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# **The TRPM8 channel forms a complex with the 5-HT<sub>1B</sub> receptor and phospholipase D that amplifies its reversal of pain hypersensitivity**

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## ABSTRACT

Effective relief from chronic hypersensitive pain states remains an unmet need. Here we report the discovery that the TRPM8 ion channel, co-operating with the 5-HT<sub>1B</sub> receptor (5-HT<sub>1B</sub>R) in a subset of sensory afferents, exerts an influence at the spinal cord level to suppress central hypersensitivity in pain processing throughout the central nervous system. Using cell line models, *ex vivo* rat neural tissue and *in vivo* pain models, we assessed functional Ca<sup>2+</sup> fluorometric responses, protein:protein interactions, immuno-localisation and reflex pain behaviours, with pharmacological and molecular interventions. We report 5-HT<sub>1B</sub>R expression in many TRPM8-containing afferents and direct interaction of these proteins in a novel multi-protein signalling complex, which includes phospholipase D1 (PLD1). We provide evidence that the 5-HT<sub>1B</sub>R activates PLD1 to subsequently activate PIP 5-kinase and generate PIP<sub>2</sub>, an allosteric enhancer of TRPM8, achieving a several-fold increase in potency of TRPM8 activation. The enhanced activation responses of synaptoneurosomes prepared from spinal cord and cortical regions of animals with a chronic inflammatory pain state are inhibited by TRPM8 activators that were applied *in vivo* topically to the skin, an effect potentiated by co-administered 5-HT<sub>1B</sub>R agonists and attenuated by 5-HT<sub>1B</sub>R antagonists, while 5-HT<sub>1B</sub>R agents alone had no detectable effect. Corresponding results are seen when assessing reflex behaviours in inflammatory and neuropathic pain models. Control experiments with alternative receptor/TRP channel combinations reveal no such synergy. Identification of this novel receptor/effector/channel complex and its impact on nociceptive processing give new insights into possible strategies for enhanced analgesia in chronic pain.

### *Keywords:*

Receptor:channel complex

Signalling

Pain

Analgesia

TRPM8

5-HT

## 1. Introduction

When chronic pain becomes established, its treatment is problematic because of the lasting molecular and functional changes that occur within the nervous system. One promising new target for chronic pain relief is the TRPM8 cation-permeable ion channel, which is expressed in a subset of primary afferent sensory neurons. TRPM8 is activated by cooling below 23°C (McKemy et al., 2002; Peier et al., 2002) and there is strong evidence for its physiological role as a cool sensor (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007; Knowlton et al., 2013). Matching an empirical understanding held since classical Greek times, activation of TRPM8 by mild cooling or the selective chemical activators icilin and menthol produces robust analgesia in chronic hypersensitive pain models (Dhaka et al., 2007; Knowlton et al., 2013; Liu et al., 2013; Proudfoot et al., 2006; Su et al., 2011) and may even attenuate some basal responses to noxious stimuli (Harrington et al., 2011; Klein et al., 2010). Correspondingly, TRPM8 activation can provide analgesia in a clinical setting (Colvin et al., 2008; Storey et al., 2010). Topical application of low concentrations of TRPM8-activating agents is an effective route for producing analgesia in both laboratory and clinical settings (Colvin et al., 2008; Proudfoot et al., 2006; Storey et al., 2010), consistent with evidence for a concentration of TRPM8-expressing nerve terminals in epidermis and around the epidermal/dermal border (Dhaka et al., 2008; Takashima et al., 2007). It appears that TRPM8-expressing afferents act centrally in the spinal cord to suppress inputs from the much larger population of nociceptive afferents (Proudfoot et al., 2006; Su et al., 2011; Zheng et al., 2010). The mechanism of interaction may involve inhibitory metabotropic glutamate receptors and GABAergic inhibitory interneurons in superficial dorsal horn (Proudfoot et al., 2006; Zheng et al., 2010), although an unidentified peripheral component may also contribute (Harrington et al., 2011). TRPM8 could play a part in the cool allodynia associated with chronic sensitised pain states (Su et al., 2011; Xing et al., 2007), which might be anticipated from its role as the primary sensor of cool temperatures, rather than implying any direct role in processes of hypersensitivity. Knockout or ablation of TRPM8 however, only partially abrogates cool allodynia (Knowlton et al., 2013). It has also been suggested that TRPM8 can contribute to noxious cold responses and peripheral administration of TRPM8 activators at high concentrations can induce hyperalgesia (Dhaka et al., 2007; Gentry et al., 2010; Klein et al., 2010; Knowlton et al., 2010; Proudfoot et al., 2006; Wasner et al., 2004). In trigeminal and dorsal root ganglia TRPM8 is selectively expressed in a small discrete subpopulation of C- and A $\delta$ -afferents, which express the Nerve Growth Factor receptor TrkA but are generally distinct from the much larger populations expressing the nociceptor markers TRPV1, TRPA1, CGRP or lectin IB4-binding sites (Dhaka et al., 2008; Kobayashi et al., 2005; McKemy et al., 2002; Peier et al., 2002; Pogorzala et al., 2013; Proudfoot et al., 2006; Takashima et al., 2007). Only a minority of somatosensory TRPM8 afferents co-express TRPV1 and these may correspond to cells with low TRPM8:Kv1 channel ratios and tetrodotoxin-resistant Na<sup>+</sup> channels that respond only to more severely cold (noxious) temperatures (Madrid et al., 2009; Sarria et al.,

2012). Elements of any noxious effects observed with high concentrations of TRPM8 activators may involve such high-threshold afferents or off-target effects on other channels. In some visceral afferents, TRPM8/TRPV1 co-existence may be more common, where TRPM8 has been suggested to act peripherally to modify nociceptive responses through an unknown mechanism (Harrington et al., 2011; Hayashi et al., 2009).

As TRPM8 activator-induced analgesia becomes more apparent in hypersensitive pain states (Proudfoot et al., 2006), we hypothesised that the TRPM8 channel may be potentiated by an endogenous factor released in chronic pain conditions. Such mediators include 5-HT, which as well as being released peripherally at sites of injury can be released from bulbospinal pathways into the spinal dorsal horn where it could potentially influence the central terminals of afferents (el-Yassir and Fleetwood-Walker, 1990; Khalil and Helme, 1990; Shu and Mendell, 1999). A variety of 5-HT receptor subtypes is expressed in dorsal root ganglia (DRG) (Chen et al., 1998; Nicholson et al., 2003; Pierce et al., 1997). One candidate with potentially appropriate characteristics is the 5-HT<sub>1B</sub> receptor (5-HT<sub>1B</sub>R), which is reported to exert an antinociceptive influence (Alhaider and Wilcox, 1993; el-Yassir et al., 1988; Granados-Soto et al., 2010; Kayser et al., 2007). Furthermore, the 5-HT<sub>1B</sub>R is associated with TrkA-positive A $\delta$ -afferents in trigeminal ganglia (Wotherspoon and Priestley, 2000), (which could in part correspond to TRPM8-containing afferents) and is more widely expressed in DRG (Classey et al., 2010), where TRPM8 expression is increased in both A $\delta$  and C-type afferents by neuropathic pain (Proudfoot et al., 2006). We therefore looked here for any functional or molecular evidence in support of a facilitatory interaction between the 5-HT<sub>1B</sub>R and TRPM8.

## **2. Materials and methods**

### *2.1. In vivo studies – pain models and drug administration*

All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines and had received approval from the University of Edinburgh ethical committee. Male Wistar rats (Harlan, UK), weight between 180-250 g, were used in all experiments and were given access to food and water *ad libitum* and housed in accordance with Home Office guidelines. Intraplantar injection of Complete Freund's Adjuvant (CFA) was used to model chronic inflammatory pain (Taurog et al., 1988). Following brief anaesthesia with a 4% isoflurane/oxygen mixture, the plantar surface of the right hindpaw was sterilised with 0.05% Hibitane (Zeneca, UK), before 150  $\mu$ l of 50% CFA (Sigma; 1 mg *Mycobacterium tuberculosis* suspension in 0.85 ml mineral oil and 0.15 ml mannide mono-oleate) in 0.9% saline was injected between the toes and towards the middle of the paw, avoiding the ankle. Ipsilateral thermal reflex hypersensitivity developed within 24 h and remained substantial for up to 2 weeks. The sciatic nerve chronic constriction injury (CCI) preparation was used as a model of chronic neuropathic pain (Bennett and Xie, 1988). Animals were anaesthetised with a 4% isoflurane/oxygen mixture (Zeneca, Cheshire, UK) before exposure of the sciatic nerve, proximal to the trifurcation at mid-thigh level. Four loose ligatures of chromic catgut (SMI

AG, Hunningen, Belgium) were tied around the nerve, separated by 1 mm. CCI rats consistently developed ipsilateral thermal reflex hypersensitivity, which peaked between days 9 and 16 post-surgery, when drug testing was carried out. For quantitative sensory testing, animals were initially habituated to the tests on a minimum of 3 occasions in order to establish consistent responses. Sensitivity to a noxious thermal stimulus was assessed by measuring paw withdrawal latency (in s) elicited by an infrared beam (55°C, Hargreaves' thermal stimulator, Linton Instrumentation, Diss, UK) directed to the mid-plantar glabrous skin of the hind paw. Withdrawal responses were only noted when they indicated clear nocifensive flicking behaviour and their latencies were recorded to the nearest 0.1 s. A maximum cut-off latency of 20 s and a minimum of 5 min recovery time between tests were established in order to prevent tissue damage. Prior to drug application, at least 3 pre-drug tests were performed to produce a mean baseline value. Testing commenced 10-15 min after drug administration under anaesthesia in order to allow full functional recovery and tests were then repeated every 10 min for up to 60 min. For intrathecal application, drugs were injected in a volume of 50 µl by percutaneous puncture into the L5/6 intervertebral space of rats anaesthetised with 3% isoflurane/oxygen (Proudfoot et al., 2006). The vehicle for drug injections was saline with up to 0.5% dimethylformamide and had no effect alone on behavioural reflex responses. For topical administration, drugs were dissolved in either 1% aqueous dimethylformamide or propan 1,2-diol (Thermo-Fisher) containing 1.5% caprolactam (Brilliance Biochemical Co Ltd, Beijing, China), a vehicle that facilitates skin penetration of hydrophilic compounds (Williams, 2003), and applied topically to cover both hindpaws for 15 min prior to tissue removal, in rats anaesthetised with 3% isoflurane/O<sub>2</sub>. The vehicles had no discernible effect alone. The following drugs were applied in appropriate combinations: the selective TRPM8 agonists, icilin, 3,4-dihydro-3-(2-hydroxyphenyl)-6-(3-nitrophenyl)-(1H)-pyrimidin-2-one (Tocris Bioscience, Bristol, UK) and l-menthol (Sigma); the selective 5-HT<sub>1B</sub>R agonist CP 94253, 5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-pyrrolo[3,2-b]pyridine hydrochloride (Tocris); the selective 5-HT<sub>1B/D</sub>R agonist, sumatriptan, 1-{3-[2-(dimethylamino)ethyl]-1H-indol-5-yl}-N-methylmethanesulfonamide succinate (Axon MedChem, Groningen, The Netherlands); the selective TRPM8 antagonist AMTB hydrochloride, *N*-(3-aminopropyl)-2-[(3-methylphenyl)methoxy]-*N*-(2-thienylmethyl)benzamide hydrochloride (Tocris); the selective 5-HT<sub>1B</sub>R antagonists, SB 224289 hydrochloride, 1'-methyl-5-[[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7 tetrahydrospiro[furo [2,3-f]indole-3,4'-piperidine hydrochloride and GR 55562 hydrochloride, 3-[3-(dimethylamino)propyl]-4-hydroxy-*N*-[4-(pyridinyl)phenyl]benzamide hydrochloride (Tocris); the selective 5-HT<sub>1D</sub>R antagonist, BRL 15572 hydrochloride, 3-[4-(4-chlorophenyl)piperazin-1-yl]-1,1-diphenyl-2-propanol hydrochloride (Tocris). The concentrations of topically applied drugs that are reached adjacent to the TRPM8-containing nerve terminals in the epidermis or around the dermal/epidermal boundary are not known but are likely to be greatly reduced compared to those delivered to the skin surface.

## 2.2. *In vivo* studies – acute behavioural effects of TRPM8 activators

To evaluate whether alternative acute responses to administration of TRPM8 activators were modulated by 5-HT<sub>1B</sub>R agents, we first quantified “wet dog shakes” induced by intraperitoneal injection of icilin (Wei, 1981; Werkheiser et al., 2009). Rats were briefly anaesthetised with 3% isoflurane/oxygen and injected at 1 ml/kg with icilin (0.5 mg/kg; 1.6 mM solution), icilin (0.5 mg/kg) plus sumatriptan (0.3 mg/kg; 1.0 mM solution), icilin (0.5 mg/kg) plus SB 224289 (3 mg/kg; 5.4 mM solution) or vehicle (1% Tween-80 with 8% dimethylformamide in de-ionised water). Doses were chosen on the basis of literature studies demonstrating efficacy (Stean et al., 2005; Vera-Portocarrero et al., 2008; Werkheiser et al., 2009). After 5 min recovery, shaking events were recorded by two independent observers over the following 20 min. In addition we investigated the nocifensive (flinching and paw lifting) behaviour elicited by direct intraplantar injection of icilin at high concentrations (Knowlton et al., 2010). Rats were briefly anaesthetised with 3% isoflurane/oxygen and received a subcutaneous intraplantar injection of 80 µg icilin in 75 µl of 1% Tween-80 with 16% dimethylformamide in de-ionised water (3.2 mM solution), either alone or additionally containing 45 µg CP 94253 (2 mM solution). Doses were chosen wherever possible on the basis of literature studies demonstrating efficacy (Gentry et al., 2010; Godinez-Chaparro et al., 2013; Knowlton et al., 2010). After 5 min recovery, flinching and paw lifting events were recorded by two independent observers over the following 20 min.

### *2.3. Dual label immunofluorescence histochemistry*

DRGs from spinal segments L4-6 were dissected, rapidly embedded in OCT cryo-sectioning medium (CellPath) and frozen on dry ice. 16 µm sections were cut by cryostat and mounted onto poly-L-lysine coated slides (VWR International). Sections were washed in PBS and then incubated for 1 h at room temperature in blocking buffer (10% normal donkey serum, 4% fish skin gelatin, 0.2% Triton X-100 in PBS) prior to overnight incubation at 4°C with a combination of primary antibodies in buffer (4% normal donkey serum, 4% fish skin gelatin, 0.2% Triton X-100 in PBS). Primary antibodies were used as follows: rabbit polyclonal anti-TRPM8 (Alomone) 1:300 and goat polyclonal anti-5-HT<sub>1B</sub>R (Santa Cruz Biotechnology) 1:150. Sections were washed in PBS and incubated for 1 h at room temperature with a combination of secondary antibodies in buffer (4% normal donkey serum, 4% fish skin gelatin in PBS). Secondary antibodies were: donkey anti-goat AlexaFluor 488 (Molecular Probes) 1:300 and donkey anti-rabbit AlexaFluor 568 (Molecular Probes) 1:600. Sections were washed a further three times in PBS and then mounted in Vectashield (Vector Labs) before visualisation through a x40 objective with oil immersion using a Zeiss LS510 Axiovert confocal microscope. Standard controls involving pre-absorption of the TRPM8 or 5-HT<sub>1B</sub>R antibody with their peptide antigen (and primary antibody omission controls) were carried out and in each case abrogated staining.

### *2.4. Cell culture*

DRGs from adult rats (normally 10-14 per animal) were dissected into Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen). After

centrifuging for 3 min at 500 g, medium was removed and collagenase (5 mg/ml) and dispase (1 mg/ml; Roche) were added before incubation at 37°C for 2 h. Following centrifugation at 500 g for 5 min, the supernatant was discarded and the precipitate was digested in 0.25% trypsin (Invitrogen) at 37°C for 8 min. Then 0.25% trypsin inhibitor (Invitrogen) was added and the preparation was centrifuged at 500 g for 5 min. The supernatant was removed and the pellet was washed with DMEM/10% FBS and re-centrifuged at 1,000 g for 5 min. After removing the supernatant, the pellet was re-suspended in DMEM/10% FBS and triturated to dissociate to a single cell suspension. This suspension was filtered through a 15% bovine serum albumin (BSA) solution (Sigma) by centrifugation at 500 g for 10 min and re-suspended in DMEM/10% FBS. The cell suspension was then purified through a Percoll solution comprising; 1 ml of 10x Hanks' Balanced Salt Solution (HBSS, Invitrogen), 1 ml 0.35% NaHCO<sub>3</sub>, 5 ml de-ionised H<sub>2</sub>O, 30 µl 1M HCl and 3 ml of 1.13 g/ml Percoll solution (Sigma), by centrifugation at 9,000 g for 10 min at 4°C. The lower Percoll layer was re-suspended in DMEM/10%FBS and centrifuged for 10 min at 9,000 g. The cell pellet was resuspended in Neurobasal medium with B-27 supplement (Invitrogen), penicillin/streptomycin (1:100), L-glutamine (1 mM, Invitrogen), NGF (50 ng/ml; Roche), GDNF (2 ng/ml; Roche) and cytosine β-D-arabino side hydrochloride (10 µM; Sigma). Following plating onto coverslips coated with poly-D-lysine (Sigma), cells were incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub> and used the following day for calcium imaging.

Clonal lines of HEK 293 cells stably expressing human TRPM8, TRPV1 or TRPA1 were generated and previously characterised. Wild-type HEK 293 cells were used in some control experiments. Cells were plated out at a density of 1.5 x 10<sup>5</sup> cells/well in 24-well plates previously coated with fibronectin (0.01 mg/ml; Sigma). Final volume was made up to 1 ml using complete DMEM without Phenol Red, containing 10% FBS and cells were then incubated at 37°C for 24 h to reach 60-80% confluency before transfection using FuGENE6 (Roche) with FLAG-tagged 5-HT<sub>1B</sub>R and HA-tagged wild-type or catalytically inactive PLD constructs or empty plasmid as appropriate (Barclay et al., 2011). In control experiments cells were transfected with 5-HT<sub>1A</sub>R or 5-HT<sub>2A</sub>R. In further studies TRPM8-expressing HEK 293 cells were co-transfected with the 5-HT<sub>1B</sub>R and wild-type PIP 5-kinase 1β, a mutant resistant to activation by the PLD product, phosphatidic acid [YKRR209VAAE, R215D]PIP 5-kinase 1β (Stace et al., 2008) or empty vector. Additional experiments were carried out in native HEK 293 cells co-transfected with the 5-HT<sub>1B</sub>R and either wild-type (rat) TRPM8 or a mutant with selectively impaired activation by PIP<sub>2</sub>, [R1008Q]TRPM8 (Rohacs et al., 2005). Cells were then grown at 37°C under 5% CO<sub>2</sub> for 48 h before assay.

### *2.5. Synaptoneurosome preparation*

New protocols were developed to enable measurement of dynamic Ca<sup>2+</sup> fluorescence responses in synaptoneurosome (resealed pre-synaptic and closely apposed post-synaptic elements) freshly prepared from CNS tissue (Hollingsworth et al., 1985; Villasana et al., 2006) using a range of technical modifications to help



preserve metabolic and ionic integrity. Appropriate ipsilateral hemisectioned spinal cord segments and bilateral frontal or somatosensory cortex regions were removed from animals under 4% isoflurane/O<sub>2</sub> anaesthesia and rapidly homogenised in ice-cold, highly oxygenated medium using a hand-held teflon-glass homogeniser. This consisted of: divalent ion-free Krebs-Henseleit buffer additionally containing HEPES (10 mM, pH 7.4), sodium pyruvate (0.5 mM), glutathione (5 µM), creatine phosphate (2 mM), magnesium chloride (5 mM) and sodium kynurenate (0.3 mM). The homogenate was rapidly syringe-filtered through 100 µm and 30 µm nylon mesh filters and then through a 5 µm pore mixed cellulose fibre matrix filter (Millipore) before centrifugation at 1,500 g for 8 min at 4°C. The pellet was resuspended in ice-cold highly oxygenated medium as above but lacking magnesium and kynurenate and containing 2 mM calcium chloride. Electron microscopic analysis of this subcellular fraction revealed the abundant presence of small dual-compartment re-sealed profiles consistent with the previously described characteristics of synaptoneuroosomes (Hollingsworth et al., 1985).

#### *2.6. Measurement of cellular and synaptoneurosome calcium fluorescence responses*

Ratiometric calcium imaging of single DRG neurons was carried out using Fura-2/AM dye (Invitrogen). Cells were loaded with 3.5 µM Fura-2/AM in DMEM supplemented with 0.1% Pluronic acid (Invitrogen) for 20 min. Following washing, coverslips were placed in a recording chamber containing 2.5 ml of DMEM. An Olympus BX50WI microscope was used for imaging (20x magnification objective lens). Pairs of images were collected every 5 s from alternating 70 ms exposures at 340 nm and 380 nm using a Photonics LPS150 monochromator and captured using a Hamamatsu ORCA-ER CCD camera. Data were analyzed using Simple-PCI software (Hamamatsu Corporation, Sewickley, PA, USA). Coverslips were superfused with DMEM buffer at 0.5 ml/min, using a gravity driven perfusion system for exchange of solutions. Temperature was maintained at 28.5°C. Following selection of a suitable field of view, recording was initiated. Only icilin-responsive cells were selected for evaluation of further treatments with other agents, alone or in combination with icilin. The effects of drugs were evaluated by paired comparisons. After a baseline period of 10 min the first icilin treatment was applied for 6 min, followed by a wash out period of at least 10-min and the second drug treatment with icilin / CP 94253 alone or icilin plus CP 94253. Following subtraction of background fluorescence, the ratio of fluorescence at 340 nm and 380 nm was calculated. Area under the curve was determined for the responses to each of the paired treatments using GraphPad Prism 4.

For calcium fluorometry in transfected TRPM8-expressing HEK 293 cells, medium was removed from each well to leave 300 µl prior to the addition of 100 µl of medium containing the visible wavelength no-wash Ca<sup>2+</sup> fluorophore, Calcium 4 (Molecular Devices, CA, USA), according to the manufacturer's instructions. The dye was dissolved in divalent-free Hanks' Balanced Salt Solution (Invitrogen) with 1.8 mM calcium chloride, 0.8 mM magnesium chloride, 25 mM HEPES pH 7.40, containing 500 µM (±) sulphinpyrazone (Sigma). Plates were incubated at 37°C for 45 min in the dark in a 95% O<sub>2</sub> / 5% CO<sub>2</sub> atmosphere, before incubation at 28°C for

5 min. Compounds were added in volumes of 1-4  $\mu$ l per well from concentrated solutions in dimethylformamide, yielding final vehicle concentrations from 0.25–1% (which had no effect alone). Intracellular  $\text{Ca}^{2+}$  fluorescence was measured at excitation 488 nm, emission 519 nm, using a Varioskan Flash fluorometric plate reader (Thermo-Scientific), as the mean of 6 readings taken at 30-s intervals from 1 min after drug addition, or in the case of real-time measurements pre/post-drug addition, by 12 successive readings at 5-s intervals. Data were collected using SkanIt software (Thermo Scientific) and analysed relative to ionomycin (5  $\mu$ M) – induced elevation of calcium fluorescence, as a benchmark positive control. The following drugs were used: icilin; menthol; the selective TRPV1 and TRPA1 activators capsaicin (Tocris) and allyl isothiocyanate (AITC, Sigma-Aldrich) respectively; CP 94253; the selective 5-HT<sub>1A</sub>R agonist, (R)-(+)-8-hydroxy-DPAT, (R)-(+)-8-hydroxy-2-dipropylaminotetralin hydrobromide (Tocris); the selective 5-HT<sub>2A/C</sub>R agonist TCB-2, (4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine (Tocris); the selective PLD inhibitors VU0155069 (Cayman Chemicals), VU0285655-1 (BML-820, Enzo Life Sciences), VU0359595 (Avanti Polar Lipids), halopemide (Cayman), raloxifene (Tocris), calphostin C (Enzo Life Sciences); the selective PKC inhibitor bisindolylmaleimide 1 (Calbiochem); the phosphoinositide-kinase inhibitor, wortmannin (Tocris); the PLC activator m-3M3FBS (Tocris) and the PLC inhibitor U-73122 (Enzo). Receptor agonists or signalling modulators were added immediately before TRP channel activators.

For experiments with freshly isolated synaptoneuroosomes, aliquots were dispensed into 24-well plates and Calcium 4 dye was added (at 2x the concentration suggested in the manufacturer's protocol). Plates were then incubated for 45 min at 37°C under 95% O<sub>2</sub> / 5% CO<sub>2</sub> before incubation at 28°C for 5 min, drug additions and then fluorometric assessment as described above. Spinal cord synaptoneuroosomes were stimulated with combined activators of nociceptive afferents, ACB (AITC/capsaicin/bradykinin) and synaptoneuroosomes from somatosensory or frontal cortex were stimulated with the broad spectrum glutamate receptor agonist, DLH (d,l-homocysteic acid). The target specificity of these stimuli was confirmed by experiments demonstrating the abrogation of responses to ACB in the presence of the TRPV1 inhibitor AMG 9810, 3  $\mu$ M, the TRPA1 inhibitor HC-030031, 10  $\mu$ M and the B<sub>2</sub> receptor antagonist HOE-140, 2  $\mu$ M, and of responses to DLH in the presence of the NMDA receptor antagonist (+) MK-801, 2  $\mu$ M and the mGluR5 antagonist MPEP, 2  $\mu$ M.

### *2.7. Immunoprecipitation and Western blot*

Cells were serum-deprived for 4 h in 10 ml of DMEM per 175 cm<sup>2</sup> flask. Cells were then homogenised in 2 ml of ice-cold IP buffer (20% glycerol, 1% CHAPS, 1% sodium deoxycholate, 1 mM sodium orthovanadate in HEPES 20 mM/NaCl 150 mM buffer, pH 7.4), with 1:100 protease inhibitor cocktail 3 (Calbiochem). Flasks were kept on ice for 30-45 min and then cells were scraped and removed. For experiments involving spinal cord tissue, segments L4-L6 were collected from animals under anaesthesia with 4% isoflurane/oxygen. In order to minimise degradation, tissue was collected onto ice-cold foil, weighed, and promptly homogenised in

20 volumes of IP buffer. For immunoprecipitation, extracts were centrifuged at 10,000 g for 10 min at 4°C to pellet remaining insoluble materials. The supernatants were then decanted and pre-cleared with Protein G-Sepharose CL-4B (20 µl 1:1 suspension in IP buffer/ml supernatant; Sigma) while rotating at 4°C for 45 min, in order to remove any proteins that non-specifically bind to the beads. Following a pulse-spin the supernatant was removed and Protein G-Sepharose beads (40 µl suspension/ml) added together with the relevant antibody. For immunoprecipitation of the FLAG-tagged 5-HT<sub>1B</sub>R, a mouse monoclonal anti-FLAG antibody (M2) was used (4 µg/ml; Sigma). For TRPM8 immunoprecipitation, a rabbit polyclonal anti-TRPM8 antibody was used (7 µg/ml; Alomone). For immunoprecipitation of HA-tagged PLD1, a mouse monoclonal anti-HA antibody, 12CA5 was used (5 µg/ml; Roche). As controls, non-immune IgG of the same species as the antibody were used for mock pull-downs. Primary antibodies and non-immune IgGs were incubated with samples overnight at 4°C. Samples were then centrifuged and the supernatants discarded. The beads were then washed three times with ice-cold IP buffer after which all liquid was removed and a volume of Laemmli buffer (85% Tris buffer (tris-hydroxymethylaminoethane, 50 mM, pH 7.4, Sigma; 5% mercaptoethanol, Sigma; 2% sodium dodecyl sulphate, SDS, Sigma) equal to that of bead suspension was added to each tube. The samples were thoroughly mixed and heated at 70°C for 10 min and then either used immediately or stored at -20 °C. The presence of the target immunoprecipitated proteins and any captured interacting proteins in pull-down samples was assessed by Western blot (Barclay et al., 2011). Proteins were separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) using the NuPage XCell *SureLock*<sup>TM</sup> Minicell gel electrophoresis system (Invitrogen). Samples (5-18 µl) were mixed with 1-2 µl loading solution (0.04% w/v Bromophenol Blue in glycerol) and added to the wells of 4–12% Bis-Tris NuPage gels. Pre-stained standard molecular weight proteins (Invitrogen or Thermo-Pierce) were run alongside. Samples were electrophoresed using NuPage MOPS running buffer (Invitrogen) under a 200 V potential difference. Proteins were transferred to polyvinylidene difluoride membrane Immobilon-P<sup>SO</sup>, Millipore) at 30 V in transfer buffer (5% NuPage transfer buffer, 10% methanol) for 90 min. The efficiency of transfer and protein loading was assessed by staining membranes with Coomassie Blue (0.1% in 30% methanol, 10% acetic acid; GE Healthcare, UK). Following de-staining with 30% methanol/10% acetic acid in de-ionised water, membranes were incubated in appropriate blocking buffers (3-5% non-fat dried milk in 0.1 M PBS or TBS with 0.1% Tween-20 (Sigma) to block non-specific binding. Membranes were then washed and incubated with rabbit polyclonal anti-FLAG (Affinity BioReagents), goat polyclonal anti-5-HT<sub>1B</sub>R (Santa Cruz Biotechnology), rabbit polyclonal anti-TRPM8 (Alomone), rabbit polyclonal anti-PLD1 (Cell Signalling Technology, CST) or peroxidase-conjugated rat monoclonal anti-HA, clone 3F10, (Roche) overnight at 4°C. Membranes were washed and incubated with peroxidase-linked secondary antibodies: goat anti-rabbit, goat anti-mouse or donkey anti-goat/sheep (each from Chemicon) made up in blocking buffer, for 50 min at room temperature. Bands were detected by peroxidase-

linked chemiluminescence and X-ray film (Lumiglo Enhanced Chemiluminescence reagent; CST and Hyperfilm; GE Healthcare).

### 2.8. Cellular phospholipase D (PLD) activity assays

Medium was removed from TRPM8-expressing or wild type HEK 293 cells previously transfected with the 5-HT<sub>1B</sub>R or the 5-HT<sub>1A</sub>R in 12-well plates and replaced with DMEM (0.5 ml) containing [<sup>3</sup>H]palmitic acid (1.5 µCi/well; Perkin Elmer Biosciences). After 18 h incubation at 37°C in a 5% CO<sub>2</sub> atmosphere, the medium was replaced with Minimum Essential Medium (MEM, 0.5 ml) containing HEPES (25 mM, pH 7.5) and fatty acid-free bovine serum albumin (0.5%). To allow assessment of the transphosphatidylation activity of PLD, butan-1-ol (30 mM) was added to each well, immediately followed by the addition of 5-HT<sub>1B</sub>R agonist. In experiments with selective PLD1 or PLD2 inhibitors, these agents were added 5 min before the butan-1-ol. Responses were terminated after 20 min by the removal of medium and addition of ice-cold methanol (0.5 ml/well). Phospholipids were extracted and [<sup>3</sup>H]phosphatidylbutanol ([<sup>3</sup>H]PtdBut) was separated on thin layer chromatography plates (LK5D; Whatman) and quantified by liquid scintillation counting as described previously (Barclay et al., 2011).

### 2.9. Cellular forskolin (FSK)-induced cyclic AMP production assays

Medium was removed from TRPM8-expressing HEK 293 cells previously transfected with the 5-HT<sub>1B</sub>R in 24-well plates and replaced with DMEM (0.5 ml). After 4 h, the medium was replaced with MEM (0.5 ml) containing HEPES (25 mM, pH 7.5) and 0.5 mM isobutylmethyl xanthine (IBMX, Sigma), a broad-spectrum phosphodiesterase inhibitor. 5-HT<sub>1B</sub>R agonist was added immediately before the adenylyl cyclase activator forskolin (5 µM, Sigma) and incubations continued for 10 min at 37°C before aspiration of medium and cell lysis in ice-cold 0.1 M HCl. The cyclic AMP (cAMP) content in aliquots of the lysis supernatant was then measured in duplicate by enzyme-linked immunoassay (EIA) kit (Cayman Chemical Company, 581001). Data were calculated as percentage of FSK-induced increment in cAMP production remaining in the presence of 5-HT<sub>1B</sub>R agonists.

### 2.10. Cellular PIP<sub>2</sub> production assays

The medium of TRPM8-expressing HEK 293 cells in 12-well plates that had been transfected with the 5-HT<sub>1B</sub>R or empty vector was replaced with MEM containing HEPES (25 mM, pH 7.5). 5-HT<sub>1B</sub>R agonist was added (with/without selective PLD1 inhibitor) and assays continued for a range of times before they were terminated by aspiration and the addition of 1 ml ice-cold trichloroacetic acid. Cells from 2 wells per assay determination were scraped and processed before extraction of acidic phospholipids and analysis by specific PIP<sub>2</sub> mass ELISA (Gray et al., 2003) kit (Echelon Biosciences, K-4500, Tebu-Bio) according to the

manufacturer's instructions. PIP<sub>2</sub> concentrations were quantified against an 8-point triplicate standard curve of 0.4-1250 pmol PIP<sub>2</sub> / assay.

### 2.11. Statistical analysis

Data in two-group format were analysed by Wilcoxon test or Student's t-test. Comparisons between more than two groups were made by One-Way ANOVA with Tukey's or Dunnett's *post hoc* test or by Two-Way ANOVA with Bonferroni's *post hoc* test. The statistical significance of changes in EC<sub>50</sub> values obtained by non-linear curve-fitting was determined by Extra Sum of Squares F Test comparing a separate EC<sub>50</sub> model to a combined global fit.

## 3. Results

### 3.1. Enhancement of TRPM8 responses to icilin by 5-HT<sub>1B</sub>R activation in DRG cells and in HEK 293 cells co-expressing TRPM8/5-HT<sub>1B</sub>R

To investigate whether TRPM8-mediated Ca<sup>2+</sup> entry into DRG cells was influenced by 5-HT<sub>1B</sub>R activation we carried out single cell fluorometric imaging using the Ca<sup>2+</sup> fluorophore Fura-2 on cultured DRG cells. Brief perfusion with a low concentration of icilin elicited elevation of intracellular Ca<sup>2+</sup> concentration in the subpopulation of DRG cells (around 10%) that express TRPM8. We used icilin rather than menthol because of its greater potency and selectivity for TRPM8. For example, icilin and menthol display EC<sub>50</sub> [95% confidence interval] values in μM of 0.31 [0.26/0.38] and 11 [7/17] at TRPM8 but 128 [114/149] and 29 [26/33] at TRPA1 in our stable TRPM8 and TRPA1-expressing HEK 293 cell lines (n=6). Fig 1A shows typical examples of subsequent responses to icilin, the selective 5-HT<sub>1B</sub>R agonist CP 94253 or the combination. Repeated stimulation with icilin alone elicited second responses that were similar to the first, CP 94253 alone had no discernible effect, but icilin caused consistently greater responses when re-applied in the presence of CP 94253. The bar chart illustrates that integrated area under the curve values for a second response to icilin plus CP 94253 were significantly greater than the first response to icilin alone. Dual label immunofluorescence histochemistry showed that the great majority (75-80%) of the small TRPM8-positive cell bodies in DRG additionally stained for the 5-HT<sub>1B</sub>R (Fig. 1B) while a variety of other DRG cells (of a range of sizes) were positive for 5-HT<sub>1B</sub>R and not TRPM8.

In order to explore the nature of the facilitatory effect of 5-HT<sub>1B</sub>R activation, we set up a cell model in which HEK 293 cells (considered to lack any endogenous 5-HT receptors (Lesage et al., 1998; Monsma et al., 1993) that had been stably transfected with TRPM8 were transiently transfected with the 5-HT<sub>1B</sub>R. Using a fluorometric plate reader assay with a visible wavelength no-wash Ca<sup>2+</sup> fluorophore, Calcium 4, more convenient for higher throughput analysis in plastic multi-well plates, we confirmed that Ca<sup>2+</sup> elevation responses to submaximal concentrations of icilin were significantly facilitated by CP 94253 (Fig. 2A). In pilot

experiments to assess post-stimulus recovery towards basal  $\text{Ca}^{2+}$  fluorescence levels, we temporarily stopped recording and manually replaced the medium, as we do not have a facility for in-fluorometer perfusion. Under these conditions, baseline fluorescence remained within 10% of prior values and the increment in fluorescence induced by 0.7  $\mu\text{M}$  icilin or 0.7  $\mu\text{M}$  icilin plus 2.5  $\mu\text{M}$  CP 94253 consistently decreased to less than 20% of its maximum within 2 min. Responses evoked by a submaximal concentration of an alternative TRPM8 activator (l)-menthol (15  $\mu\text{M}$ ) were also significantly enhanced by CP 94253 (2.5  $\mu\text{M}$ ), from  $45.1 \pm 3.3$  to  $71.1 \pm 9.8\%$  of the maximal response to 500  $\mu\text{M}$  (l)-menthol, (means  $\pm$  SEM,  $n=6$ ,  $p = 0.0173$  by Student's t-test). CP 94253 (2.5  $\mu\text{M}$ ) caused a significant 3.32-fold increase in the apparent potency of icilin, shifting its mean  $\text{EC}_{50}$  [95% confidence interval] from 0.313 [0.289/0.339] to 0.094 [0.080/0.118]  $\mu\text{M}$  ( $p < 0.001$ ), with no discernible change in maximum response but significant increments at individual submaximal concentrations (Fig. 2B, Table 1). The selective 5-HT<sub>1B/1D</sub>R agonist sumatriptan (2  $\mu\text{M}$ ) had a similar effect (Table 1), while neither CP 94253 nor sumatriptan alone (0.02-2.5  $\mu\text{M}$ ) caused any discernible change in  $\text{Ca}^{2+}$  fluorescence in TRPM8-expressing HEK 293 cells that were transfected with the 5-HT<sub>1B</sub>R ( $n=4$  in each case). The effects of CP 94253 and sumatriptan on TRPM8 responses to icilin were significantly reversed in the presence of a selective 5-HT<sub>1B</sub>R antagonist SB 224289 (5  $\mu\text{M}$ ), (Fig. 2A, Table 1). SB 224289 (3-100  $\mu\text{M}$ ) or a further 5-HT<sub>1B</sub>R antagonist GR 55562 (5-200  $\mu\text{M}$ ) had no discernible effect alone on  $\text{Ca}^{2+}$  fluorescence responses to icilin (1  $\mu\text{M}$ ) in 5-HT<sub>1B</sub>R plus TRPM8-expressing HEK 293 cells ( $n=5$ ), whereas the selective TRPM8 antagonist AMTB caused complete, concentration-dependent inhibition of responses, with a mean  $\text{IC}_{50}$  [95% confidence interval] of 8.04 [7.78/8.28]  $\mu\text{M}$  ( $n=5$ ). The interaction between TRPM8 and the 5-HT<sub>1B</sub>R seems to be specific for this combination as icilin responses in TRPM8 cells were unaffected by activation of transfected 5-HT<sub>1A</sub>R and responses to allyl isothiocyanate (AITC) in TRPA1 cells or capsaicin in TRPV1 cells were unaffected by 5-HT<sub>1B</sub>R activation, although, in agreement with an earlier report (Ohta et al., 2006) 5-HT<sub>2A</sub>R activation facilitated capsaicin responses (Table 1).

### 3.2. Physical association of the 5-HT<sub>1B</sub>R and the TRPM8 channel

In a small number of cases, G protein-coupled receptors (GPCRs) have been reported to associate physically with ion channels to form channel-regulating complexes (Davare et al., 2001; Lavine et al., 2002; Li et al., 2010; Shukla et al., 2010). There is also evidence, from the TRPV1 channel in particular, that GPCR-controlled signalling enzymes and their adapter proteins may bind to the channel (Chaudhury et al., 2011; Zhang et al., 2008) or that GPCR-generated second messengers may directly modulate it (Kim et al., 2009). We explored here whether a direct interaction may occur between the 5-HT<sub>1B</sub>R and the TRPM8 channel. When FLAG-tagged 5-HT<sub>1B</sub>Rs were transiently expressed in the stable TRPM8-expressing HEK 293 cell line, we identified capture of FLAG-5-HT<sub>1B</sub>R in TRPM8-directed pulldowns but not in non-immune IgG controls (Fig. 3A). This was corroborated by the converse experiment in which TRPM8-immunoreactivity was specifically captured in

FLAG-5-HT<sub>1B</sub>R pulldowns but not controls (Fig. 3B). Prior exposure to receptor agonist did not appear to be necessary. Despite the fact that native tissues express these antigens only in low abundance, we carried out corresponding *in vivo* experiments using spinal cord lysates. Although more non-specific and reagent-associated bands were evident, these confirmed that 5-HT<sub>1B</sub>R-directed pulldowns compared to non-immune IgG controls specifically captured native TRPM8 immunoreactivity (Fig. 3C). Converse experiments are illustrated in Fig. 6E. These findings support the idea that stable complexes can be formed between 5-HT<sub>1B</sub>Rs and TRPM8 ion channels, both in a cell line model and *in vivo*.

### 3.3. Signalling events underlying the functional influence of the 5-HT<sub>1B</sub>R on the TRPM8 channel

The 5-HT<sub>1B</sub>R is primarily coupled to G proteins of the G<sub>i</sub>/G<sub>o</sub> family, leading to inhibition of adenylyl cyclase and opening of K<sup>+</sup> channels, but can activate additional intracellular signalling pathways, including PLD (Hinton et al., 1999). PLD has been implicated in the regulation of TRPC3 channels by GPCRs, although the molecular basis is not clear (Glitsch, 2010; Kwan et al., 2009). Second messenger-regulated kinases generally seem to exert inhibitory effects on TRPM8 (De Petrocellis et al., 2007; Premkumar et al., 2005; Sarria and Gu, 2010). It has been reported that the alpha subunit of G<sub>q</sub> (G $\alpha_q$ ) can interact with TRPM8 to directly cause channel inhibition (Zhang et al., 2012), although a contrasting report describes G $\alpha_q$ -dependent activation of PLC to hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) and lead to Ca<sup>2+</sup> mobilisation (Klasen et al., 2012). There is a strong consensus of evidence that maintenance of optimal TRPM8 channel function is dependent on adequate levels of PIP<sub>2</sub> being present (Daniels et al., 2009; Liu and Qin, 2005; Rohacs et al., 2005). Notably, PLD activity increases the rate of PIP<sub>2</sub> synthesis by upregulation of the PIP<sub>2</sub>-synthetic enzyme, phosphatidylinositol 4-phosphate (PIP) 5-kinase (Cockcroft, 2009). We therefore asked whether elevation of PIP<sub>2</sub> levels by 5-HT<sub>1B</sub>R-mediated PLD activity might be a plausible mechanism for the potentiation of TRPM8 function. Using stable TRPM8-expressing cells, transiently transfected with the 5-HT<sub>1B</sub>R, we took both pharmacological and molecular approaches to assess whether PLD and potentially its role in generating PIP<sub>2</sub> might be involved in the enhancement by CP 94253 of Ca<sup>2+</sup> responses to a low concentration of icilin (Fig. 4). The effect of CP 94253 was significantly attenuated in the presence of selective PLD inhibitors; VU0155069, halopemide, raloxifene and calphostin C (Scott et al., 2009), at concentrations that had no effect alone (Fig. 4A). As calphostin C can also inhibit PKC following photoactivation, we confirmed that the PKC inhibitor bisindolylmaleimide 1 did not mimic the effect of the PLD inhibitors. To address whether modulation of PIP<sub>2</sub> levels might be important, we firstly tested wortmannin (an inhibitor, not only of phosphatidylinositol (PI) 3-kinase, but also less potently, of PI 4-kinase, which is essential for PIP<sub>2</sub> synthesis) and found that this too reversed the effect of CP 94253. In addition, a synthetic activator of phospholipase C (PLC), (which breaks down PIP<sub>2</sub>), m-3M3FBS, abrogated the effect of CP 94253, whereas a PLC- $\beta$  inhibitor, U-73122, caused a modest but significant facilitation. To further specify the role of PLD in 5-HT<sub>1B</sub>R-mediated facilitation of

TRPM8, we tested the effects of new highly selective inhibitors of PLD1 and PLD2 isoforms VU0359595 and VU0285655-1, respectively on CP 94253-induced enhancement of  $\text{Ca}^{2+}$  responses to icilin (Fig. 4B). The effect of CP 94253 but not that of icilin alone was potently inhibited by VU0359595 with a mean  $\text{IC}_{50}$  [95% confidence interval] of 14.1 [7.8/25.7] nM but showed an  $\text{IC}_{50}$  for VU0285655-1 of  $>10 \mu\text{M}$ . Given the high affinity of VU0359595 and its marked 1700-fold selectivity for PLD1 over PLD2 (Lewis et al., 2009), together with similar but more modest 163-fold selectivity of VU0155069 (Scott et al., 2009), compared to the 21-fold selectivity of VU0285655-1 for PLD2 over PLD1 (Lavieri et al., 2009), these observations clearly implicate PLD1 rather than PLD2. In further experiments, we co-transfected dominant negative constructs of PLD1 or PLD2 and showed that only the dysfunctional PLD1 construct significantly attenuated the CP 94253 facilitation of icilin responses (Fig. 4C). Using a cellular PLD activity assay in 5-HT<sub>1B</sub>R-transfected TRPM8-expressing HEK 293 cells we demonstrated that CP 94253 (0.005-2  $\mu\text{M}$ ) showed robust concentration-dependent activation of [<sup>3</sup>H]phosphatidylbutanol ([<sup>3</sup>H]PtdBut) production with a mean  $\text{EC}_{50}$  [95% confidence interval] of 34.3[14.1/83.2] nM and mean maximal response ( $\pm$  SEM) of  $2.39 \pm 0.14$  fold of basal (n=4). Responses to 2  $\mu\text{M}$  CP 94253 were significantly attenuated (by  $77.0 \pm 11.1\%$ ) in the presence of 100 nM VU0359595 (p=0.0049, Student's t-test, n=4) but not discernibly altered in the presence of 1  $\mu\text{M}$  VU0285655-1. As a comparison we measured the conventional Gi/o-mediated signalling output of the 5-HT<sub>1B</sub>R as inhibition of 5  $\mu\text{M}$  forskolin-induced cAMP production. This showed concentration-dependent responses to CP 94253 across a similar concentration range with a mean  $\text{IC}_{50}$  [95% confidence interval] of 13.1[4.3/40.0] nM and mean maximal response ( $\pm$  SEM) of  $45.9 \pm 6.1\%$  reduction from control (n=4). These experiments indicate that PLD1 but not PLD2 is a mediator of 5-HT<sub>1B</sub>R facilitation of TRPM8 channel function and point to the idea that its influence on PIP<sub>2</sub> levels may underly this effect.

To address directly candidate events occurring downstream of PLD1 activation by the 5-HT<sub>1B</sub>R we evaluated the role of PIP 5-kinase, PIP<sub>2</sub> production and PIP<sub>2</sub> action on the TRPM8 channel. 5-HT<sub>1B</sub>R plus TRPM8-expressing HEK 293 cells were co-transfected with wild-type PIP 5-kinase 1 $\beta$ , a negative mutant construct defective in activation by the PLD product phosphatidic acid [YKRR209VAAE, R215D]PIP 5-kinase 1 $\beta$  (Stace et al., 2008) or empty vector (Fig. 5A). The facilitation of  $\text{Ca}^{2+}$  fluorescence responses caused by CP 94253 in vector-transfected cells was maintained in the presence of wild-type PIP 5-kinase but strongly and significantly attenuated in the presence of the mutant construct. This indicates that PIP 5-kinase recognition of and activation by phosphatidic acid is a key step in the mechanism of 5-HT<sub>1B</sub>R facilitation of the TRPM8 channel. Measurement of the time course of PIP<sub>2</sub> concentration by PIP<sub>2</sub> mass ELISA (Gray et al., 2003) showed a significant but transient peak in the first 5-10 min of stimulation with CP 94253 in TRPM8-expressing HEK 293 cells that had been transfected with 5-HT<sub>1B</sub>R but not those transfected with vector alone (Fig. 5B). The peak in PIP<sub>2</sub> production was largely and significantly attenuated in the presence of a low



concentration of the PLD1-selective inhibitor VU0359595 (100 nM), indicating that 5-HT<sub>1B</sub>R-induced PIP<sub>2</sub> production is dependent on PLD1 activity. To further assess the role of PIP<sub>2</sub> modulation of the channel in the regulatory influence of the 5-HT<sub>1B</sub>R, we co-transfected native HEK 293 cells with the 5-HT<sub>1B</sub>R and either wild-type (rat) TRPM8 or a mutant construct, [R1008Q]TRPM8, in which PIP<sub>2</sub> recognition and channel activation is notably reduced (Rohacs et al., 2005). The mutant construct showed reduced maximal Ca<sup>2+</sup> responses to the TRPM8 activator icilin (55.1 ± 1.2% of control at 200 μM icilin, mean ± SEM, n=6, p=0.0003, Student's t-test) together with a marked reduction in potency (EC<sub>50</sub> [95% confidence interval] of 9.4 [8.1/11.0] μM for the mutant construct compared to 0.17 [0.15/0.19] μM for wild-type TRPM8, consistent with previous characterisation (Rohacs et al., 2005). When CP 94253 was added together with a submaximal concentration of icilin in wild-type TRPM8 cells, Ca<sup>2+</sup> fluorescence responses were significantly increased (0.3 μM icilin alone, 55.6 ± 11.7% of maximal response; 0.3 μM icilin plus 2.5 μM CP 94253, 90.8 ± 6.1%, means ± SEM, n=6, p=0.024, Student's t-test). In contrast, CP 94253 had no significant effect on responses of the mutant channel with reduced PIP<sub>2</sub> responsiveness to a submaximal concentration of icilin (10 μM icilin alone, 25.4 ± 11.4% of maximal response; 10 μM icilin plus 2.5 μM CP 94253, 32.4 ± 8.1%, means ± SEM, n=6, p=0.6159, Student's t-test). These data firmly corroborate the idea that the PIP<sub>2</sub> recognition site on the TRPM8 channel is a key component in the facilitatory influence exerted by 5-HT<sub>1B</sub>R activation.

### *3.4. Phospholipase D1 is physically associated with the 5-HT<sub>1B</sub>R and TRPM8 channel*

As several GPCRs, including 5-HTR subtypes, interact directly with PLD (Barclay et al., 2011) we explored whether the 5-HT<sub>1B</sub>R might also interact with PLD1 in particular, especially when associated with TRPM8. PLD contributes to GPCR regulation of TRPC3 channels (Glitsch, 2010; Kwan et al., 2009) but there is no prior evidence of direct PLD:channel interaction. Using TRPM8-expressing HEK 293 cells co-transfected with FLAG-5-HT<sub>1B</sub>R and HA-PLD1, we specifically captured HA-PLD1 in TRPM8-directed pulldowns but not non-immune IgG controls (Fig. 6A). This interaction was corroborated by converse experiments in which TRPM8 was specifically captured in HA-directed pulldowns but not controls (Fig. 6B). In addition FLAG-directed pulldowns captured HA-PLD1 (Fig. 6C) and HA-directed pulldowns captured 5-HT<sub>1B</sub>R (Fig. 6D). In spinal cord lysates, despite the presence of various non-specific and reagent-associated bands, we identified that TRPM8-directed pulldowns compared to non-immune IgG controls specifically captured native PLD1 and 5-HT<sub>1B</sub>R (Fig. 6E). Native 5-HT<sub>1B</sub>R pulldowns are illustrated in Fig. 3C but we did not attempt to carry out native PLD1-directed pulldowns because of the lack of adequate reagents and the low level yet ubiquitous expression of PLD isoforms. These findings establish directly the existence of a regulatory complex between a GPCR, a TRP channel and PLD both in cell models and *in vivo*.

### *3.5. 5-HT<sub>1B</sub>R activation in vivo potentiates TRPM8-mediated inhibition of pain state-associated neural hyper-responsiveness and reflex pain behaviours*

To evaluate whether 5-HT<sub>1B</sub>Rs could facilitate the anti-nociceptive influence of TRPM8 that has been described *in vivo* (Dhaka et al., 2007; Knowlton et al., 2013; Liu et al., 2013; Proudfoot et al., 2006; Su et al., 2011) we took two approaches. Firstly, we examined dynamic changes in receptor-mediated Ca<sup>2+</sup> fluorescence responses of synaptoneurosomes freshly isolated from CNS regions of animals with pain states, or controls, that had been previously treated *in vivo* by topical application of drugs to the skin of the hindpaws. Synaptoneurosomes from spinal cord were stimulated directly with a combination of agents that act on nociceptive afferents (the TRPA1 activator, AITC, 20 μM; the TRPV1 activator, capsaicin, 10 μM; the B<sub>1/2</sub> receptor agonist, bradykinin, 10 μM; ACB), with the Ca<sup>2+</sup> ionophore, ionomycin, 5 μM (as a maximal positive control) or with vehicle. Synaptoneurosomes from somatosensory cortex and frontal cortex were correspondingly stimulated with a broad spectrum glutamate receptor agonist (d,l-homocysteic acid; DLH, 6.25 μM). Figure 7A illustrates raw Ca<sup>2+</sup> fluorescence recordings of synaptoneurosomes prepared from spinal cord ipsilateral to intraplantar injection of Complete Freund's Adjuvant (CFA) into one hindpaw 24 h previously to model inflammatory pain. ACB or ionomycin were added immediately before the recording period to aliquots of the preparations in parallel wells. Basal and also, after an initial rise, stimulated wells showed a gradual but consistent decline in fluorescence, thought to reflect disturbance of the settled synaptoneurosome layer due to the movement of the plate during the fluorometer reading cycle. Ionomycin induced a marked and maintained increment in Ca<sup>2+</sup> fluorescence which was mimicked to around half of its magnitude by ACB, with both of these responses showing consistent statistical significance compared to basal. The response to ACB was significantly attenuated by the inhibitor of synaptic vesicle exocytosis, tetanus toxin (5 nM, 45 min), indicating that at least part of the ACB-induced increment in Ca<sup>2+</sup> fluorescence was likely to involve transmission within the preparation. Figure 7B, showing ACB- or DLH-induced increments in Ca<sup>2+</sup> fluorescence above basal, normalised to ionomycin-induced increments, illustrates that CFA, 24 h previously, significantly amplified stimulus-evoked responses to ACB in spinal cord (on ipsilateral, and to a lesser extent contralateral, sides) and to DLH in both cortical regions. This accords with the widespread activating changes elicited by CFA at not only spinal but also supraspinal levels of the CNS (Samad et al., 2001). Each of these enhancements was reversed by topical administration of icilin (200 μM in aqueous 1% dimethylformamide) to the hindpaws for 15 min under general anaesthesia prior to tissue removal. As described previously (Proudfoot et al., 2006), the concentration of icilin reaching TRPM8 on fine afferent terminals largely around the epidermal/dermal border is expected to be many fold less than that externally applied. Mean ACB-evoked Ca<sup>2+</sup> fluorescence responses in ipsilateral spinal cord synaptoneurosomes, averaged over 6 readings at 30-s intervals starting 1 min into the recording period and normalised to maximal ionomycin-evoked responses, were 15.3 ± 2.7% (naïve); 34.4 ± 7.0% (CFA); 12.2 ± 3.1% (CFA with topical icilin). Analysis by One-Way ANOVA with Tukey's *post hoc* test indicated that the CFA-induced enhancement of responses and the reversal of this following icilin were both statistically significant (p<0.05, n=4-5). Vehicle had no discernible effect on responses from CFA-injected

animals ( $37.0 \pm 8.1\%$ ,  $n=4$ ). Similarly in synaptoneurosomes from somatosensory and frontal cortex, responses to DLH were  $12.5 \pm 1.1\%$  and  $13.4 \pm 0.6\%$  (naïve);  $19.6 \pm 3.0\%$  and  $22.4 \pm 2.4\%$  (CFA);  $6.4 \pm 4.2\%$  and  $10.0 \pm 3.5\%$  (CFA with topical icilin) respectively, with icilin reversal of the enhancement being statistically significant in each case ( $p<0.05$  and  $p<0.01$ ,  $n=3-5$ ).

To investigate whether 5-HT<sub>1B</sub>R activation could potentiate this TRPM8-mediated influence we tested lower concentrations of icilin in combination with the 5-HT<sub>1B/1D</sub>R agonist sumatriptan in a topical vehicle (propan 1,2-diol containing 1.5% caprolactam) that has been previously shown to enhance skin penetration of compounds including sumatriptan (Femenia-Font et al., 2006; Williams, 2003) and is compatible with icilin uptake via this route (Proudfoot et al., 2006). Figure 7C shows that the enhanced mean Ca<sup>2+</sup> fluorescence responses of ipsilateral spinal cord synaptoneurosomes to ACB in CFA-injected animals were significantly reduced by topical administration *in vivo* of icilin (50 µM) plus sumatriptan (100 µM) prior to tissue removal, whereas either drug alone or vehicle had no significant effect. The combined effect of icilin plus sumatriptan was significantly reversed by topical *in vivo* co-administration of the 5-HT<sub>1B</sub>R antagonist SB 224289 (120 µM). Furthermore, while a relatively low topical concentration of menthol (1 mM) did not significantly reduce ACB responses, in the additional presence of sumatriptan (100 µM), statistically significant inhibition was observed, as seen with icilin. Similarly in somatosensory and frontal cortex, the enhanced responses to DLH seen following CFA injection were significantly reduced by topically applied icilin (110 µM) plus sumatriptan (165 µM), neither of which had a significant effect alone. Thus the widespread CNS hyper-responsiveness caused by inflammation can be attenuated by peripheral topical administration of TRPM8 agonist *in vivo* and this effect is amplified by co-activation of 5-HT<sub>1B</sub>Rs *in vivo*.

We further asked whether co-activation of the 5-HT<sub>1B</sub>R might actually make a contribution to TRPM8 achieving its full analgesic effect in chronic pain. CFA-injected animals were treated *in vivo* with 200 µM icilin applied topically to the hindpaws before measuring the Ca<sup>2+</sup> fluorescence responses of spinal cord synaptoneurosomes to ACB. This slightly higher concentration of icilin alone caused significant reversal of hyper-responsiveness, which was significantly attenuated by the selective 5-HT<sub>1B</sub>R antagonists SB 224289, 120 µM or GR 55562, 200 µM, included in the topically applied solution, and almost completely abrogated by inclusion of the selective TRPM8 inhibitor AMTB, 200 µM but not affected by the 5-HT<sub>1D</sub>R-selective antagonist BRL 15572, 150 µM (Fig. 8). None of the antagonists topically applied alone had any discernible effect on spinal cord synaptoneurosomes Ca<sup>2+</sup> responses to ACB in CFA-treated animals. Icilin, SB 224289 and AMTB were also tested in naïve animals, where they displayed no discernible effect. Furthermore the 5-HT<sub>1B</sub>R antagonists had no direct effect that could be detected on icilin responses in TRPM8-expressing HEK 293 cells whether or not these were transfected with the 5-HT<sub>1B</sub>R. This suggests that TRPM8 requires the co-activation of the 5-HT<sub>1B</sub>R in order to fully exert its reversal of chronic pain-associated hyper-responsiveness in the CNS.

In addition, we explored whether reflex pain behaviours in chronic pain models were affected in a similar way. Figure 9A shows that in CFA-injected animals, the ipsilateral reduction in paw withdrawal latency to noxious heat (Hargreaves' test) (Proudfoot et al., 2006) was significantly reversed towards contralateral and naive values by the *in vivo* topical administration of icilin (50  $\mu$ M) plus sumatriptan (100  $\mu$ M), whereas the effects of either alone were not significant. Figure 9B shows that a higher concentration of topically applied icilin (200  $\mu$ M) significantly reversed CFA-induced hypersensitivity on its own and that this effect of icilin was strongly attenuated in the additional presence of SB 224289 (120  $\mu$ M), which had no discernible effect alone. The synergy between low topical concentrations of icilin and sumatriptan in reversing CFA-induced hypersensitivity was replicated with an alternative TRPM8 activator, menthol (1 mM), which caused significant reversal only in the presence of sumatriptan (Fig. 9C). Figure 9D further shows that in the CCI model of neuropathic pain the sensitised ipsilateral Hargreaves' responses were only marginally reduced by a low intrathecal dose of icilin (2.5 nmole) but this became statistically significant when icilin was co-administered with CP 94253 (2.5 nmole), which had no significant effect alone. The newly identified TRPM8:5-HT<sub>1B</sub>R:PLD1 signalling complex thus appears to play an important physiological role *in vivo* in enabling 5-HT<sub>1B</sub>R amplification of the analgesia exerted by TRPM8 on sensitised pain behaviours and further seems to be necessary for TRPM8 to exert its full impact on chronic pain.

In control experiments we investigated whether alternative acute behavioural responses to icilin administration were affected by 5-HT<sub>1B</sub>R agents. Firstly we investigated "wet dog shakes" induced by intraperitoneal injection of icilin (Wei, 1981; Werkheiser et al., 2009). Icilin (0.5 mg/kg elicited  $65.4 \pm 8.3$  shaking/shuddering events from 5-25 min following injection (mean  $\pm$  SEM, n=5), a response that was not discernibly altered in the presence of either sumatriptan (0.3 mg/kg;  $60.6 \pm 9.0$  events, n=4) or SB 224289 (3 mg/kg;  $56.7 \pm 8.2$  events, n=4). Injection of vehicle elicited no events. The lack of any significant effect (One-Way ANOVA with Dunnett's *post hoc* test) indicates that pharmacological interventions at the 5-HT<sub>1B</sub>R do not impact on this behavioural response to intraperitoneal icilin, at least under the conditions tested. Secondly as nocifensive behaviours can be elicited by direct intraplantar administration of high concentrations of icilin (Knowlton et al., 2010) and response thresholds to thermal and mechanical stimuli are reduced at very high icilin or menthol concentrations (Dhaka et al., 2007; Gentry et al., 2010; Proudfoot et al., 2006), we investigated whether such responses were modified by 5-HT<sub>1B</sub>R activation. Intraplantar icilin (80  $\mu$ g) elicited  $42.5 \pm 4.6$  flinching or paw lifting events from 5-25 min following injection (mean  $\pm$  SEM, n=4), whereas in the additional presence of CP 94253 (45  $\mu$ g) the response was partially but significantly reduced to  $26.6 \pm 4.0$  events (n=4; p=0.0393, Student's t-test). Injection of vehicle did not elicit any discernible response. These observations are consistent with a recent report describing attenuation of formalin-induced nocifensive responses by intraplantar 5-HT<sub>1B</sub>R agonists (Godinez-Chaparro 2013) and indicate that 5-HT<sub>1B</sub>R agonists act to

attenuate rather than exacerbate nocifensive responses elicited by intraplantar injection of high concentrations of TRPM8 agonist.

#### 4. Discussion

To address the hypothesis that functional activation of the TRPM8 ion channel might be facilitated by a GPCR such as the 5-HT<sub>1B</sub>R, we carried out a range of *in vitro* and *in vivo* experiments. Using single cell Ca<sup>2+</sup> fluorometry on isolated DRG cells we identified the subpopulation that responded to the TRPM8 agonist icilin and then showed that they displayed a much greater response to a subsequent challenge with icilin plus a selective 5-HT<sub>1B</sub>R agonist (CP 94253), which had no effect alone. Dual label immunofluorescence revealed that around 80% of the small TRPM8-positive cells in DRG also express the 5-HT<sub>1B</sub>R. We therefore set up a cell line model of HEK 293 cells stably expressing TRPM8 that were transiently transfected with the 5-HT<sub>1B</sub>R. Their Ca<sup>2+</sup> fluorescence responses to a low concentration of icilin or menthol were strongly increased in the presence of CP 94253 or the selective 5-HT<sub>1B/1D</sub>R agonist sumatriptan and the potentiation was abrogated by the selective 5-HT<sub>1B</sub>R antagonist SB 224289. In contrast the 5-HT<sub>1A</sub>R was unable to enhance TRPM8 responses and the 5-HT<sub>1B</sub>R was unable to enhance TRPV1 or TRPA1 responses, emphasising the specificity of the interaction observed here. There are several examples of GPCRs forming physical complexes with Ca<sup>2+</sup> and K<sup>+</sup> channels (Davare et al., 2001; Lavine et al., 2002; Li et al., 2010) but only a single report of direct association with a TRP family channel, in which the angiotensin AT<sub>1A</sub>R forms a complex with TRPV4 and  $\beta$ -arrestin 1 in vascular smooth muscle cells (Shukla et al., 2010). We found compelling evidence here from co-immunoprecipitation in the cell model and *in vivo* that the 5-HT<sub>1B</sub>R was directly associated with TRPM8.

We next considered whether signalling by the 5-HT<sub>1B</sub>R might mediate its facilitatory influence on TRPM8. In the cell model, CP 94253-enhanced icilin-evoked Ca<sup>2+</sup> fluorescence responses were challenged with key pharmacological and molecular reagents. Several signalling pathways have been reported to modulate TRPM8. G $\alpha_q$  is reported to interact directly with TRPM8 and inhibit the channel through an unknown mechanism that may not involve activation of PLC- $\beta$  and PIP<sub>2</sub> hydrolysis (Zhang et al., 2012). However a contrasting report indicates that interaction with TRPM8 causes activation of G $\alpha_q$  and downstream PLC signalling (Klasen et al., 2012). PKC inhibits channel amplitude or activation threshold in response to menthol or cold and promotes desensitisation (Premkumar et al., 2005; Sarria and Gu, 2010). PKA does not affect responses to relatively high concentrations of menthol (Bavencoffe et al., 2010; Sarria and Gu, 2010), but can inhibit responses to submaximal concentrations of icilin or menthol (De Petrocellis et al., 2007). Lysophospholipids generated by phospholipase A<sub>2</sub> can facilitate TRPM8 activation, whereas polyunsaturated fatty acids that are produced concomitantly exert inhibitory effects (Andersson et al., 2007; Bavencoffe et al., 2011). Raised cytosolic Ca<sup>2+</sup> concentrations may contribute to channel desensitisation by PKC activation or depletion of PIP<sub>2</sub> (Rohacs et al., 2005), which is an essential co-factor for TRPM8 channel function. Reasoning that processes leading to

increased PIP<sub>2</sub> levels such as PLD activation (Cockcroft, 2009) could potentially be involved in the facilitatory influence of the 5-HT<sub>1B</sub>R on TRPM8, we showed that selective inhibitors of PLD blocked the effect of CP 94253. The effects of wortmannin, an inhibitor of PI 4-kinase, needed for PIP<sub>2</sub> production and m-3M3FBS / U-73122, an activator and inhibitor respectively of PLC, which breaks down PIP<sub>2</sub>, were each consistent with this hypothesis. PLD1 rather than PLD2 was implicated by reversal of the 5-HT<sub>1B</sub>R effect in the presence of selective pharmacological inhibitors for PLD1 but not PLD2 and by negative mutant PLD constructs. The 5-HT<sub>1B</sub>R agonist CP 94253 caused robust activation of cellular PLD1 through a similar concentration range to its classical G<sub>i/o</sub>-mediated response of inhibiting forskolin-stimulated cAMP production. Further co-immunoprecipitation experiments revealed that the key signalling enzyme PLD1 was additionally docked in the 5-HT<sub>1B</sub>R:TRPM8 complex, both in the cell model and *in vivo*. In addition, we provided direct evidence for the subsequent involvement of PIP 5-kinase in the PLD1-dependent facilitatory effect of 5-HT<sub>1B</sub>R on the channel as the effect was abrogated by transfection of a phosphatidic acid-unresponsive PIP 5-kinase mutant. Furthermore we showed that 5-HT<sub>1B</sub>R activation caused a transient rise in cellular PIP<sub>2</sub> concentration that was prevented by PLD1 inhibitor and demonstrated that a TRPM8 mutant construct with greatly reduced PIP<sub>2</sub> responsiveness failed to show the 5-HT<sub>1B</sub>R-mediated facilitation seen with the wild-type channel. Taken together, these findings point strongly to the existence of a 5-HT<sub>1B</sub>R:TRPM8:PLD1 complex that can achieve upregulation of TRPM8 function by PLD signalling leading to increased levels of PIP<sub>2</sub>; a positive allosteric modulator of channel function.

To examine the physiological significance of 5-HT<sub>1B</sub>R:TRPM8 interactions we carried out experiments in both inflammatory (CFA) and neuropathic (CCI) pain models, assessing the impact on pain state-associated hyper-responsiveness to excitatory stimuli in *ex vivo* synaptoneuroosomes or on sensitised pain behaviours *in vivo*. We administered 5-HT<sub>1B</sub>R agonists and TRPM8 activators topically to the skin of the hindpaws or intrathecally to the spinal cord. A selective 5-HT<sub>1B/1D</sub>R agonist sumatriptan amplified the effect of low concentrations of icilin or menthol in reversing CFA-induced hyper-responsiveness of synaptoneuroosomes from spinal and supraspinal CNS regions, with 5-HT<sub>1B</sub>R specificity confirmed through reversal by SB 224289. At slightly higher concentrations, icilin alone produced a similar outcome, which could be attenuated by SB 224289 or GR 55562, indicating a functional dependence of TRPM8 on the associated 5-HT<sub>1B</sub>R in order to achieve its full influence of reversing hypersensitivity in chronic pain. Icilin, SB 224289 or the TRPM8 antagonist AMTB alone had no effect on responsiveness of spinal cord synaptoneuroosomes from naive animals, suggesting that TRPM8 or tonic activation of the TRPM8/5-HT<sub>1B</sub>R complex exert a substantive modulatory influence only in chronic pain states. These observations were paralleled by matching reversals of sensitised pain behaviours both in the CFA model following topical application of agents to the hindpaw skin and in the CCI model following intrathecal drug administration (accessing peripheral and central terminal regions of TRPM8 afferents respectively). Again, icilin or menthol in combination with 5-HT<sub>1B</sub>R agonist, slightly higher

levels of icilin alone or SB 224289 alone had no effect on contralateral behavioural responses. Thus the TRPM8:5-HT<sub>1B</sub>R:PLD1 complex in sensory afferents appears to play an important role in the analgesic effect of TRPM8 activation *in vivo*, particularly in chronic sensitised pain states. Our data showing that the efficacy of slightly higher concentrations of icilin alone, in the CFA model of inflammatory pain, is attenuated by 5-HT<sub>1B</sub>R antagonist suggest tonic activation of the 5-HT<sub>1B</sub>R component of the complex in the chronic pain state. 5-HT that could achieve this activation is likely to be released into the peripheral inflammatory milieu from various sources including platelets (Khalil and Helme, 1990; Shu and Mendell, 1999). It is possible that central spinal release of 5-HT in pain states could also affect the TRPM8:5-HT<sub>1B</sub>R:PLD1 complex on central terminals of relevant afferents but our experiments did not address that question, as the topical application of drugs to the hindpaw is unlikely to achieve effective concentrations in spinal cord.

The 5-HT<sub>1B</sub>R facilitation of TRPM8 function in non-nociceptive afferents that exert anti-hypersensitivity/analgesic actions appears to contrast with effects on other acute behavioural responses to icilin that are seen after its direct administration at much higher concentrations, yet are TRPM8-dependent and thought to be peripherally mediated (Colburn et al., 2007; Dhaka et al., 2007; Knowlton et al., 2010; Knowlton et al., 2013; Pogorzala et al., 2013). In the “wet dog shakes” model of shaking/shuddering induced by intraperitoneal injection of icilin (Wei, 1981; Werkheiser et al., 2009), co-administration of 5-HT<sub>1B</sub>R agonist or antagonist (at doses known to be efficacious in other contexts) had no apparent effect. In a model of nocifensive behaviour induced by intraplantar injection of icilin (Knowlton et al., 2010), co-administration of 5-HT<sub>1B</sub>R agonist attenuated rather than exacerbated the response. In these models the concentrations of icilin solution (1.6-3.2 mM) directly applied to the peritoneal space (where the drug may potentially access visceral afferents) or close to cutaneous afferent terminals within the skin are far higher than those (50-200  $\mu$ M), which when applied topically to the outside of the skin showed useful anti-hypersensitivity/analgesic actions, either alone or in combination with 5-HT<sub>1B</sub>R agonist. It is further likely that after topical application the concentrations of drugs actually reaching TRPM8 afferents at the epidermal/dermal border are greatly reduced by limited penetration of the stratum corneum and dilution due to diffusion. Likewise, the concentration of 5-HT<sub>1B</sub>R agonist that attenuated nocifensive responses in the intraplantar model (2 mM) is considerably higher than the low topical concentrations (100-165  $\mu$ M) that had no significant anti-hypersensitivity/analgesic effect alone but displayed remarkable synergy with low concentrations of TRPM8 activators. Taken together these findings indicate that while 5-HT<sub>1B</sub>R agonists have the advantageous effect of amplifying the anti-hypersensitivity/analgesic effects of low levels of TRPM8 activator, presumably acting on non-nociceptive afferents, they either do not affect or attenuate the unfavourable, potentially nociceptor-associated effects (“wet dog shakes” or intraplantar nocifensive response, respectively) elicited by direct administration of very high icilin concentrations. This profile emphasises the therapeutic potential of co-administration of low levels of TRPM8 activators and 5-HT<sub>1B</sub>R agonists in the treatment of chronic pain, especially via the topical route.

Analgesic actions of triptans alone have been reported but these generally appear to be limited to supraspinal levels; for example, following systemic administration, triptans attenuate nociceptive responses of single trigeminal but not spinal dorsal horn neurons (Cumberbatch et al., 1998). Triptan attenuation of non-cranial inflammatory and capsaicin-induced hyperalgesia has been described however, with evidence for both central and peripheral sites of action (Loyd et al., 2012; Nikai et al., 2008) but this displays a predominantly 5-HT<sub>1D</sub>-like profile, although 5-HT<sub>1B</sub>R(s) may contribute (Godinez-Chaparro et al., 2013). The actions of 5-HT<sub>1B/1D</sub> agents here display a distinct 5-HT<sub>1B</sub>R rather than 5-HT<sub>1D</sub>R profile and are seen exclusively in co-operation with TRPM8. Triptans are important clinically in the treatment of migraine where their target 5-HT<sub>1B/1D</sub>Rs, present on meningeal afferents are thought to play a key role in the mechanism of action (Levy et al., 2004), although receptors at other peripheral and central locations may also be important. Interestingly, recent genome-wide association studies have implicated a single nucleotide polymorphism close to the TRPM8 gene as a genetic risk factor for migraine (Chasman et al., 2011; Esserlind et al., 2013). In addition, a preliminary clinical trial has reported the TRPM8 agonist menthol to be efficacious in the treatment of migraine (Borhani Haghighi et al., 2010). These reports raise the possibility that TRPM8:5-HT<sub>1B</sub>R interactions, similar to those described here in somatosensory afferents, might also occur in cerebrovascular afferents and in support of this hypothesis we observed co-expression of 5-HT<sub>1B</sub>R in around 80% of TRPM8-expressing cells in trigeminal ganglion.

In summary we show here, in cell models, in *ex vivo* synaptic preparations and *in vivo*, that TRPM8 and the 5-HT<sub>1B</sub>R interact directly in a novel signalling complex that incorporates PLD1, which mediates the facilitatory influence of the 5-HT<sub>1B</sub>R on TRPM8 by means of its product phosphatidic acid acting on PIP 5-kinase to increase production of PIP<sub>2</sub>, an allosteric facilitator of the TRPM8 channel. This amplification of TRPM8 action by the 5-HT<sub>1B</sub>R is important in TRPM8 reversal of pain state-induced synaptic hyper-responsiveness at both spinal and supraspinal levels and in the analgesic actions that TRPM8 activators exert over chronic pain. The observation that a 5-HT<sub>1B</sub>R antagonist can attenuate anti-hypersensitivity/analgesic effects of slightly higher levels of TRPM8 activator alone suggests that there may be some tonic activation of the 5-HT<sub>1B</sub>R component of this complex in chronic pain states. Overall, these findings suggest that combination therapy with TRPM8 activators and 5-HT<sub>1B</sub>R agonists (including via a topical route) could have the potential to provide more efficacious analgesia in the treatment of chronic pain states.

### **Conflict of interest**

The authors declare they have no conflict of interest.

### **Author contributions**



S.F-W. and R.M. conceived and designed the study; I.V-F., L.S., H.J., J.C., A.A., H.G., R.R., P.H., B.T., R.M. and S. F-W. performed research and analyzed the data; S.F-W. and R.M. wrote the paper.

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## Figure Legends

**Fig. 1.** Facilitation of TRPM8-mediated  $\text{Ca}^{2+}$  elevation responses by 5-HT<sub>1B</sub>R activation and co-expression of TRPM8/5-HT<sub>1B</sub>R in DRG cells. (A) Single cultured DRG cells were loaded with Fura-2/AM and their 340/380 nm fluorescence ratio was measured every 5-s through consecutive recording periods. Cells were initially superfused with 2.5  $\mu\text{M}$  icilin to identify responsive cells (~10% cells), then after washout and recovery, a second stimulus was given. The traces show typical examples of subsequent responses to 2.5  $\mu\text{M}$  icilin (n=12), 3  $\mu\text{M}$  CP 94253 (n=4) or the combination (n=19), taken from three independent preparations. Examples of either oscillatory or stable responses were seen in each case but no differences were noted in the way they responded to pharmacological agents. The bar chart shows mean  $\pm$  SEM values for integrated area under the curve (AUC) of second responses normalised to initial icilin alone responses. \* indicates a significantly greater second response (with icilin plus CP 94253) compared to the first icilin alone response (p=0.0059, Wilcoxon test). Second responses to icilin alone were not significantly different from first responses and CP 94253 alone did not increase  $\text{Ca}^{2+}$  fluorescence. (B) Dual label immunofluorescence confocal images of lumbar (L4-6) DRG cells labelled for 5-HT<sub>1B</sub>R (green) and TRPM8 (red). Only around 10% of cells, which were small in size, were positive for TRPM8, whereas a larger number of cells of different sizes were positive for the 5-HT<sub>1B</sub>R. Most TRPM8-positive cells were also positive for the 5-HT<sub>1B</sub>R (71%, 87% and 79% in naïve, CCI and CFA animals, respectively, with cell counts of  $53 \pm 7$ , mean  $\pm$  SEM, from at least 3 separate animals in each case). Scale bar 20  $\mu\text{m}$ . The representative images were from an animal 24 h following CFA.

**Fig. 2.** Facilitation of TRPM8-mediated  $\text{Ca}^{2+}$  fluorescence responses by 5-HT<sub>1B</sub>R activation in the HEK 293 cell model. Stable TRPM8-expressing HEK 293 cells were transiently transfected with 5-HT<sub>1B</sub>R, loaded with Calcium 4 dye and responses measured by fluorometric plate reader (A) Shows acute time-course responses with readings taken every 5 s and drugs added at the arrow (means  $\pm$  SEM, n = 5). Icilin (0.7  $\mu\text{M}$ , ●, ici) caused a significant increase (\*p<0.01 compared to CP 94253 (2.5  $\mu\text{M}$ , ■, CP), which had no discernible effect alone. The combination of icilin plus CP 94253 (▲, ici CP) significantly amplified the effect seen with icilin († p<0.01) and this increment was reversed in the presence of the selective 5-HT<sub>1B</sub>R antagonist, SB 224289 (5  $\mu\text{M}$ , ▼, ici CP SB), ‡ p<0.01. Statistical analysis was by Two-Way ANOVA with Bonferroni's *post hoc* test. (B) Shows concentration-response curves to icilin in the absence (●, control) or presence (▲, CP) of 2.5  $\mu\text{M}$  CP 94253 (means  $\pm$  SEM, n = 6). CP 94253 caused significant increases in responses at individual icilin concentrations († p<0.05), but no change in maximal response (Two-Way ANOVA with Bonferroni's *post hoc* test) and a significant 3.32-fold reduction in EC<sub>50</sub> (Extra Sum of Squares F Test, see Table 1).



**Fig. 3.** Physical association of 5-HT<sub>1B</sub>R and TRPM8 in HEK 293 cells and *in vivo*. TRPM8-expressing HEK 293 cells transiently transfected with FLAG-tagged 5-HT<sub>1B</sub>R, or spinal cord segments L4-6 were solubilised and analysed by immunoprecipitation/immunoblot to identify interacting proteins, pointed out by arrows. (A) In transfected HEK 293 cells, TRPM8 pulldown but not non-immune IgG captured TRPM8 (~115 kDa, left panel) and also FLAG-5-HT<sub>1B</sub>R (~42 kDa, right panel) with only minor non-specific bands evident. (B) In HEK 293 cells, FLAG pulldown but not non-immune IgG captured FLAG-5-HT<sub>1B</sub>R (~42 kDa, left panel) and also TRPM8 (~115 kDa, right panel). A larger unidentified band at 95 kDa, potentially a receptor dimer, was seen in the FLAG antibody blot. (C) In spinal cord lysates, 5-HT<sub>1B</sub>R pulldown but only minimally non-immune IgG captured native 5-HT<sub>1B</sub>R (~42 kDa, left panel) and also TRPM8 (~115 kDa, right panel). In the 5-HT<sub>1B</sub>R blot non-specific bands were seen around 25 kDa, which were thought to reflect reagent light chain IgG. In the TRPM8 blot a number of non-specific bands were seen especially around 50 kDa and 25 kDa, which were thought to reflect reagent IgG, as well as an unidentified band around 85 kDa. The same membranes were re-probed for both antigens in each case. Similar observations were made in at least 3 separate experiments.

**Fig. 4.** Signalling events underlying functional interaction between 5-HT<sub>1B</sub>R and TRPM8. Stable TRPM8-expressing HEK 293 cells transiently transfected with the 5-HT<sub>1B</sub>R were loaded with Calcium 4. Responses to submaximal concentration of icilin with/without CP 94253 were measured by fluorometric plate reader in the presence of signalling pathway modulators (A, B) or transfected negative mutant PLD1 or PLD2 constructs (C). In (A) and (C) white columns indicate icilin alone (0.25 μM) while grey columns indicate icilin (0.25 μM) plus CP 94253 (2 μM). Signalling modulators were:- the selective PLD inhibitors VU0155069 (VU1, 0.5 μM), halopemide (Hal, 4 μM), raloxifene (Ral, 25 μM), calphostin C (Cal C, 2.5 μM); the selective PKC inhibitor bisindolylmaleimide 1 (BIM1, 2.5 μM); the phosphatidylinositol kinase inhibitor wortmannin (Wor, 30 μM); the PLC activator m-3M3FBS (m3M3, 2.5 μM); the PLC-β inhibitor U-73122 (U73, 10 μM). Values are means ± SEM, n = 5. Data were analysed by One-Way ANOVA with Tukey's *post hoc* test. \*\*\* indicates responses significantly greater than corresponding icilin alone (p<0.001). ††† indicates responses significantly less than icilin alone or icilin plus CP 94253 (p<0.001). ‡ indicates responses significantly greater than icilin plus CP 94253 (p<0.05). In (B) cells were stimulated with 0.25 μM icilin or 0.25 μM icilin plus 2.5 μM CP 94253 in the presence of increasing concentrations of PLD1-selective inhibitor VU0359595 (● and ▼ respectively), or PLD2-selective inhibitor VU0285655-1 (■ and ▲ respectively). Values are means ± SEM (n=4). Responses to icilin plus CP 94253 (but not icilin alone) were significantly reduced in the presence of VU0359595 (\* and \*\* indicate p<0.05 and p<0.01 respectively; One-Way ANOVA with Dunnett's *post-hoc* test), whereas VU0285655-1 had no significant effect. The mean IC<sub>50</sub> [95% confidence interval] for VU0359595 was 14.1[7.8/25.7] nM. In (C) cells were co-transfected with catalytically inactive constructs of PLD1 ([K898R]PLD1, Δ PLD1), PLD2 ([K758R]PLD2, Δ PLD2) or empty vector. \*\*\* Indicates responses

significantly greater than corresponding icilin alone ( $p < 0.001$ ), ††† indicates responses significantly less than icilin plus CP 94253 groups transfected with empty vector or mutant PLD2 construct ( $p < 0.001$ , One-Way ANOVA with Tukey's *post hoc* test).

**Fig. 5.** Involvement of PIP 5-kinase and PIP<sub>2</sub> in 5-HT<sub>1B</sub>R facilitation of TRPM8 function. (A) Ca<sup>2+</sup> fluorescence responses to 0.3 μM icilin (white columns) or 0.3 μM icilin plus 2.5 μM CP 94253 (grey columns) were recorded from TRPM8/5-HT<sub>1B</sub>R-expressing HEK 293 cells that had been co-transfected with wild-type PIP 5-kinase, [YKRR209VAEE, R215D]PIP 5-kinase (a phosphatidic acid-unresponsive mutant) or empty vector. Values are means ± SEM (n=6). Responses to icilin were significantly facilitated by CP 94253 in cells that had been transfected with empty vector (\*\*\*\*  $p < 0.001$ ) or wild-type PIP 5-kinase (\*\*  $p < 0.01$ ) but not with mutant PIP 5-kinase, whereas responses to icilin plus CP 94253 were significantly reduced by mutant PIP5-kinase compared to the other conditions (††  $p < 0.01$ , One-Way ANOVA with Tukey's *post hoc* test in each case). (B) The time course of CP 94253 (2.5 μM)-induced changes in cellular PIP<sub>2</sub> levels was measured by PIP<sub>2</sub> mass ELISA in control TRPM8-expressing HEK 293 cells (■) and cells transfected with 5-HT<sub>1B</sub>R, either in the absence (▲) or the presence (▼) of the PLD1 inhibitor VU0359595 (100 nM). Values are means ± SEM (n=4). In 5-HT<sub>1B</sub>R-transfected cells, CP 94253 caused a significant but transient increase in PIP<sub>2</sub> levels (\*  $p < 0.05$  and \*\*  $p < 0.01$ ), while these increases were significantly reduced by VU0359595 (†  $p < 0.05$  and ††  $p < 0.01$ , respectively; Two-Way ANOVA with Bonferroni's *post hoc* test in each case). (C) The concentration-dependence of icilin-induced Ca<sup>2+</sup> fluorescence responses was measured in HEK 293 cells transfected with the 5-HT<sub>1B</sub>R and either wild-type (rat) TRPM8 (●) or [R1008Q] TRPM8 mutant, which displays impaired activation by PIP<sub>2</sub> (■). Values are means ± SEM (n=6). The mutant channel showed a reduced maximal response ( $p = 0.0003$  at 200 μM icilin, Student's t-test) together with a reduced potency of activation, EC<sub>50</sub> [95% confidence interval] of 9.4[8.1/11.0] μM for the mutant compared to 0.17[0.15/0.19] μM for the wild-type. The mutant TRPM8 channel response to 10 μM icilin (~EC<sub>50</sub>; ▼) was not significantly altered in the presence of 2.5 μM CP 94253, whereas the wild-type channel response to 0.3 μM icilin (~EC<sub>50</sub>; ▲) was significantly increased (\*  $p < 0.05$ , Student's t-test).

**Fig. 6.** Physical association of PLD1 with both the 5-HT<sub>1B</sub>R and TRPM8 in HEK 293 cells and *in vivo*. TRPM8-expressing HEK 293 cells transiently transfected with FLAG-tagged 5-HT<sub>1B</sub>R and HA-tagged PLD1, or spinal cord segments L4-6, were solubilised and analysed by immunoprecipitation/immunoblot to identify interacting proteins, pointed out by arrows. (A) and (B) In transfected HEK 293 cells, TRPM8 pulldown but not non-immune IgG captured TRPM8 (~110 kDa, A left panel) and also HA-PLD1 (~95 kDa, A right panel). Correspondingly, HA pulldown but not non-immune IgG captured HA-PLD1 (~95 kDa, B left panel) and also TRPM8 (~110 kDa, B right panel). The same membranes were re-probed for both antigens in each case. (C) and (D) In HEK 293 cells FLAG pulldown but not non-immune IgG captured 5-HT<sub>1B</sub>R (~42 kDa, C left half-

panel) and also HA-PLD1 (~95 kDa, C right half-panel). Correspondingly, HA pulldown but not non-immune IgG captured HA-PLD1 (~95 kDa, D left half-panel) and also 5-HT<sub>1B</sub>R (~42 kDa, D right half-panel). The half-panels represent the same lanes of membrane cut into low and high molecular weight regions, which were separately probed for the two antigens. (E) In spinal cord lysates TRPM8 pulldown but only minimally non-immune IgG captured TRPM8 (~115 kDa, left panel), PLD1 (~95 kDa, centre panel) and 5-HT<sub>1B</sub>R (~42 kDa, right panel). A number of non-specific bands were seen especially around 50 kDa and 25 kDa, which were thought to represent reagent IgG as well as an unidentified band around 85 kDa. The blots were carried out on replicates of the same samples run on the same gel, with the membrane subsequently cut up to be separately probed for each antigen. In each case, similar observations were made in at least 3 separate experiments.

**Fig. 7.** TRPM8-induced reversal of pain state-induced hypersensitivity in spinal, or supraspinal, *ex vivo* synaptoneurosome preparations is potentiated by 5-HT<sub>1B</sub>R agonists. Synaptoneurosome preparations were prepared from lumbar spinal cord, somatosensory cortex or frontal cortex of rats injected 24 h previously with CFA into one hindpaw or controls. After loading with Calcium 4 in multiwell plates, Ca<sup>2+</sup> fluorescence responses were recorded in spinal cord to ACB (a combined stimulus of noxious agents: allyl isothiocyanate, 20 μM; capsaicin, 10 μM; bradykinin, 10 μM) and in somatosensory or frontal cortex to d,l-homocysteic acid (DLH, 6.25 μM). In each case responses were compared to maximal Ca<sup>2+</sup> fluorescence responses induced by ionomycin (5 μM). (A) shows an example of raw fluorescence records, taken from spinal cord synaptoneurosome ipsilateral to CFA with stimuli added at the start of the recording period and readings taken every 30-s over 4 min (means ± SEM, n=3). Ionomycin caused a marked increase in Ca<sup>2+</sup> fluorescence above basal (\*\* p<0.01), which was mimicked to approximately half of that extent by ACB (\* p<0.05, \*\* p<0.01). The ACB response was significantly reduced by the inhibitor of synaptic vesicle exocytosis, tetanus toxin (TeTx, 5 nM, 45 min) († p<0.05). Statistical analysis was by Two-Way ANOVA with Bonferroni's *post hoc* test. (B) shows responses to ACB in spinal cord, or DLH in cortical regions, normalised to corresponding ionomycin-induced increments in each case (means ± SEM, n=5-11). Responses were significantly amplified following CFA in ipsilateral (and to a lesser extent contralateral) spinal cord, somatosensory and frontal cortex (\* p<0.05, \*\* p<0.01; Two-Way ANOVA with Bonferroni's *post hoc* test). (C) shows results of similar experiments from rats in which drugs were applied topically to the hindpaws in a vehicle of 1.5% caprolactam in propan 1,2-diol under anaesthesia for 15 min. Vehicle treatment had no discernible effect. Mean ACB/DLH-evoked responses of synaptoneurosome through readings 2-7 were normalised to ionomycin responses, (means ± SEM, n=3-6). In each region CFA treatment significantly amplified ACB/DLH responses (\* p<0.05, \*\* p<0.01). While moderate topical concentrations of icilin (ici, 50 μM for spinal cord, 110 μM for cortex) or sumatriptan (sum, 100 μM for spinal cord, 165 μM for cortex) had no significant effect, in combination they caused robust inhibition of CFA-amplified responses (†† p<0.01). In spinal cord this effect was reversed by topical SB

224289 (120  $\mu\text{M}$ , †††  $p<0.01$ ) and mimicked by topical menthol (1mM) with sumatriptan (100  $\mu\text{M}$ , (††  $p<0.01$ ). Statistical analysis was by One-Way ANOVA with Tukey's *post hoc* test.

**Fig. 8.** Reversal of pain state-induced hypersensitivity by slightly more intense activation of TRPM8 alone is attenuated by 5-HT<sub>1B</sub>R antagonists. Synaptoneurosomes were prepared from ipsilateral spinal cord of CFA-injected/control rats treated with topical icilin with/without 5-HT<sub>1B/1D</sub>R or TRPM8 antagonists. Mean Ca<sup>2+</sup> fluorescence responses to ACB (n=4-7) were calculated as in Fig. 7. CFA caused significant amplification of ACB responses (\*  $p<0.05$ ). A higher concentration of icilin (ici, 200  $\mu\text{M}$ ) caused robust inhibition of CFA-amplified responses (††  $p<0.01$ ). The effect of icilin was significantly reversed by 5-HT<sub>1B</sub>R antagonists SB 224289 (120  $\mu\text{M}$ ) or GR 55562 (200  $\mu\text{M}$ ) and TRPM8 antagonist AMTB (200  $\mu\text{M}$ ), (†  $p<0.05$ , ††  $p<0.01$ ). The 5-HT<sub>1B</sub>R antagonist BRL 15572 (150  $\mu\text{M}$ ) was ineffective. Icilin, SB 224289 and AMTB had no discernible effect on ACB responses from naïve animals. Statistical analysis was by One-Way ANOVA with Dunnett's *post hoc* test.

**Fig. 9.** TRPM8-mediated reversal of pain state-induced hypersensitivity in reflex withdrawal behaviours *in vivo* is modulated by 5-HT<sub>1B</sub>R agents. In the CFA model of inflammatory pain (A-C) or the CCI model of neuropathic pain (D) TRPM8/5-HT<sub>1B</sub>R agents were delivered topically in propan 1,2-diol with 1.5% caprolactam, under 15 min anaesthesia or by acute intrathecal injection in saline with 0.5% dimethylformamide. Paw withdrawal responses to noxious radiant heat (Hargreaves' test) were measured (means  $\pm$  SEM, n=3-6) with white symbols showing responses ipsilateral to injury and black symbols contralateral. Vehicles alone had no discernible effect. Statistical analysis of TRPM8/5-HT<sub>1B</sub>R agonist effects compared to baseline values was by One-Way ANOVA with Dunnett's *post hoc* test, while reversal of agonist effects by antagonist was assessed by Two-Way ANOVA with Bonferroni's *post hoc* test. (A) While low topical concentrations of icilin (ici, 50  $\mu\text{M}$ , ○) or sumatriptan (sum, 100  $\mu\text{M}$ , △) alone had no discernible effect, in combination (ici sum, □) they significantly attenuated ipsilateral hypersensitivity compared to baseline (\*  $p<0.05$ , \*\*  $p<0.01$ ). This effect was significantly reversed in the additional presence of SB 224289 (ici sum SB, 120  $\mu\text{M}$ , ▽), (†  $p<0.05$ , ††  $p<0.01$ ). (B) A higher topical concentration of icilin alone (ici, 200 $\mu\text{M}$ , □) significantly attenuated ipsilateral hypersensitivity compared to baseline (\*  $p<0.05$ ). This effect was significantly reversed in the additional presence of SB 224289 (ici SB, ▽), (†  $p<0.05$ ), whereas SB alone (SB, ◇) had no effect. (C) While low topical concentrations of menthol (men, 1mM, ○) or sumatriptan alone (Fig. 9A) had no discernible effect, in combination (men sum, □) they significantly attenuated ipsilateral hypersensitivity compared to baseline (\*  $p<0.05$ , \*\*  $p<0.01$ ). (D) While low intrathecal doses of icilin (ici, 2.5 nmole, ○) or CP 94253 (CP, 2.5 nmole, △) had no significant effect, in combination (ici CP, □) they significantly attenuated ipsilateral hypersensitivity compared to baseline (\*  $p<0.05$ ). No significant changes in contralateral responses were observed during any of the experiments.

**Table 1: Effects of 5-HT receptor subtypes on Ca<sup>2+</sup> fluorescence responses elicited by different TRP channels in HEK 293 cells**

TRP channel (activator)	5-HT receptor (agonist, concentration)	EC <sub>50</sub> (μM) for TRP channel activator (mean [95% confidence interval])		
		Control	+ 5-HTR agonist	Mean fold change in potency
TRPM8 (icilin)	5-HT <sub>1B</sub> (CP 94253, 2.5 μM)	0.313 [0.289/0.339]	0.094 [0.080/0.118]	x 3.32 **
	5-HT <sub>1B</sub> (sumatriptan, 2 μM)	0.288 [0.258/0.320]	0.099 [0.076/0.129]	x 2.90 **
	5-HT <sub>1A</sub> ((R)-(+)-8-OH DPAT, 2 μM)	0.298 [0.267/0.334]	0.275 [0.238/0.317]	x 1.08 <sup>ns</sup>
TRPV1 (capsaicin)	5-HT <sub>1B</sub> (CP 94253, 2.5 μM)	0.119 [0.111/0.129]	0.143 [0.106/0.193]	x 0.83 <sup>ns</sup>
	5-HT <sub>2A</sub> (TCB-2, 2 μM)	0.128 [0.119/0.138]	0.062 [0.052/0.075]	x 2.06 **
TRPA1 (AITC)	5-HT <sub>1B</sub> (CP 94253, 2.5 μM)	2.35 [2.02/2.74]	2.16 [1.97/2.36]	x 1.09 <sup>ns</sup>

HEK 293 cell lines stably expressing TRPM8, TRPV1 or TRPA1 were transiently transfected with different 5-HT receptor subtypes and Ca<sup>2+</sup> fluorescence responses to selective channel activators were recorded. Concentration-response curves were measured in the absence or presence of selective 5-HTR agonists, as in Fig. 2B (means ± SEM, n=6). None of the curves showed any change in maximal responses but the potency of icilin at TRPM8 was significantly increased by 5-HT<sub>1B</sub>R activation and that of capsaicin at TRPV1 was increased by activation of 5-HT<sub>2A</sub>R. The statistical significance of changes in EC<sub>50</sub> was determined by Extra Sum of Squares F Test, comparing a separate EC<sub>50</sub> model to a combined global fit (\*\* p<0.01, ns p>0.05). The 2.90-fold increased potency of icilin at TRPM8 caused by sumatriptan was reversed by the 5-HT<sub>1B</sub>R antagonist SB 224289 (5 μM), leaving only a 1.16-fold shift in apparent potency of icilin, which was not statistically significant.

Fig.1.

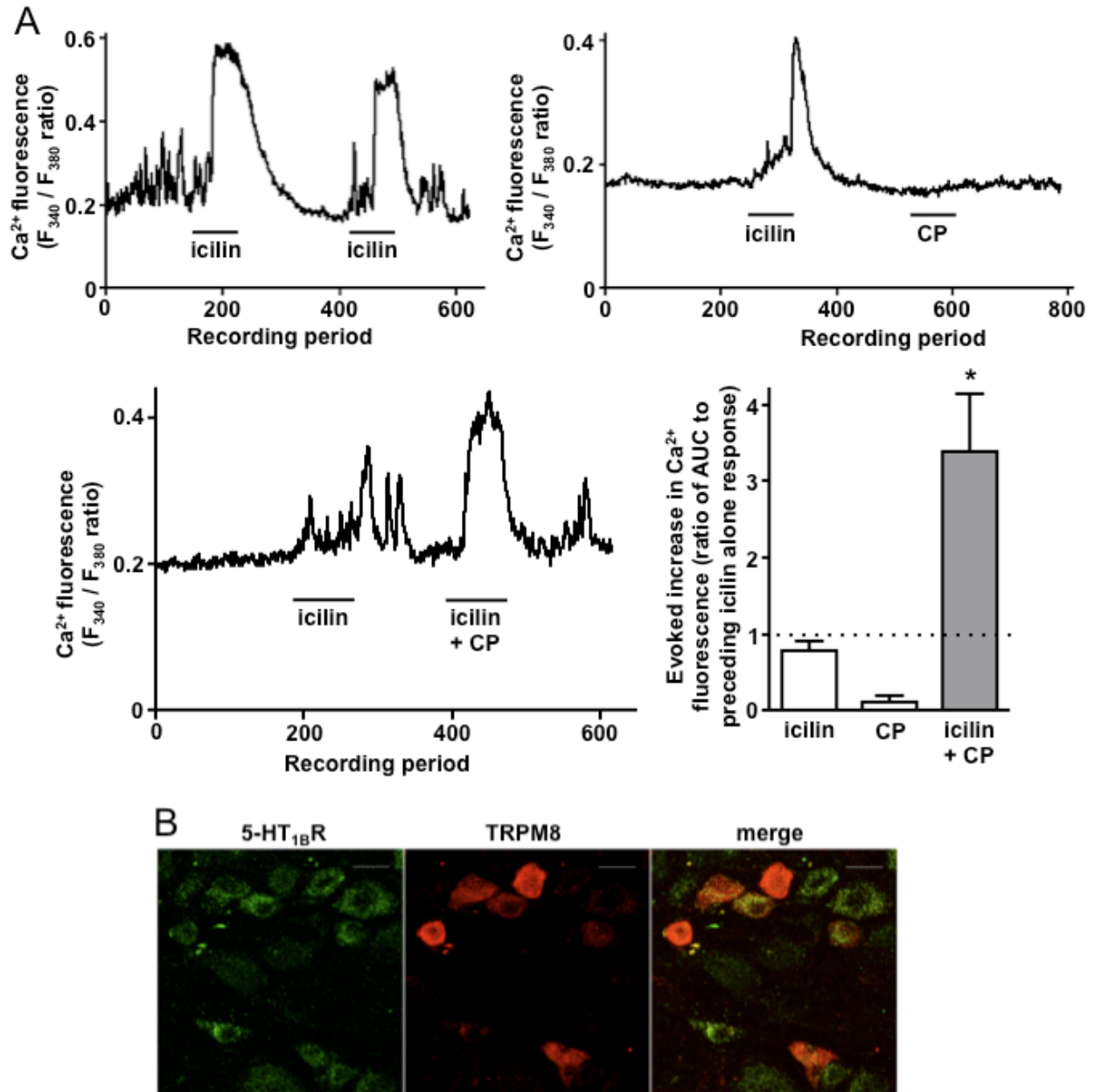


Fig.2.

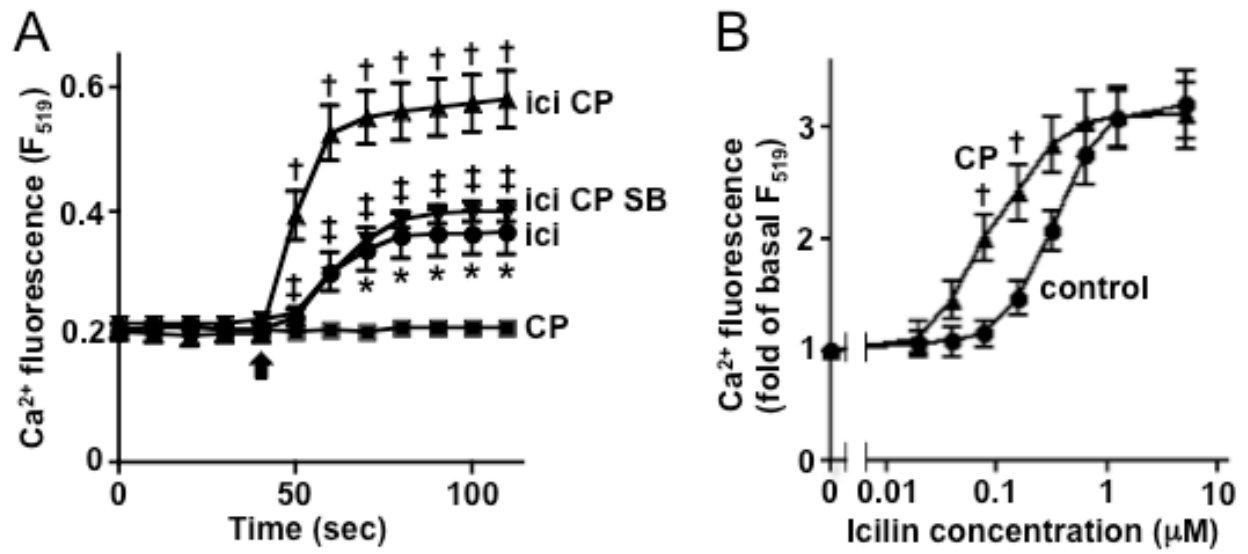


Fig.3.

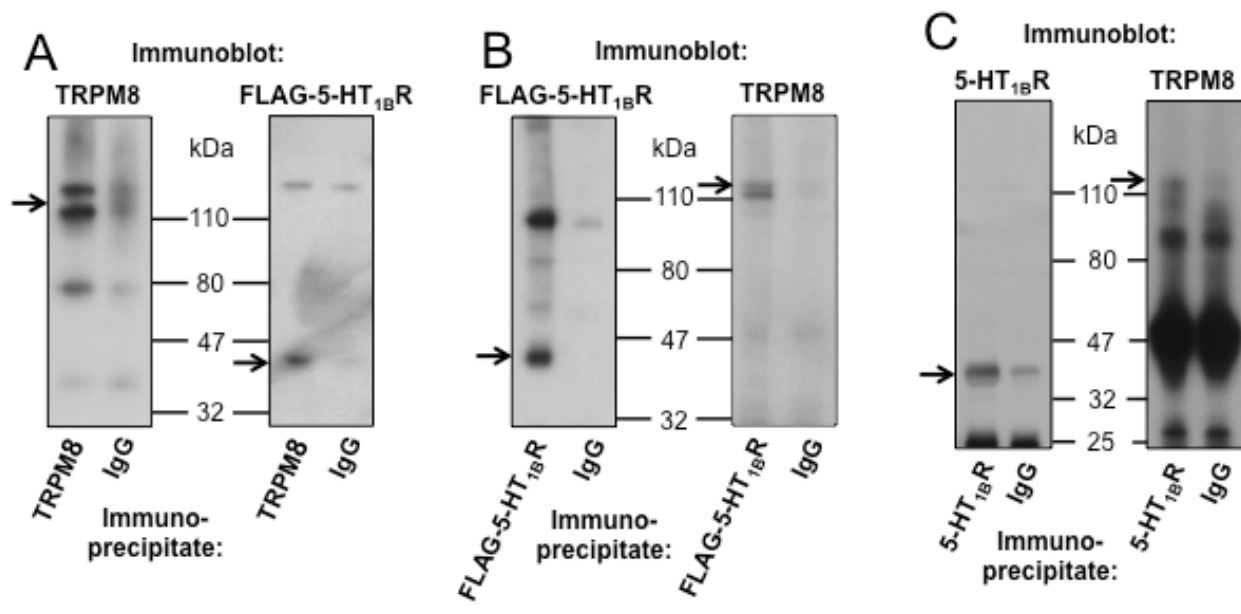




Fig.4.

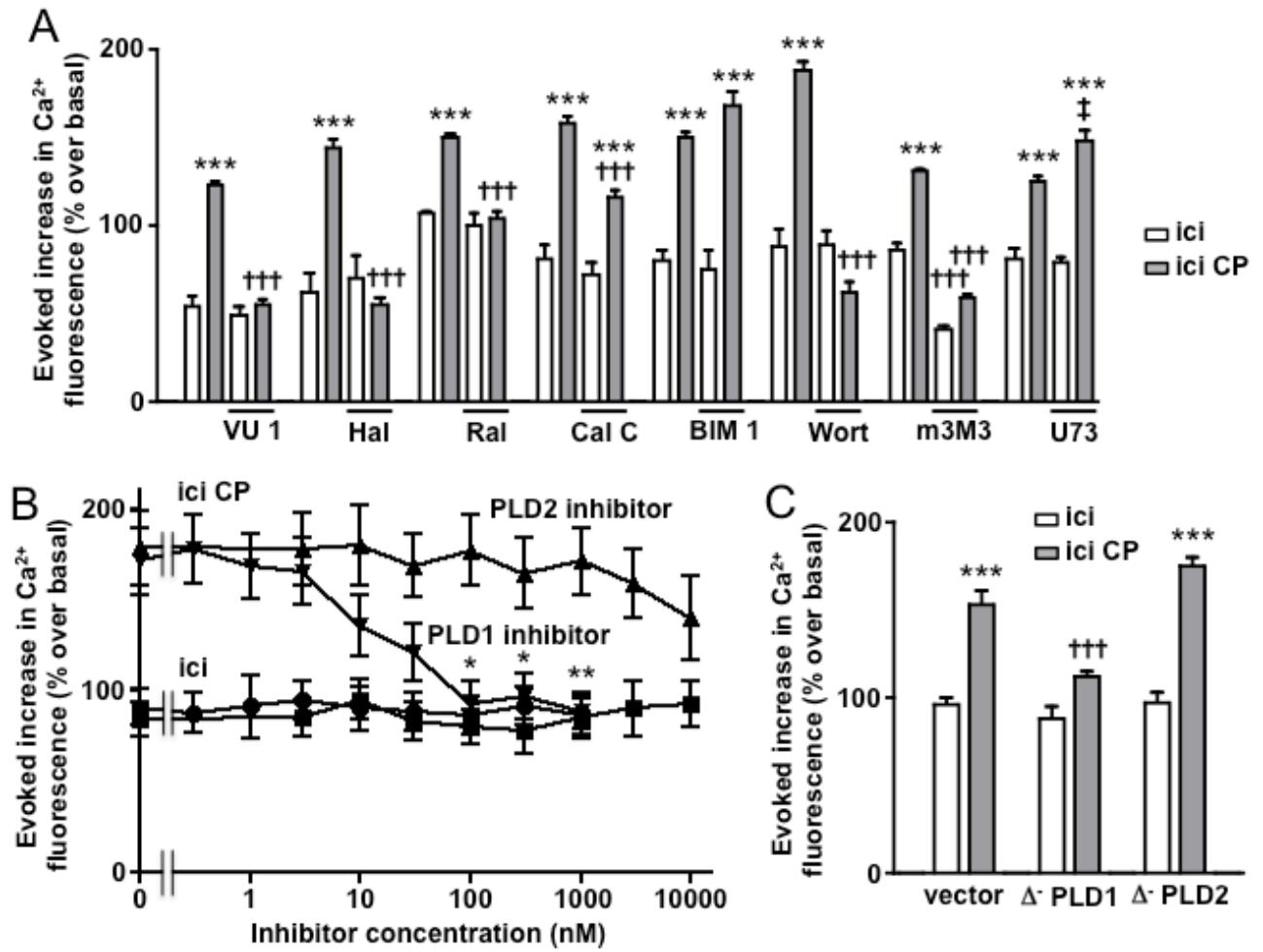


Fig.5.

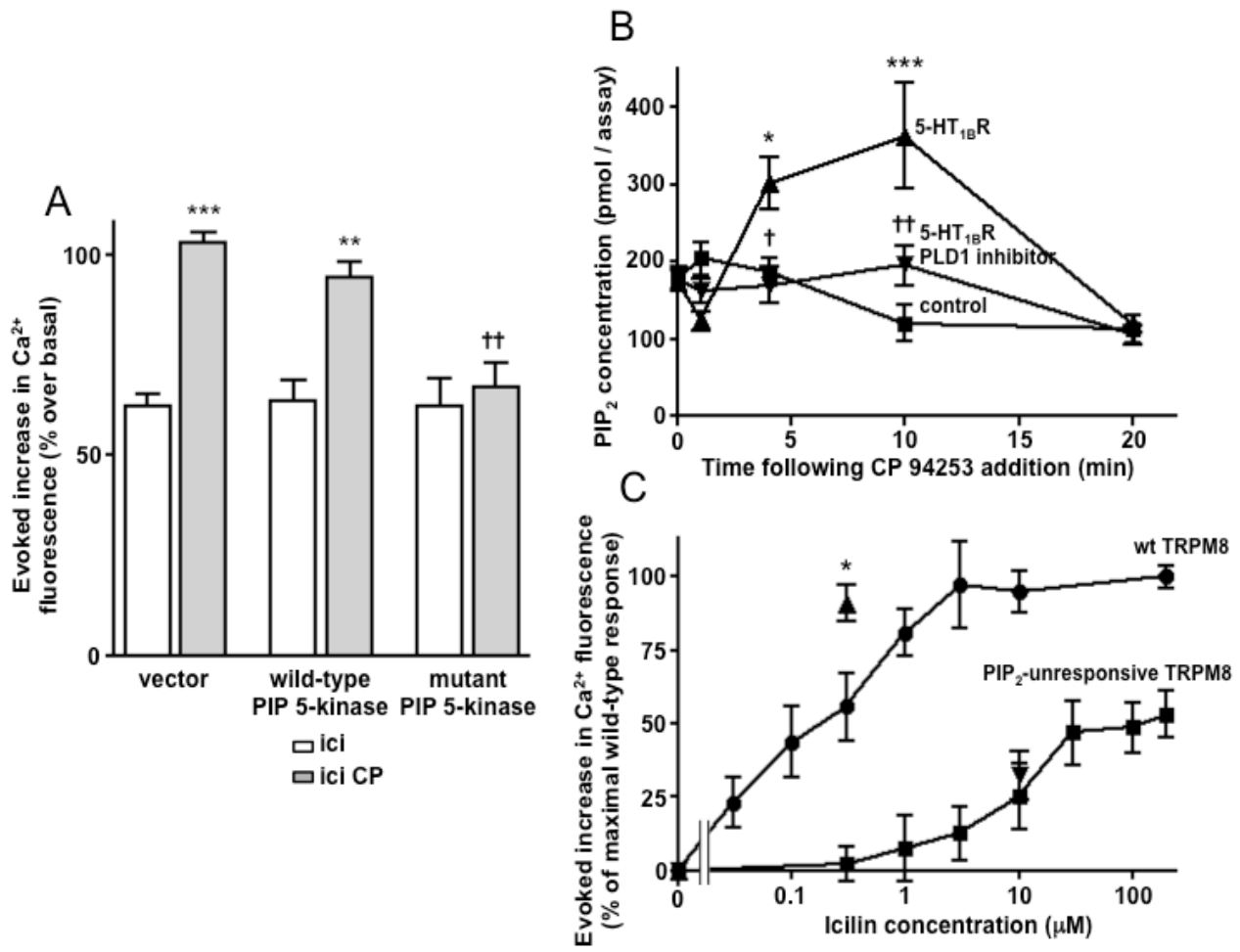


Fig.6.

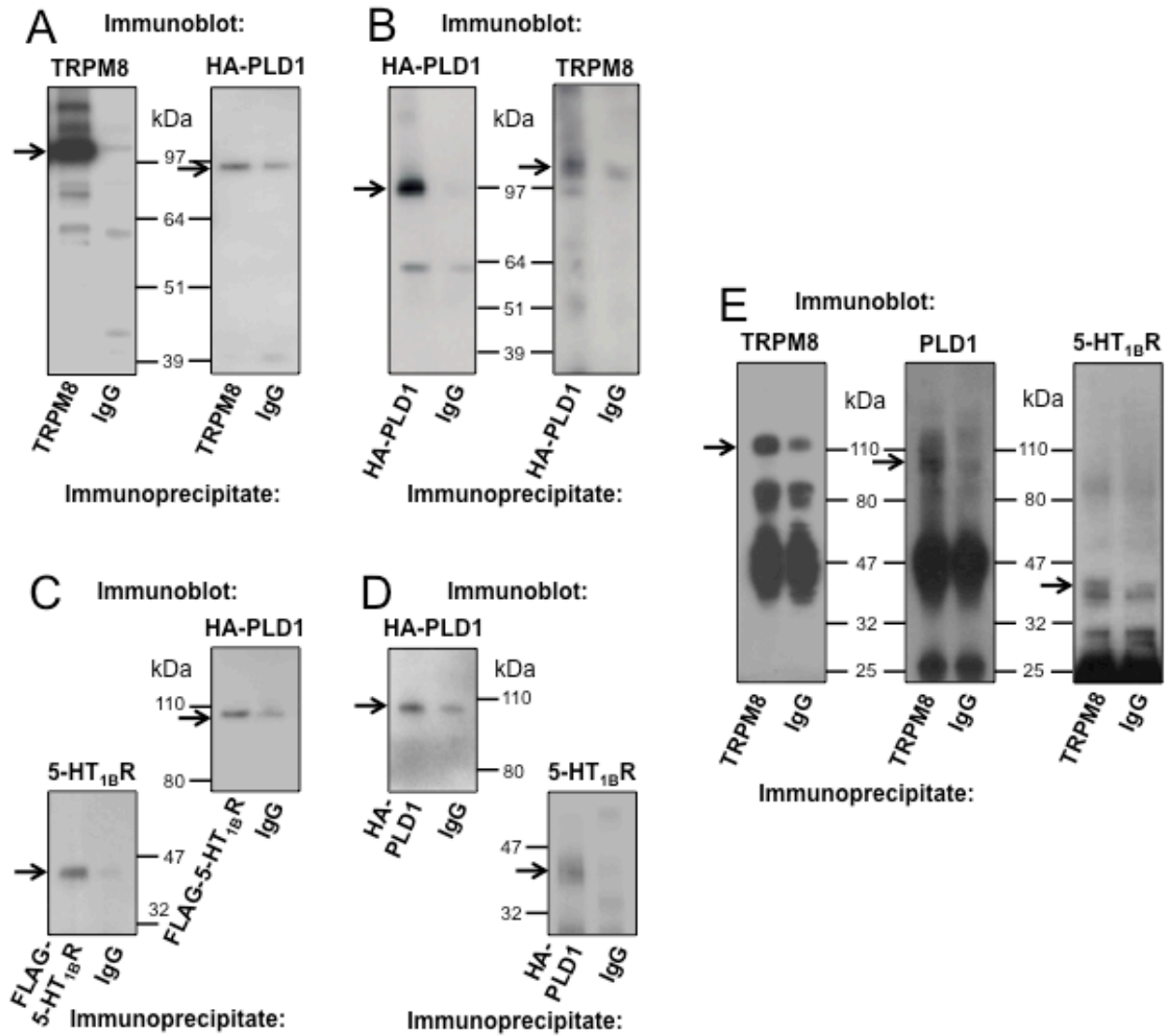


Fig.7.

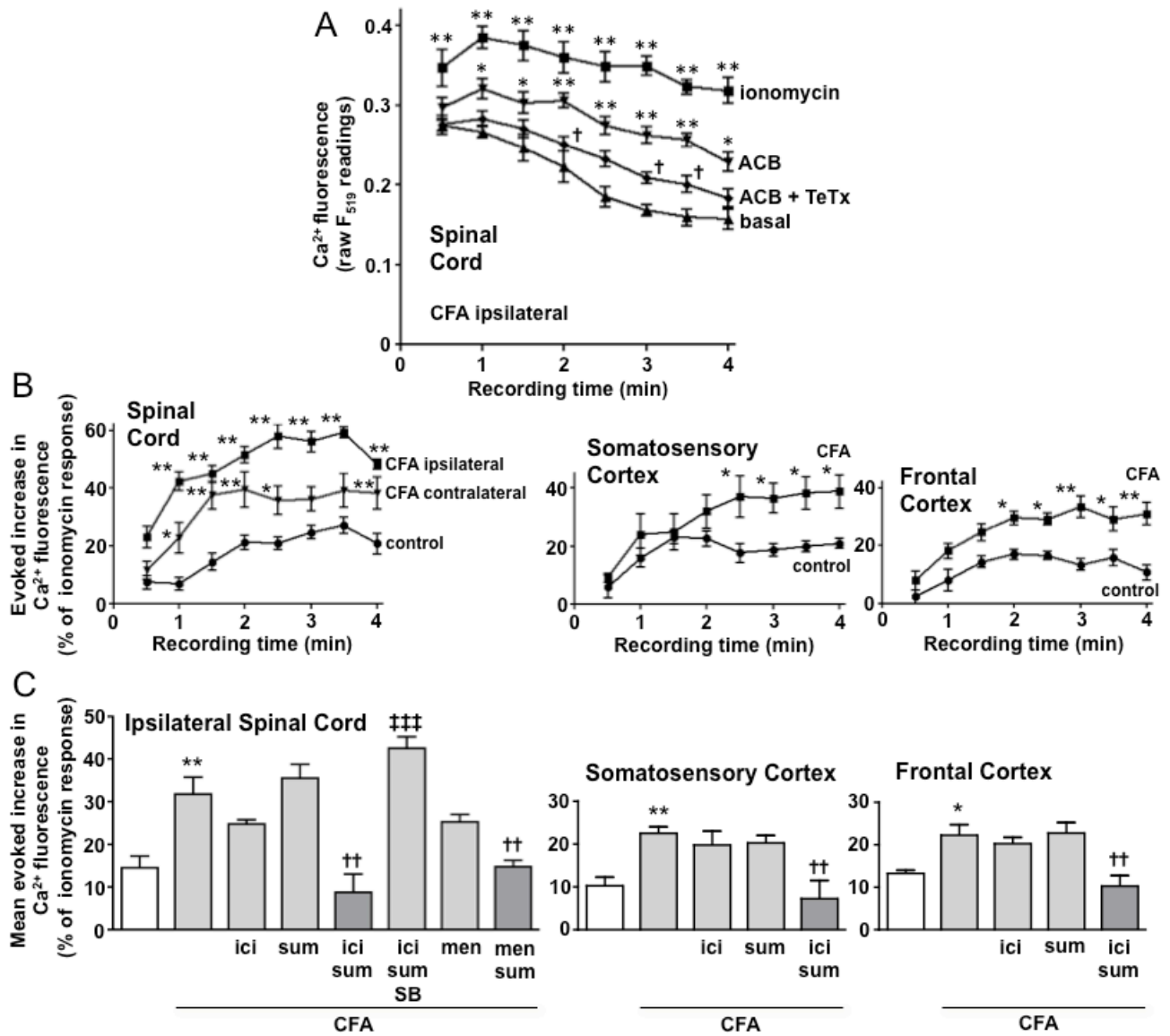


Fig.8.

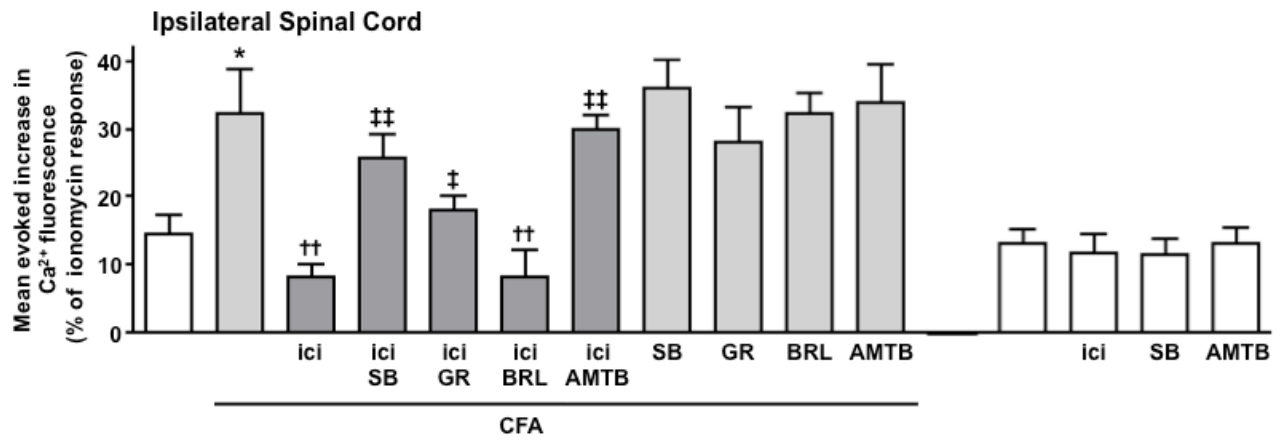


Fig.9.

