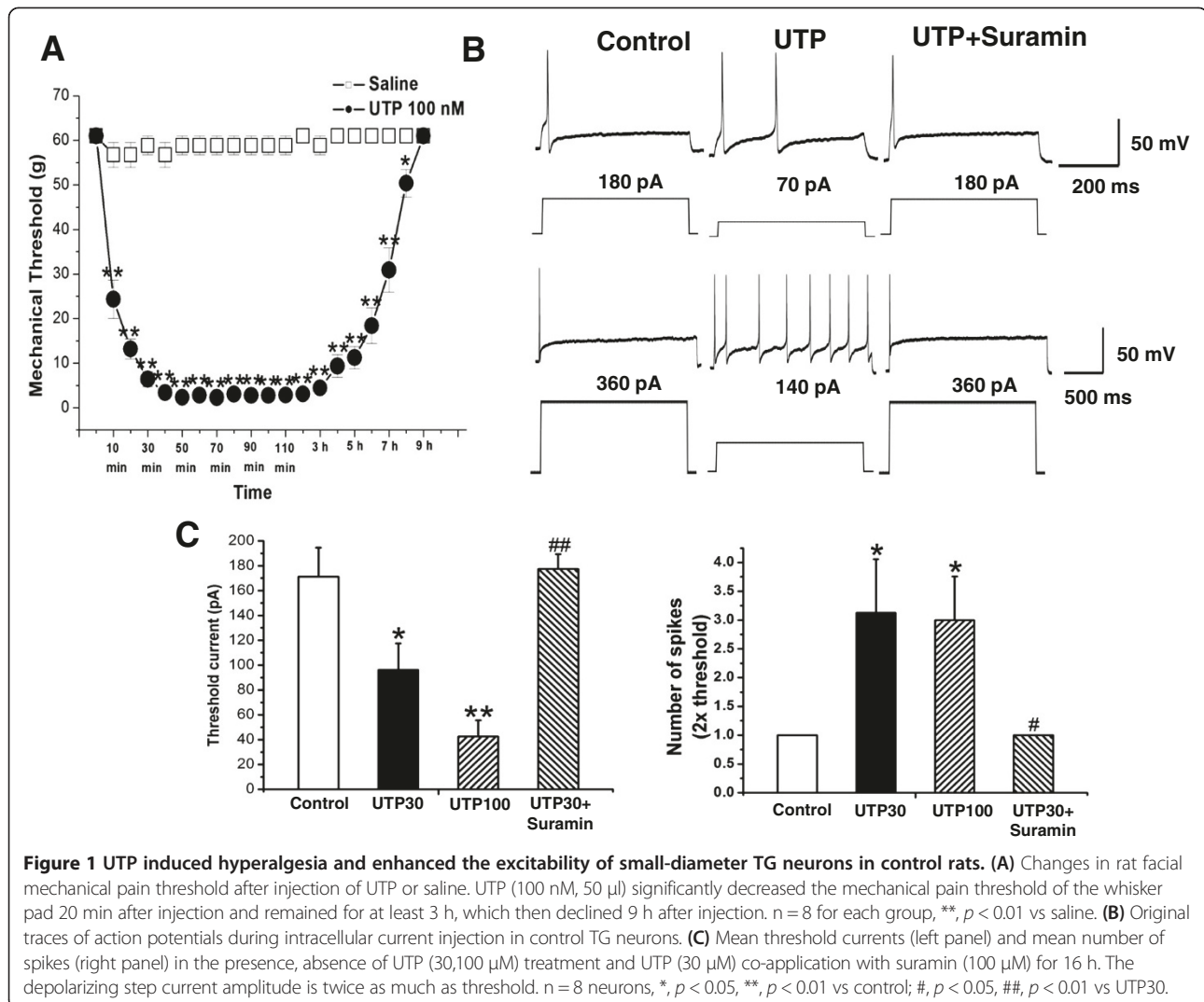
Double-immunofluorescence staining for P2Y<sub>2</sub> receptors and Kv1.4 or Kv3.4 or Kv4.2 or Kv4.3 was performed on TG neurons in sham and ION-CCI groups. In the sham group, 272 out of 301 (90.4%) P2Y<sub>2</sub> receptor-positive neurons were Kv1.4 positive; 302 out of 307 (98.1%) P2Y<sub>2</sub> receptor-positive neurons were Kv3.4 positive; 274 out of 325 (84.3%) P2Y<sub>2</sub> receptor-positive neurons were Kv4.2 positive; and 159 out of 201 (79.1%) P2Y<sub>2</sub> receptor-positive neurons were Kv4.3 positive. Most of them had soma diameters ranging from 25 to 34  $\mu$ m ( $n = 294$  neurons).

##### UTP induces hyperalgesia in control rats

We investigated the role of UTP, an agonist of P2Y<sub>2</sub> and P2Y<sub>4</sub> receptors on control rats. UTP (100 nM, 50  $\mu$ l) significantly decreased the mechanical pain threshold of the whisker pad 20 min after injection and this remained at least for 3 h (Figure 1A,  $n = 8$  for each group,  $p < 0.01$ ); it then declined 9 h after the injection (Figure 1A).

##### UTP enhances the excitability of small-diameter TG neurons in control rats

Based on the results of the animal behavioral test, we explored the effect of P2Y<sub>2</sub> receptor activation on the electrophysiological properties of small-diameter TG neurons of rats. Action potentials generated in these TG neurons by square-pulse stimulation are illustrated in



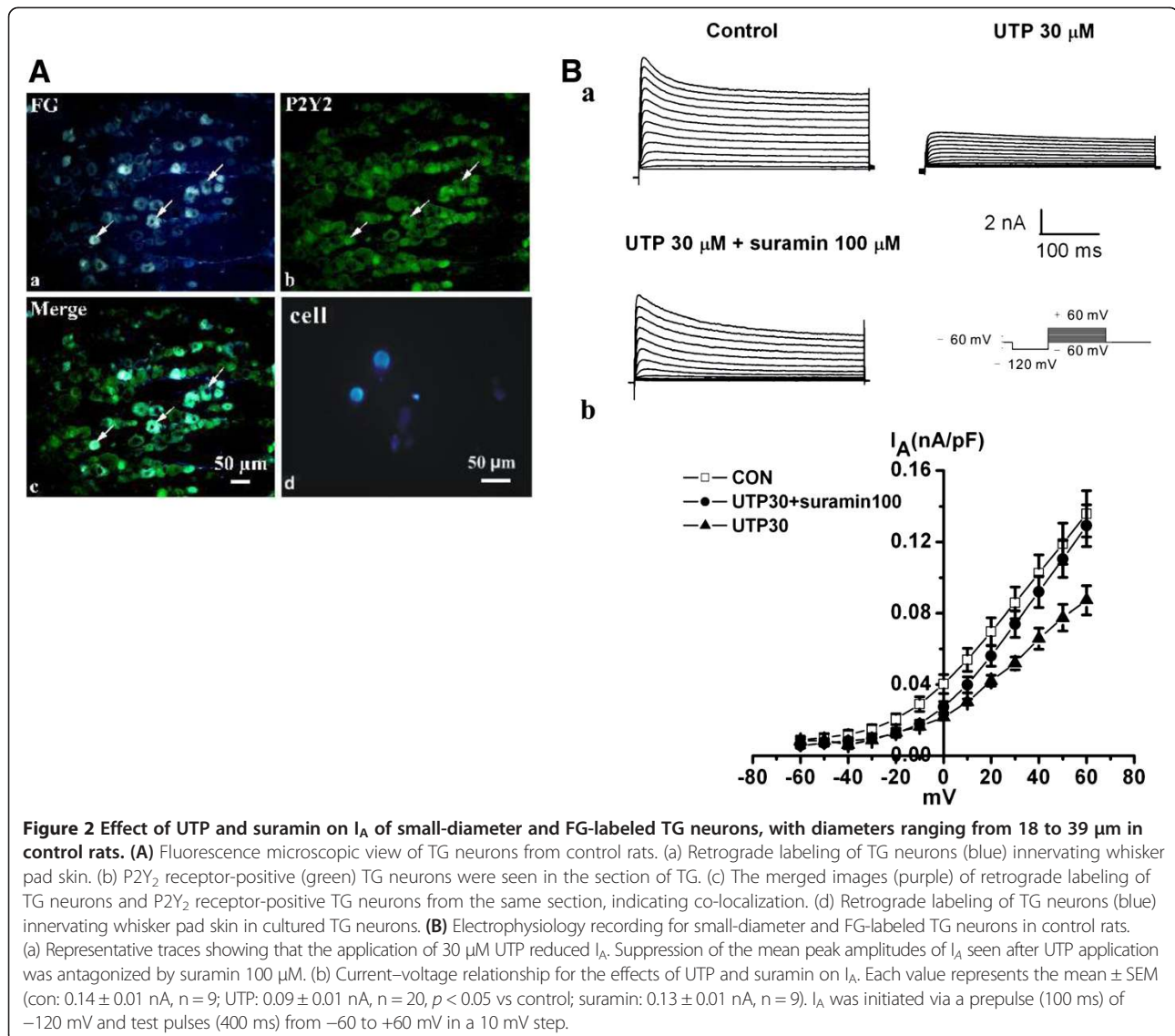
**Figure 1** UTP induced hyperalgesia and enhanced the excitability of small-diameter TG neurons in control rats. **(A)** Changes in rat facial mechanical pain threshold after injection of UTP or saline. UTP (100 nM, 50  $\mu$ l) significantly decreased the mechanical pain threshold of the whisker pad 20 min after injection and remained for at least 3 h, which then declined 9 h after injection.  $n = 8$  for each group, \*\*,  $p < 0.01$  vs saline. **(B)** Original traces of action potentials during intracellular current injection in control TG neurons. **(C)** Mean threshold currents (left panel) and mean number of spikes (right panel) in the presence, absence of UTP (30,100  $\mu$ M) treatment and UTP (30  $\mu$ M) co-application with suramin (100  $\mu$ M) for 16 h. The depolarizing step current amplitude is twice as much as threshold.  $n = 8$  neurons, \*,  $p < 0.05$ , \*\*,  $p < 0.01$  vs control; #,  $p < 0.05$ , ##,  $p < 0.01$  vs UTP30.

Figure 1B. Incubation of TG neurons from control rats with UTP 30 or 100  $\mu$ M for 16 h, caused dose-dependent decrease in the mean threshold intensities for evoking action potentials (UTP30 group:  $96.3 \pm 21.2$  pA; UTP100 group:  $42.5 \pm 13.1$  pA; control group:  $171.3 \pm 23.2$  pA;  $n = 8$ , Figure 1B,C,  $p < 0.05$  or  $p < 0.01$  vs control). The decrease of mean threshold intensities for evoking action potentials by UTP 30  $\mu$ M was reversed by co-incubated with suramin 100  $\mu$ M (UTP30 group:  $96.3 \pm 21.2$  pA; suramin group:  $177.5 \pm 11.8$  pA;  $n = 8$ , Figure 1B,C,  $p < 0.05$ ). As shown in Figure 1C, the mean number of spikes evoked in the UTP-incubated TG neurons during depolarizing step pulses at  $2 \times$  threshold was significantly higher in the UTP-incubated TG neurons than those in the control neurons but did not show any dose-dependent changes (control: 1 spikes/400 ms; UTP-incubated TG neurons (30 or 100  $\mu$ M):  $3.1 \pm 0.9$  spikes/400 ms or  $3.0 \pm 0.8$  spikes/400 ms;  $n = 8$ ,  $p < 0.05$ ). The increase of mean number of spikes by UTP 30  $\mu$ M

was blocked by co-incubated with suramin 100  $\mu$ M (Figure 1B,C,  $n = 8$ ,  $p < 0.05$  vs UTP 30 group).

**Activation of P2Y<sub>2</sub> receptors mediates a functional inhibition of I<sub>A</sub> channels by UTP in FG-labeled small-diameter TG neurons in control rats**

FG-labeled TG neurons are illustrated in Figure 2A. We observed whether activation of P2Y<sub>2</sub> receptors could functionally inhibit I<sub>A</sub> subunits in these TG neurons. For voltage-clamp experiments, typical waveforms of depolarization-activated I<sub>A</sub> are shown in Figure 2B. After incubation with UTP (30  $\mu$ M) for 16 h, the mean peak amplitude of I<sub>A</sub> was significantly suppressed compared with that of control ( $0.12 \pm 0.01$  nA vs  $0.06 \pm 0.01$  nA,  $n = 12$ ,  $p < 0.01$ ). The suppression of peak amplitudes of I<sub>A</sub> by UTP (30  $\mu$ M) was then blocked by co-application of suramin (Figure 2B, con:  $0.14 \pm 0.01$  nA,  $n = 9$ ; UTP:  $0.09 \pm 0.01$  nA,  $n = 20$ ,  $p < 0.05$ ; suramin:  $0.13 \pm 0.01$  nA,  $n = 9$ ,



$p < 0.01$ ). We did not see any dose-dependent changes in  $I_A$  when using UTP 100  $\mu\text{M}$  ( $0.08 \pm 0.01$  nA,  $n = 20$ ).

In order to observe whether other pain-related P2 receptors were involved in the inhibition of  $I_A$ ,  $\alpha, \beta$ -meATP, a  $P2X_3$  and  $P2X_{2/3}$  receptor agonist, and 2-MeSADP, a  $P2Y_1$  receptor agonist, were used. We did not find any changes in  $I_A$  following application of either  $\alpha, \beta$ -meATP or 2-MeSADP, respectively (Figure 3). This implied that  $P2X_1$ ,  $P2X_3$ ,  $P2Y_1$ ,  $P2Y_{12}$  and  $P2Y_{13}$  receptors were not involved.

#### UTP-induced reduction in the expression levels of $I_A$ subunits (Kv1.4 or Kv3.4 or Kv4.2 and Kv4.3) in control TG neurons via $P2Y_2$ receptors

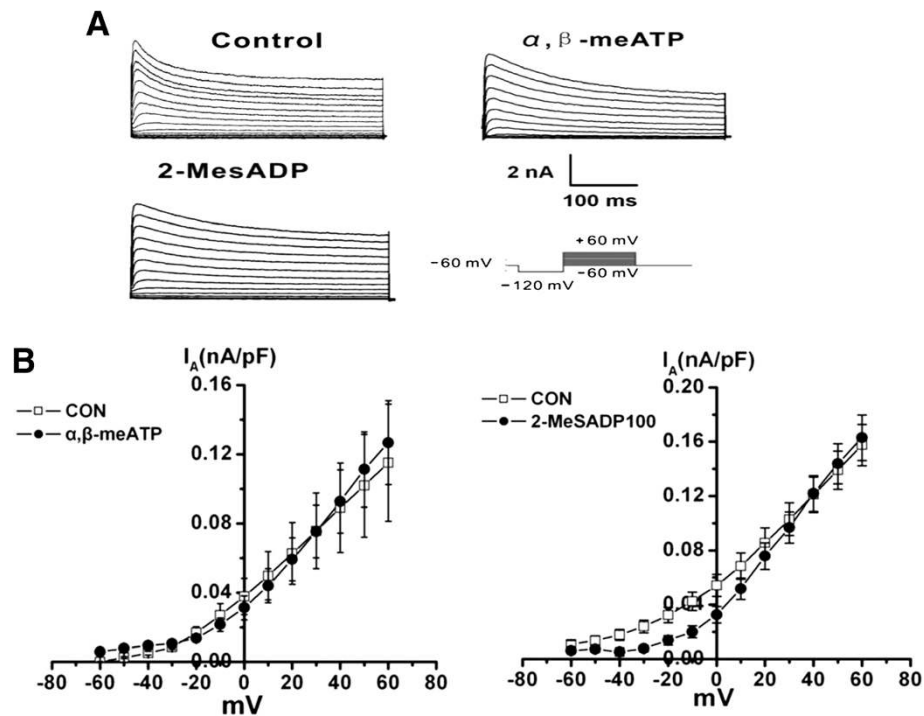
Firstly, we performed double immunofluorescent staining for  $P2Y_2$  receptors and Kv1.4 or Kv3.4 or Kv4.2 or Kv4.3 on TG neurons in rats, respectively. The results showed that the  $P2Y_2$  receptor-positive TG neurons also expressed

Kv1.4, Kv3.4, Kv4.2 and Kv4.3 (Figure 4A,  $n = 5$  rats), respectively. We further found that UTP induced a significant decrease in the expression of Kv1.4, Kv3.4, Kv4.2, and Kv4.3 mRNA in TG (Figure 4B,  $n = 10$  samples in each group,  $p < 0.01$ ,  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.01$  vs sham group). Treatment with suramin (100  $\mu\text{M}$ ) in the UTP (30  $\mu\text{M}$ )-incubated TG neurons for 16 h in control rats reversed the decrease of the expression of Kv1.4, Kv3.4, Kv4.2, and Kv4.3 mRNA (Figure 4B,  $n = 10$  samples in each group,  $p < 0.01$ ,  $p < 0.01$ ,  $p < 0.01$ ,  $p < 0.01$  vs UTP group).

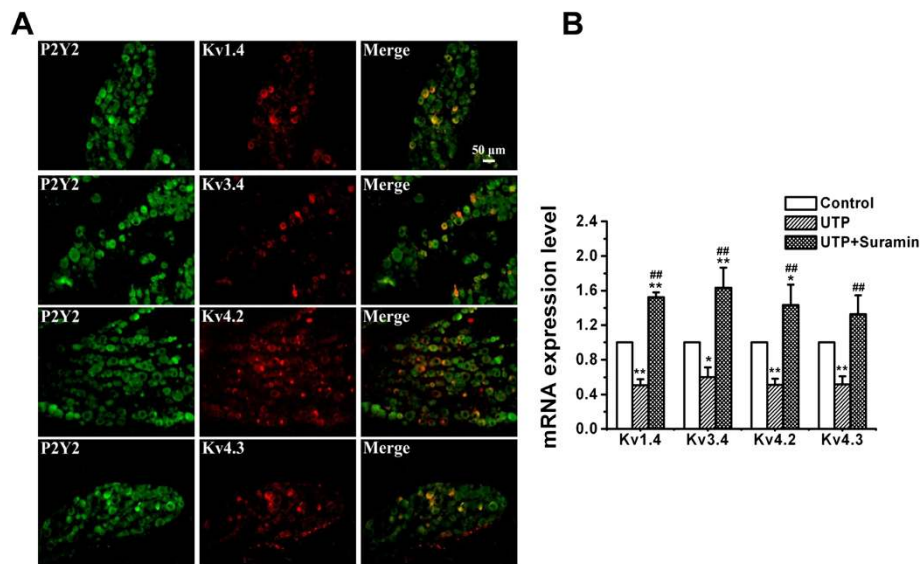
#### Effects of $P2Y_2$ receptors on Kv1.4, Kv3.4, Kv4.2 and Kv4.3 in ION-CCI rat TG neurons

##### The role of $P2Y_2$ receptors on mechanical allodynia in ION-CCI rats

The effects of suramin on the mechanical pain threshold of ION-CCI rats were determined. As shown in Figure 5A,

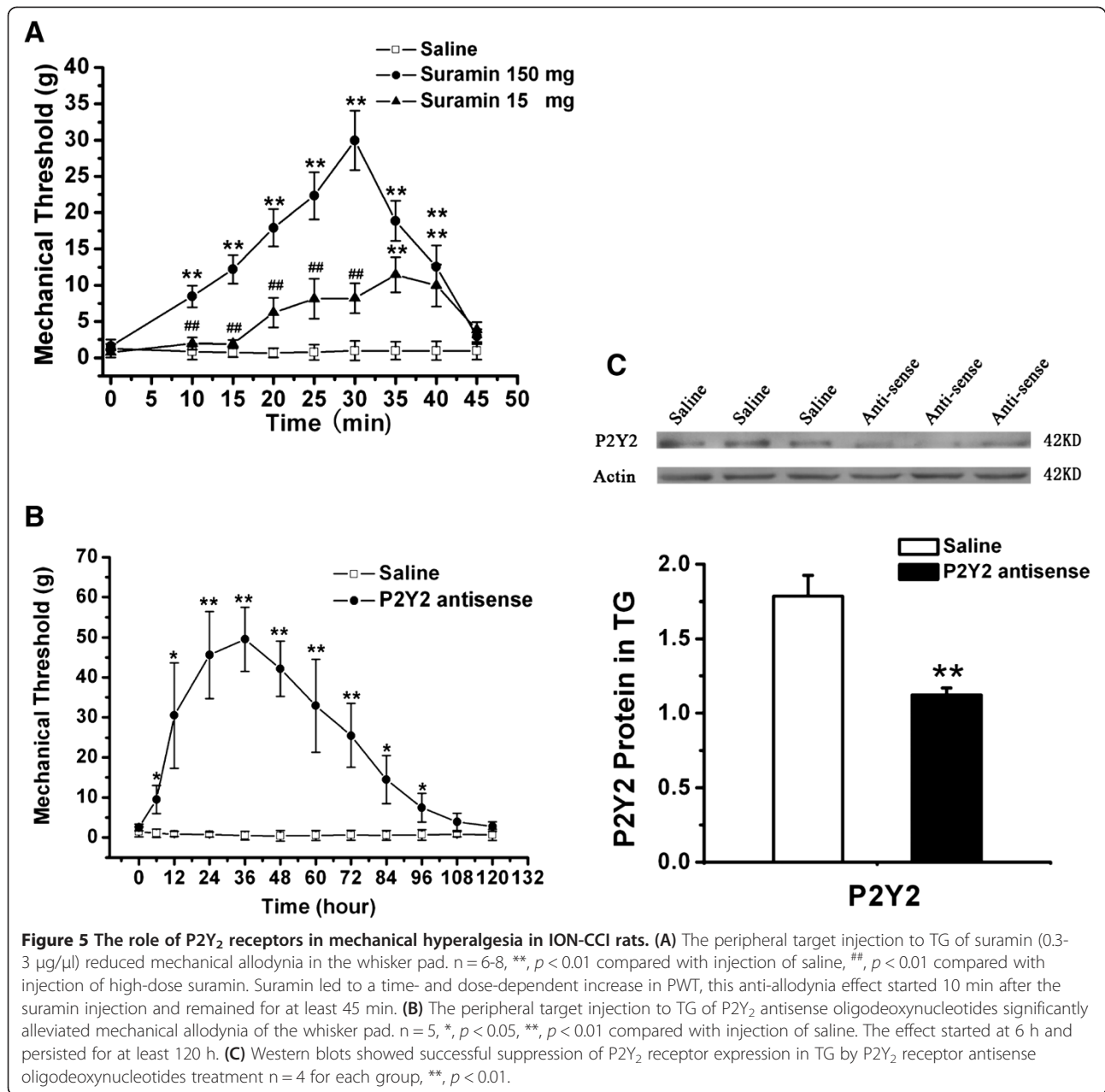


**Figure 3** Effect of  $\alpha, \beta$ -meATP and 2-MeSADP on  $I_A$  of small-diameter TG neurons in control rats. **(A)**  $I_A$  was initiated via a prepulse (100 ms) of  $-120$  mV and test pulses (400 ms) from  $-60$  to  $+60$  mV in a 10 mV step. Original traces showing that the application of  $10 \mu\text{M}$   $\alpha, \beta$ -meATP and  $100 \mu\text{M}$  2-MeSADP for 16 h did not suppress  $I_A$ . **(B)** Current-voltage relationship for both on  $I_A$ . Each value represents the mean  $\pm$  SEM (Con:  $0.14 \pm 0.01$  nA,  $n = 20$ ;  $\alpha, \beta$ -meATP:  $0.13 \pm 0.02$  nA, 2-MeSADP:  $0.13 \pm 0.02$  nA,  $n = 11$ ,  $p > 0.05$ ).



**Figure 4** Effect of UTP on the expression levels of  $I_A$  subunits in control TG neurons. **(A)** Double-immunostaining revealed the expression of Kv1.4, Kv3.4, Kv4.2 and Kv4.3 subunits in P2Y<sub>2</sub> receptor-positive neurons in the ION-C1I TG sections. The P2Y<sub>2</sub> receptor-positive TG neurons also expressed Kv1.4, Kv3.4, Kv4.2 and Kv4.3, respectively,  $n = 5$  rats. **(B)** Reduction in the mRNA levels of  $I_A$  subunits by UTP in cultured TG neurons from control rats. Treatment with suramin ( $100 \mu\text{M}$ ) in the UTP-incubated ( $30 \mu\text{M}$ ) TG neurons for 16 h in control rats reversed the decrease in the mRNA levels of Kv1.4, Kv3.4, Kv4.2, and Kv4.3 subunits.  $n = 10$  samples in each group, \*,  $p < 0.05$ , \*\*,  $p < 0.01$  vs control; ##,  $p < 0.01$  vs UTP.





suramin (15,150 mg) led to a time- and dose-dependent increase in PWT (saline:  $0.96 \pm 0.5$  g, n = 8; suramin 15 mg:  $11.45 \pm 2.4$  g, n = 6 rats; suramin 150 mg:  $29.96 \pm 4.1$  g, n = 7) compared with that of control (ION-CCI) rats (*p* < 0.01). This anti-allodynia effect started 10 min after the suramin injection and remained at least 45 min. Further, we injected P2Y<sub>2</sub> receptor AS-ODN twice a day for 2 days through the peripheral target injection to TG via the infraorbital foramen and then determined whether it could improve neuropathic pain 9 days after injection. The PWT of whisker pad was significantly increased after

injection of P2Y<sub>2</sub> receptor AS-ODN, compared with that of the control (ION-CCI) rats (Figure 5B, *p* < 0.05). The effect started at 6 h and persisted for at least 120 h (Figure 5B, saline:  $0.49 \pm 0.1$  g, n = 7 rats; AS-ODN:  $49.54 \pm 8.0$  g, n = 5). To confirm that P2Y<sub>2</sub> receptor AS-ODN had knocked down the expression of P2Y<sub>2</sub> receptor, the expression of P2Y<sub>2</sub> receptor after P2Y<sub>2</sub> receptor AS-ODN injection was investigated. Compared with that in the saline group, injection of P2Y<sub>2</sub> receptor AS-ODN significantly reduced P2Y<sub>2</sub> receptor protein expression (n = 4, *p* < 0.01, Figure 5C).

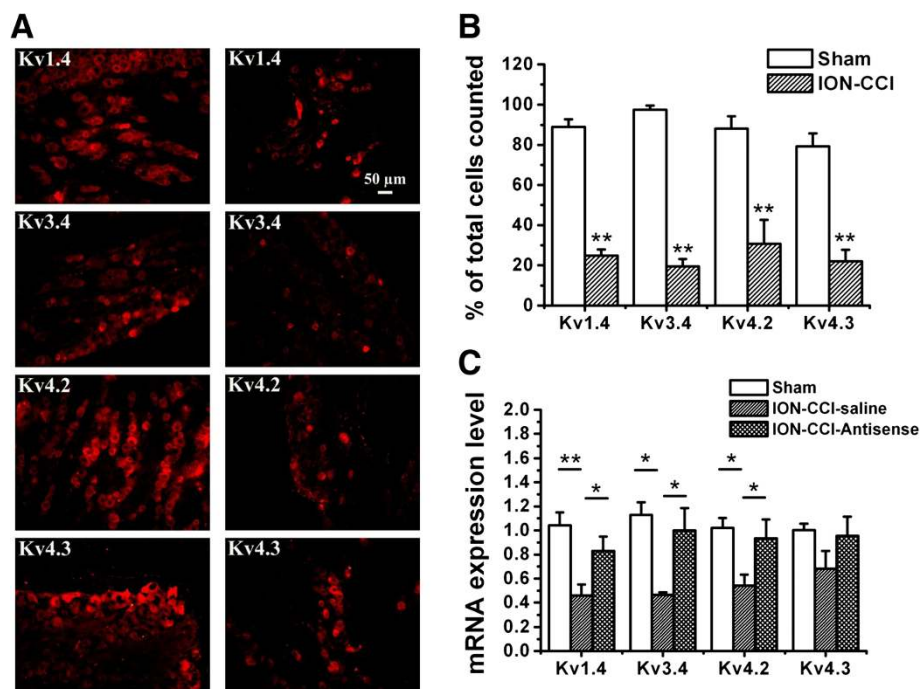
***P2Y<sub>2</sub> receptor antisense reverses the decreased expression of Kv1.4 or Kv3.4 or Kv4.2 or Kv4.3 on P2Y<sub>2</sub>-positive TG neurons after ION-CCI***

To determine whether I<sub>A</sub>-related subunits, Kv1.4, Kv3.4, Kv4.2 and Kv4.3, were changed after ION-CCI, double-immunofluorescence staining for P2Y<sub>2</sub> receptors and Kv1.4 or Kv3.4 or Kv4.2 or Kv4.3 was performed on TG neurons in sham and ION-CCI groups. In the sham group, 272 out of 301 (90.4%) P2Y<sub>2</sub> receptor-positive neurons were Kv1.4 positive; 302 out of 307 (98.1%) P2Y<sub>2</sub> receptor-positive neurons were Kv3.4 positive; 274 out of 325 (84.3%) P2Y<sub>2</sub> receptor-positive neurons were Kv4.2 positive; and 159 out of 201 (79.1%) P2Y<sub>2</sub> receptor-positive neurons were Kv4.3 positive. In the ION-CCI group, 42 out of 180 (23.3%) P2Y<sub>2</sub> receptor-positive neurons were Kv1.4 positive; 42 out of 245 (17.1%) P2Y<sub>2</sub> receptor-positive neurons were Kv3.4 positive; 66 out of 302 (21.9%) P2Y<sub>2</sub> receptor-positive neurons were Kv4.2 positive; and 31 out of 166 (18.7%) P2Y<sub>2</sub> receptor-positive neurons were Kv4.3 positive (Figure 6A). The number of Kv1.4, Kv3.4, Kv4.2 and Kv4.3 subunits on P2Y<sub>2</sub> receptor-positive TG neurons was significantly decreased in the ION-CCI group compared with

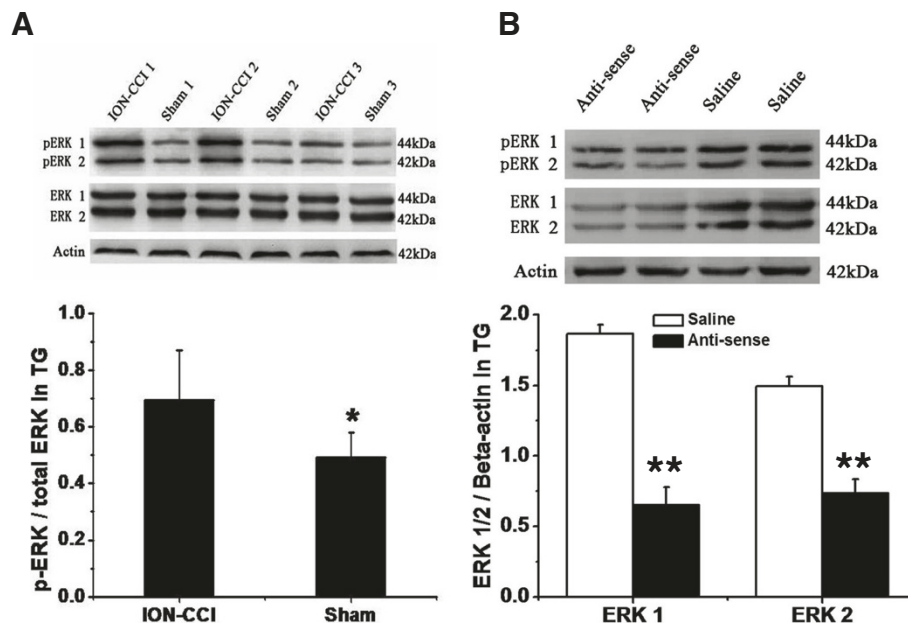
that in the sham group (Figure 6B,  $p < 0.01$ ,  $p < 0.01$ ,  $p < 0.01$ ,  $p < 0.01$ ). Furthermore, the expression of Kv1.4, Kv3.4 and Kv4.2 in TG neurons was significantly decreased in ION-CCI group compared with that in sham group (Figure 6B,  $p < 0.01$ ,  $p < 0.05$ ,  $p < 0.05$ ). Treatment with P2Y<sub>2</sub> receptor AS-ODN (15 μg/50 μl) significantly reversed the reduction of Kv1.4, Kv3.4 and Kv4.2 mRNA expression after ION-CCI (Figure 6C,  $p < 0.05$ ,  $p < 0.05$ ,  $p < 0.05$ ). However, the expression of Kv4.3 mRNA was not different among three groups (Figure 6C,  $p > 0.05$ ).

***Activation of P2Y<sub>2</sub> receptors mediates an inhibition of I<sub>A</sub> channels through ERK pathways on small-diameter TG neurons in control rats***

Western blot results showed that the level of ERK1/2 phosphorylation was significantly increased in the ipsilateral TG after ION-CCI compared with that from the sham groups (Figure 7A,  $n = 5$  for each group,  $p < 0.05$ ). It has been reported that ERK activation contributes to changes in membrane excitability as a result of direct or indirect phosphorylation of kinases, key receptors, and ion channels [30]. ERK inhibitors (U0126 or PD98059) enhance A-type potassium currents in dorsal horn neurons



**Figure 6** Difference of I<sub>A</sub> channel expression in TG between sham and ION-CCI rats. **(A)** Double-immunostaining for P2Y<sub>2</sub> receptors and Kv1.4 or Kv3.4 or Kv4.2 or Kv4.3 on TG neurons in sham and ION-CCI sections, respectively. **(B)** Percentages of numbers of Kv1.4, Kv3.4, Kv4.2 and Kv4.3 subunits in P2Y<sub>2</sub> receptor-positive neurons are significantly decreased in TG from ION-CCI rats compared with sham rats.  $n = 4$  rats, \*\*,  $p < 0.01$ . **(C)** Changes in the mRNA levels of I<sub>A</sub> subunits in TG after P2Y<sub>2</sub> receptor antisense oligodeoxynucleotides treatment. The mRNA levels of Kv1.4, Kv3.4 and Kv4.2 were significantly decreased in the saline group of ION-CCI rats compared with the sham rats. They were reversed after P2Y<sub>2</sub> receptor antisense oligodeoxynucleotides treatment.  $n = 5-9$  rats, \*,  $p < 0.05$ , \*\*,  $p < 0.01$  compared with saline groups. There was no difference in the levels of Kv4.3 mRNA among the groups.  $n = 6-8$  rats,  $p > 0.05$ .



**Figure 7** Role of ERK pathway in activation of P2Y<sub>2</sub> receptors mediates an inhibition of I<sub>A</sub> channels on small-diameter TG neurons in control rats. **(A)** Comparison of the phosphorylation of ERK1/2 in TG from sham and ION-CCI rats. Western blot results showed that the level of ERK1/2 phosphorylation was significantly increased in the ipsilateral TG after ION-CCI, compared with that from the sham group. *n* = 5 for each group \*, *p* < 0.05. **(B)** In TG from ION-CCI rats, treatment with P2Y<sub>2</sub> receptor antisense oligodeoxynucleotides (15 μg/50 μl) significantly decreased the expression of ERK protein in TG. *n* = 5 for each group, \*\*, *p* < 0.01.

of the spinal cord [40,41], indicating the possible modulation of ERK in potassium channels. We therefore tested whether the presence of an ERK inhibitor, U0126, would modulate the effect of UTP on trigeminal pain perception. In TG from ION-CCI rats, treatment with P2Y<sub>2</sub> receptor AS-ODN (15 μg/50 μl) significantly decreased ERK expression at the protein level (Figure 7B, *n* = 5 for each group, *p* < 0.01). The inhibitory effect of UTP on I<sub>A</sub> was significantly reversed (Figure 8A). The mean peak amplitude of was reversed to 0.15 ± 0.03 nA in the U0126 group (100 μM, *n* = 11), which was significantly different from that of the UTP group (30 μM, 0.09 ± 0.01 nA, *n* = 20, *p* < 0.05, Figure 8B). Further, in the cultured ION-CCI TG neurons, in the presence of U0126 (100 μM), the mean threshold intensities for evoking action potentials was significantly increased (control: 85 ± 14 pA; U0126: 182.9 ± 12.1 pA; *n* = 8, Figure 8C,D, *p* < 0.01 vs control), while the number of action potentials was significantly decreased (Figure 8C,D, *n* = 7, *p* < 0.05 vs control).

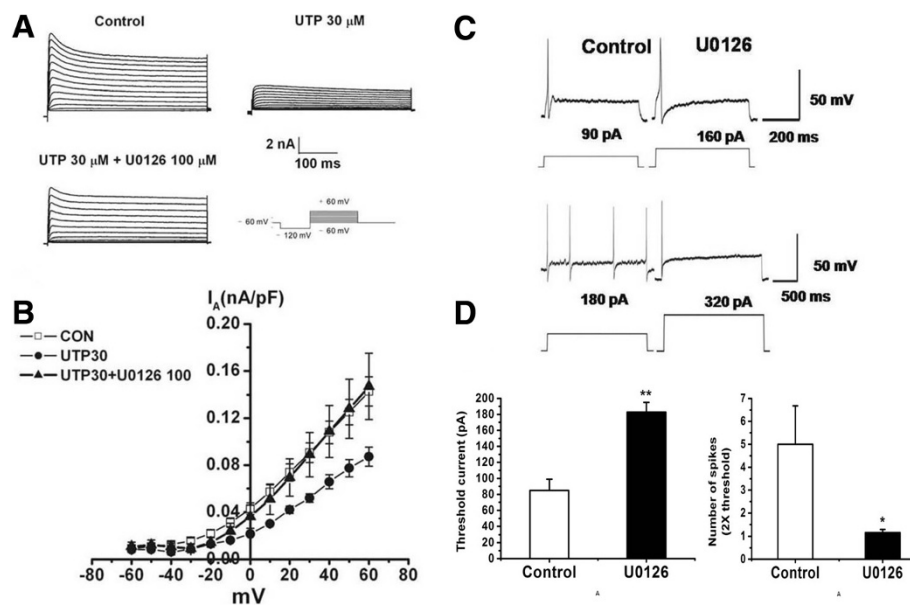
## Discussion

Four major findings arise from this study, 1) P2Y<sub>2</sub> receptors and Kv1.4, Kv3.4, Kv4.2 or Kv4.3 channels were co-expressed in rat TG neurons. The expression of Kv1.4, Kv3.4, Kv4.2 or Kv4.3 on P2Y<sub>2</sub> receptor-positive TG neurons was significantly decreased after ION-CCI; 2) UTP application enhanced the excitability of control

TG neurons and depressed the I<sub>A</sub> currents, which could be reversed by suramin; 3) activation of P2Y<sub>2</sub> receptors down-regulated mRNA expression and function of Kv1.4, Kv3.4, Kv4.2 and Kv4.3 on TG neurons in control rats; 4) after the expression of P2Y<sub>2</sub> receptors was suppressed by AS-ODN treatment, mechanical allodynia was reduced and mRNA levels of Kv1.4 and Kv3.4 and Kv4.2 were increased in ION-CCI rats. These results provide evidence that the down-regulation of I<sub>A</sub>-related potassium channels by activation of P2Y<sub>2</sub>Rs in TG neurons potentiates neuronal excitability which then contributes to trigeminal neuropathic pain.

## Activation of P2Y<sub>2</sub> receptors enhances TG neuron excitability through suppression of I<sub>A</sub> channels in control rats

Growing evidence indicates that P2X and P2Y receptor-mediated signaling critically contributes to the development and maintenance of neuropathic pain [12,13]. Here, we have demonstrated that activation of P2Y<sub>2</sub> receptors leads to a significant increase in the excitability of TG neurons. Previous studies have shown that an increase in membrane excitability in DRG neurons was a cellular-correlate of enhanced nociceptive behavior [18,42,43]. ATP is a non-selective agonist for several ionotropic P2X and metabotropic P2Y receptor subtypes [44]. Usually, ATP released from healthy cells plays a



**Figure 8** Effects of UTP and U0126 on  $I_A$  and action potentials of small-diameter TG neurons in control rats. (A)  $I_A$  was initiated via a prepulse (100 ms) of  $-120$  mV and test pulses (400 ms) from  $-60$  to  $+60$  mV in a 10 mV step. Original traces showing that the application of 30  $\mu$ M UTP reduced  $I_A$ . Suppression of the mean peak amplitudes of  $I_A$  seen after UTP application was antagonized by the presence of U0126 100  $\mu$ M. (B) Current-voltage relationship for the effect of UTP (30  $\mu$ M) and co-application with U0126 (100  $\mu$ M) on  $I_A$ . The mean peak amplitude of  $I_A$  was reversed to  $0.15 \pm 0.03$  nA in the U0126 group, which was significantly different from that of the UTP group (Con:  $0.14 \pm 0.01$  nA,  $n = 20$ ; U0126:  $0.15 \pm 0.03$  nA,  $n = 11$ ; UTP:  $0.09 \pm 0.01$  nA,  $n = 20$ ,  $p < 0.05$  vs U0126). (C) Original traces of action potentials during intracellular current injection in ION-CCI TG neurons. (D) Mean threshold currents (left panel,  $n = 8$  neurons) and mean number of spikes (right panel,  $n = 7$  neurons) in the presence and absence of U0126 (100  $\mu$ M) treatment for 16 h. The depolarizing step current amplitude is twice that of the threshold, \*,  $p < 0.05$ , \*\*,  $p < 0.01$  vs control.

physiological role [45]. In pathological conditions, ATP release can be evoked from sensory neurons and it produces fast excitatory potentials in DRG cells [46]. Thus, endogenously released ATP from damaged cells [47] may contribute to the ectopic firing of A $\beta$  and A $\delta$  neurons and lead to the development of allodynia [48]. In the present study, ATP caused a large decrease in the mean threshold intensities for evoking action potentials and a significant increase in the mean number of spikes in control TG neurons, which is consistent with a previous study [3]. Although there are (controversial) conflicting outcomes following the use of UTP via P2Y<sub>2</sub> receptors for neuropathic pain [16-19,48], this study demonstrated that UTP caused a large decrease in the mean threshold intensities for evoking action potentials and a significant increase in the mean number of spikes in control TG neurons. UTP has a similar effect on sensory neurons and thus plays a key role in the development of mechanical allodynia [48]. These results suggest that nucleotides enhance the excitability of TG neurons, probably via both P2Y<sub>2</sub> and P2Y<sub>4</sub> receptors, because UTP is a P2Y<sub>2</sub>/P2Y<sub>4</sub> receptor agonist.

Kv channels are crucial in the control of neuronal excitability, and their down-regulation leads to an increase of neuronal excitability [26,49,50]. Homomeric Kv1.4

channels predominate in A $\delta$  and C fibers arising from small-diameter DRG neurons [28]. Morgan et al. [51] reported that Kv1.4 and Kv4.2, which form transient (A-type) K<sup>+</sup> channels, may regulate synaptic transmission via presynaptic or postsynaptic mechanisms, respectively. The present electrophysiological study found that UTP mediated a functional inhibition of  $I_A$  channels in FG-labeled small-diameter TG neurons in control rats. UTP-induced depression of  $I_A$  was blocked by suramin, hence, the P2Y<sub>2</sub> nucleotide receptor must have contributed for the following reasons: (1) UTP, a P2Y<sub>2</sub>/P2Y<sub>4</sub> receptor agonist enhanced the excitability of TG neurons and inhibited  $I_A$ . (2) ATP and UTP were about equipotent as observed for rat P2Y<sub>2</sub> and P2Y<sub>4</sub> receptors [52]. (3) Suramin, which is a relatively selective antagonist of P2Y<sub>2</sub> receptors reversed the UTP-induced inhibition of  $I_A$  [7,53]. (4)  $\alpha,\beta$ -meATP, a P2X<sub>3</sub> and P2X<sub>2/3</sub> receptor agonist and 2-MeSADP, a P2Y<sub>1</sub> receptor agonist did not inhibit  $I_A$ . Thus activation of P2Y<sub>2</sub> receptors enhanced excitability of TG neurons probably by suppressing  $I_A$ .

Inhibition of  $I_A$  can increase the firing frequency and broaden the action potential leading to increased Ca<sup>2+</sup> influx and neurotransmitter release [33,50,54]. The Kv subunits, Kv1.4, Kv3.4, Kv4.2, and Kv4.3, could be dominant in contributing to  $I_A$ . Kv3.4 was expressed mainly



by nociceptive DRG neurons where Kv4.3 appeared selectively in the soma of a subset of non-peptidergic nociceptive DRG neurons, and reduced expression of Kv4.3 in pain-sensing neurons may induce neuropathic pain [26]. Hu et al. [55] found that genetic elimination of Kv4.2 reduced  $I_A$  and increased excitability of dorsal horn neurons. The expression of mRNA for Kv1.4, Kv3.4, Kv4.2, and Kv4.3 was markedly reduced in diabetic neuropathic rats [27]. Combined with our electrophysiological data, the down-regulation of  $I_A$  subunits, including mRNA for Kv1.4, Kv3.4, Kv4.2, and Kv4.3, after application of UTP, could account for the reduced  $I_A$  observed in UTP-incubated small diameter TG neurons from control rats. Suramin reversed the UTP-induced effect on TG neurons in control rats, further suggesting that P2Y<sub>2</sub> receptors were involved.

#### The involvement of P2Y<sub>2</sub> receptors in mechanical allodynia in ION-CCI rats

In this study, we found the expression of Kv1.4, Kv3.4, Kv4.2 and Kv4.3 on P2Y<sub>2</sub> receptor-positive TG neurons significantly decreased after ION-CCI compared with those in the sham group. Expression of P2Y<sub>2</sub> receptors, Kv1.4, Kv3.4, Kv4.2 and Kv4.3 was significantly reduced in ION-CCI rats. These data imply that  $I_A$  channel expression levels of nociceptors and nerve ligation-induced neuropathic pain could be closely related.

The present study showed that activation of P2Y<sub>2</sub> receptors could suppress  $I_A$  channels in control rats, which might be one of the mechanisms of hyperexcitability of TG neurons after UTP application. We hypothesized that block of P2Y<sub>2</sub> receptors could relieve trigeminal neuropathic pain. Firstly, we confirmed that suramin led to a time- and dose-dependent decrease in pain-related behavior of ION-CCI rats. Some similar observations were reported concerning the analgesic effects of suramin in animal pain models [56,57]. Because suramin is an antagonist of P2Y receptors except P2Y<sub>4</sub> and P2Y<sub>6</sub> receptors [57], the results suggest that P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>11</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub> receptors could affect pain-related behavior in ION-CCI rats. Considering the effect of UTP in control rats, we concluded that P2Y<sub>2</sub> receptors were probably involved in ION-CCI-induced pain behavior. Secondly, injection of P2Y<sub>2</sub> receptor AS-ODN significantly alleviated mechanical hypersensitivity 6 h after injection, which remained until 120 h. The results further support that block of P2Y<sub>2</sub> receptors could relieve trigeminal neuropathic pain.

To test whether there is a correlation between mechanical sensitivity and  $I_A$  channel expression, we measured the mRNA levels of the  $I_A$ -related potassium channels, Kv1.4, Kv3.4, Kv4.2 and Kv4.3, in TG neurons before and 36 h after P2Y<sub>2</sub> receptor AS-ODN treatment. The mRNA expressions of Kv1.4, Kv3.4 and Kv4.2

subunits were markedly reduced after ION-CCI, which were then reversed after selective knockdown of P2Y<sub>2</sub> receptor gene expression. It has been reported that there is a close relationship between P2Y and Kv channels. ATP and UTP reversibly inhibited the voltage-gated K<sup>+</sup> currents in *Xenopus* embryo spinal neurons [58]. KCNQ1/KCNE1 K<sup>+</sup> channels and P2Y<sub>4</sub> receptors are co-expressed from the time of birth in the apical membrane of rat strial marginal cells [59]. Purinergic P2Y agonists suppress M currents ( $I_M$ ), which are generated by Kv7 [18,60]. Our results suggest that activation of P2Y<sub>2</sub> receptors could result in the development of mechanical hypersensitivity, a major symptom of neuropathic pain, which could be as a result of the suppression of the mRNA expression of Kv1.4, Kv3.4 and Kv4.2 subunits.

In the present study, the expressions of Kv4.3 in mRNA and protein levels were decreased after application of UTP in cultured TG neurons from control rats (Figure 6A,B), but did not change in TG after ION-CCI (Figure 6C). This may be because: (1) Kv4.3 channels were not prominent in the development of allodynia in ION-CCI rats; and (2) an increase of Kv4.3 channels in glial cells surrounding the neurons in TG compensated for the changes in TG neurons after ION-CCI. Expression of  $I_A$ -related K<sub>V</sub> channels, such as Kv4.1, in glial cells suggests that glial cells also play an important role in chronic pain [61,62]. Further research of Kv channels on TG glial cells is required to explain how  $I_A$  channels are involved in trigeminal neuropathic pain.

#### ERK1/2 is the downstream kinase for the effect of P2Y<sub>2</sub> receptors on $I_A$ channels

P2Y<sub>2</sub> receptors are G protein-coupled receptors that usually activate PLC-β via G<sub>αq</sub>, which results in the release of intracellular Ca<sup>2+</sup> and activation of PKC [63]. These events further activate extracellular signal-regulated kinase (ERK), including ERK1 and ERK2 [64-66]. ERK and Kv4.2 have a functional link at both the cellular and behavioral levels [67]. Phosphorylation of Kv4.2 by PKC enhanced ERK phosphorylation of the channel *in vitro*. These findings suggest the possibility that Kv4.2 is a locus for PKC and ERK cross-talk [68]. Kv4.3 positive neurons also expressed ERK2 and mGluR5, suggesting that Kv4.3 subunits could be involved in pain modulation [69]. In line with the previous report [70], we found that ION-CCI significantly increased the level of ERK1/2 phosphorylation in TGs. Evidence provided in this study further suggests that the inhibition of  $I_A$  channels through P2Y<sub>2</sub> receptors is modulated by ERK signaling after ION-CCI. First,  $I_A$  was significantly inhibited by UTP, which could be reversed when ERK signaling was blocked by U0126. Second, in ION-CCI rats, the expression of ERK in protein level was increased and the mRNA expressions of Kv1.4, Kv3.4 and Kv4.2 subunits were

decreased, which were then reversed by P2Y<sub>2</sub> receptor AS-ODN treatment. A recent study has shown that the PI3K/Akt signaling pathway can be activated by P2Y<sub>2</sub> receptors [71]. The PI3K/Akt signaling pathway and Kv channels are both involved in the same disease [72]. Although we could not exclude that other pathways contribute to this effect of UTP, the ERK signaling pathway might be one of the downstream pathways for the effect of P2Y<sub>2</sub> receptors on I<sub>A</sub> channels, which might contribute to the development of trigeminal neuropathic pain.

In the present study, the effect of UTP on mechanical pain threshold in normal rats started from 10 min, suggesting the pathway without alterations of gene expression. The possibilities could be through facilitating homomeric P2X<sub>2</sub> [19], P2X<sub>3</sub> [19,73], or TRPV1 receptors [16,74]. Further, the long-term effect of UTP (more than 30 min) in pain behavior study and antisense oligodeoxynucleotides effect on ION-CCI rats indicate the alterations of gene expression. Although the underlying mechanisms are not fully understood, inhibition of P2Y<sub>2</sub> receptors leads to down-regulation of ERK-mediated phosphorylation and increase of the expression of I<sub>A</sub>-related Kv channels in trigeminal ganglion neurons, which might contribute to the clinical treatment of trigeminal neuropathic pain. Taken together, these data suggest that P2Y<sub>2</sub> receptors on TG might play an important role in initiating and maintaining the allodynia in trigeminal neuropathic pain.

#### Abbreviations

UTP: Uridine 5'-triphosphate; ATP: Adenosine 5'-triphosphate; ION-CCI: Chronic constriction nerve injury of the infraorbital nerve; TG: Trigeminal ganglion; AS-ODN: Antisense oligodeoxynucleotides.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

NL designed and contributed all experiments. NL and ZYL performed pain behavioral tests, immunohistochemical staining, and patch-clamp recording, and analysed these experiments data. LHY performed QT-RT-PCR, and Western-blot and analysed these experiments data. BM and XMD contributed to all experiments design and supervised research. NL wrote the manuscript. GB and BM revised the manuscript. Above all authors contributed read and approved the final manuscript.

#### Acknowledgement

This study was supported by the Key Program of Shanghai Science and Technology Committee (No. 08JC1405400) and Program of Changjiang Scholar and Innovative Team. We are deeply grateful to Dr. Gillian E. Knight (from the Autonomic Neuroscience Centre, University College Medical School, UK) for her kind assistance in English writing.

#### Author details

<sup>1</sup>Department of Physiology, The Key Laboratory of Molecular Neurobiology, Ministry of Education, Second Military Medical University, Shanghai 200433, P.R. China. <sup>2</sup>Department of Anesthesiology, Changhai hospital, Second Military Medical University, Shanghai 200433, P.R. China. <sup>3</sup>Autonomic Neuroscience Centre, University College Medical School, Royal Free Campus, Rowland Hill Street, London NW3 2PF, UK. <sup>4</sup>Department of Pharmacology and Therapeutics, University of Melbourne, Grattan Street, Parkville, Victoria 3010, Australia.

Received: 7 October 2013 Accepted: 4 March 2014

Published: 18 March 2014

#### References

1. DaSilva AF, DosSantos MF: The role of sensory fiber demography in trigeminal and postherpetic neuralgias. *J Dent Res* 2012, **91**(1):17-24.
2. Bennett GJ, Xie YK: A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 1988, **33**(1):87-107.
3. Kitagawa J, Takeda M, Suzuki I, Kadoi J, Tsuboi Y, Honda K, Matsumoto S, Nakagawa H, Tanabe A, Iwata K: Mechanisms involved in modulation of trigeminal primary afferent activity in rats with peripheral mononeuropathy. *Eur J Neurosci* 2006, **24**(7):1976-1986.
4. Ma F, Zhang L, Westlund KN: Trigeminal nerve injury ErbB3/ErbB2 promotes mechanical hypersensitivity. *Anesthesiology* 2012, **117**(2):381-388.
5. Yeon KY, Chung G, Kim YH, Hwang JH, Davies AJ, Park MK, Ahn DK, Kim JS, Jung SJ, Oh SB: Eugenol reverses mechanical allodynia after peripheral nerve injury by inhibiting hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. *Pain* 2011, **152**(9):2108-2116.
6. Nakai K, Nakae A, Oba S, Mashimo T, Ueda K: P2X4 receptor expression in a rat model of trigeminal neuropathic pain. *Neuroreport* 2010, **21**(8):559-563.
7. Latremoliere A, Mauborgne A, Masson J, Bourgoin S, Kayser V, Hamon M, Pohl M: Differential implication of proinflammatory cytokine interleukin-6 in the development of cephalic versus extracephalic neuropathic pain in rats. *J Neurosci* 2008, **28**(34):8489-8501.
8. Love S, Coakham HB: Trigeminal neuralgia: pathology and pathogenesis. *Brain* 2001, **124**:2347-2360.
9. Lazarowski ER, Boucher RC, Harden TK: Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol Pharmacol* 2003, **64**(4):785-795.
10. Collier HO, James GW, Schneider C: Antagonism by aspirin and fenamates of bronchoconstriction and nociception induced by adenosine-5'-triphosphate. *Nature* 1966, **212**(5060):411-412.
11. Bleehen T, Keele CA: Observations on the algogenic actions of adenosine compounds on the human blister base preparation. *Pain* 1977, **3**(4):367-377.
12. Wirkner K, Sperlagh B, Illes P: P2X3 receptor involvement in pain states. *Mol Neurobiol* 2007, **36**(2):165-183.
13. Burnstock G: Purinergic receptors and pain. *Curr Pharm Des* 2009, **15**(15):1717-1735.
14. Ruan HZ, Burnstock G: Localisation of P2Y1 and P2Y4 receptors in dorsal root, nodose and trigeminal ganglia of the rat. *Histochem Cell Biol* 2003, **120**(5):415-426.
15. Gerevich Z, Illes P: P2Y receptors and pain transmission. *Purinergic Signal* 2004, **1**(1):3-10.
16. Lakshmi S, Joshi PG: Co-activation of P2Y2 receptor and TRPV channel by ATP: implications for ATP induced pain. *Cell Mol Neurobiol* 2005, **25**(5):819-832.
17. Stucky CL, Medler KA, Molliver DC: The P2Y agonist UTP activates cutaneous afferent fibers. *Pain* 2004, **109**(1-2):36-44.
18. Yousuf A, Klinger F, Schicker K, Boehm S: Nucleotides control the excitability of sensory neurons via two P2Y receptors and a bifurcated signaling cascade. *Pain* 2011, **152**(8):1899-1908.
19. Chen X, Molliver DC, Gebhart GF: The P2Y2 receptor sensitizes mouse bladder sensory neurons and facilitates purinergic currents. *J Neurosci* 2010, **30**(6):2365-2372.
20. Ando RD, Mehesz B, Gyires K, Illes P, Sperlagh B: A comparative analysis of the activity of ligands acting at P2X and P2Y receptor subtypes in models of neuropathic, acute and inflammatory pain. *Br J Pharmacol* 2010, **159**(5):1106-1117.
21. Okada M, Nakagawa T, Minami M, Satoh M: Analgesic effects of intrathecal administration of P2Y nucleotide receptor agonists UTP and UDP in normal and neuropathic pain model rats. *J Pharmacol Exp Ther* 2002, **303**(1):66-73.
22. Ficker E, Heinemann U: Slow and fast transient potassium currents in cultured rat hippocampal cells. *J Physiol* 1992, **445**:431-455.
23. Wu LG, Saggau P: Presynaptic inhibition of elicited neurotransmitter release. *Trends Neurosci* 1997, **20**(5):204-212.

24. Yoshida S, Matsumoto S: Effects of alpha-dendrotoxin on K<sup>+</sup> currents and action potentials in tetrodotoxin-resistant adult rat trigeminal ganglion neurons. *J Pharmacol Exp Ther* 2005, **314**(1):437–445.
25. Vydyanathan A, Wu ZZ, Chen SR, Pan HL: A-type voltage-gated K<sup>+</sup> currents influence firing properties of isolectin B4-positive but not isolectin B4-negative primary sensory neurons. *J Neurophysiol* 2005, **93**(6):3401–3409.
26. Chien LY, Cheng JK, Chu D, Cheng CF, Tsaur ML: Reduced expression of A-type potassium channels in primary sensory neurons induces mechanical hypersensitivity. *J Neurosci* 2007, **27**(37):9855–9865.
27. Cao XH, Byun HS, Chen SR, Cai YQ, Pan HL: Reduction in voltage-gated K<sup>+</sup> channel activity in primary sensory neurons in painful diabetic neuropathy: role of brain-derived neurotrophic factor. *J Neurochem* 2010, **114**(5):1460–1475.
28. Rasband MN, Park EW, Vanderah TW, Lai J, Porreca F, Trimmer JS: Distinct potassium channels on pain-sensing neurons. *Proc Natl Acad Sci USA* 2001, **98**(23):13373–13378.
29. Takeda M, Tanimoto T, Ikeda M, Kadoi J, Matsumoto S: Activator of GABAB receptor inhibits the excitability of rat small diameter trigeminal root ganglion neurons. *Neuroscience* 2004, **123**(2):491–505.
30. Tsuboi Y, Takeda M, Tanimoto T, Ikeda M, Matsumoto S, Kitagawa J, Teramoto K, Simizu K, Yamazaki Y, Shima A, Ren K, Iwata K: Alteration of the second branch of the trigeminal nerve activity following inferior alveolar nerve transection in rats. *Pain* 2004, **111**(3):323–334.
31. Neubert JK, Mannes AJ, Keller J, Wexel M, Iadarola MJ, Caudle RM: Peripheral targeting of the trigeminal ganglion via the infraorbital foramen as a therapeutic strategy. *Brain Res Brain Res Protoc* 2005, **15**(3):119–126.
32. Seye CI, Kong Q, Erb L, Garrad RC, Krugh B, Wang M, Turner JT, Sturek M, González FA, Weisman GA: Functional P2Y2 nucleotide receptors mediate uridine 5'-triphosphate-induced intimal hyperplasia in collared rabbit carotid arteries. *Circulation* 2002, **106**(21):2720–2726.
33. Imamura Y, Kawamoto H, Nakanishi O: Characterization of heat-hyperalgesia in an experimental trigeminal neuropathy in rats. *Exp Brain Res* 1997, **116**(1):97–103.
34. Ambalavanar R, Moritani M, Moutanni A, Gangula P, Yallampalli C, Dessem D: Deep tissue inflammation upregulates neuropeptides and evokes nociceptive behaviors which are modulated by a neuropeptide antagonist. *Pain* 2006, **120**(1–2):53–68.
35. Chichorro JG, Zampronio AR, Rae GA: Endothelin ET(B) receptor antagonist reduces mechanical allodynia in rats with trigeminal neuropathic pain. *Exp Biol Med (Maywood)* 2006, **231**(6):1136–1140.
36. Takeda M, Tanimoto T, Ikeda M, Nasu M, Kadoi J, Yoshida S, Matsumoto S: Enhanced excitability of rat trigeminal root ganglion neurons via decrease in A-type potassium currents following temporomandibular joint inflammation. *Neuroscience* 2006, **138**(2):621–630.
37. Ma B, Yu LH, Fan J, Ni X, Burnstock G: Pharmacological properties of P2 receptors on rat otic parasympathetic ganglion neurons. *Life Sci* 2008, **83**(5–6):185–191.
38. Liu L, Simon SA: Modulation of IA currents by capsaicin in rat trigeminal ganglion neurons. *J Neurophysiol* 2003, **89**(3):1387–1401.
39. Takeda M, Kitagawa J, Takahashi M, Matsumoto S: Activation of interleukin-1beta receptor suppresses the voltage-gated potassium currents in the small-diameter trigeminal ganglion neurons following peripheral inflammation. *Pain* 2008, **139**(3):594–602.
40. Hu HJ, Glauner KS, Gereau RW: ERK integrates PKA and PKC signaling in superficial dorsal horn neurons. I. Modulation of A-type K<sup>+</sup> currents. *J Neurophysiol* 2003, **90**(3):1671–1679.
41. Karim F, Hu HJ, Adwanikar H, Kaplan D, Gereau RW: Impaired inflammatory pain and thermal hyperalgesia in mice expressing neuron-specific dominant negative mitogen activated protein kinase kinase (MEK). *Mol Pain* 2006, **2**:2.
42. Brown DA, Passmore GM: Some new insights into the molecular mechanisms of pain perception. *J Clin Invest* 2010, **120**(5):1380–1383.
43. Liu B, Linley JE, Du X, Zhang X, Ooi L, Zhang H, Gamper N: The acute nociceptive signals induced by bradykinin in rat sensory neurons are mediated by inhibition of M-type K<sup>+</sup> channels and activation of Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels. *J Clin Invest* 2010, **120**(4):1240–1252.
44. Ralevic V, Burnstock G: Receptors for purines and pyrimidines. *Pharmacol Rev* 1998, **50**(3):413–492.
45. Bodin P, Burnstock G: Purinergic signalling: ATP release. *Neurochem Res* 2001, **26**(8–9):959–969.
46. Jahr CE, Jessell TM: ATP excites a subpopulation of rat dorsal horn neurones. *Nature* 1983, **304**(5928):730–733.
47. Cook SP, McCleskey EW: Cell damage excites nociceptors through release of cytosolic ATP. *Pain* 2002, **95**(1–2):41–47.
48. Woolf CJ: Dissecting out mechanisms responsible for peripheral neuropathic pain: implications for diagnosis and therapy. *Life Sci* 2004, **74**(21):2605–2610.
49. Chi XX, Nicol GD: Manipulation of the potassium channel Kv1.1 and its effect on neuronal excitability in rat sensory neurons. *J Neurophysiol* 2007, **98**(5):2683–2692.
50. Catacuzzeno L, Fioretti B, Pietrobon D, Franciolini F: The differential expression of low-threshold K<sup>+</sup> currents generates distinct firing patterns in different subtypes of adult mouse trigeminal ganglion neurones. *J Physiol* 2008, **586**(Pt 21):5101–5118.
51. Sheng M, Tsaur ML, Jan YN, Jan LY: Subcellular segregation of two A-type K<sup>+</sup> channel proteins in rat central neurons. *Neuron* 1992, **9**(2):271–284.
52. von Kugelgen I: Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. *Pharmacol Ther* 2006, **110**(3):415–432.
53. Moriyama T, Iida T, Kobayashi K, Higashi T, Fukuoka T, Tsumura H, Leon C, Suzuki N, Inoue K, Gachet C, Noquchi K, Tominaga M: Possible involvement of P2Y2 metabotropic receptors in ATP-induced transient receptor potential vanilloid receptor 1-mediated thermal hypersensitivity. *J Neurosci* 2003, **23**(14):6058–6062.
54. Hoffman DA, Magee JC, Colbert CM, Johnston D: K<sup>+</sup> channel regulation of signal propagation in dendrites of hippocampal pyramidal neurons. *Nature* 1997, **387**(6636):869–875.
55. Hu HJ, Carrasquillo Y, Karim F, Jung WE, Nerbonne JM, Schwarz TL, 4th Gereau RW: The kv4.2 potassium channel subunit is required for pain plasticity. *Neuron* 2006, **50**(1):89–100.
56. Gu JG, Bardoni R, Magherini PC, MacDermott AB: Effects of the P2-purinoceptor antagonists suramin and pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid on glutamatergic synaptic transmission in rat dorsal horn neurons of the spinal cord. *Neurosci Lett* 1998, **253**(3):167–170.
57. Wu Y, Willcockson HH, Maixner W, Light AR: Suramin inhibits spinal cord microglia activation and long-term hyperalgesia induced by formalin injection. *J Pain* 2004, **5**(1):48–55.
58. Brown P, Dale N: Modulation of K(+) currents in Xenopus spinal neurons by p2y receptors: a role for ATP and ADP in motor pattern generation. *J Physiol* 2002, **540**(Pt 3):843–850.
59. Hur DG, Lee JH, Oh SH, Kim YH, Shin DH, Chang SO, Kim CS: KCNQ1/KCNE1 K<sup>+</sup> channel and P2Y4 receptor are co-expressed from the time of birth in the apical membrane of rat strial marginal cells. *Acta Otolaryngol Suppl* 2007, **558**:30–35.
60. Hernandez CC, Zaika O, Tolstykh GP, Shapiro MS: Regulation of neural KCNQ channels: signalling pathways, structural motifs and functional implications. *J Physiol* 2008, **586**(7):1811–1821.
61. McMahon SB, Malcangio M: Current challenges in glia-pain biology. *Neuron* 2009, **64**(1):46–54.
62. Milligan ED, Watkins LR: Pathological and protective roles of glia in chronic pain. *Nat Rev Neurosci* 2009, **10**(1):23–36.
63. Burnstock G: Purine and pyrimidine receptors. *Cell Mol Life Sci* 2007, **64**:1471–1483.
64. Arthur DB, Georgi S, Akassoglou K, Insel PA: Inhibition of apoptosis by P2Y2 receptor activation: novel pathways for neuronal survival. *J Neurosci* 2006, **26**(14):3798–3804.
65. Soltoff SP, Avraham H, Avraham S, Cantley LC: Activation of P2Y2 receptors by UTP and ATP stimulates mitogen-activated kinase activity through a pathway that involves related adhesion focal tyrosine kinase and protein kinase C. *J Biol Chem* 1998, **273**(5):2653–2660.
66. Choi RC, Chu GK, Siow NL, Yung AW, Yung LY, Lee PS, Lo CC, Simon J, Dong TT, Barnard EA, Tsim KW: Activation of UTP-sensitive P2Y2 receptor induces the expression of cholinergic genes in cultured cortical neurons: a signaling cascade triggered by Ca<sup>2+</sup> mobilization and extracellular regulated kinase phosphorylation. *Mol Pharmacol* 2013, **84**(1):50–61.
67. Hu HJ, Alter BJ, Carrasquillo Y, Qiu CS, Gereau RW: Metabotropic glutamate receptor 5 modulates nociceptive plasticity via extracellular signal-regulated kinase-Kv4.2 signaling in spinal cord dorsal horn neurons. *J Neurosci* 2007, **27**(48):13181–13191.

68. Schrader LA, Ren Y, Cheng F, Bui D, Sweatt JD, Anderson AE: **Kv4.2 is a locus for PKC and ERK/MAPK cross-talk.** *Biochem J* 2009, **417**(3):705–715.
69. Huang HY, Cheng JK, Shih YH, Chen PH, Wang CL, Tsaur ML: **Expression of A-type K channel alpha subunits Kv 4.2 and Kv 4.3 in rat spinal lamina II excitatory interneurons and colocalization with pain-modulating molecules.** *Eur J Neurosci* 2005, **22**(5):1149–1157.
70. Alvarez P, Dieb W, Hafidi A, Voisin DL, Dallel R: **Insular cortex representation of dynamic mechanical allodynia in trigeminal neuropathic rats.** *Neurobiol Dis* 2009, **33**(1):89–95.
71. Katz S, Ayala V, Santillan G, Boland R: **Activation of the PI3K/Akt signaling pathway through P2Y(2) receptors by extracellular ATP is involved in osteoblastic cell proliferation.** *Arch Biochem Biophys* 2011, **513**(2):144–152.
72. Mukai Y, Shimokawa H, Matoba T, Hiroki J, Kunihiro I, Fujiki T, Takeshita A: **Acute vasodilator effects of HMG-CoA reductase inhibitors: involvement of PI3-kinase/Akt pathway and Kv channels.** *J Cardiovasc Pharmacol* 2003, **42**(1):118–124.
73. Wirkner K, Stanchev D, Köles L, Klebingat M, Dihazi H, Flehmig G, Vial C, Evans RJ, Furst S, Mager PP, Eschrich K, Illes P: **Regulation of human recombinant P2X3 receptors by ecto-protein kinase C.** *J Neurosci* 2005, **25**:7734–7742.
74. Malin SA, Davis BM, Koerber HR, Reynolds IJ, Albers KM, Molliver DC: **Thermal nociception and TRPV1 function are attenuated in mice lacking the nucleotide receptor P2Y2.** *Pain* 2008, **15**(138(3)):484–496.

doi:10.1186/1744-8069-10-21

**Cite this article as:** Li et al.: Inhibition of G protein-coupled P2Y<sub>2</sub> receptor induced analgesia in a rat model of trigeminal neuropathic pain. *Molecular Pain* 2014 **10**:21.

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