



Serotonin Receptor 2B Mediates Mechanical Hyperalgesia by Regulating Transient Receptor Potential Vanilloid 1

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Received: 27 August 2015 / Accepted: 24 November 2015 / Published online: 3 December 2015
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Abstract Serotonin [5-hydroxytryptamine (5-HT)], an inflammatory mediator, contributes to inflammatory pain. The presence of multiple 5-HT subtype receptors on peripheral and central nociceptors complicates the role of 5-HT in pain. Previously, we found that 5-HT_{2B/2C} antagonist could block 5-HT-induced mechanical hyperalgesia. However, the types of neurons or circuits underlying this effect remained unsolved. Here, we demonstrate that the G_{q/11}-phospholipase C β -protein kinase C ϵ (PKC ϵ) pathway mediated by 5-HT_{2B} is involved in 5-HT-induced mechanical hyperalgesia in mice. Administration of a transient receptor potential vanilloid 1 (TRPV1) antagonist inhibited the 5-HT-induced mechanical hyperalgesia. 5-HT injection enhanced 5-HT- and capsaicin-evoked calcium signals specifically in isolectin B₄ (IB₄)-

negative neurons; signals were inhibited by a 5-HT_{2B/2C} antagonist and PKC ϵ blocker. Thus, 5-HT_{2B} mediates 5-HT-induced mechanical hyperalgesia by regulating TRPV1 function.

Keywords Serotonin · 5-HT_{2B} · Mechanical hyperalgesia · Protein kinase C ϵ · Transient receptor potential vanilloid 1

Introduction

Serotonin [5-hydroxytryptamine (5-HT)], released from platelets, mast cells, and endothelial cells into the inflamed site, is an important inflammatory mediator causing pain and hyperalgesia (Dray 1995; Sommer 2004). More than one subtype of serotonin receptor is present in peripheral nociceptive afferents (Hoyer et al. 2002), so each receptor may have distinct functions in 5-HT-induced pain. Use of specific receptor agonists, antagonists, or gene-targeting techniques have clarified the roles of 5-HT-receptor subtypes in pain. 5-HT-induced mechanical hyperalgesia is inhibited by 5-HT_{2B/2C} antagonist but not 5-HT_{1A}, 5-HT_{2A}, or 5-HT₃ antagonist (Lin et al. 2011). A formalin model further revealed that 5-HT₄, 5-HT₆, and 5-HT₇ are required for maintaining secondary mechanical allodynia and hyperalgesia (Godinez-chaparro et al. 2012). These findings suggest that 5-HT_{2B/2C} could be involved in inducing mechanical hyperalgesia, whereas 5-HT_{4/6/7} are responsible for maintaining the hyperalgesia. However, the mechanisms of inducing and maintaining mechanical hyperalgesia remain unsolved.

Several lines of evidence have suggested that G_{q/11}, G_i, protein kinase C ϵ (PKC ϵ), or phospholipase C β (PLC β) is involved in mechanical hyperalgesia (Khasar et al. 1999; Joseph et al. 2007; Dina et al. 2009; Joseph and Levine 2010; Tappe-Theodor et al. 2012). Thus, mechanical

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hyperalgesia may be mediated by a $G_{q/11}$ - or G_i -PLC β -PKC ϵ pathway. The *in vitro* studies found that 5-HT₂ receptors activate $G_{q/11}$ protein, thus leading to PKC activation (Loric et al. 1995; Lin et al. 2011). so 5-HT_{2B/2C} may mediate 5-HT-induced mechanical hyperalgesia through a $G_{q/11}$ -PKC pathway.

Studies in mice lacking transient receptor potential vanilloid 1 (TRPV1) gene revealed that TRPV1 is involved in thermal nociception and hyperalgesia (Caterina et al. 2000; Davis et al. 2000). but several lines of evidence suggest that TRPV1 is involved in mechanical hyperalgesia. TRPV1 antagonists inhibit CFA-, capsaicin, or acid-induced mechanical hyperalgesia (Gavva et al. 2005; Honore et al. 2005; Cui et al. 2006; Chen et al. 2014). Spinal activation of TRPV1 leads to mechanical allodynia, and TRPV1 antagonist can reverse this effect (Kim et al. 2012). Endogenous activation of spinal TRPV1 could be due to $G_{q/11}$ -coupled receptors or arachidonic acid metabolites (Gibson et al. 2008; Kim et al. 2009; Kim et al. 2012). 5-HT potentiates TRPV1 function through protein kinase A (PKA) or PKC (Sugiuar et al. 2004; Ohta et al. 2006). Whether TRPV1 is involved in 5-HT-induced mechanical hyperalgesia is unknown.

In this study, we demonstrated that 5-HT-induced mechanical hyperalgesia is regulated by a 5-HT_{2B}- $G_{q/11}$ -PLC β -PKC ϵ pathway. Administration of 5-HT strongly enhanced 5-HT- and capsaicin-induced calcium signals in IB₄-negative neurons, and enhanced signals were regulated by the 5-HT_{2B}- $G_{q/11}$ -PKC ϵ pathway. Interestingly, 5-HT-induced mechanical hyperalgesia was also inhibited by TRPV1 antagonist or in mice lacking *TRPV1* gene. 5-HT-induced mechanical hyperalgesia may be mediated by a 5-HT_{2B}- $G_{q/11}$ -PLC β -PKC ϵ pathway via regulating TRPV1 function.

Experimental Procedures

Animals

Male CD1 mice (8–12 weeks old) were bred and cared for in accordance with the Guide for the Use of Laboratory Animals (National Academy Press, Washington, DC). Animal experimental procedures were approved by the local animal use committee (IACUC, National Central University, Taiwan). TRPV1^{-/-} mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and backcrossed to CD-1 mice for at least ten generations to generate outbred TRPV1^{+/-} mice. The TRPV1^{+/+}, TRPV1^{+/-}, and TRPV1^{-/-} mice were offspring of a TRPV1^{+/-} intercross. The genotyping primer sequences were 5'-C A C G A G A C T A G T G A G A C G T G / 5'-TCCTCATGCACTTCAGGAAA for TRPV1^{-/-} mice and 5'-C C T G C T C A A C A T G C T C A T T G / 5'-TCCTCATGCACTTCAGGAAA for TRPV1^{+/+} mice.

Agents

5-HT, pertussis toxin (PTX) (Lin et al. 2011; Dina et al. 2009). capsaicin, capsazepine (*N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbo thioamide) (Zhang et al. 2007). and U73122 (1-[6-[[[(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione) (Hou et al. 2004; Joseph et al. 2007; Lin et al. 2011) were from Sigma (St. Louis, MO). Granisetron hydrochloride (1-methyl-*N*-[(3-endo)-9-methyl-9-azabicyclo[3.3.1]non-3-yl]-1H-indazole-3-carboxamide hydrochloride) (Kayser et al. 2007; Lin et al. 2011). SB206553 hydrochloride (3,5-dihydro-5-methyl-*N*-3-pyridinylbenzo[1,2-*b*:4,5-*b'*]dipyrrole-1(2H)-carboxamide hydrochloride)[7], H89 dihydrochloride (*N*-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride) (Zhang et al. 2007; Chen et al. 2008). SQ22536 (9-(tetrahydro-2-furanyl)-9H-purin-6-amine) (Villarreal et al. 2009; Sachs et al. 2009) and RS127445 hydrochloride (4-(4-fluoro-1-naphthalenyl)-6-(1-methylethyl)-2-pyrimidinamine hydrochloride)(Urtikova et al. 2012) were from Tocris Bioscience (Bristol, UK). PKC ϵ V₁₋₂ peptide conjugated with protein transduction domain of TAT protein (CYGRKKRRQRRR-CEAVSLKPT, TAT-PKC ϵ V₁₋₂) (Schwarze et al. 1999; Parada et al. 2005; Sachs et al. 2009) was kindly provided by KAI Pharmaceuticals, Inc. (CA, USA). For animal experiments, all drugs or peptides were diluted in saline before injection. For cell experiments, all drugs or peptides were diluted in (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)/2-ethanesulfonic acid (MES) buffer (125 mM NaCl, 1 mM KCl, 5 mM CaCl₂, 1 mM MgCl₂, 8 mM glucose, 10 mM HEPES, and 15 mM MES, pH 7.6).

Behavioral Tests

Pain-behavioral tests were described previously (Lin et al. 2011). Briefly, male CD-1, TRPV1^{+/+}, TRPV1^{+/-}, and TRPV1^{-/-} mice (8–12 weeks old) were intraplantarly injected with 25 μ l 5-HT, receptor antagonists or inhibitors, then underwent animal behavioral tests for withdrawal thresholds to mechanical stimuli applied to the hindpaw (von Frey filaments, Touch-Test; North Coast Medical, Morgan Hill, CA). Mice ($n = 6$ per group) were pre-trained for 2 h each day for 2 days before the test. A series of von Frey fibers (0.6, 1.4, 2.0, 4 g) were applied to the plantar surface of each hindpaw five times at 5-s intervals after injections. The paw withdrawal threshold (PWT) was determined when paw withdrawal was observed in more than three of five applications.

Primary Cell Culture

Primary dorsal root ganglia (DRG) were cultured as described (Lin et al. 2011). Briefly, mice were pre-injected with or without 5-HT_{2B/2C} antagonist SB206553, then with 5-HT. After

30 min, mouse lumbar 4–6 DRGs were collected in pre-warmed serum-free Dulbecco's modified Eagle's medium (DMEM). After collagenase IA and trypsin treatments, cells were washed in medium and re-suspended in 2 ml serum-free DMEM, then dissociated into single cells by mechanical titration. Cell suspension was slowly dropped into 10 ml serum-free DMEM. After 3–5 min, the cell suspension on the top (~10 ml) was collected and centrifuged at $1224\times g$ for 5 min. The cell pellet was suspended and mixed in 400 μ l DMEM containing 10 % fetal bovine serum (FBS) and seeded on 100 μ g/ml poly-L-lysine-coated 24-mm coverslips. After incubation at 37 °C for 2 h, cells were supplemented with 1.5 ml DMEM containing 10 % FBS and maintained at 37 °C for 12 to 14 h before intracellular Ca^{2+} imaging.

Intracellular Calcium Imaging

Intracellular calcium imaging was performed as described (Chen et al. 2009; Lin et al. 2011). Primary cultured neurons grown on coverslips were washed once with serum-free medium and pre-incubated at 37 °C with 1.25 μ M Fura-2 acetoxymethyl ester (Fura-2-AM; Molecular Probes) for 40 min in HEPES/MES buffer. Coverslips were assembled into culture wells and supplemented with 500 μ l HEPES/MES buffer. Cells were stimulated with 500 μ l HEPES/MES buffer containing 2-fold concentrations of 5-HT, antagonists, or inhibitors, then underwent intracellular calcium recording with use of a Zeiss inverted microscope equipped with a xenon lamp. Cell images were taken with use of a Zeiss Plan-Apo 63X oil-immersion objective lens. Fura-2-AM fluorescence was measured by 10 Hz alternating-wavelength time scanning, with 340 and 380 nm excitation and 510 nm emission. The fluorescence ratio at two excitation wavelengths (340/380 nm, Ca^{2+} -bound Fura-2-AM/free Fura-2-AM) was recorded and analyzed. After recording, cells were stained with IB₄-FITC conjugates (5 μ g/ml) for 15 min and washed with phosphate-buffered saline. IB₄-FITC-labeled cells were identified by use of a FITC filter at 480 nm excitation and 535 nm emission.

In Situ Hybridization and Immunohistochemistry

In situ hybridization and immunohistochemistry were performed as previously described (Lin et al. 2011). Briefly, lumbar 4 DRG tissues were frozen and sectioned in 12- μ m-thick slices. Sections were hybridized with 5-HT_{2B}-digoxigenin-UTP (dig, Roche)-labeled complementary RNA (cRNA) probes, followed by detection with an alkaline-phosphatase-conjugated anti-dig antibody (Roche). Some sections involved direct staining with IB₄-FITC conjugates (12.5 μ g/ml, Sigma). The specimens were examined by use of a $\times 20$ objective with a fluorescence microscope (Zeiss,

Axiovert 200, Germany). The digitized images were captured and the neuron size was measured by MetaMorph software.

Statistical Analysis

All data are presented as mean \pm SEM. One- or two-way analysis of variance (ANOVA) with post hoc Bonferroni test was used to compare results from multiple groups. The statistically significant levels were set at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results

5-HT-Induced Mechanical Hyperalgesia Is Regulated by the 5-HT_{2B}-Gq-PLC β -PKC ϵ Pathway

We previously found that 5-HT-induced mechanical hyperalgesia was inhibited by the 5-HT_{2B/2C} antagonist SB206553 (Lin et al. 2011). To determine whether 5-HT_{2B} or 5-HT_{2C} mediated the 5-HT-induced mechanical hyperalgesia, we administered the selective antagonist of 5-HT_{2B} (RS127445). The effect doses for RS127445 were 0.25 and 0.5 nmol (Fig. 1a). In agreement with the previous study (Lin et al. 2011), 5-HT injection into the mouse hindpaw induced mechanical hyperalgesia within 30 min, which disappeared at 4 h (Fig. 1c). Administration of RS127445 at the dose of 0.5 nmol completely inhibited mechanical hyperalgesia induced by 5-HT (Fig. 1d). It is confirmed that 5-HT_{2B} mediated 5-HT-induced mechanical hyperalgesia. 5-HT binding to 5-HT_{2B} receptors activated G_q protein, thus activating PLC β , then PKC and increased intracellular calcium concentration (Loric et al. 1995; Lin et al. 2011). To further confirm that the G_q-PLC β -PKC pathway is involved in 5-HT_{2B}-mediated mechanical hyperalgesia, we tested the doses of PKC ϵ or PKA inhibitors. PKC ϵ or PKA inhibitors were injected before 5-HT injection, and mechanical hyperalgesia was tested at 30 min after 5-HT injection. PKA inhibitor (H89) did not block 5-HT-induced hyperalgesia even in 2.5 nmol dose (Fig. 1b), but 1.25 nmol of PKA inhibitor was able to reduce CFA-induced mechanical hyperalgesia (data not shown). The dose of 1.25 nmol for PKA was used in the following experiments. PKC ϵ inhibitor (PKC ϵ I, peptide V₁₋₂) reduced 5-HT-induced hyperalgesia at the dose of 0.75 nmol and completely block hyperalgesia at 1.25 nmol. The dose of 1.25 nmol for PKC ϵ I was used in the following experiments (Fig. 1b). We then examined the effects of PKAI and PKC ϵ I at different times after 5-HT injection. With injection of 1.25 nmol PKC ϵ I before 5-HT injection, the hyperalgesia to mechanical stimuli was completely inhibited within 30 min (3.67 ± 0.33 g on the PKC ϵ I-injected ipsilateral paw and 1.42 ± 0.15 g on the 5-HT-injected ipsilateral paw, Fig. 1e) and the inhibition lasted at least 4 h. Similar experiments blocking

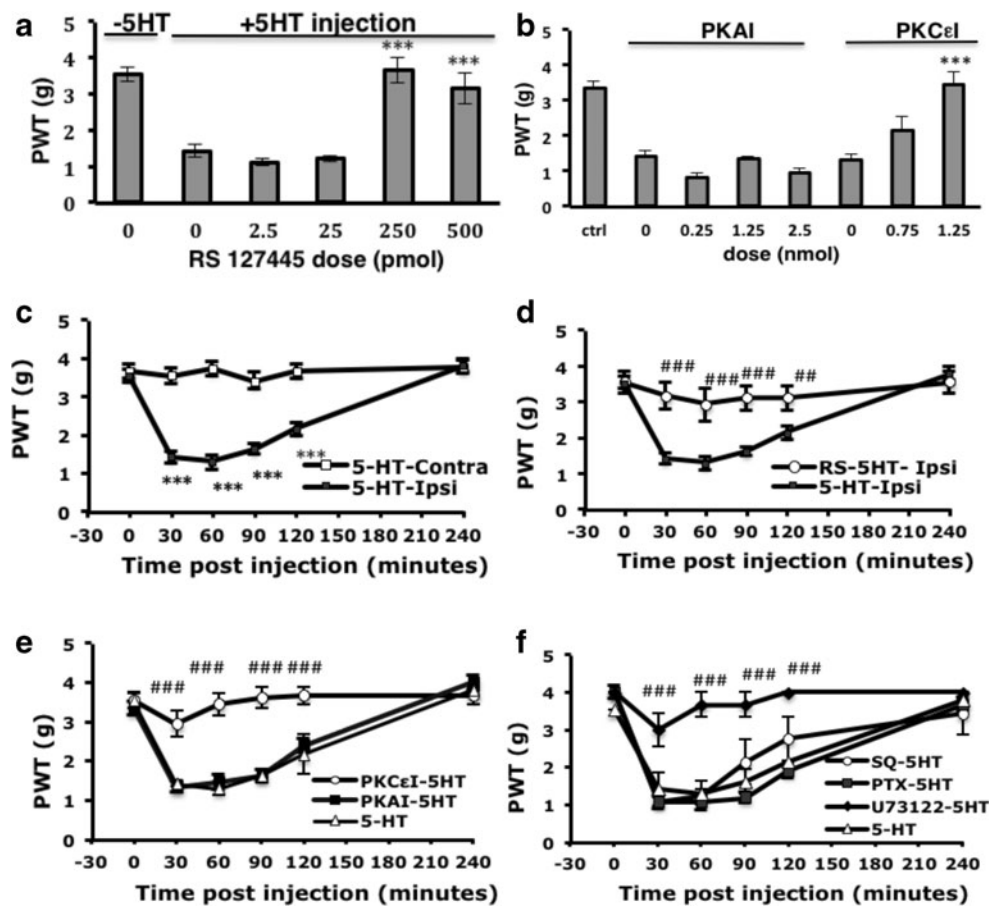


Fig. 1 5-HT_{2B} mediates 5-HT-induced mechanical hyperalgesia through phospholipase C β (*PLC* β) and protein kinase C ϵ (*PKC* ϵ). **a** Wild-type CD1 mice (8–12 weeks old) were intraplantarly injected with different doses of 5-HT_{2B} antagonist (RS127445 (*RS*)) before 5-HT injection. Mechanical tests were performed and the threshold of paw withdrawal (*PWT*) was measured at 30 min. Data are mean \pm SEM *PWT* of total tested mice ($n \geq 6$ per group). Comparisons between inhibitor-injected groups and inhibitor-uninjected groups were done by one-way ANOVA with a post hoc Bonferroni test. $***p < 0.001$. **b** Mice were intraplantarly injected with 25 μ l of different doses of PKA inhibitor (*PKAI*; H89) or *PKC* ϵ inhibitor (*PKC* ϵ I; TAT-*PKC* ϵ V₁₋₂) before 5-HT injection. The *PWT* was measured at 30 min (for *PKAI*) or 60 min (for *PKC* ϵ I) after 5-HT injection. Inhibitor-injected groups were compared with inhibitor-uninjected groups by one-way ANOVA with a post hoc Bonferroni test. $***p < 0.001$. **c** Mice were injected with 5-HT, followed by mechanical tests at different times. Ipsilateral *PWT*s were compared with contralateral *PWT*s by two-way ANOVA with a post hoc Bonferroni test.

$***p < 0.001$. **d** Mice were pre-injected with *RS* (20 μ M) before 5-HT injection, followed by mechanical tests at different times. Comparisons between the ipsilateral *PWT*s of 5-HT-injected and *RS*/5-HT-injected animals were done by two-way ANOVA with a post hoc Bonferroni test. $##p < 0.01$; $###p < 0.001$. **e** Mice were pre-injected with *PKC* ϵ I (50 μ M) or *PKAI* (50 μ M) before 5-HT injection. *PWT* for ipsilateral paw was measured at different times. The ipsilateral *PWT*s of 5-HT/inhibitor- were compared with the ipsilateral *PWT*s of 5-HT-injected animals by two-way ANOVA with a post hoc Bonferroni test. $##p < 0.01$; $***p < 0.001$; $###p < 0.001$. **f** Mice were pre-injected with AC inhibitor (*SQ*22536 (*SQ*), 1 mM), G_i inhibitor (pertussis toxin (*PTX*), 4 ng/ μ l) or *PLC* β inhibitor (*U73122*, 500 μ M) before 5-HT injection. *PWT* for ipsilateral paw was measured at different times. Comparisons between ipsilateral *PWT*s of 5-HT/inhibitor- and ipsilateral *PWT*s of 5-HT-injected animals (*number sign*), were done by two-way ANOVA with a post hoc Bonferroni test. $###p < 0.001$

PKA with H89 could not inhibit 5-HT-induced mechanical hyperalgesia (1.33 ± 0.07 g on the *PKAI*-injected ipsilateral paw Fig. 1e). We then examined the involvement of G-protein signaling in 5-HT-induced mechanical hyperalgesia. The effective doses for *SQ*22536, *U73122*, and *PTX* were first tested in CFA model (data not shown). The dose of 5.1 μ g of adenylyl cyclase (AC) inhibitor *SQ*22536, 5.8 μ g of *PLC* β inhibitor (*U73122*), or 100 ng of G_{i/o} protein inhibitor (*PTX*) was injected into mouse paw. Only *PLC* β blocker but not AC or G_{i/o} protein inhibitor could inhibit 5-HT-induced

mechanical hyperalgesia (Fig. 1f). Therefore, 5-HT-induced mechanical hyperalgesia was mediated by the 5-HT_{2B}-G_q-*PLC* β -*PKC* ϵ pathway.

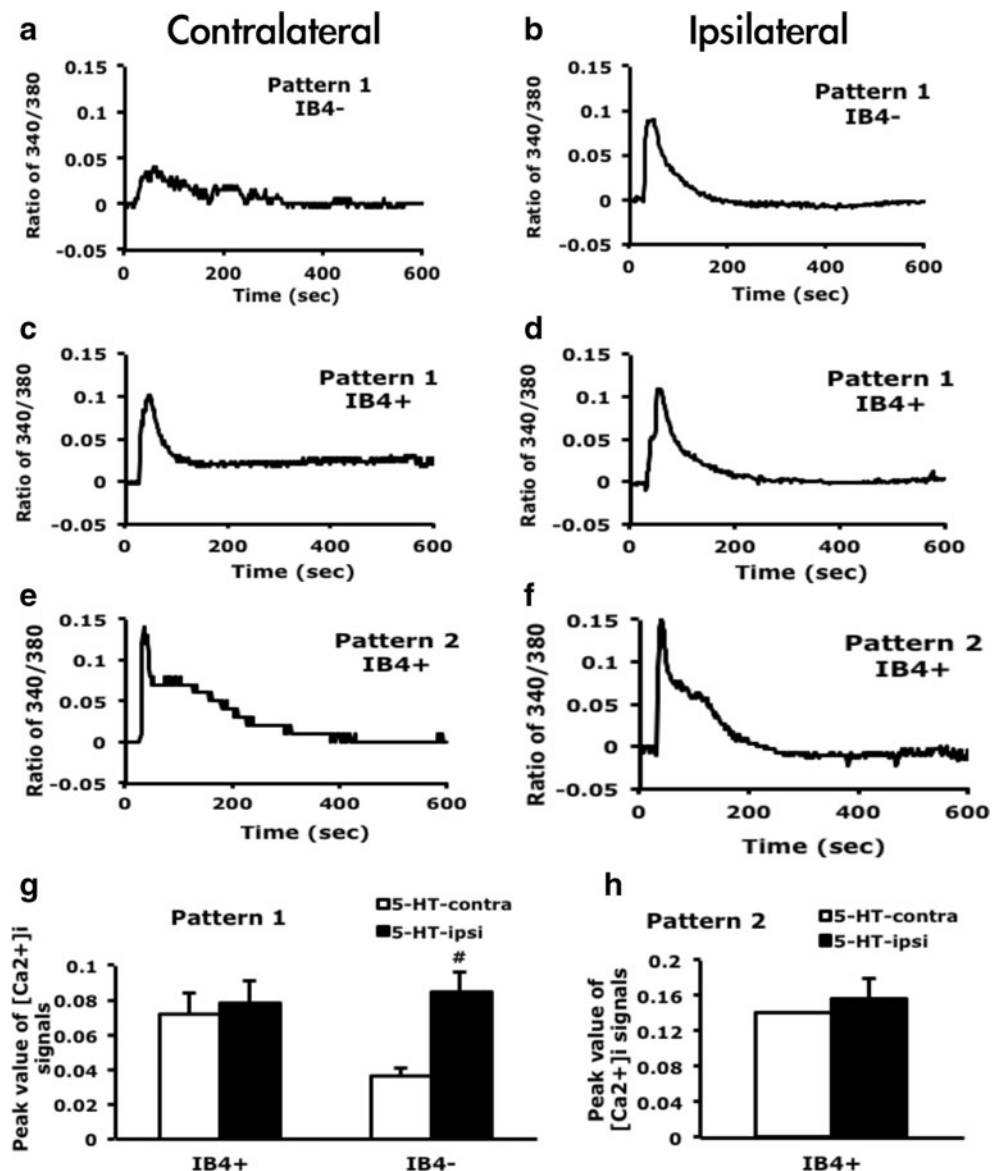
Enhanced 5-HT-Induced Calcium Signals Are Regulated by 5-HT_{2B}-*PLC* β -*PKC* ϵ Signaling

In a previous study (Lin et al. 2011), we found that 5-HT-induced calcium signals in cultured DRG neurons had two patterns: (1) transient increase and (2) transient increase and

sustained return. Only pattern 1 was significantly enhanced after 5-HT injection. However, the subsets of DRG neurons with the enhanced signals remained unknown. We cultured DRG neurons from mice after 5-HT injection and examined 5-HT-induced calcium signals. Consistent with previous findings, 5-HT-induced calcium signals had 2 patterns. Pattern 2 occurred only in IB₄-positive neurons, but pattern 1 appeared in both IB₄-positive and IB₄-negative neurons (Fig. 2a–f). The peak of pattern 2 signals did not change after 5-HT injection (Fig. 2h). After 5-HT injection, pattern 1 signals were 3-fold enhanced in IB₄-negative neurons (0.035 ± 0.021 contralateral side vs. 0.106 ± 0.022 ipsilateral side), but remained unchanged in IB₄-positive neurons (Fig. 2g). Although pattern 1 signals in IB₄-positive neurons were not enhanced, the number of 5-HT responsive neurons was increased (2.5 % contralateral side vs. 5.2 % ipsilateral side). The number of 5-HT-

responsive IB₄-negative neurons was also increased (1.7 vs. 5.9 %). 5-HT-responsive neurons were 10 to 25 μm in diameter, with most being 10 to 15 μm in diameter and increased in proportion after 5-HT injection (total responsive neurons are 5.1 % on contralateral and 13.3 % on ipsilateral sides, Fig. 3a, b). In situ hybridization revealed that neurons expressing 5-HT_{2B} were 5 to 35 μm in diameter, with most 10 to 25 μm (Fig. 3c). The size of 5-HT_{2B}-expressing neurons was correlated with the size of 5-HT-responsive neurons, which suggested that 5-HT_{2B} mediated 5-HT-induced signals in small-diameter neurons (10 to 25 μm). Immunostaining of DRG sections revealed that among the 10- to 25-μm diameter neurons, IB₄-positive immunoreaction was mainly located in neurons 15 to 25 μm in diameter (Fig. 3d). This finding was consistent with IB₄ staining of cultured DRG neurons. 5-HT-responsive neurons <15 μm were mainly IB₄ negative. 5-HT-

Fig. 2 IB₄-negative neurons show enhanced calcium signals after 5-HT injection. Lumbar 4–6 dorsal root ganglia (DRG) ipsilateral or contralateral to the 5-HT-injected mouse paw were cultured for 12 h, then stimulated with 5-HT (1 μM), and intracellular calcium change was recorded for 600 s. After recording, neurons were pulse labeled with IB₄-FITC (5 μg/ml) for 10 min. Time-dependent mean calcium increase in IB₄-negative (IB₄⁻) neurons (a, b) and IB₄-positive (IB₄⁺) neurons (c–f). The peak values were represented in the histograms (g, h). Comparisons between ipsilateral and contralateral DRG neurons of 5-HT-injected animals were done by two-way ANOVA with a post hoc Bonferroni test. #*p* < 0.05



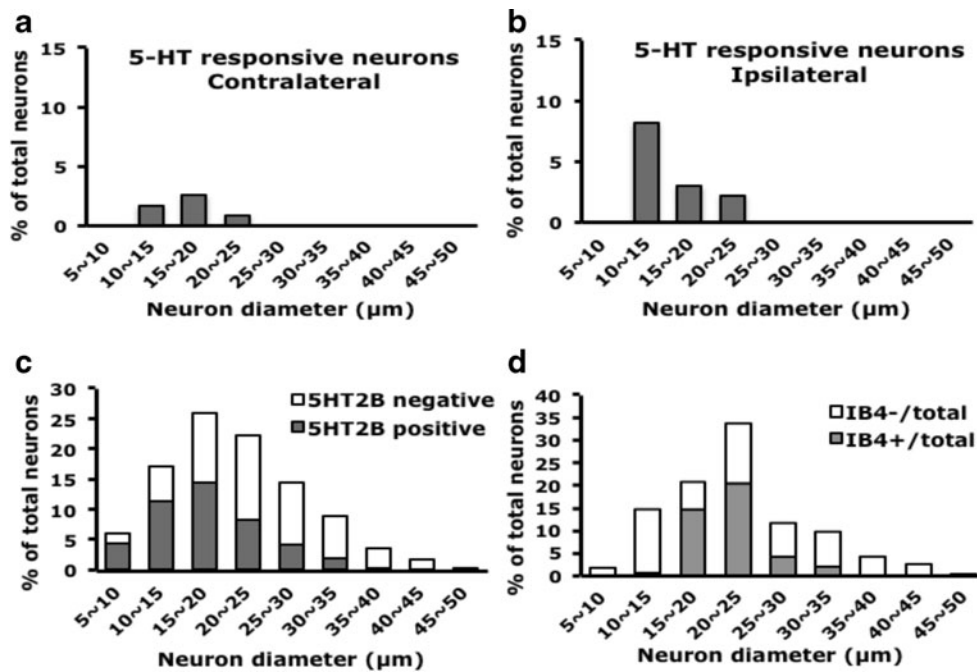


Fig. 3 5-HT-responsive neurons are small-diameter neurons. **a, b** Lumbar 4–6 DRG ipsilateral or contralateral to the 5-HT-injected mouse paw were cultured for 12 h, then stimulated with 5-HT (1 μM), and intracellular calcium change was recorded for 600 s. The diameter of neurons was measured by MetaMorph software. Histograms show proportion of 5-HT-responsive neurons to total recorded neurons. **c, d**

Lumbar DRG tissues were sectioned and hybridized with DIG-labeled 5-HT_{2B} antisense cRNA probes (**c**) or directly stained with IB₄-FITC conjugates (**d**). The diameter of neurons was measured by MetaMorph software. Histograms show proportion of **c** 5-HT_{2B}-positive neurons to total neurons and **d** IB₄-positive or IB₄-negative subpopulations to respective total neurons

enhanced calcium signals were also in IB₄-negative population (Fig. 2h).

Consistent with previous findings (Lin et al. 2011), injection of a 5-HT_{2B/2C} antagonist inhibited pattern 1 signals and the transient signals in pattern 2; pattern 1 signals were completely inhibited in both IB₄-positive and IB₄-negative neurons (Fig. 4a). The number of 5-HT-responsive neurons was also decreased (1.3 % with SB206553 and 5-HT injection vs. 13 % with 5-HT injection).

To examine whether 5-HT-induced pattern 1 calcium signals depend on PLCβ, we treated neurons with the PLCβ inhibitor, U73122. Signal pattern 1 was inhibited by the addition of U73122 in both IB₄-positive and IB₄-negative neurons (Fig. 4b), suggesting that pattern 1 calcium signals are regulated by PLCβ. We then used the PKCε blocker PKCεI to examine 5-HT-induced calcium signals. PKCεI completely inhibited 5-HT-induced calcium signals in IB₄-negative neurons, but only partially blocked calcium signals in IB₄-positive neurons (Fig. 4c).

Enhanced 5-HT-Induced Calcium Signals Are Regulated by a 5-HT_{2B}-PKCε-Dependent Pathway

In IB₄-positive neurons, 5-HT-induced calcium signaling remained unchanged in some 5-HT-responsive neurons with EGTA treatment to remove extracellular calcium (Fig. 5a, b), but was completely inhibited in some other neurons (Fig. 5c, d). In IB₄-negative neurons, 5-HT-induced signals were

sensitive to removal of extracellular calcium (Fig. 5e, f). Therefore, calcium signals from some 5-HT-responsive IB₄-positive neurons are from the endoplasmic reticulum (ER) and are directly induced by 5-HT₂ activation. However, calcium signals from other 5-HT-responsive IB₄-positive neurons and all 5-HT-responsive IB₄-negative neurons are from extracellular pools through unknown channels. It may explain that 5-HT-induced calcium signals were only partially inhibited by PKCεI in IB₄-positive neurons because 5-HT_{2B}-mediated calcium release from ER is insensitive to EGTA. In IB₄-negative neurons, 5-HT-induced calcium signals were from extracellular pools and regulated by PKCε.

One possibility for calcium influx is due to 5-HT₃. In IB₄-negative neurons, the addition of a 5-HT₃ antagonist granisetron could not inhibit 5-HT-induced calcium signals (Fig. 5g). In IB₄-positive neurons, 5-HT-induced calcium signals were inhibited by the 5-HT₃ antagonist in some neurons but remained unchanged in some neurons (Fig. 5g). The partial inhibition of granisetron in IB₄-positive neurons suggested that calcium signals were partially sensitive to EGTA.

Thus, in IB₄-positive neurons, some 5-HT-induced calcium signals are released from an internal Ca²⁺ store that is directly induced by 5-HT_{2B/2C} activation and some from extracellular influx through 5-HT₃ or other calcium channels regulated by a 5-HT_{2B/2C}-PKCε pathway. In IB₄-negative neurons, all 5-HT-induced calcium signals are from Ca²⁺ influx and are regulated by a 5-HT_{2B/2C}-PKCε pathway.

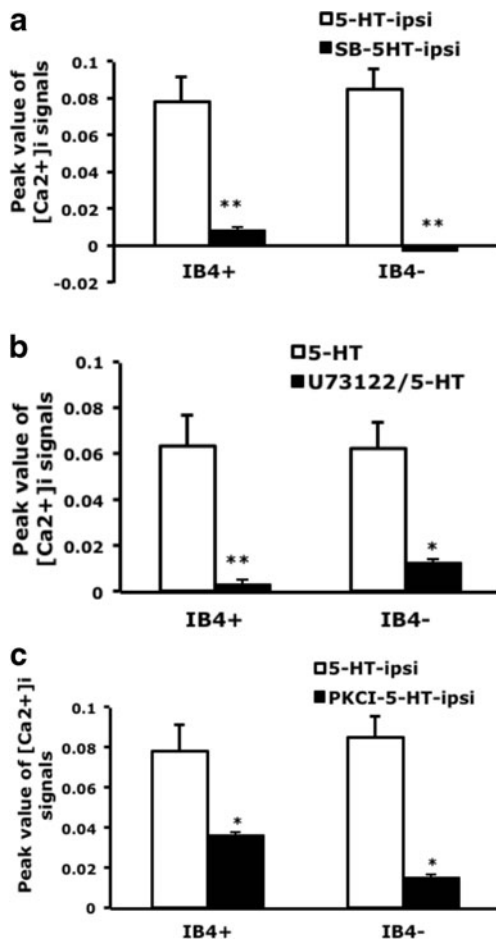


Fig. 4 Pattern 1 signals are inhibited by 5-HT_{2B/2C} PLC β and PKC ϵ inhibitors. **a** Mice were injected with 5-HT or SB206553 (10 μ M)/5-HT. At 30 min, lumbar 4–6 DRG ipsilateral to the 5-HT-injected paw and SB206553/5-HT-injected paw were taken and cultured for 12 h. Neurons were then stimulated with 5-HT (1 μ M), and intracellular calcium change was recorded for 600 s. After recording, the neurons were pulse-labeled with IB₄-FITC (5 μ g/ml) for 10 min. Data are mean \pm SEM peak values of [Ca²⁺]_i signals (\sim 35 s after the addition of 5-HT). Comparison between 5-HT-injected and SB206553-5-HT-injected neurons was done by two-way ANOVA with a post hoc Bonferroni test. $**p < 0.01$. **b** DRG neurons were pre-incubated with the PLC β inhibitor U73122 (5 μ M) for 5 min, then stimulated with 5-HT (1 μ M); calcium signals were recorded for 400 s. U73122-treated groups were compared with untreated groups by two-way ANOVA with a post hoc Bonferroni test. $*p < 0.05$; $**p < 0.01$. **c** Mice were injected with 5-HT or PKC ϵ I (50 μ M)/5-HT. Lumbar 4–6 DRG were cultured and stimulated with 5-HT (1 μ M); calcium signals were recorded for 200 seconds. Comparison between 5-HT-injected and PKC ϵ I-5-HT-injected groups was done by two-way ANOVA with a post hoc Bonferroni test. $*p < 0.05$

TRPV1 Is Involved in 5-HT-Induced Mechanical Hyperalgesia

Previous studies suggested that TRPV1 function is enhanced by 5-HT (Sugiuar et al. 2004; Ohta et al. 2006) and TRPV1 is involved in capsaicin-, acid-, or CFA-induced mechanical hyperalgesia (Gavva et al. 2005; Honore et al. 2005; Cui et al. 2006; Chen et al. 2014). Thus, we examined whether

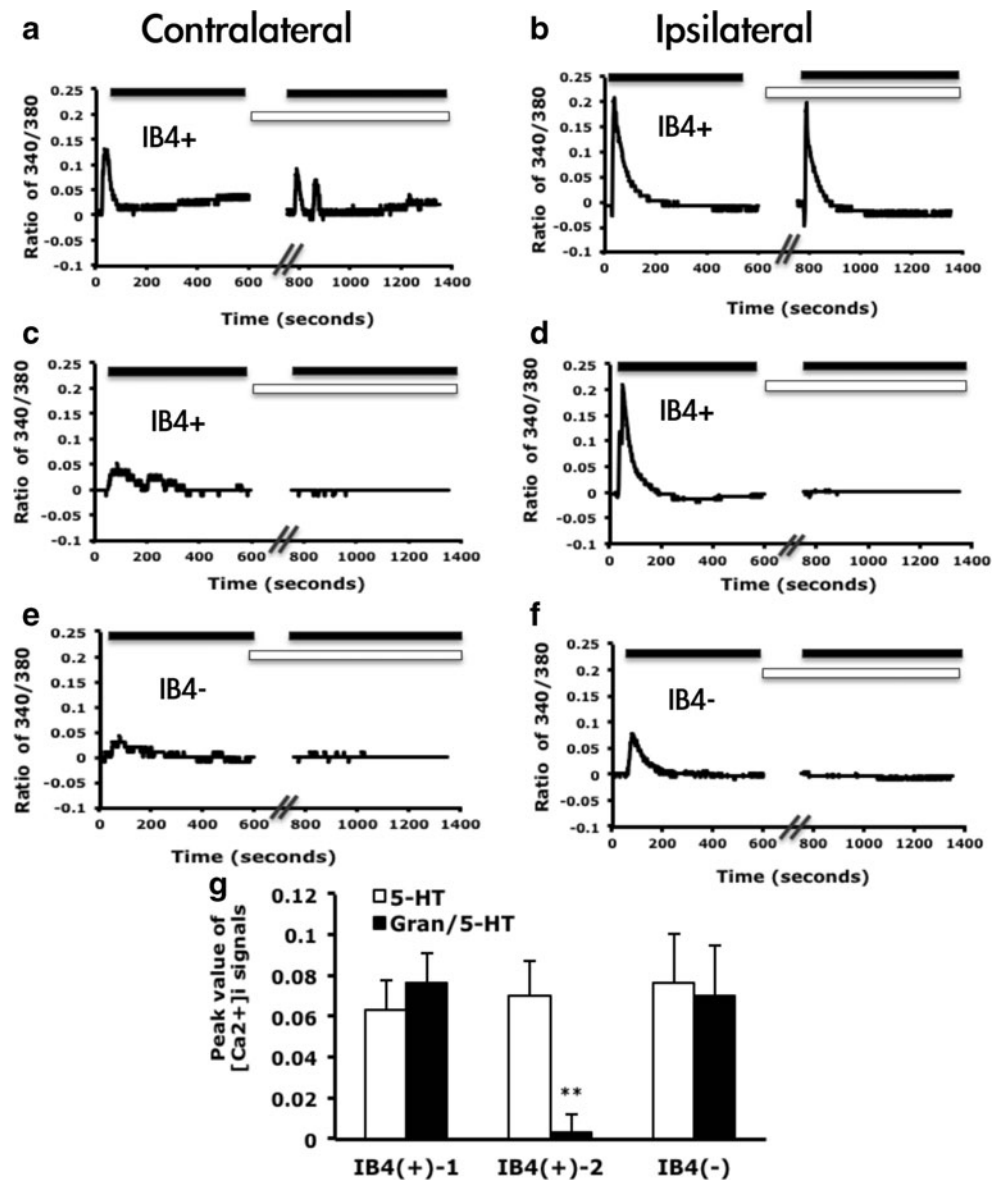
TRPV1 is involved in 5-HT-induced mechanical hyperalgesia. The administration of the TRPV1 antagonist capsazepine (0.25 nmol) before 5-HT injection completely inhibited hyperalgesia to mechanical stimuli within 30 min and lasted at least 4 h (Fig. 6a). We further validated the role of TRPV1 in 5-HT-induced mechanical hyperalgesia using TRPV1-deficient mice. 5-HT injection induced mechanical hyperalgesia in TRPV1^{+/-} and TRPV1^{+/+} but not TRPV1^{-/-} mice (Fig. 6b). The results suggested that TRPV1 participates 5-HT-induced mechanical hyperalgesia.

We next examined whether TRPV1 function is regulated by 5-HT_{2B}-mediated signaling. Capsaicin-induced calcium signals were greater in IB₄-positive than -negative neurons (0.155 ± 0.042 vs. 0.051 ± 0.09 contralateral side; Fig. 7a), which agrees with previous findings (Petruska et al. 2000, 2002; Liu et al. 2004). After 5-HT injection, capsaicin-induced calcium signals were increased in IB₄-negative neurons (0.245 ± 0.044 ipsilateral side vs. 0.051 ± 0.09 contralateral side) but not IB₄-positive neurons (Fig. 7a). Injection of 5-HT_{2B/2C} antagonist inhibited the 5-HT-enhanced capsaicin-evoked signals in IB₄-negative but not IB₄-positive neurons (Fig. 7b), which suggests that 5-HT-enhanced TRPV1 function in IB₄ negative is regulated by 5-HT_{2B/2C}. We further examined whether PKC ϵ regulates enhanced TRPV1 function. Capsaicin-evoked signals were also inhibited by PKC ϵ I in IB₄-negative neurons (Fig. 7c). Accordingly, these results suggests that 5-HT enhances TRPV1 function through the 5-HT_{2B}-PKC ϵ pathway.

Discussion

Our previous findings suggested that 5-HT_{2B/2C} antagonist inhibited 5-HT-induced mechanical hyperalgesia. In this study, we demonstrated that 5-HT_{2B}-PLC β -PKC ϵ pathway and TRPV1 function are involved in 5-HT-induced mechanical hyperalgesia because administration of antagonists for 5-HT_{2B}, PLC β , PKC ϵ , or TRPV1 inhibited 5-HT-induced mechanical hyperalgesia. In ipsilateral DRG neurons, 5-HT injection increased 5-HT or capsaicin-induced calcium signals mainly in IB₄-negative neurons, which was regulated by the 5-HT_{2B}-PLC β -PKC ϵ pathway. 5-HT injection caused unilateral hyperalgesia in injected site and facilitated 5-HT signaling in ipsilateral DRG. It is likely that plastic changes occur only in injected peripheral terminal of nociceptors and in ipsilateral DRG, resulting in peripheral sensitization, inducing ipsilateral hyperalgesia. Because no facilitated 5-HT responsiveness occurred in contralateral DRG, it is indicated that no signal was passed to the contralateral side. Thus only unilateral hyperalgesia was observed after 5-HT injection. A possible mechanism is that initial insult (5-HT) induces activation of 5-HT_{2B}-PLC β -PKC ϵ in the peripheral terminal of the nociceptor. Activation of this pathway could relieve TRPV1 from

Fig. 5 5-HT-induced calcium signals have different sensitivities to EGTA. Lumbar 4–6 DRG ipsilateral or contralateral to the 5-HT-injected mouse paw were cultured for 12 h, then stimulated with 5-HT (1 μ M), and intracellular calcium change was recorded for 600 s. After a washing, neurons were pre-incubated with EGTA (2 mM) for 10 min (a–f) or with 5-HT₃ antagonist (Granisetron (*Gran*), 1 μ M, g) for 5 min, then stimulated with 5-HT (1 μ M); calcium signals were recorded for 1400 or 400 s, respectively. Neurons were then pulse labeled with IB₄-FITC (5 μ g/ml) for 10 min. Time-dependent mean calcium increase in IB₄-positive (a–d) and IB₄-negative neurons (e–f). *White bars* indicate the presence of EGTA, *black bars* indicate the time for addition of 5-HT (1 μ M). **g** Granisetron-treated responses were divided into two types: unchanged (type 1) and decreased (type 2). Comparison between 5-HT treatment alone and Granisetron/5-HT-treated groups was done by one-way ANOVA with a post hoc Bonferroni test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$



PIP₂ inhibition (Chuang et al. 2001) to produce peripheral sensitization that increases the number of neurons responding to 5-HT and facilitates 5-HT or capsaicin-induced signaling in IB₄-negative neurons (Fig. 8). Thus, 5-HT-induced mechanical hyperalgesia may be mainly mediated by 5-HT_{2B}-G_q-PLC β -PKC ϵ signaling by regulating TRPV1 function.

We previously suggested that 5-HT_{2B} mediates 5-HT-induced mechanical hyperalgesia using 5-HT_{2B/2C} antagonist, SB206553 (Lin et al. 2011). Interestingly, Urtikova et al. (2012) found antinociceptive effects of 5-HT_{2B} on neuropathic pain in rats, using 5-HT_{2B} selective antagonist RS127445. We used RS127445 in our mouse model but found that 0.25 and 0.5 nmol RS127445 blocked 5-HT-induced mechanical hyperalgesia. These results confirmed our previous suggestion that 5-HT_{2B} plays a pro-nociceptive role to mediate 5-HT-

induced mechanical hyperalgesia, although 5-HT_{2C} involvement cannot be completely ignored given that a number of studies have shown that 5-HT_{2C} receptor has pronociceptive or antinociceptive effects (Jeong et al. 2004; Nakajima et al. 2009; Nakai et al. 2010). Previous findings suggested that PLC β , G_{q/11}, G_i, PKC ϵ are involved in carrageenan- or PGE₂-induced mechanical hyperalgesia (Khasar et al. 1999; Joseph et al. 2007; Dina et al. 2009; Tappe-Theodor et al. 2012). Blocking PLC β or PKC ϵ but not G_i or AC specifically inhibited 5-HT-induced mechanical hyperalgesia, which further indicates that the G_q-PLC β -PKC ϵ pathway is involved in 5-HT-induced mechanical hyperalgesia. Activation of 5-HT_{2B} induces G_q-PLC β -PKC ϵ signaling (Loric et al. 1995; Lin et al. 2011). The dependency of 5-HT-induced mechanical hyperalgesia on the

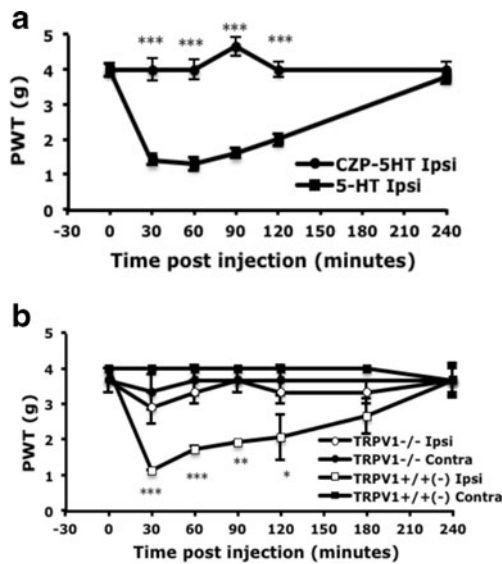


Fig. 6 5-HT-induced mechanical hyperalgesia is inhibited by peripheral injection of TRPV1 antagonist or in mice lacking TRPV1. **a** Wild-type CD1 mice (8–12 weeks old) were pre-injected with or without 25 μ l TRPV1 antagonist (capsazepine (CZP), 10 μ M), then with 25 μ l 5-HT (10 μ M). **b** TRPV1^{+/+}, TRPV1^{+/-}, and TRPV1^{-/-} mice (8–16 weeks old) were injected with 25 μ l 5-HT (10 μ M). The threshold of paw withdrawal (PWT) was measured before ($t=0$) and after injection. Data are mean \pm SEM PWT of total tested mice ($n=6$ per group). Data for TRPV1^{+/+} and TRPV1^{+/-} mice are grouped together. Comparison between 5-HT-injected and CZP-5HT-injected animals or between ipsilateral side of TRPV1^{+/+} and TRPV1^{-/-} animals was done by two-way ANOVA with a post hoc Bonferroni test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

G $_q$ -PLC β -PKC ϵ pathway gives further evidence of the involvement of 5-HT $_{2B}$. Clearly, 5-HT $_{2B}$ has pro-nociceptive rather than antinociceptive effects in 5-HT-induced mechanical hyperalgesia. The antinociceptive roles of 5-HT $_{2B}$ on neuropathic pain may be influenced by other inflammatory mediators or immune cells as suggested previously (Urtikova et al. 2012).

Administration of 5-HT into mice increased the number of DRG neurons responding to 5-HT (Fig. 3) in both IB $_4$ -positive and IB $_4$ -negative neurons, suggesting that both types of neurons are involved in 5-HT-induced pain. However, 5-HT-evoked transient calcium signals (pattern 1) were enhanced in only IB $_4$ -negative neurons. 5-HT-evoked calcium signals were greater in IB $_4$ -positive than -negative neurons before 5-HT injection, which may explain the enhanced calcium signals found only in IB $_4$ -negative neurons. IB $_4$ -positive and IB $_4$ -negative neurons may play distinct roles in 5-HT-induced pain. Injection of 5-HT $_{2B/2C}$ antagonist inhibited pattern 1 transient signals in both types of neurons. Given that the 5-HT $_{2B/2C}$ antagonist blocks only 5-HT-induced mechanical hyperalgesia (Lin et al., 2011), pattern 1 calcium signals inhibited by 5-HT $_{2B/2C}$ antagonist may be involved in 5-HT-induced mechanical hyperalgesia. Accordingly, both IB $_4$ -positive and IB $_4$ -negative neurons are involved, although pattern 1 signals in IB $_4$ -positive neurons were not enhanced. Stucky

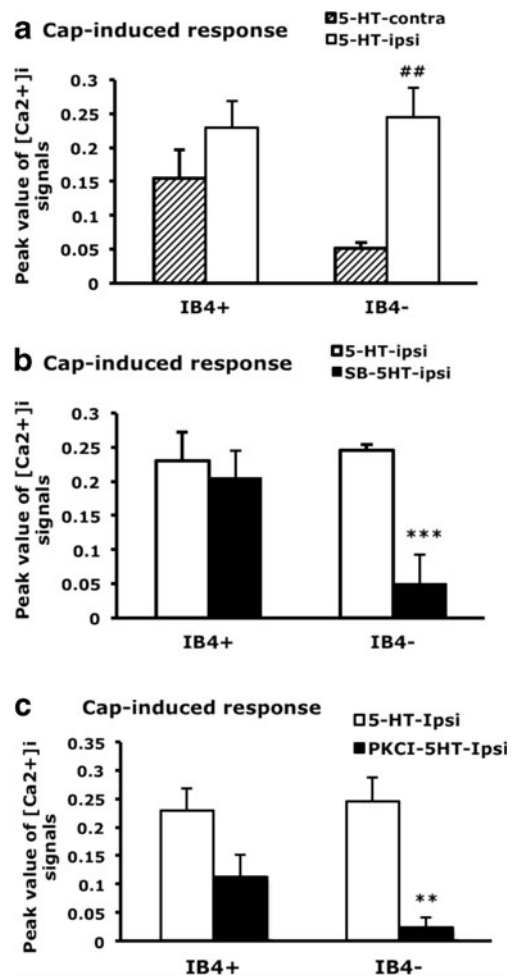


Fig. 7 Capsaicin-induced calcium influx is enhanced by 5-HT and inhibited by 5-HT $_{2B/2C}$ antagonist and PKC ϵ blocker. Lumbar 4–6 DRG ipsilateral or contralateral to the 5-HT-injected, SB206553-5-HT, or PKC ϵ I-5-HT-injected mouse paw were cultured for 12 h, then stimulated with capsaicin (100 nM), and intracellular calcium change was recorded for 600 s. After recording, neurons were pulse labeled with IB $_4$ -FITC (5 μ g/ml) for 10 min. Peak values of [Ca $^{2+}$] $_i$ signals (approximately 35 s after the addition of agonists) represent data points as indicated. Comparisons between ipsilateral and contralateral sides of 5-HT-injected animals (number sign), between ipsilateral side of 5-HT-injected and SB206553-5-HT-injected animals (asterisk) or between ipsilateral side of 5-HT-injected and PKC ϵ I-5-HT-injected animals (asterisk) were done by two-way ANOVA with a post hoc Bonferroni test. ## $p < 0.01$; * $p < 0.01$; *** $p < 0.001$

and Lewin (1999) suggested that IB $_4$ -negative neurons have lower action potential (AP) threshold and shorter AP duration than IB $_4$ -positive neurons: IB $_4$ -negative neurons could be essential in transducing information about stimuli, but IB $_4$ -positive neurons could be more important for transmission of information at the first central synapse. 5-HT-induced mechanical hyperalgesia is not a chronic effect (lasts 2 h). Therefore, 5-HT injection greatly enhanced calcium signals in IB $_4$ -negative neurons, which may be responsive for transducing 5-HT stimuli, rather than in IB $_4$ -positive neurons, which may be important in synaptic transmission. Both types

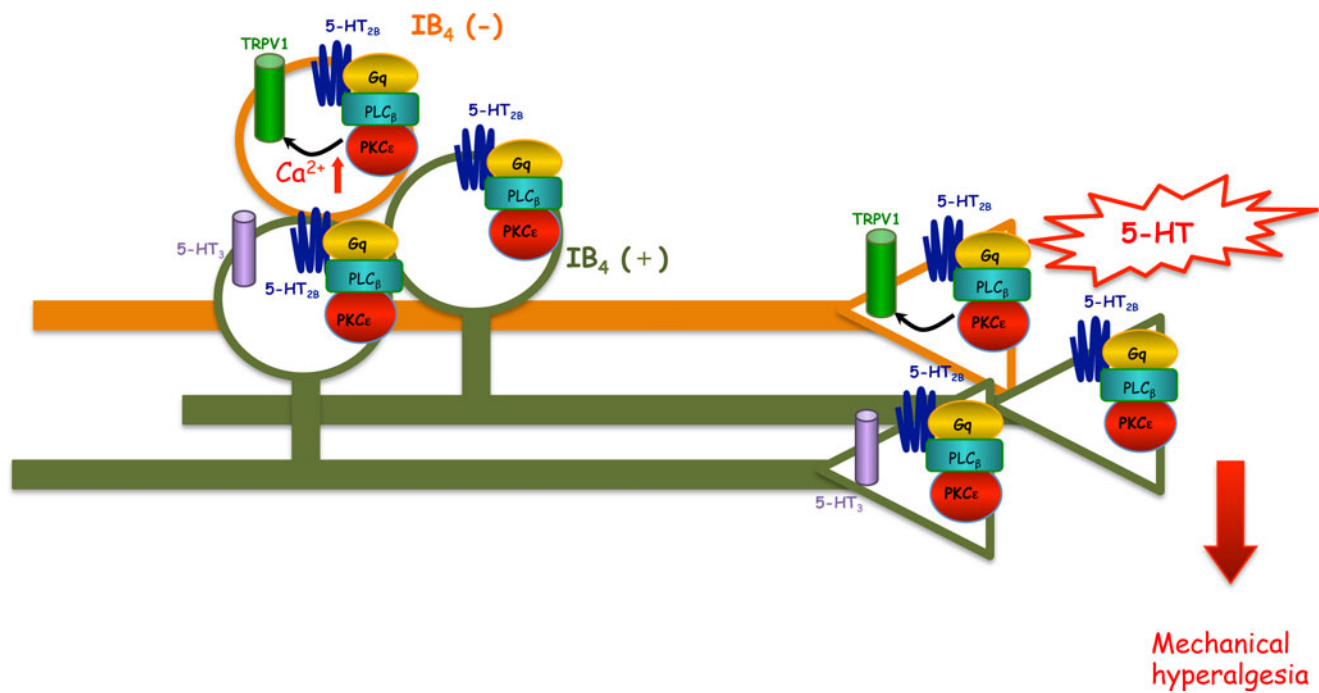


Fig. 8 Putative model of 5-HT-induced mechanical hyperalgesia through 5-HT_{2B}-mediated pathways. In the periphery of IB₄-negative neurons, 5-HT_{2B} responds to 5-HT to activate G_q protein and PLCβ, thus activating PKCε. PKCε activation enhances TRPV1 function, thereby leading to mechanical hyperalgesia. In the cell body of IB₄-negative neurons, peripheral 5-HT stimulation increases the number of 5-HT-responsive neurons and 5-HT-evoked calcium signals. Enhanced calcium signals

could be due to regulation of TRPV1 by 5-HT_{2B}-PLCβ-PKCε pathway. In the periphery of IB₄-positive neurons, 5-HT_{2B} responds to 5-HT to activate G_q protein, PLCβ, and PKCε, thereby leading to mechanical hyperalgesia. In the cell body of IB₄-positive neurons, peripheral 5-HT stimulation increases the number of 5-HT-responsive neurons

of neurons participate in mechanical hyperalgesia, so the signals can be inhibited by 5-HT_{2B/2C} antagonist.

In IB₄-negative neurons, inhibition of 5-HT_{2B}, PKCε and PLCβ completely blocked 5-HT-induced pattern 1 calcium signals, so pattern 1 signals may be regulated by the 5-HT_{2B}-PLCβ-PKCε pathway. Pattern 1 calcium increase was also sensitive to the addition of EGTA, which indicates that the increase was due to calcium influx through calcium channels. However, the calcium influx was not through 5-HT₃ receptor because 5-HT₃ receptor antagonist, granisetron, did not block the calcium influx. 5-HT-induced calcium influx may occur through other calcium channels in IB₄-negative neurons. In contrast, IB₄-positive neurons have more complicated responses. 5-HT-evoked calcium signals were completely inhibited by 5-HT_{2B/2C} antagonist and PLCβ, but partially inhibited by PKCεI, suggesting that calcium signals were partially regulated by 5-HT_{2B}-PLCβ-PKCε pathways. Since calcium signals in some IB₄-positive neurons were sensitive to EGTA, it indicated that the part of calcium signals was from calcium influx. Calcium signals unregulated by PKCε could directly result from 5-HT_{2B/2C} activation that leads to calcium release from ER store, which are insensitive to EGTA. Calcium signals regulated by PKCε could be from voltage-gated calcium channels or other calcium channels. Interestingly, 5-HT₃ antagonist specifically inhibited calcium

signals of some IB₄-positive neurons. Thus, calcium influx regulated by 5-HT_{2B}-PLCβ-PKCε pathway is more likely from 5-HT₃ channel. 5-HT₃ was previously found not involved in 5-HT-induced mechanical hyperalgesia (Lin et al. 2011) but is involved in 5-HT-induced thermal hyperalgesia (Lloyd et al. 2012). Although 5-HT₃ antagonist cannot completely block 5-HT-induced mechanical hyperalgesia, it shortens the hyperalgesia response (Lin et al. 2011), which agrees with our finding that pattern 1 signals in some IB₄-positive neurons were mediated by 5-HT₃ because such neurons are important in synaptic transmission (Stucky and Lewin 1999). 5-HT₃ could be involved in maintaining mechanical hyperalgesia, which is also regulated by 5-HT_{2B}-PLCβ-PKCε pathway.

Serotonin has been shown to enhance TRPV1 functions (Sugiuar et al. 2004; Ohta et al. 2006). As expected, we found that 5-HT injection enhanced capsaicin-evoked calcium signals but only in IB₄-negative not IB₄-positive neurons. Interestingly, capsaicin-evoked calcium signals were greater in IB₄-positive than IB₄-negative neurons before 5-HT injection, which is consistent with the study in rats (Liu et al. 2004) but not mice (Dirajlal et al. 2003; Breese et al. 2005). The difference could be due to the outbred mice we used. The increased capsaicin-evoked calcium signals in IB₄-negative neurons explain the effect of 5-HT injection only in IB₄-

negative neurons. The capsaicin-evoked signals in IB₄-negative neurons were greatly inhibited by 5-HT_{2B/2C} antagonism and PKC ϵ blockage, which suggests that 5-HT-enhanced capsaicin-evoked calcium signals were regulated by the 5-HT_{2B/2C}-PKC ϵ pathway. Capsaicin-sensitive IB₄-negative neurons have lower mechanical threshold and larger mechanical evoked currents than capsaicin-sensitive IB₄-positive neurons (Drew et al. 2002). Therefore, 5-HT-enhanced capsaicin-evoked calcium signals in IB₄-negative neurons may be important in 5-HT-induced mechanical hyperalgesia. Administration of a TRPV1 antagonist before 5-HT injection in mice specifically inhibited 5-HT-induced mechanical hyperalgesia. Mice lacking the TRPV1 gene did not show mechanical hyperalgesia in response to 5-HT stimuli, which suggests that TRPV1 is involved in 5-HT-induced mechanical hyperalgesia. TRPV1 involvement in mechanical hyperalgesia is surprising but not impossible. Several lines of evidence have proposed that TRPV1 antagonists inhibit capsaicin-, acid-, or CFA-induced mechanical hyperalgesia (Gavva et al. 2005; Honore et al. 2005; Cui et al. 2006; Chen et al. 2014). Kim et al. (2012) proposed that spinal TRPV1 plays critical roles in mediating neuropathic mechanical allodynia. Activation of spinal TRPV1 could be due to G_{q/11}-coupled receptors or arachidonic acid (AA) metabolites (Gibson et al. 2008; Kim et al. 2009; Kim et al. 2012). Given that 5-HT₂ receptor activation can activate phospholipase A2, thus leading to AA release (Tournois et al. 1998), peripheral 5-HT_{2B} activation by 5-HT may induce AA release to activate peripheral TRPV1, thus affecting mechanical hyperalgesia.

Although calcium signals regulated by 5-HT_{2B} are critical for 5-HT-induced mechanical hyperalgesia, sodium currents may also have important roles in mechanical hyperalgesia. Several lines of evidence have suggested that 5-HT increases tetrodotoxin-resistant (TTX-R) I_{Na} currents (Gold et al. 1996). Na_v1.8 (a TTX-R channels) is related to inflammatory hyperalgesia (Abrahamsen et al. 2008; Yen et al. 2009), and PKC can modulate TTX-R I_{Na} currents (Gold et al. 1998; Cang et al. 2009). Therefore, 5-HT_{2B}-G_q-PLC β -PKC ϵ signaling could regulate voltage-gated Na⁺ channels to affect mechanical hyperalgesia.

In conclusion, our study demonstrates that 5-HT_{2B}-G_q-PLC β -PKC ϵ signaling and TRPV1 function are involved in 5-HT-induced mechanical hyperalgesia. Peripheral 5-HT stimulation increased the responsiveness of DRG neurons to 5-HT. The number of both IB₄-positive and IB₄-negative neurons responding to 5-HT were increased. 5-HT or capsaicin-evoked calcium signals were increased in IB₄-negative neurons by 5-HT_{2B}-G_q-PLC β -PKC ϵ pathway. Accordingly, 5-HT-induced mechanical hyperalgesia may be mainly mediated by 5-HT_{2B}-G_q-PLC β -PKC ϵ signaling by regulating TRPV1 function.

Acknowledgments This work was supported by funds from the National Science Council, Taiwan (grant no. NSC 99-2320-B-008-001-

MY3, NSC 102-2321-B-008-001, and NSC102-2320-B-001-021-MY3). We thank KAI pharmaceuticals, Inc., USA for providing PKC ϵ -inhibited peptide.

Authors' Roles YSS participated in the study design, performed the experimental procedures, and was primary author of the manuscript. YYC performed the animal experiments. SYL performed the primary cultures and calcium imaging experiments. CCC generated TRPV1^{-/-} knockout mice and participated data interpretation and manuscript writing. WHS conceived the study and participated in its design, coordination, and data interpretation and contributed to writing the manuscript.

Conflict of Interest The authors declare no competing financial interests.

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