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Thermal Nociception and TRPV1 Function are Attenuated in Mice Lacking the Nucleotide Receptor P2Y₂

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Abstract

Recent studies indicate that ATP and UTP act at G protein-coupled (P2Y) nucleotide receptors to excite nociceptive sensory neurons; nucleotides also potentiate signaling through the pro-nociceptive capsaicin receptor, TRPV1. We demonstrate here that P2Y₂ is the principal UTP receptor in somatosensory neurons: P2Y₂ is highly expressed in dorsal root ganglia and P2Y₂(^{-/-}) mice showed profound deficits in UTP-evoked calcium transients and potentiation of capsaicin responses. P2Y₂(^{-/-}) mice were also deficient in the detection of painful heat: baseline thermal response latencies were increased and mutant mice failed to develop thermal hypersensitivity in response to inflammatory injury (injection of complete Freund's adjuvant into the hindpaw). P2Y₂ was the only Gq-coupled P2Y receptor examined that showed an increase in DRG mRNA levels in response to inflammation. Surprisingly, TRPV1 function was also attenuated in P2Y₂(^{-/-}) mice, as measured by the frequency and magnitude of capsaicin responses *in vitro* and behavioral responses to capsaicin administration *in vivo*. However, TRPV1 mRNA levels and immunoreactivity were not reduced, and behavioral sensitivity to capsaicin could be largely restored in P2Y₂(^{-/-}) mice by pretreatment with bradykinin, suggesting that normal function of TRPV1 requires ongoing modulation by G protein-coupled receptors. These results indicate that nucleotide signaling through P2Y₂ plays a key role in thermal nociception.

BACKGROUND

Primary afferent nociceptors convey information about tissue-damaging stimuli to the CNS, giving rise to the sensation of pain. Extracellular nucleotides act at two families of receptors expressed by nociceptors: the P2X family of ATP-gated (purinergic) ion channels [23], and the P2Y family of purine- and pyrimidine-activated G protein-coupled receptors [29;42]. Metabotropic nucleotide signaling in sensory neurons has been shown to cause release of intracellular Ca⁺⁺ stores, neuropeptide secretion and potentiation of signaling through the capsaicin, acid and heat-gated ion channel, TRPV1 [39;53;22;31]. We have previously reported that ATP and UTP evoke persistent action potential firing through a P2Y receptor, most likely P2Y₂, in isolated sensory neurons *in vitro* and in identified nociceptors in an isolated skin nerve

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preparation. These results suggest a biphasic response to ATP presentation in sensory neurons: an early transient burst of action potentials mediated by P2X receptors, followed by a sustained phase of action potential firing and enhancement of neuronal sensitivity by activation of P2Y receptors. RNA for four Gq-coupled P2Y family members (P2Y₁, P2Y₂, P2Y₄ and P2Y₆) has been identified in sensory ganglia [39;38] but functional characterization of P2Y family members has been impeded by the limited selectivity of agonists and antagonists for these receptors [37;49]. Therefore, we examined mice with a deletion in the P2Y₂ locus to evaluate the contribution of P2Y₂ to nociception. Here, we report that P2Y₂ is highly expressed in dorsal root ganglia (DRG), and most UTP-evoked Ca⁺⁺ responses in DRG neurons are lost in the absence of P2Y₂. Unexpectedly, P2Y₂(^{-/-}) mice have significant deficits in thermal sensation, including decreased baseline sensitivity to noxious heat, resistance to the development of inflammatory hyperalgesia and a significant reduction in responsiveness to the algogenic compound capsaicin, despite normal distribution of the capsaicin receptor, TRPV1. Capsaicin responsiveness was largely restored by pretreatment with bradykinin, another Gq-coupled receptor agonist known to potentiate TRPV1 function. Our results demonstrate that P2Y₂ is essential for both acute noxious thermal sensation and inflammation-evoked thermal hyperalgesia; these data also suggest a critical role for ongoing P2Y₂ signaling in the normal function of TRPV1.

MATERIALS AND METHODS

Mice

Adult male wildtype (WT) C57/Bl6 mice or P2Y₂ null mutant (P2Y₂(^{-/-})) mice bred onto the C57B/6 background (>6 generations) were used for all experiments. P2Y₂(^{-/-}) mice were generously provided by Dr. Beverly Koller (University of North Carolina, Chapel Hill); they were maintained as homozygotes, bred normally and were grossly normal behaviorally and anatomically [12;20;1]. Mice were housed in group cages (except during behavioral experiments), maintained on a 12:12 hour light-dark cycle in a temperature controlled environment (20.5°C) and given food and water *ad libitum*. These studies were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the guidelines of the Committee for Research and Ethical Issues of IASP.

Real-Time PCR

Real-time PCR analysis was carried out as previously described [30]. Briefly, hindpaw inflammation was induced in mice of each genotype by injection of complete Freund's adjuvant (CFA) (see below). 1, 4, 7 or 14 days after CFA injection (5 mice/genotype/each time point), mice were deeply anesthetized with Avertin (i.p. injection of 2.5% avertin (2,2,2-tribromoethanol and tert-amyl alcohol) diluted in 0.9% saline; 20ml/g body weight) and killed by transcardial perfusion with 4°C isotonic saline. L3, L4 and L5 dorsal root ganglia were dissected bilaterally and collected on dry ice. DRG tissue was also collected from uninjected mice (5 mice for each genotype). To isolate mRNA, frozen tissue samples were placed in 1 ml Trizol reagent (Invitrogen, Carlsbad, CA), homogenized, extracted in chloroform and separated in phase lock gel tubes (Eppendorf, Hamburg, Germany). RNA was precipitated in isopropanol at -20°C for 1 hour then on dry ice for 1 hour, then washed with 75% ethanol and resuspended in water. RNA quality was determined using an Agilent (Palo Alto, CA) 2100 Bioanalyzer according to the manufacturers instructions and quantity was determined using the 260 nm absorbance recorded by a spectrophotometer. Extracted RNA was treated with DNase (Invitrogen) to remove genomic DNA (1 µl DNase, 2µl 10X DNase buffer, 0.25µl RNasin/5µg RNA in H₂O, 20 µl total/reaction). RNA was then reverse-transcribed using Invitrogen Superscript II reverse transcriptase according to the manufacturer's instructions. Negative control reactions were run without mRNA to test for contamination. After PCR

amplification, a dissociation curve was plotted against melting temperature to ensure amplification of a single product.

Analysis—The thermal cycler (Applied Biosystems 5700 real-time thermal cycler driven by ABI Prism 7000SDS software) measures the relative fluorescence of SYBR Green bound to double-stranded DNA compared to a passive reference for each cycle. Threshold cycle (Ct) values, the fractional cycle number in which SYBR Green fluorescence rises above background, are recorded as a measure of template concentration. Relative fold changes in RNA levels were calculated by the $\Delta\Delta C_t$ method using p53-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference standard: Ct values from triplicate samples were averaged and subtracted from the reference standard, yielding ΔC_t . The difference between the ΔC_t of the experimental and control groups was then calculated ($\Delta\Delta C_t$) and the percent change in mRNA abundance was determined as $2^{-\Delta\Delta C_t}$. Statistical significance was determined by ANOVA using the Statview software package. P2Y receptor expression levels were normalized to P2Y₂ and reported as the percent of P2Y₂ expression levels, or as the percent change in mRNA levels compared to the pre-injection baseline in mice receiving CFA.

Calcium Imaging

Adult mice were given an overdose of avertin anesthetic and perfused transcardially with 4°C Ca⁺⁺/Mg⁺⁺-free Hank's basic salt solution (HBSS). All cervical, thoracic and lumbar DRGs were rapidly dissected in HBSS and dissociated as previously described [28]. Ca⁺⁺ imaging was performed 18–24 hours after plating. Cells were loaded with 2 μ M Fura-2 in HBSS with 5 mg/ml bovine serum albumin for 30 minutes at 37°C, then mounted on a microscope stage with constantly flowing HBSS at 5 ml/minute. Perfusion rate was controlled with a gravity flow system (Warner VC-66) and perfusate temperature was maintained at 30°C using heated stage and in-line heating system (Warner PH1, SHM-6, TC344B). Drugs were delivered with a computer-controlled rapid-switching local perfusion system. Firmly-attached, refractile cells were identified as regions of interest in the software (Simple PCI, C-Imaging). Absorbance data at 340 and 380 nm were collected once per second and the relative fluorescence (ratio 340/380) was plotted against time. Only neurons that responded to an initial 2 second pulse of 50mM K⁺ were further evaluated. Ca⁺⁺ transients were examined in response to brief (2 second) application of agonists. Transients were considered positive responses if the change in fluorescence ratio ($\Delta F_{340/380}$) between the baseline and the transient peak was > 0.1; transients were easily distinguished from optical noise (which was always < 0.02 $\Delta F_{340/380}$ in our experiments). Ca⁺⁺ responses were quantified as the percentage of cells per mouse responding to a given agonist and the amplitude, area under the curve, latency and duration of the response using Excel macros written for this purpose. Statistical significance was evaluated using the student-*t* test; p values are reported in the text. Drugs were purchased from Sigma. Capsaicin was dissolved in 1-methyl-2-pyrrolidinone as a 10mM stock solution; 1.0 μ M capsaicin was made fresh daily in HBSS. Nucleotides (ATP, UTP, ADP and UDP) were received on dry ice, diluted to 10mM stocks in nuclease-free H₂O (Ambion) upon arrival, aliquoted into small volumes and maintained at –80°C. Aliquots were used once and any remainder discarded.

Behavioral Paradigms

Thermal Sensitivity

Hargreaves Test: WT and P2Y₂ knockout mice (n=10/genotype) were placed in individual plexiglass chambers on a glass plate maintained at 30°C and allowed to acclimate for one hour. In all behavioral experiments, the experimenters were blinded to the genotype of the mice. Response latencies to noxious thermal stimulation were measured by applying a radiant heat stimulus (15% intensity on the Hargreaves apparatus; IITC Inc.) to each hindpaw. The heat

source was activated with an electric trigger coupled to a timer, and the latency to stimulus response (flinching or lifting the paw) was recorded to the nearest 0.1 second.

Cold Plate Test: The cold plate test was performed as previously described [10]. Briefly, mice were placed in a Plexiglass container with an ice floor, and the latency to the first nocifensive response (foot lifting and jumping) and the number of responses in 60 seconds was quantified.

Mechanical Sensitivity (von Frey Filament Test)—Mice were placed in individual plexiglass chambers on a wire mesh and allowed to acclimate for one hour. Mechanical sensitivity was evaluated using a series of calibrated von Frey filaments of graded tension. The 50% response threshold was determined by applying filaments to the mid-plantar region of each hindpaw 5 times for each filament, applying fibers of increasing and decreasing diameter around the response threshold (the up-down method [7]) to determine the stimulus (in grams) that evoked a response 50% of the time. Allodynia was measured as the percent decrease in 50% threshold relative to the mouse's baseline threshold prior to inflammation.

ATP-Evoked Hyperalgesia—The Hargreaves test was performed as described above (n=10 mice/genotype) before and after unilateral injection of ATP into the hindpaw. After baseline responses were obtained, mice were lightly restrained and injected with 10nmol ATP in 20μl saline using a 30 gauge needle and returned to the chambers. Mice were then retested 10 and 30 minutes after injection of ATP and the latency to withdrawal (to radiant heat) of the injected paw was recorded.

Capsaicin-Evoked Behavior—WT and P2Y₂(-/-) mice (n=9/genotype/treatment) were injected in the plantar surface of the hindpaw with saline or bradykinin (3μg in 5μl saline) 10 minutes prior to injection of capsaicin (1μg capsaicin in 15μl saline with 10% ethanol, 0.5% TWEEN-20) into the same hindpaw, using a microsyringe with a 30 gauge needle. Nocifensive responses were quantified for 2 minutes after capsaicin injection. Heat response latencies were measured 10, 30 and 60 minutes after capsaicin injection using a Hargreaves apparatus.

Capsaicin Aversion Drinking Test—WT and P2Y₂(-/-) mice (n=10/genotype) were housed with 2 water bottles, one containing distilled water plus capsaicin dissolved in ethanol, the other containing distilled water with an equal concentration of ethanol. Mice were allowed to drink freely; the volume remaining was measured by weight and bottles refilled daily. Measurements were taken for three days for each concentration of capsaicin, 2μM and 500nM. Solutions were made daily from a stock solution of 3.3mM capsaicin dissolved in 30% ethanol. After the experiment, the average amount of capsaicin drunk each day was calculated and compared between WT and P2Y₂(-/-) mice.

Inflammation—An emulsion of complete Freund's adjuvant (CFA) was prepared by thoroughly mixing equal volumes of sterile saline and complete Freund's adjuvant (heat-killed and dried *Mycobacterium tuberculosis* in paraffin oil and mannide monooleate) (Sigma, St. Louis, MO). For behavioral analysis, ten mice of each genotype received a subcutaneous injection of CFA emulsion (20 μl) in the plantar surface of both hindpaws [21]. The same animals were tested for thermal hypersensitivity using the Hargreaves apparatus and mechanical allodynia using von Frey hair analysis 1, 4 or 15 days after CFA injection. Edema was evaluated by measuring paw thickness with a caliper micrometer.

Immunocytochemistry

Mice were given an overdose of Avertin anesthetic and killed by transcardial perfusion with 4°C saline followed by 4% paraformaldehyde. Tissue was rapidly dissected, placed in 25% sucrose overnight and frozen in OCT mounting medium. Section were cut at 12 μm (DRG) or

20 μ m (spinal cord, skin) on a cryostat and collected on Superfrost microscope slides, then kept at -20°C until used. Slides were placed in blocking solution containing 2% normal donkey serum, 0.2% Triton X-100 in PBS for 30 minutes, then incubated in the same solution with rabbit anti-TRPV1 antibody (Oncogene Research, 1:500) overnight at room temperature. The TRPV1 antibody was applied with a cocktail of mouse N52 anti-neurofilament (1:2000) and mouse anti-peripherin (Chemicon, 1:300) as a pan-neuronal counter-stain for cell counting [18]. Next, slides were washed 3 times for 3 minutes in PBS and incubated for 30 minutes in secondary antibodies diluted 1:200 in blocking solution. Secondary antibodies were donkey anti-rabbit or donkey anti-mouse conjugated to CY3 or CY2 (Jackson ImmunoResearch). Slides were washed 3 times in PBS, dipped in water and cover-slipped in Slow fade anti-bleaching solution in glycerol (Molecular Probes), then photographed under epifluorescence or confocal microscopy with a digital camera and collected using Adobe Photoshop software. No staining was evident with the secondary antibody alone or when TRPV1 staining was examined in DRG sections from TRPV1(−/−) mice (generously provided by Dr. Michael J. Caterina, Johns Hopkins University). The percentage of TRPV1-immunoreactive (TRPV1-IR) neurons in WT and P2Y₂(−/−) mice was determined using systematic random sampling [35]. Six evenly-spaced sections (every nth section, where n = total number of sections/6) from L4 DRG were chosen, starting with a randomly-chosen section between 1 and n. Sections were photographed for TRPV1-IR and the counterstain and the percentage of TRPV1-IR versus counterstained neurons with clearly defined nuclei was determined for both genotypes (n = 3 mice/genotype, 3216 neurons (WT), 2159 neurons (P2Y₂(−/−)).

RESULTS

P2Y Expression in Naïve and Inflamed Sensory Neurons

To probe the relative expression levels of UTP-responsive P2Y receptors in sensory neurons (see Table 1) expression levels of the known G_q-coupled P2Y receptors (P2Y₁, P2Y₂, P2Y₄ and P2Y₆) in lumbar DRG were determined and compared using real-time PCR. P2Y₁₁ was not included in this study because our results (BLAST search of the mouse genome with human and canine P2Y₁₁) and others indicate that mice do not possess the gene for this receptor [48]. All tested P2Y family members were expressed in DRG, but the levels of expression varied widely: P2Y₂~P2Y₁>>P2Y₆>>P2Y₄. For comparison, the relative expression level of another G_q-coupled receptor was examined, the metabotropic glutamate receptor mGluR5; this receptor is also implicated in nociceptive signaling [3;50]. Interestingly, both P2Y₁ and P2Y₂ mRNAs are significantly (p < .001) more abundant than mGluR5. However, these differences must be interpreted conservatively as baseline comparisons between genes are sensitive to differences in primer efficiency.

Comparison of P2Y mRNA levels between WT and P2Y₂(−/−) mice revealed that P2Y₂ expression is eliminated in P2Y₂(−/−) mice. Next, P2Y mRNA levels were examined in response to inflammation evoked by injection of complete Freund's adjuvant (CFA) into the hindpaw. P2Y₂ was the only family member that showed increased expression in response to inflammation: P2Y₂ was upregulated 150% 1 day after CFA injection, then increased to more than 300% above baseline after 4 days before returning to baseline 7 days after injection (Figure 1). Upregulation of P2Y₂ expression is consistent with an enhanced role for this receptor during inflammation. Interestingly, P2Y₁, P2Y₄ and P2Y₆, were downregulated during inflammation.

P2Y₂ Mediates Most UTP Responses in Sensory Neurons

G protein-coupled receptors (GPCRs) coupled to G_q evoke the release of Ca⁺⁺ from intracellular stores through the activation of phospholipase C and generation of IP₃. We have proposed that P2Y₂ is the principal UTP receptor in sensory neurons [42]. To determine whether P2Y₂ is required for UTP signaling, increases in intracellular Ca⁺⁺ evoked by 100 μ M

ATP or UTP were examined using Fura-2 Ca^{++} imaging in DRG neurons acutely isolated from WT and $\text{P2Y}_2(-/-)$ mice. Representative traces are shown in Figure 2A and B; mean $\pm$ SE data are shown in Table 2. The preferred agonists for P2Y_1 (ADP) and P2Y_6 (UDP) were also tested in WT and $\text{P2Y}_2(-/-)$ backgrounds (Figure 2). In WT mice, ATP (acting at P2X as well as P2Y receptors) and UTP (selective for P2Y receptors) evoked Ca^{++} transients in $66 \pm 4\%$ and $41 \pm 9\%$ of sensory neurons, respectively. In WT cells, UTP responses were significantly ($p < .001$) delayed with respect to ATP responses (time to peak: ATP $35 \pm 3\text{s}$, UTP $74 \pm 11\text{s}$). These data are consistent with a model in which ATP responses are a combination of rapidly-activating P2X and slowly-activating P2Y components, while UTP responses are P2Y -only (see also Figure 2B from Molliver et al., 2002). Confirmation of this model will require further study. In contrast, UTP-evoked responses were seen in only $7 \pm 4\%$ of neurons from $\text{P2Y}_2(-/-)$ mice. Of these remaining responses, both peak (ΔF : wt 0.33 ± 0.07 , $\text{Y}_2(-/-)$ 0.1 ± 0.01) and area (ΔF_a : wt 12 ± 2 , $\text{Y}_2(-/-)$ 3 ± 1) measures were significantly ($p < .001$) reduced compared to WT UTP responses. These data indicate that P2Y_2 mediates UTP-evoked Ca^{++} responses in the great majority of sensory neurons. P2Y_2 is also activated by ATP, but neither the frequency ($65 \pm 5\%$) nor the peak magnitude of ATP responses were reduced in $\text{P2Y}_2(-/-)$ DRG cells (see Table 2). However, ATP response areas were significantly reduced (ΔF_a : wt 12 ± 2 ; $\text{Y}_2(-/-)$ 7 ± 1 ; $p < .001$). ATP responses in $\text{P2Y}_2(-/-)$ cells tended to be more brief than those in WT cells (compare Figure 2A and B, left panels), and this difference underlies the significant reduction in ATP response area. This result indicates that P2Y_2 contributes a significant component of the ATP response in sensory neurons. Importantly, responses to the non- P2Y_2 agonists ADP and UDP were unaltered in frequency (see Figure 2) or magnitude (see Table 2) in $\text{P2Y}_2(-/-)$ DRG cells.

P2Y_2 Potentiates TRPV1 Activity in Isolated Sensory Neurons

Moriyama et al. [31] reported that UTP potentiates signaling through TRPV1. To determine the role of P2Y_2 in basal TRPV1 activity, Ca^{++} responses to brief (2s) application of $1\mu\text{M}$ capsaicin were recorded in sensory neurons isolated from WT and $\text{P2Y}_2(-/-)$ mice. Representative data are shown in Figure 3A. Strikingly, the percentage of capsaicin-responsive neurons in $\text{P2Y}_2(-/-)$ mice was reduced by more than 50% (Figure 3B). The amplitude of the remaining capsaicin responses was diminished by more than 80% compared to responses obtained in WT neurons (Figure 3C). These data suggest that normal function of TRPV1 requires tonic modulation by P2Y_2 . To examine acute nucleotide enhancement of TRPV1 signaling, capsaicin responses were measured before and after a 30 second perfusion with $100\mu\text{M}$ ATP or UTP (Figure 4; data are summarized in Table 3). In WT neurons, exposure to ATP or UTP potentiated most ($>60\%$) subsequent capsaicin responses. In some cases, cells that did not display responses to an initial application of capsaicin became responsive after exposure to nucleotides. Nucleotide (ATP or UTP) potentiation of capsaicin responses was profoundly reduced in neurons isolated from $\text{P2Y}_2(-/-)$ mice: the proportion of the remaining capsaicin-responsive neurons exhibiting nucleotide potentiation was decreased by more than 50%, and the magnitude of the potentiation was greatly reduced (see Table 3). These data suggest that P2Y_2 is the dominant effector of TRPV1 potentiation by nucleotides and that TRPV1 function is substantially reduced in the absence of P2Y_2 .

The reduction in capsaicin responsiveness in $\text{P2Y}_2(-/-)$ neurons could be explained by reduced TRPV1 expression or by a reduction in the function of existing TRPV1. To probe TRPV1 expression, TRPV1 immunoreactivity was analyzed in WT and $\text{P2Y}_2(-/-)$ L4 DRG and central projections in the dorsal horn of the L4 spinal cord. Representative sections are shown Figure 5. There was no discernable qualitative decrease in the distribution of TRPV1-immunoreactive axons in the dorsal horn or footpad, or in the percentage of TRPV1- positive cell bodies in DRG (Table 4). Furthermore, whole DRG mRNA levels for TRPV1 were not different in

mutant mice (see Table 4). Therefore, attenuation of capsaicin responsiveness must be due to a change in TRPV1 function rather than in the expression of the receptor.

P2Y₂ Is Required for Acute and Inflammatory Thermal Hyperalgesia

Injection of ATP or UTP into the mouse hindpaw causes acute thermal hypersensitivity. This response requires TRPV1 expression and likely results from the nucleotide-evoked potentiation of TRPV1 signaling [31]. To determine whether P2Y₂ mediates ATP-evoked hyperalgesia, we examined thermal response latencies using the Hargreaves test in WT and P2Y₂(^{-/-}) mice before and after injection of ATP into the plantar surface of the hindpaw (Figure 6). Surprisingly, baseline responses of naïve P2Y₂(^{-/-}) mice to noxious heat were significantly ($p < .03$) longer than those of WT mice, indicating a deficit in baseline noxious thermal sensation. As expected, ATP significantly ($p < .04$) decreased heat response latencies in WT mice 10 and 30 minutes after injection. However, responses of P2Y₂(^{-/-}) mice were not significantly altered by ATP, indicating that ATP-evoked heat hyperalgesia requires P2Y₂.

Injection of complete Freund's adjuvant (CFA) in the hindpaw causes persistent thermal and mechanical allodynia lasting for days [15;54]. A requirement for TRPV1 in the development of inflammatory thermal hyperalgesia has been shown previously [6;14]. Therefore, we tested the thermal and mechanical sensitivity of P2Y₂(^{-/-}) mice in this model of persistent inflammatory pain. Mean thermal response latencies are shown in Figure 7. Both WT and P2Y₂(^{-/-}) mice developed substantial edema following CFA injection (naïve: 2.25 ± 0.01 mm; day 3: WT 3.25 ± 0.12 mm, P2Y₂(^{-/-}) 3.40 ± 0.07 mm; $p < 0.001$ for each genotype vs. naïve paw measurements). Both WT and P2Y₂(^{-/-}) mice exhibited decreased mechanical thresholds three days after CFA injection (naïve: WT 2.64 ± 0.3 g, P2Y₂(^{-/-}) 2.48 ± 0.4 g; day 3: WT 1.47 ± 0.42 g, P2Y₂(^{-/-}) 0.81 ± 0.02 g; $p < 0.005$ for each genotype vs. naïve thresholds). There was no significant difference in the extent of mechanical hypersensitivity between WT and P2Y₂(^{-/-}) mice. However, only WT mice showed significant hypersensitivity to noxious heat. These results indicate that P2Y₂ signaling is not necessary for edema or mechanical allodynia, but is required for the development of inflammatory heat hyperalgesia.

Responses to noxious cold were also examined in P2Y₂(^{-/-}) and WT mice on a cold block apparatus ($n = 10$ mice/genotype). Neither latency to response (WT 15.0 ± 1.96 sec; P2Y₂(^{-/-}) 13.6 ± 2.21 sec) nor the number of responses in 1 minute (WT 16.20 ± 1.36 ; P2Y₂(^{-/-}) 16.20 ± 2.17) were significantly different in P2Y₂(^{-/-}) mice. Therefore, P2Y₂ does not appear to play a role in the detection of noxious cold stimuli.

P2Y₂ Regulates TRPV1 Function in Naïve Mice

Two paradigms were used to examine directly whether the deficits in TRPV1 signaling observed in isolated P2Y₂(^{-/-}) neurons lead to a whole-animal behavioral phenotype: a capsaicin aversion drinking test [6;40], and injection of capsaicin into the hindpaw. In the drinking test, mice were given a choice between two identical water bottles: one containing dilute capsaicin and one containing water alone. The amount of water consumed each day was quantified and results are shown in Table 5. There was no difference in total water consumption between strains, and both WT and P2Y₂(^{-/-}) mice preferred capsaicin-free water. However, P2Y₂(^{-/-}) mice drank significantly more 500nM ($p < 0.05$) and 2 μ M ($p < 0.01$) capsaicin than WT mice, indicating that the mutant mice have a reduced aversion to capsaicin.

Injection of capsaicin into the plantar surface of the hindpaw caused nocifensive behavior and thermal hyperalgesia in WT mice (Figure 8). In P2Y₂(^{-/-}) mice, the frequency of nocifensive behavior (Figure 8A) and the duration and magnitude of the thermal hyperalgesia (Figure 8B) were significantly less than in WT mice. As TRPV1 immunoreactivity is still present in DRG

cell bodies and terminals of P2Y₂(^{-/-}) mice, we conclude that regulation by Gq-coupled receptors is required for normal TRPV1 function. To test this hypothesis directly, we examined capsaicin responses in WT and P2Y₂(^{-/-}) mice following acute activation of another Gq-coupled receptor known to cause thermal hyperalgesia through the potentiation of TRPV1, the bradykinin receptor B2 [16;2]. Bradykinin was injected into the hindpaw of WT and P2Y₂(^{-/-}) mice 10 minutes prior to capsaicin injection. Bradykinin alone did not cause nocifensive behavior, nor did bradykinin increase the frequency of nocifensive behaviors following capsaicin injection in WT mice (Figure 8A). However, in P2Y₂(^{-/-}) mice, bradykinin injection significantly ($p < 0.001$) increased the number of capsaicin responses elicited; following bradykinin injection, responses were very similar in WT and P2Y₂(^{-/-}) mice. Bradykinin also prolonged the thermal hyperalgesia evoked by capsaicin injection in WT mice (Figure 8B). In P2Y₂(^{-/-}) mice, bradykinin preadministration increased capsaicin-induced thermal hyperalgesia to near-WT levels, although the duration of hyperalgesia remained shorter. These results suggest that TRPV1 function can be largely restored in P2Y₂(^{-/-}) mice by activation of other Gq-coupled receptors co-expressed with TRPV1.

DISCUSSION

In this study, we have used genetic, behavioral and physiological approaches to investigate the role of nucleotide signaling through P2Y₂ in basal nociception, as well as in the generation of inflammatory hypersensitivity. P2Y₂ is highly expressed in DRG and is the only P2Y receptor examined that is upregulated in response to inflammation of the hindpaw. Mice lacking P2Y₂ show reduced sensitivity to noxious thermal stimulation and fail to develop either acute (ATP-evoked) hyperalgesia or persistent (inflammation-evoked) hyperalgesia. Sensory neurons from P2Y₂(^{-/-}) mice display a profound reduction in responsiveness to capsaicin, although TRPV1 mRNA and immunoreactivity are not reduced. Taken together, our results suggest that P2Y₂ is a critical component of thermal nociception and is also required for normal TRPV1 function.

P2Y₂ and Nucleotide Signaling in Sensory Neurons

Several studies suggest that P2Y receptors contribute to nociceptive signaling. The P2Y₂/P2Y₄ agonist UTP causes depolarization and action potential firing in dissociated sensory neurons *in vitro* and in identified nociceptors in the skin-nerve preparation [29;42]. UTP also potentiates capsaicin-evoked neuropeptide release [22] and has been implicated in transcriptional plasticity in nociceptors [29]. Here, we show that P2Y₂ is the principal receptor mediating UTP-evoked increases in intracellular [Ca⁺⁺] in sensory neurons. Responses to ATP, which activates both P2X and P2Y receptors, were not reduced in frequency in P2Y₂(^{-/-}) neurons, presumably due to overlap in expression between P2X receptors and P2Y₂.

A small subset of neurons in P2Y₂(^{-/-}) mice retained UTP-evoked responses, most likely via P2Y₄, which has a pharmacological profile similar to that of P2Y₂ [48]. The percentage of total DRG neurons showing potentiation of capsaicin responses by UTP in P2Y₂(^{-/-}) mice (32% of 21%, Table 3) is consistent with the percentage of neurons that displayed UTP-evoked Ca⁺⁺ transients in mutant mice (7%, Figure 2), suggesting that an alternate UTP receptor, probably P2Y₄, functions in a small population of TRPV1-expressing neurons. However, this P2Y₂-independent pathway was relatively ineffective at both cellular and behavioral levels, as the magnitude of potentiation was very small in the absence of P2Y₂ (Table 3), and P2Y₂(^{-/-}) mice did not develop significant thermal hyperalgesia in response to injection of ATP or CFA. Our finding that P2Y₄ is downregulated after CFA injection provides further evidence that this receptor is not a major contributor to nociceptor sensitization.

Real-time PCR results confirm previous reports that P2Y₁ is highly expressed in lumbar DRG as well as P2Y₂ [32;31;38]. However, available evidence suggests that P2Y₁ and P2Y₂ are not

widely co-expressed [24]. In mice, P2Y₂ appears to be extensively co-localized with TRPV1 and CGRP, whereas P2Y₁ shows little overlap with TRPV1 but co-localizes with labeling by the plant lectin IB4, which identifies CGRP-negative C-fibers [31]. Tominaga and colleagues initially implicated P2Y₁ in the potentiation of TRPV1 currents by ATP, but later demonstrated that TRPV1 potentiation and ATP-evoked hyperalgesia persist in P2Y₁ knockout mice [45; 31].

The preferred agonist for P2Y₁ is ADP; depending on the cellular environment in which the receptor is expressed, ATP has been reported to be a partial agonist or an antagonist for P2Y₁ [37;5]). In this study, ADP-evoked Ca⁺⁺ transients were elicited in approximately 40% of sensory neurons, but rarely co-localized with capsaicin-evoked transients (not shown). Furthermore, we found that P2Y₁ mRNA levels were decreased throughout the first week after CFA injection, suggesting that P2Y₁ function decreases during inflammatory hyperalgesia.

P2Y₂ is Required for the Development of Thermal Hyperalgesia

Nucleotide signaling is proposed to play a role in both acute pain and chronic hyperalgesia. ATP is a candidate messenger of tissue damage, released through either passive or active mechanisms from damaged cells and/or through vesicular release from peripheral sympathetic and sensory axons [11;4]. Our results implicate P2Y₂ in the pain of ATP injection, a phenomenon originally attributed exclusively to P2X receptors [19]. Mice lacking P2X₃ have reduced (but not abolished) acute nocifensive responses to ATP injection [9], however the thermal hyperalgesia evident 10 and 30 minutes following ATP injection is dependent upon TRPV1 rather than P2X₃ [9;19]. We demonstrate here that the protracted thermal hyperalgesia resulting from ATP injection requires P2Y₂ as well as TRPV1. Thus, pain evoked by ATP injection *in vivo* appears to be mediated by both P2X and P2Y receptors. *In vitro*, ATP and UTP potentiated Ca⁺⁺ transients evoked by capsaicin in WT sensory neurons, however little potentiation was observed in P2Y₂(-/-) neurons. ATP evokes Ca⁺⁺ transients in P2Y₂-/- neurons via P2X receptors, but does not potentiate TRPV1 in these cells, indicating that Ca⁺⁺ influx alone is not sufficient to potentiate TRPV1 function. These data suggest that P2Y₂ potentiates TRPV1 function to produce acute thermal hyperalgesia.

Injection of CFA into the hindpaw causes a localized inflammatory response including edema and thermal and mechanical hyperalgesia. Previous studies have reported that thermal hyperalgesia resulting from inflammation does not develop in TRPV1(-/-) mice, although mechanical sensitization does occur [6;14]. In P2Y₂(-/-) mice, we found a similar phenotype: mechanical allodynia was similar to that seen in WT mice, but thermal hyperalgesia did not develop. In contrast, P2X₃(-/-) mice show enhanced thermal hyperalgesia in response to CFA injection [41]. Consistent with a role for P2Y₂ in persistent inflammatory pain, P2Y₂ mRNA is upregulated 3-fold in WT DRG in response to CFA injection. Therefore, P2Y₂ signaling is likely enhanced in sensory neurons during inflammation. Together, our results suggest a model in which P2Y₂ modulates TRPV1 activity to produce thermal hyperalgesia in both acute and persistent pain states.

The mechanism for P2Y₂-mediated potentiation of TRPV1 has not yet been determined; *in vitro*, P2Y₂ activation has been shown to lower the temperature threshold for TRPV1 activation, possibly resulting in channel opening at body temperature [31;26]. Other G_q-coupled receptors also modulate TRPV1 function, including serotonin receptors [43], the protease-activated receptor PAR-2 [13], the prokineticin receptor PKR1 [46] and bradykinin receptors [8;44]. These studies suggest that modulation of TRPV1 occurs both through depletion of plasma membrane PIP₂, which may inhibit TRPV1 [8;36], and by phosphorylation through the activation of a signaling pathway requiring protein kinase C [47;34;31]. Alternatively, potentiation of TRPV1 signaling may be mediated by phosphorylation-dependent insertion of the channel into the plasma membrane [52]. Additional mechanisms

may be involved as well: a recent report indicates that P2Y₂ can enhance nerve growth factor signaling through its receptor tyrosine kinase, TrkA [1], which also potentiates TRPV1-encoded currents [25;17]. However, TrkA activation does not affect the temperature threshold of TRPV1, suggesting that P2Y₂ and TrkA potentiate TRPV1 function through different mechanisms [25;17].

In this study, behavioral responsiveness to capsaicin absent in P2Y₂(^{-/-}) mice was partially restored by administration of bradykinin. Several reports indicate that bradykinin potentiates TRPV1 signaling *in vitro* and produces heat hyperalgesia that is mediated by TRPV1 *in vivo* [16;2]. Our findings support a model in which ongoing P2Y₂ signaling determines basal TRPV1 activity under normal conditions and enhances TRPV1 function during inflammation. Reports that nucleotides are constitutively released by a variety of cell types provide a source for modulating nucleotides [27]. In the absence of P2Y₂, an alternate effector of Gq signaling (bradykinin receptor) was able to acutely restore TRPV1 function. Intriguingly, a recent analysis of mice with a null mutation for the Gq-coupled prokineticin receptor PKR1 also found a decrease in capsaicin responsiveness [33]. It is not clear why multiple Gq-coupled receptors would be required for the full expression of TRPV1 function; it may be that different Gq-coupled receptors modulate TRPV1 signaling in different subsets of sensory neurons, or alternatively that multiple receptors cooperate in the same neurons to modulate TRPV1 function. Future studies will address the nature of the interaction between P2Y₂ and TRPV1.

P2Y₂ Regulates Noxious Heat Thresholds

P2Y₂(^{-/-}) mice exhibited significantly longer response latencies to noxious heat (but not cold) than WT mice, indicating a significant reduction in heat sensation. This sensory deficit may be a consequence of attenuated TRPV1 signaling, given that capsaicin responsiveness was substantially reduced in P2Y₂(^{-/-}) mice both *in vitro* and *in vivo*. However, although TRPV1 plays a critical role in inflammatory thermal hyperalgesia, reports conflict over the importance of TRPV1 for baseline thermal sensation. Caterina and colleagues reported that naïve TRPV1(^{-/-}) mice are less responsive to noxious heat stimulation than WT mice, using hotplate, Hargreaves and tail-immersion tests [6]. Other investigators found no thermal phenotype in naïve TRPV1(^{-/-}) mice [14] and no alteration of baseline heat response properties in TRPV1(^{-/-}) cutaneous nociceptors [51]. Detection of heat is likely to be dependent on an array of sensors that act in concert to determine thermal sensitivity; P2Y₂ signaling may provide a TRPV1-independent mechanism contributing to baseline noxious heat sensation. Indeed, cultured keratinocytes release ATP in response to heat in a graded, temperature-dependent fashion (S. Mandadi, M. Tominaga, personal communication). Thus, skin cells and cutaneous afferent terminals may act as an integrated thermal transduction unit, using nucleotides as chemical messengers. This hypothesis is supported by the discovery that P2X₃(^{-/-}) mice show a deficit in warmth detection, suggesting that P2X₃ receptors also contribute to the transduction of thermal stimuli [41]. Our results indicate that nucleotide signaling through P2Y₂ is an essential component of both baseline thermal nociception and thermal hyperalgesia.

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Figure 1. P2Y₂ mRNA is Upregulated in Response to Inflammation

Graph illustrates percent changes in mRNA levels over time after induction of a localized inflammatory response by injection of CFA into the hindpaw. mRNA levels for the 4 Gq-coupled P2Y receptors known to be expressed in mouse were measured by real-time PCR in L3–5 DRG from WT mice before (baseline) and 1, 4, 7 and 14 days after injection with complete Freund's adjuvant (CFA). P2Y₂ was the only receptor significantly upregulated after injection, peaking at 4 days. P2Y₁ was significantly down-regulated 1 day after injection. These results indicate that P2Y₂ expression is dynamically regulated in response to inflammation (*p < 0.05, **p < 0.005 vs. baseline for each gene).

Figure 2. ATP- and UTP-evoked Ca^{++} Transients are Attenuated in $\text{P2Y}_2(-/-)$ Mice

Increases in intracellular free Ca^{++} were measured in isolated DRG neurons in response to brief (2 second) focal application of P2Y agonists. Each agonist was singly applied on separate coverslips. In neurons from WT mice (A), application of 100 μM ATP, UTP, ADP or UDP yielded robust Ca^{++} transients. Results obtained from $\text{P2Y}_2(-/-)$ DRG neurons are shown in (B). Interestingly, the percentage of UTP-responsive neurons and the UTP response magnitudes were significantly (* $p < 0.01$) reduced in neurons from $\text{P2Y}_2(-/-)$ mice (Table 2). Responses to the application of 100 μM ADP or UDP were not different in frequency or magnitude (Table 2) in neurons isolated from $\text{P2Y}_2(-/-)$ and WT mice. Results were obtained from 6 mice for each agonist/genotype.

Figure 3. Diminished TRPV1 signaling in sensory neurons from P2Y₂(-/-) mice

Ca^{++} transients evoked by a brief (2 second) application of 1 μM capsaicin were measured by Fura-2 Ca^{++} imaging in isolated DRG neurons. Representative traces are shown in **A**. In neurons from P2Y₂(-/-) mice, both the percentage of capsaicin-responsive neurons (**B**) and the magnitude of remaining responses (**C**) were significantly (***) $p < 0.001$ less than those seen in WT neurons, suggesting an attenuation of TRPV1 signaling in P2Y₂(-/-) sensory neurons. Results were obtained from 5 mice per genotype; data are reported as mean $\pm$ SE.

Figure 4. Potentiation of TRPV1 signaling by nucleotides is greatly reduced in P2Y₂^{-/-} mice
 Ca⁺⁺ imaging techniques were used to investigate the potentiation of capsaicin-evoked Ca⁺⁺ transients by ATP and UTP in isolated neurons from WT and P2Y₂^{-/-} mice. Capsaicin was delivered for 2 seconds at 10 minute intervals. Significant desensitization was consistently observed in the 2nd capsaicin response. 100μM ATP or UTP was then applied for 30 seconds at 3 minutes before the 3rd capsaicin pulse, and the change in magnitude between the 2nd and 3rd capsaicin responses measured. In WT neurons (**A**), application of ATP (**left panel**) or UTP (**right panel**) routinely resulted in a significant potentiation of the capsaicin response (compare 2nd and 3rd capsaicin responses in **A**). Nucleotide potentiation of capsaicin responses was largely absent in P2Y₂^{-/-} neurons (**B**). Data are summarized in Table 3.

Figure 5. No Reduction of TRPV1-Expressing Neurons in P2Y₂(-/-) Mice

Immunocytochemical staining for TRPV1 in sections of L4 dorsal root ganglion (DRG), spinal cord dorsal horn (dorsal horn) and glabrous skin of the hindpaw (skin) from WT and P2Y₂(-/-) mice is shown. Staining in DRG sections contained many brightly-labeled small-diameter neurons and some more faintly-labeled cells, while a dense band of staining was evident in the superficial dorsal horn. In skin sections, labeled axons were visible in the dermal plexus (Der) and in axons penetrating the epidermis (Epi). Sections from WT and P2Y₂(-/-) mice were not distinguishable on the basis of TRPV1 staining. There was no reduction in the percentage of TRPV1-immunoreactive DRG neurons in P2Y₂(-/-) mice; indeed, the percentage of labeled neurons was slightly higher in mutant mice than in WT (see Table 5). Scale bars = 50μm.

Figure 6. P2Y₂(-/-) mice show deficits in noxious heat sensation and ATP-evoked thermal hyperalgesia

Response latency to a radiant heat stimulus (normalized to WT baseline) was measured in WT and P2Y₂(-/-) mice before and after injection of 10nmol ATP into the plantar surface of the hindpaw. Baseline response latencies of P2Y₂(-/-) mice were significantly ($p < 0.03$) longer than those of WT mice, indicating a deficit in baseline thermal responsiveness. In WT mice, ATP injection significantly ($*p < 0.04$) decreased the latency of responses to a radiant heat stimulus (thermal hyperalgesia) 10 and 30 minutes after injection. P2Y₂(-/-) mice did not show significant changes in thermal response latency after injection of ATP and were significantly different from WT mice at these time points ($p < 0.01$). (n = 10 mice/genotype)

Figure 7. P2Y₂(-/-) mice fail to develop thermal hyperalgesia during CFA-evoked inflammation
 Three days after injection of 20μl complete Freund's adjuvant (CFA 3) into mouse hindpaws, WT mice exhibited substantial edema, mechanical allodynia (see Results) and persistent thermal hyperalgesia (*p < 0.005 vs. naïve WT mice, BL = pre-injection baseline). P2Y₂(-/-) mice also developed edema and mechanical allodynia but failed to develop significant thermal hyperalgesia (n = 10 mice/genotype).

Figure 8. P2Y₂(-/-) deficits in behavioral sensitivity to capsaicin are restored by pretreatment with bradykinin

WT and P2Y₂(-/-) mice (n=9/genotype/treatment) received an injection of 3μg bradykinin or saline in one hindpaw, followed by capsaicin 10 minutes later. Nocifensive behaviors (**A**) were quantified for 2 minutes following capsaicin injection, and heat hyperalgesia (**B**) was measured with a Hargreaves apparatus 10, 30 and 60 minutes after capsaicin injection. P2Y₂(-/-) mice showed significantly fewer nocifensive responses than WT mice in response to capsaicin alone, but this deficit was eliminated by prior treatment with bradykinin. Similarly, WT mice showed more significant heat hyperalgesia following capsaicin injection than P2Y₂(-/-) mice, and bradykinin pretreatment (dotted lines) potentiated capsaicin-induced heat hyperalgesia in P2Y₂(-/-) mice to WT levels. (* p < 0.05, ** p < 0.01, *** p < 0.001 vs. P2Y₂(-/-) baseline; ★ p < 0.01 vs WT capsaicin without bradykinin)

Table 1

Relative Abundance of P2Y Receptor mRNA.

Receptor	Preferred Agonists	ΔC_T WT	Abundance Relative to P2Y ₂	ΔC_T P2Y ₂ ^{-/-}
P2Y ₁	ADP	6.22 ± 0.11	96.8%	6.48 ± 0.03
P2Y ₂	ATP/UTP	6.17 ± 0.08	100%	---
P2Y ₄	ATP/UTP	13.63 ± 0.31	0.6%	17.19 ± 0.57 [*]
P2Y ₆	UDP	10.92 ± 0.14	3.7%	11.21 ± 0.45
mGluR5	glutamate	10.28 ± 0.29	5.8%	---

* $p < 0.002$ vs. WT ΔC_T ; data presented as mean ± SE

Relative expression levels of P2Y family members expressed as the cycle threshold (C_T), the normalized threshold (ΔC_T : C_T value minus the C_T for the standard, GAPDH), and as the relative mRNA abundance normalized to P2Y₂. Another G_q-coupled receptor implicated in nociceptive signaling in DRG neurons, the metabotropic glutamate receptor mGluR5, is provided for comparison. All reactions were run using cDNA samples from the same animals (n=5). See Ralevic and Burnstock (1998) for a review of P2Y agonists. P2Y₁ and P2Y₂ are the most abundant of the 4 G_q-coupled receptors.

Table 2**Nucleotide Response Properties in Sensory Neurons**

		WT	P2Y₂(-/-)
ATP	ΔF	0.34 ± 0.04	0.31 ± 0.02
	ΔF_a	12 ± 2	$7 \pm 1^*$
	n (cells)	108	109
UTP	ΔF	0.33 ± 0.07	$0.10 \pm 0.01^*$
	ΔF_a	12 ± 2	$3 \pm 1^*$
	n (cells)	111	105
ADP	ΔF	0.25 ± 0.06	0.26 ± 0.04
	n (cells)	27	36
UDP	ΔF	0.27 ± 0.12	0.21 ± 0.1
	n (cells)	18	15

* $p < 0.001$ vs. WT; n(mice) = 6 for each agonist/genotype, data presented as mean $\pm$ SE

Values reported are mean peak response (ΔF) and mean response area (ΔF_a) of Ca^{++} transients elicited in DRG neurons by the presentation of the indicated agonist. Agonists were applied individually on separate coverslips. ATP and UTP responses were significantly ($p < 0.001$) reduced in P2Y₂(-/-) cells, while responses to ADP and UDP were not altered. Representative traces and percentages of responsive neurons are shown in Figure 2.

Table 3
Potentiation of Capsaicin Responses by UTP and ATP Requires P2Y₂

	% Capsaicin Responders in DRG	% Capsaicin Responders Potentiated by UTP	Potentiation Magnitude (UTP)	% Capsaicin Responders Potentiated by ATP	Potentiation Magnitude (ATP)
WT n (cells)	49 ± 5% 39/80	71 ± 13% 27/39	4.28 ± .98 27	64 ± 10% 35/56	3.67 ± 0.35 35
P2Y ₂ (-/-) n (cells)	21 ± 4%* 22/105	32 ± 12%* 7/22	1.31 ± .29* 7	30 ± 9%* 15/51	1.22 ± 0.24* 15

* p < 0.02 vs. WT; n(mice) = 5 for each genotype; data presented as mean ± SE.

Values are the percentage of cells that respond to capsaicin, and the percentage of those cells in which the second capsaicin response is potentiated by prior ATP or UTP presentation. Potentiation is quantified as the ratio of response area ($\Delta F_{a2}/\Delta F_{a1}$). Potentiation of capsaicin responses by UTP or ATP is common in WT cells and significantly ($p < 0.02$) diminished in frequency and magnitude in P2Y₂(-/-) neurons. Note that there are significantly ($p < 0.02$) fewer capsaicin-responsive sensory neurons in P2Y₂(-/-) mice. Representative traces are shown in Figure 4.

Table 4TRPV1 mRNA and protein are not reduced in P2Y₂(-/-) DRG.

	Ct	ΔCt	% WT mRNA	% Neurons TRPV1-IR
wt	21.29 ± 0.12	4.45 ± 0.14	100.0%	37.4 ± 1.8
P2Y ₂ (-/-)	21.37 ± 0.13	4.53 ± 0.09	94.5%	42.9 ± 2.6 *

* p < 0.05 vs. WT, data presented as mean ± SE

Values presented are mean cycle threshold (C_T), normalized threshold (ΔC_T: C_T value minus the C_T for the standard GAPDH), and relative mRNA abundance normalized to TRPV1 expression in WT mice. This quantitative real-time PCR analysis revealed no significant difference in TRPV1 mRNA abundance between WT and P2Y₂(-/-) L3-5 DRG (n = 5 mice/genotype). Immunocytochemistry revealed a small but significant increase in the percentage of L4 DRG neurons positively labeled for TRPV1 (TRPV1-IR) in P2Y₂(-/-) mice compared to WT (n = 3 mice/genotype).

Table 5**P2Y₂(-/-) Mice Are Less Sensitive to Oral Capsaicin.**

	2.0μM Capsaicin	0.5μM Capsaicin
WT	15.0 ± 1.0%	30.0 ± 3.6%
P2Y₂(-/-)	19.8 ± 2.1% *	41.2 ± 2.0% **

*
p < 0.05**
p < 0.01 vs. WT, n (mice) = 10 per genotype, data presented as mean ± SE

Values reported are mean amounts of capsaicin-containing water consumed as a percentage of total water consumption. WT and P2Y₂(-/-) mice were given a choice of two water bottles, one containing either 500nM 2μM capsaicin and one containing water alone (plus diluent). Water consumption from each bottle was measured daily for three days. P2Y₂(-/-) mice drank significantly more capsaicin-containing water at both concentrations than WT mice. Total water consumption was not different between WT and P2Y₂(-/-) mice.