Differential chemosensory function and receptor expression of splanchnic and pelvic colonic afferents in mice

Stuart M. Brierley[1,4], R. Carter, W. Jones III[2,3], Linjing Xu[2], David R. Robinson[6,7], Gareth A. Hicks[7], G. F. Gebhart[2] and L. Ashley Blackshaw[1,4,5]

[1]*Nerve–Gut Research Laboratory, Department of Gastroenterology, Hepatology and General Medicine, Royal Adelaide Hospital, Adelaide, South Australia, 5000, Australia*
[2]*Department of Pharmacology and* [3]*Medical Scientist Training Program, University of Iowa, Iowa City, IA 52242, USA*
[4]*Discipline of Physiology, School of Molecular and Biomedical Sciences and* [5]*Department of Medicine, University of Adelaide, Adelaide, South Australia, 5005, Australia*
[6]*Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1 PD, UK*
[7]*Neurology and GI Centre of Excellence for Drug Discovery, GlaxoSmithKline Research & Development Limited, New Frontiers Science Park, Third Avenue, Harlow, Essex CM19 5AW, UK*

Lumbar splanchnic (LSN) and sacral pelvic (PN) nerves convey different mechanosensory information from the colon to the spinal cord. Here we determined whether these pathways also differ in their chemosensitivity and receptor expression. Using an *in vitro* mouse colon preparation, individual primary afferents were tested with selective P2X and transient receptor potential vanilloid receptor 1 (TRPV1) receptor ligands. Afferent cell bodies in thoracolumbar and lumbosacral dorsal root ganglia (DRG) were retrogradely labelled from the colon and analysed for $P2X_3$- and TRPV1-like immunoreactivity (LI). Forty per cent of LSN afferents responded to α,β-methylene adenosine 5′-triphosphate (α,β-meATP; 1 mM), an effect that was concentration dependent and reversed by the P2X antagonist pyridoxyl5-phosphate 6-azophenyl-2′,4′-disulphonic acid (PPADS) (100 μM). Significantly fewer PN afferents (7%) responded to α,β-meATP. Correspondingly, 36% of colonic thoracolumbar DRG neurones exhibited $P2X_3$-LI compared with only 19% of colonic lumbosacral neurones. Capsaicin (3 μM) excited 61% of LSN afferents and 47% of PN afferents; 82% of thoracolumbar and 50% of lumbosacral colonic DRG neurones displayed TRPV1-LI. Mechanically insensitive afferents were recruited by α,β-meATP or capsaicin, and were almost exclusive to the LSN. Capsaicin-responsive LSN afferents displayed marked mechanical desensitization after responding to capsaicin, which did not occur in capsaicin-responsive PN afferents. Therefore, colonic LSN and PN pathways differ in their chemosensitivity to known noxious stimuli and their corresponding receptor expression. As these pathways relay information that may relate to symptoms in functional gastrointestinal disease, these results may have implications for the efficacy of therapies targeting receptor modulation.

(Resubmitted 2 May 2005; accepted 6 June 2005; first published online 9 June 2005)
Corresponding author S. M. Brierley: Nerve-Gut Research Laboratory, Level 1 Hanson Institute, Frome Road, Adelaide, South Australia, Australia. Email: stuart.brierley@adelaide.edu.au

One of the most common pain syndromes seen in the clinic is irritable bowel syndrome (IBS) (Camilleri, 2001). Enhanced visceral sensation and abdominal pain are hallmarks of this disease (Ritchie, 1973; Naliboff *et al.* 1997; Camilleri, 2001), and identification of drugs that can normalize symptom occurrence has been considered critical in the search for effective IBS therapies (Mayer, 2001). Pain is the symptom that affects quality of life the most (Gralnek *et al.* 2000), therefore it is a logical approach to investigate receptors that have been strongly implicated in pain in other systems. Thus the $P2X_3$ and transient receptor potential vanilloid receptor 1 (TRPV1) (formerly known as VR1) receptors are prime candidates for study.

$P2X_3$ is a member of the P2X purinoceptor family of ATP-gated ion channels (North, 2002, 2004), while TRPV1 belongs to the transient receptor potential (TRP) channel family and is activated by heat, protons and vanilloid ligands such as capsaicin, the pungent ingredient in chillies

 DOI: 10.1113/jphysiol.2005.089714

(Caterina *et al.* 1997; Caterina & Julius, 2001). Both of these receptors are predominantly expressed in small-diameter primary afferent neurones (C-fibres) (Chen *et al.* 1995; Bradbury *et al.* 1998; Vulchanova *et al.* 1998; Guo *et al.* 1999; Caterina *et al.* 2000; Davis *et al.* 2000). Both $P2X_3$ and TRPV1 have been strongly implicated in nociception and pain (Caterina *et al.* 1997, 2000; Davis *et al.* 2000; Caterina & Julius, 2001; North, 2002, 2004), although these studies have been performed in levels of dorsal root ganglia that are devoid of colonic innervation (Ness & Gebhart, 1988; Robinson *et al.* 2004). Notably, from a clinical gastroenterology point of view, expression of both $P2X_3$ and TRPV1 is increased in colonic nerve fibres of patients with inflammatory bowel disease (Yiangou *et al.* 2001*a*,*b*; Geppetti & Trevisani, 2004).

Activation of $P2X_3$ and TRPV1 receptors, by the selective agonists α,β-methylene adenosine 5′-triphosphate (α,β-meATP) and capsaicin, respectively, will excite gastrointestinal afferents (Kirkup *et al.* 1999; Page *et al.* 2002; Wynn *et al.* 2003, 2004; Zagorodnyuk *et al.* 2003), including colonic afferents (Lynn & Blackshaw, 1999; Wynn *et al.* 2003). However, the extrinsic spinal innervation of the colon makes the issue more complex as sensory information from the distal colon and rectum travels to the central nervous system through two distinct anatomical pathways: the lumbar splanchnic nerves (LSN), which terminate in the thoracolumbar spinal cord, and the paired sacral pelvic nerves (PN), which terminate in the lumbosacral spinal cord. Mechanosensitive afferents have been identified in both of these nerve supplies (Blumberg *et al.* 1983; Haupt *et al.* 1983; Janig & Koltzenburg, 1991; Sengupta & Gebhart, 1994; Lynn & Blackshaw, 1999; Hicks *et al.* 2002; Brierley *et al.* 2004). Recent evidence suggests that LSN and PN afferents carry markedly different types of information, indicating that these pathways are distinct, both anatomically and functionally, and likely to serve unique roles in the detection of mechanical stimuli in the distal colon (Brierley *et al.* 2004). Despite considerable investigation into the responses of afferents from either pathway to various chemical stimuli, including these agonists (Kirkup *et al.* 2001; Blackshaw & Gebhart, 2002), to date the roles of $P2X_3$ and TRPV1 have not been investigated together in an individual pathway, nor has a direct comparison of the chemosensory properties and receptor expression displayed by these two pathways been made in the same species. This is a fundamental gap in our understanding, as sensitization of mechanoreceptors and perhaps chemoreceptors may give rise to the abdominal pain and discomfort experienced by IBS patients (Mayer & Gebhart, 1993). To date no study has made a direct comparison of the chemosensitivity of splanchnic and pelvic afferents innervating the colon in any species, nor has there been a direct comparison of $P2X_3$ and TRPV1 receptor expression between identified thoracolumbar and lumbosacral colonic neurones. Thus the present study had two purposes: (1) to address the lack of knowledge concerning the comparative chemosensitivity of LSN and PN colonic afferents from the distal colon by using a novel *in vitro* electrophysiological approach; and (2) to compare directly receptor expression of retrogradely labelled LSN and PN colonic neurones in thoracolumbar and lumbosacral dorsal root ganglia (DRG). Our data indicate differences in the chemosensitivity of afferents in the LSN and PN pathways, as more of the chemosensory afferents responding to α,β-meATP and capsaicin are found in the LSN pathway, and this is reflected in expression of $P2X_3$ and TRPV1 receptors by their cell bodies in the DRG.

Methods

All electrophysiological experiments were performed in accordance with the guidelines of the Animal Ethics Committees of the Institute for Medical and Veterinary Science and the University of Adelaide, Adelaide, Australia, and the Institutional Animal Care and Use Committee of The University of Iowa, Iowa City, Iowa, USA. All procedures in the UK were carried out in strict accordance with local, internal, and UK Home Office regulations (Animals (Scientific Procedures) Act 1986), and were performed under sterile conditions within a designated animal procedure room.

In vitro mouse colonic primary afferent preparations

Dissections were carried out in male and female mice (C57BL/6; 20–30 g) according to protocols described in detail previously (Brierley *et al.* 2004). Briefly, mice were killed by CO_2 inhalation and cervical dislocation, the colon (5–6 cm) and mesentery (containing the lumbar colonic nerves) removed intact with either the attached neurovascular bundle containing the inferior mesenteric ganglion (IMG) and LSN or in separate preparations with the major pelvic ganglion (MPG) and PN. The tissue was transferred to ice-cold Krebs solution and, following further dissection, the distal colon was opened longitudinally along the antimesenteric border to orientate lumbar colonic insertions to lie along the edge of the open preparation. The tissue was pinned flat, mucosal side up, in a specialized organ bath consisting of two adjacent compartments machined from clear acrylic (Danz Instrument Service, Adelaide, South Australia), the floors of which were lined with Sylgard (Dow Corning Corp., Midland, MI, USA). The PN or neurovascular bundle containing the LSN was extended from the tissue compartment into the recording compartment where it was laid onto a mirror. A movable wall with a small 'mouse hole' (to allow passage of the nerves) was lowered into position and the recording chamber filled with paraffin oil.

The colonic compartment was superfused with a modified Krebs solution (mM: 117.9 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4(H_2O)_7$, 2.5 $CaCl_2$, 11.1 D-glucose, 2 sodium butyrate, and 20 sodium acetate), bubbled with carbogen (95% O_2, 5% CO_2) at a temperature of 34°C. All preparations contained the L-type calcium channel antagonist nifedipine (1 μM) to suppress smooth muscle activity, and the prostaglandin synthesis inhibitor indometacin (3 μM) to suppress potential inhibitory actions of endogenous prostaglandins (Lynn & Blackshaw, 1999; Brierley *et al.* 2004). Under a dissecting microscope, the LSN were dissected away from the neurovascular bundle and the nerve sheath surrounding the LSN or PN peeled gently back to expose the nerve trunk. Using fine forceps, the nerve trunk was teased apart into 6–10 bundles that were placed individually onto a platinum recording electrode. A platinum reference electrode rested on the mirror in a small pool of Krebs solution adjacent to the recording electrode.

Characterization of lumbar splanchnic and sacral pelvic serosal afferents

Colonic afferents were characterized using the classification system previously applied in mouse and rat colon (Lynn & Blackshaw, 1999; Hicks *et al.* 2002; Brierley *et al.* 2004). Receptive fields were identified by systematically stroking the mucosal surface with a brush of sufficient stiffness to activate all types of mechano-sensitive afferent. In the present study we focused on serosal afferents as we have previously shown that they are by far the most conserved afferent class shared between the LSN and PN pathways (Brierley *et al.* 2004). For this reason we focused on this afferent class in testing and comparing afferent chemosensitivity. Serosal afferents were classified in both pathways by their graded response to perpendicular probing with calibrated von Frey hairs (70, 160, 400, 1000 and 2000 mg; each force applied three times for a period of 3 s) and their insensitivity to circular stretch (1–5 g) and fine mucosal stroking (10 mg) (Brierley *et al.* 2004).

Drug addition to receptive fields

Chemosensitivity of LSN and PN serosal afferents to the P2X-purinoceptor agonist α,β-meATP (1 mM) and the TRPV1 agonist capsaicin (3 μM) were determined after mechanical thresholds and stimulus response functions had been established. These concentrations were chosen as they elicit powerful responses in similar preparations (Lynn & Blackshaw, 1999; Page *et al.* 2002). A small metal ring was placed over the receptive field of interest, residual Krebs was aspirated, and chemical stimuli were applied to the mucosal surface for 2 min before being aspirated from the ring. This route of administration has been previously shown to activate serosal afferents reproducibly (Lynn & Blackshaw, 1999; Hicks *et al.* 2002). In all experiments, the mechanical sensitivity of receptive fields to probing (2000 mg von Frey hair) was re-determined between each drug application, to determine possible alterations in mechanical sensitivity and ensure continued viability of the unit under investigation. A probing stimulus of 2000 mg was chosen because it was reproducibly effective in activating all LSN and PN serosal afferents. After removal of α,β-meATP from around the receptive field, the afferents were allowed to return to a normal baseline level of activity before capsaicin addition. Drugs were administered in this order to avoid damage or desensitization. Moreover, only one concentration of capsaicin was used, to avoid desensitization of responses. In separate experiments pyridoxyl 5-phosphate 6-azophenyl-2′,4′-disulphonic acid (PPADS) was administered to the bath during application of α,β-meATP and mechanical stimuli to determine the role of P2X receptors in the effect of the agonist and in LSN mechanotransduction. PPADS at a concentration of 100 μM was chosen as it has been previously shown to block the effects of α,β-meATP on rat colonic pelvic distension-sensitive afferents (Wynn *et al.* 2003, 2004). Responses to chemicals were counted when a maintained >25% increase in discharge above basal levels occurred (Hicks *et al.* 2002).

Electrophysiological data recording and analysis

Electrical signals generated by nerve fibres placed on the platinum recording electrode were fed into a differential amplifier, filtered, sampled (20 kHz) using a 1401 interface (Cambridge Electronic Design, Cambridge, UK) and stored on a PC for off-line analysis. The amplified signal was also used for on-line audio monitoring. Action potentials were analysed off-line using the Spike 2 wave-mark function and discriminated as single units on the basis of distinguishable waveform, amplitude and duration. Data are expressed as mean ± S.E.M. *n* indicates the number of individual afferents. Adaptation profiles to probing were calculated as the mean number of spikes per 100 ms bin over the entire 3 s of a 1 g probing stimulus. Data were analysed using Prism 4 software (GraphPad Software, San Diego, CA, USA), and where appropriate, were analysed using a two-way analysis of variance (ANOVA) with Bonferroni *post hoc* tests (to determine significant differences between curves) or paired and unpaired *t* tests. Linear regression analysis was used to compare slopes of adaptation profiles. Fisher's exact tests were used to compare significance differences between stimulus thresholds, responders and non-responders to chemical stimuli. Not all PN serosal afferents are

Table 1. Different patterns of chemosensitivity displayed by individual LSN and PN serosal afferents and mechanically insensitive afferents recruited in response to α,β-meATP (1 mM) and capsaicin (3 μM)

	n (%) of serosal afferents in each classification		*n* (%) of recruited afferents in each classification	
Responsiveness to agonist	Splanchnic nerve	Pelvic nerve	Splanchnic nerve	Pelvic nerve
α,β-meATP: −ve capsacin: −ve	14 (45)	6 (50)	n/a	n/a
α,β-meATP: +ve capsacin: +ve	9 (29)	0 (0)	5 (42)	0 (0)
α,β-meATP: −ve capsacin: +ve	7 (23)	5 (42)	5 (42)	1 (100)
α,β-meATP: +ve capsacin: −ve	1 (3)	1 (8)	2 (16)	0 (0)

Note not all PN serosal afferents are included in this comparison as some individual afferents could not be tested with both agonists. n/a, not applicable.

included in the individual serosal afferent chemosensitivity comparison (Table 1) as some individual afferents could not be tested with both agonists. Differences were considered significant at a level of $P < 0.05$.

Drugs

Stock solutions of all drugs were kept frozen and diluted to their final concentration in Krebs solution. α,β-meATP, capsaicin and PPADS were obtained from Sigma-Aldrich.

Retrograde labelling

Seven adult (six weeks old) male mice were used for surgical injection of the fluorescent retrograde neuronal tracer, Fast Blue (FB; diamidino compound 253/50, CAS# 74749-42-1; EMS-Chemie, Groß-Umstadt, Germany). Animals were anaesthetized with isofluorane (5% induction using an induction box, 2% maintenance) and, following midline laparotomy, four to eight injections (approximately 5 μl, Hamilton syringe with a 25-gauge needle) of FB (2–5% in sterile saline) were made within the wall of the descending colon. The viscera were carefully rinsed with sterile saline after each injection to ensure that dye was not incorporated into structures other than the colon wall, and the muscle and skin were then sutured closed.

Following surgery, but prior to regaining consciousness, mice were given an analgesic (50 μl of 30 μg ml^{-1} subcutaneous buprenorphine (Vetergesic) Reckitt Benckiser Healthcare (UK) Ltd). Recovery for all animals was under constant observation in a warm environment. Their health, including body weight, was recorded for the following three days, during which time additional soft food was provided to supplement their normal diet.

Dorsal root ganglion tissue preparation

At 9–21 days postoperatively, animals were killed humanely with a rising concentration of carbon dioxide. Following decapitation, DRG from bilateral spinal levels T8–L1 and L6–S1, previously shown to be retrogradely labelled from the colon (Ness & Gebhart, 1988; Robinson *et al.* 2004), were removed and fixed in 4% paraformaldehyde for 2–4 h (4°C). The DRG were rinsed in Dulbecco's phosphate-buffered saline (D-PBS) before cryoprotection in sucrose (30%, 4°C) for two days, after which excess liquid was removed with the corner of a paper towel. DRG were then snap frozen by brief immersion into 2-methylbutane (−30°C), and stored individually at −70°C.

Each ganglion was mounted in Tissue-Tek O.C.T. embedding medium (Sakura, Zoeterwoude, the Netherlands) and sectioned at −30°C in a Leica CM3000 cryostat. DRG were not thawed until sectioned and placed on a slide (Superfrost Plus, BDH, Poole, UK). Sections were cut (10–14 μm) and thaw-mounted non-serially onto one or more series of six slides (to reduce the probability that any cell would appear on the same slide more than once). Slides were stored in the dark (−20°C) until required.

Immunohistochemistry

After dilution series optimization experiments, selected slides were processed for immunohistochemistry. Unless stated, all stages were performed at room temperature, and all washes were in TNT wash buffer (120 mM NaCl, 50 mM pH 7.5 Trizma, 0.05% Tween-20; 1 mM) under gentle agitation (3 × 10 min). Once defrosted, slides were washed before a 30 min incubation in 10% normal goat serum (NGS; Vector Laboratories, Inc., Burlingame, CA, USA) to reduce non-specific binding, and then overnight at 4°C in the primary antibody ($P2X_3$ receptor, in-house raised (Kidd *et al.* 1998), 1 : 1000; or capsaicin receptor (TRPV1), Calbiochem, San Diego, CA, USA, 1 : 50. Slides were then washed and incubated with a secondary antibody (Alexa Fluor 488 1 : 200; Molecular Probes, Inc., Eugene, OR, USA) for 2 h. All antibodies were made up in 2% NGS.

Following a final wash, any excess fluid was shaken from the slides, which were then mounted, coverslipped, and viewed on a Leica DMR microscope with epifluorescence unit and A4, and I3 filters for observation of FB and Alexa Fluor 488, respectively. Digital images were captured using a Leica DC200 digital camera.

DRG sections were devoid of specific labelling in the absence of any primary antibody. Previous studies in mouse tissues have shown the $P2X_3$ primary antibody used in the present study to be a specific tool for the study of this receptor (Kidd *et al.* 1998; Souslova *et al.* 2000), and experiments in which the TRPV1 primary antibody was preincubated with its immunizing peptide showed no labelling of DRG neurones, indicating the specificity of the antibody for this sequence. $P2X_3$ and TRPV1 dual labelling was not possible as both antibodies were raised in the rabbit, and reliable staining was not observed during preliminary studies with alternative antibodies.

Labelled cells were analysed using ImageJ with its Sync Windows plugin (Wayne Rasband, National Institutes of Health, USA; http://rsb.info.nih.gov/ij/). The distribution of retrogradely labelled mouse colonic neurones is based around two peaks: one broad, covering T8–L1 (Robinson *et al.* 2004), representing the LSN innervation, and the other restricted to L6 and S1 (Robinson *et al.* 2004), representing the PN innervation; thus many more DRG neurones were examined from LSN than from PN levels.

Results

Mechanosensory properties of lumbar splanchnic & sacral pelvic afferents

Serosal afferents were recorded from both the LSN and PN pathways that had receptive fields located on the colonic wall. As we have shown previously these were activated reproducibly by perpendicular probing of their receptive field with calibrated von Frey hairs and did not respond to colonic stretch (1–5 g) or stroking of the colonic mucosa with a 10 mg von Frey hair (Fig. 1*A* and *B*). We corroborated our previous data showing that the dynamic properties of these afferents differed considerably between the two pathways. Despite the fact that serosal afferents from both pathways displayed graded responses to increasing probing stimuli, PN afferents were significantly more sensitive to probing than LSN afferents, displaying significantly greater stimulus response functions (Fig. 1*C*). In addition PN serosal afferents displayed a significantly greater magnitude of response to probing with 400, 1000 and 2000 mg von Frey hairs (Fig. 1*C*). PN and LSN serosal afferents also displayed significantly different adaptation profiles to probing with a 1000 mg von Frey hair (Fig. 1*D*). Pelvic serosal afferents displayed a more maintained response to probing than their LSN counterparts, indicated by significantly shallower linear regression slopes (Fig. 1*D*). Pelvic serosal afferents also displayed significantly lower stimulus thresholds to probing, as almost twice as many PN afferents responded to lower probing stimuli (70, 160 and 400 mg), while 100% of PN serosal afferents responded to a 1000 mg probing stimulus compared with only 89% being activated in the LSN pathway (Fig. 1*E*). In summary, consistent with our previous report (Brierley *et al.* 2004), we observed that PN serosal afferents required lower intensity probing stimuli to be activated, evoked larger responses to probing stimuli, and displayed a more maintained response to probing than LSN serosal afferents.

Chemosensory properties of lumbar splanchnic and sacral pelvic afferents

Lumbar splanchnic responses to α,β-methyleneATP. Ten of the 31 (32%) LSN serosal mechanoreceptive afferents tested responded to α,β-meATP (1 mM) (Figs 2*A* and 3*Ai*). They displayed a rapid excitation of discharge, after a short latency (5.3 ± 1.6 s; Fig. 3*C*), that was normally short in duration (6.6 ± 3.3 s; Fig. 3*D*), before discharge returned to basal levels (Fig. 3*Ai*). In all cases (10/10), the response was reproducible on a second application of α,β-meATP (1 mM) three minutes later. Splanchnic serosal afferents responded to α,β-meATP in a concentration-dependent manner ($EC50 = 21.2\ \mu M$, Fig. 3*E*), and in separate experiments this response was blocked by the non-selective P2X-receptor antagonist PPADS (100 μM (Wynn *et al.* 2003, 2004) for 15 min, $P < 0.001$, *t* test, $n = 6$, Fig. 3*F*. No mechanical sensitization or desensitization was observed after α,β-meATP, as probing with a 2000 mg von Frey hair after the removal of α,β-meATP gave the same response to probing before and after exposure (Fig. 4*A*). Afferents responding to α,β-meATP and those not responding were similarly unaffected in their mechanosensitivity ($P > 0.05$, *t* test, data not shown). In separate experiments PPADS (100 μM) did not alter the mechanosensitivity of LSN afferents ($n = 6$, $P > 0.05$, *t* test, Fig. 4*C*).

Recruitment of mechanically insensitive lumbar splanchnic afferents by α,β-meATP. On seven occasions during testing of a mechanically sensitive serosal afferent, another separate mechanically insensitive chemosensitive unit was recruited (Fig. 5*A*). Mechanically insensitive afferents were also recruited following capsaicin addition (see later), giving a total population of 43 LSN afferents in this investigation. Therefore, in total 17 of 43 (40%; Fig. 2*A*) LSN afferents responded to α,β-meATP (1 mM). Recruited afferents displayed reproducible responses to α,β-meATP with similar latencies (4.7 ± 2.7 s) to

mechanically sensitive serosal afferents; however, they displayed significantly longer durations of response (37.3 ± 9.8 s; $P < 0.01$, *t* test). Recruited afferents did not subsequently become mechanosensitive.

Sacral pelvic responses to α,β-meATP. In contrast to LSN afferents, fewer (1 of 15; 7%) PN serosal mechanoreceptive afferents responded to α,β-meATP (1 mM) (Fig. 2*B* and Fig. 3*Bi*). Furthermore, no PN afferents were recruited during the addition of α,β-meATP. As seen in the LSN pathway, mechanosensitivity was unaltered in afferents that did not respond to α,β-meATP ($P > 0.05$, *t* test, Fig. 4*A*). Due to the low number of PN afferents responding to α,β-meATP, no statistical comparison of response latency and duration could be performed between pathways. However, it is clear that more LSN

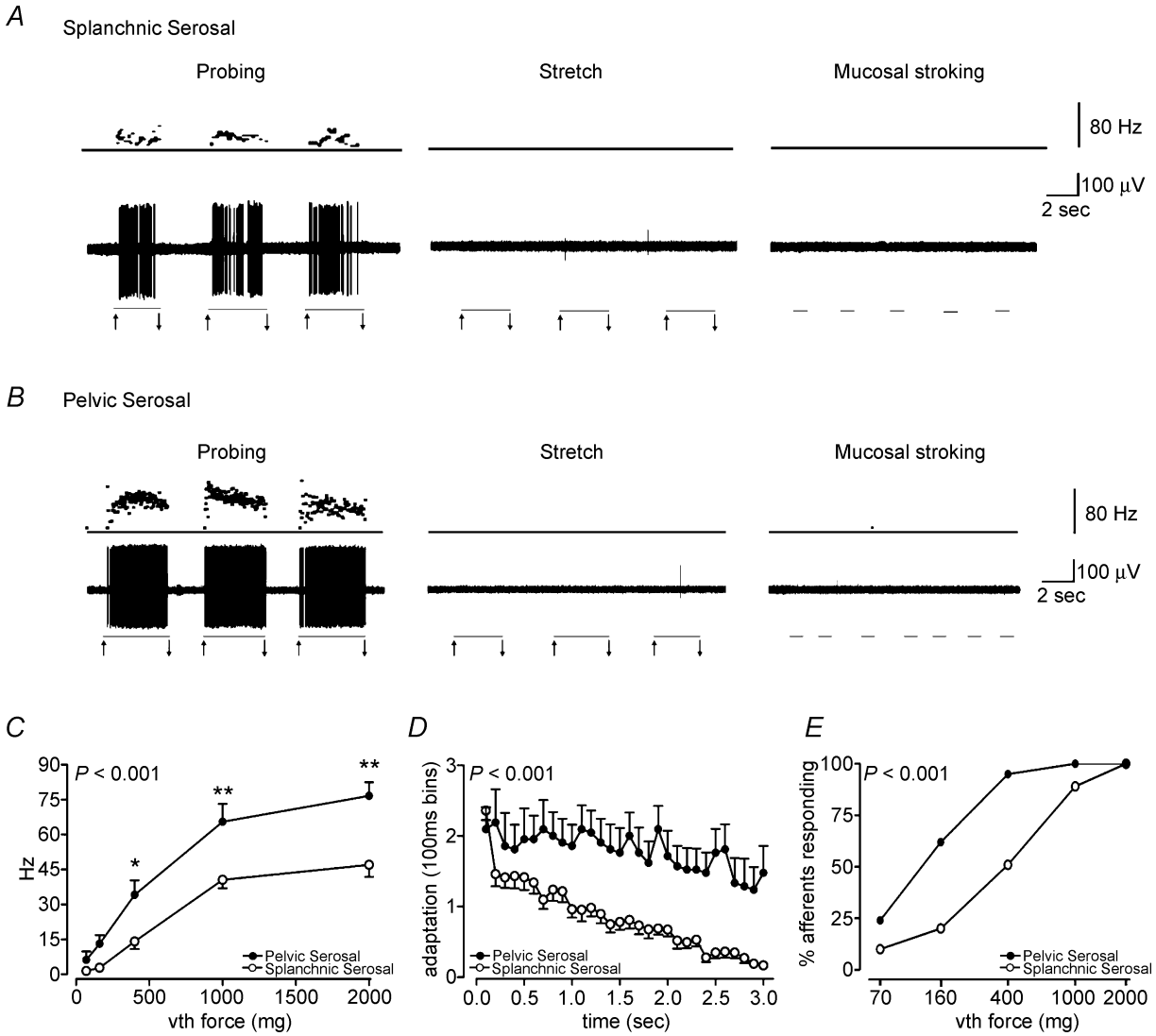

Figure 1. Mechanosensory properties of splanchnic and pelvic serosal afferents
A, splanchnic and *B*, pelvic serosal afferents were activated only by probing of their receptive fields and were insensitive to circular stretch (1–5 g) and fine mucosal stroking (10 mg); note the difference in magnitude of response to a 2000 mg von Frey hair. *C*, stimulus–response functions of LSN and PN serosal afferents to increasing probing stimuli (70–2000 mg). Pelvic serosal afferents were significantly more sensitive to probing displaying greater stimulus response functions ($P < 0.001$, two-way ANOVA, LSN $n = 31$ *versus* PN $n = 21$) (*$P < 0.01$ Bonferroni *post hoc* test, data from 400 mg stimulus; **$P < 0.001$, Bonferroni *post hoc* test, data from 1000 and 2000 mg stimulus). *D*, pelvic serosal afferents displayed a more maintained adaptation response profile ($P < 0.001$, two-way ANOVA, LSN $n = 31$ *versus* PN $n = 21$) displaying a significantly shallower slope ($P < 0.001$; linear regression; PN; -0.23 ± 0.03 *versus* LSN; -0.52 ± 0.03). *E*, pelvic serosal afferents display lower stimulus thresholds as almost twice the amount of PN serosal afferents respond to lower probing stimuli (160 and 400 mg; $P < 0.001$, Fisher's exact test). Data are mean ± S.E.M.

afferents in total are responsive to α,β-meATP than PN afferents ($P < 0.05$, Fisher's exact test).

Lumbar splanchnic responses to capsaicin. Sixteen of 31 (52%) LSN serosal mechanoreceptive afferents responded to capsaicin (3 μM) (Fig. 2*C* and Fig 3*Aii*). They displayed a powerful excitation of discharge after a short latency (4.3 ± 1.1 s; Fig. 3*C*). The average duration of response was 35.7 ± 8.3 s before discharge returned to basal levels (Fig. 3*D* and *Aii*). Capsaicin-responsive LSN serosal afferents displayed pronounced mechanical desensitization when reprobed with a 2000 mg von Frey Hair after exposure to capsaicin ($P < 0.05$, *t* test, Fig. 4*B*). By contrast, capsaicin-unresponsive LSN serosal afferents did not show mechanical desensitization ($P > 0.05$, *t* test, data not shown).

Recruitment of mechanically insensitive lumbar splanchnic afferents by capsaicin. Five of the seven mechanically insensitive afferents recruited by α,β-meATP also responded to capsaicin (3 μM, Fig. 5*B*). In addition to these, five other mechanically insensitive units were recruited by capsaicin (3 μM) that were insensitive to α,β-meATP. In total 26 of 43 (61%) LSN afferents responded to capsaicin (3 μM; Fig. 2*C*). Recruited units displayed similar latencies (2.7 ± 1.3 s) and duration of

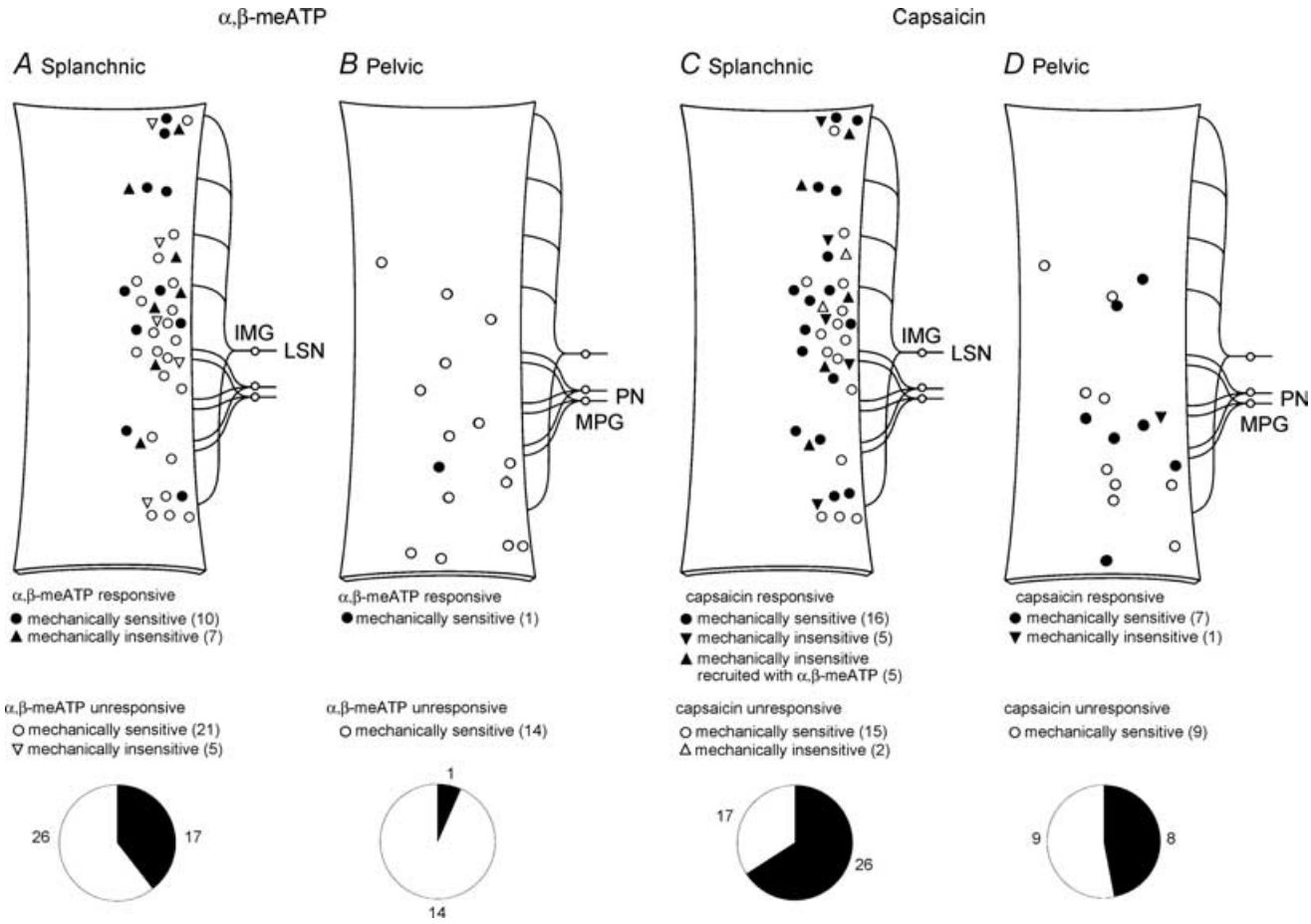

Figure 2. Proportions and distributions of splanchnic and pelvic afferents responding to α,β-meATP capsaicin

A, 40% (17 of 43) of LSN afferents responded to α,β-meATP (1 mM). Ten of these were mechanically sensitive serosal afferents, seven others were recruited additionally during these tests and it was not possible to activate them mechanically. *B*, by contrast only 7% (1 of 15) of PN serosal afferents responded to α,β-meATP (1 mM). In contrast to LSN afferents, no PN afferents were recruited during the addition of α,β-meATP. *C*, in total 26 of 43 (61%) LSN afferents responded to capsaicin (3 μM), 16 of these were serosal afferents and five were mechanically insensitive afferents that were also recruited by α,β-meATP. Additional to these were five afferents recruited by capsaicin (3 μM) which were insensitive to α,β-meATP. *D*, in contrast, only 47% (8 of 17) of PN afferents responded to capsaicin (3 μM). Seven of these were serosal afferents and only one additional mechanically insensitive afferent was recruited by capsaicin. IMG: inferior mesenteric ganglion; LSN: lumbar splanchnic nerve; PN: pelvic nerve; MPG: major pelvic ganglion.

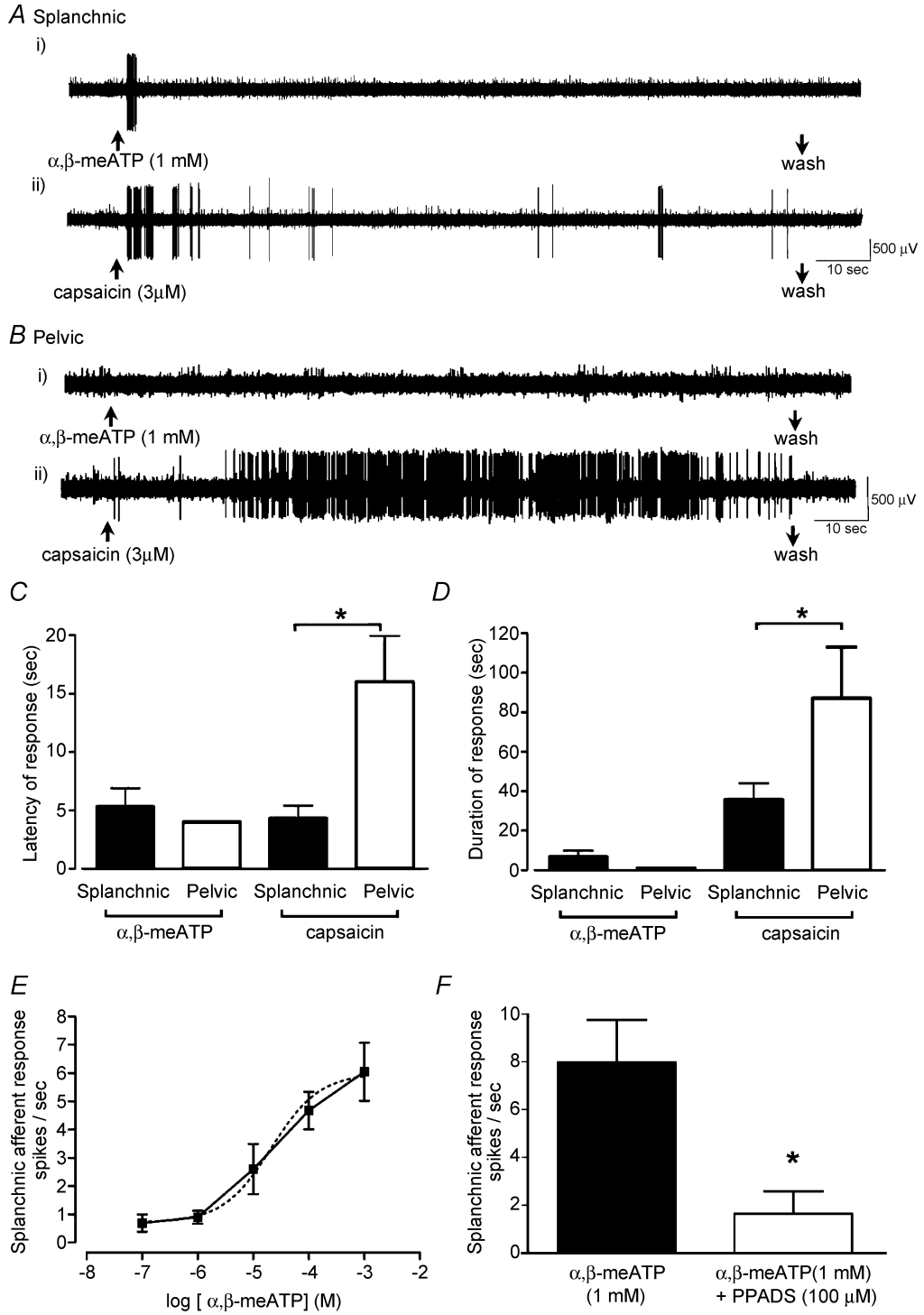

Figure 3. Splanchnic and pelvic afferent responses to α,β-meATP and capsaicin
A, example of a LSN serosal afferent responding to α,β-meATP (1 mM; *i*) and to capsaicin (3 μM; *ii*). *B*, example of a PN serosal afferent that was unresponsive to α,β-meATP (1 mM; *i*), but that did respond to capsaicin (3 μM; *ii*). *C*, latency of response of LSN and PN afferents that responded to α,β-meATP and capsaicin. Pelvic afferents took a significantly longer time to respond to capsaicin compared with LSN afferents ($^*P < 0.05$, PN $n = 7$ *versus* LSN $n = 16$, *t* test). *D*, duration of response of LSN and PN afferents that responded to α,β-meATP and capsaicin. Pelvic afferents displayed significantly longer durations of response to capsaicin ($^*P < 0.05$, PN $n = 7$ *versus* LSN $n = 16$, *t* test). *E*, α,β-meATP caused a concentration-dependent excitation of LSN serosal afferents (EC_{50}: 21.2 μM, broken line indicates sigmoidal dose–response curve, $n = 6$). *F*, the LSN serosal afferent response to α,β-meATP (1 mM) was blocked by the non-selective P2X receptor antagonist PPADS (100 μM; $n = 6$). Note only one PN afferent responded to α,β-meATP. Data are mean ± S.E.M.

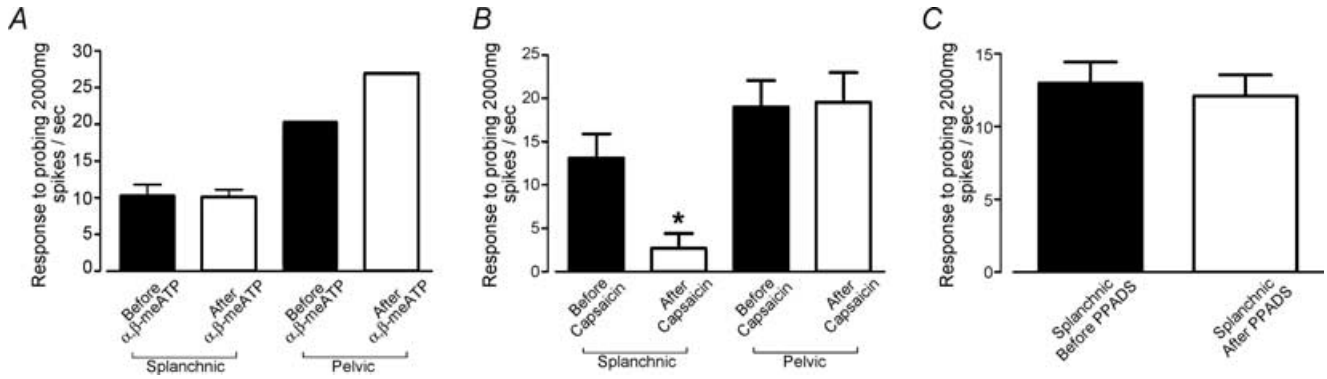

Figure 4. Capsaicin-induced mechanical desensitization of splanchnic afferents
A, splanchnic afferents did not display altered mechanosensitivity after responding to α,β-meATP. (LSN $n = 10$; Note only one PN afferent responded to α,β-meATP). *B*, capsaicin-responsive LSN afferents displayed significant mechanical desensitization after responding to capsaicin (3 μM; $P < 0.01$, $n = 16$, *t* test). *Significant difference between before and after capsaicin in splanchnic pathway. This is in direct contrast to capsaicin-responsive PN afferents which did not display altered mechanosensitivity ($P > 0.05$, $n = 7$, *t* test). *C*, PPADS (100 μM) did not affect the mechanosensitivity of LSN afferents ($P > 0.05$, $n = 6$). Afferents were mechanically tested with a 2000 mg von Frey hair before and after drug addition α,β-meATP (*A*),and capsaicin (*B*) or PPADS (*C*).

responses (50.3 ± 7.8 s; $P > 0.05$, *t* test) to mechanically sensitive, capsaicin-responsive serosal afferents. Recruited afferents did not subsequently become mechanosensitive.

Sacral pelvic responses to capsaicin. In contrast to the LSN afferent responses, fewer (7 of 16; 44%) PN serosal mechanoreceptive afferents responded to capsaicin (3 μM) (Fig. 2*D*). They displayed a powerful excitation

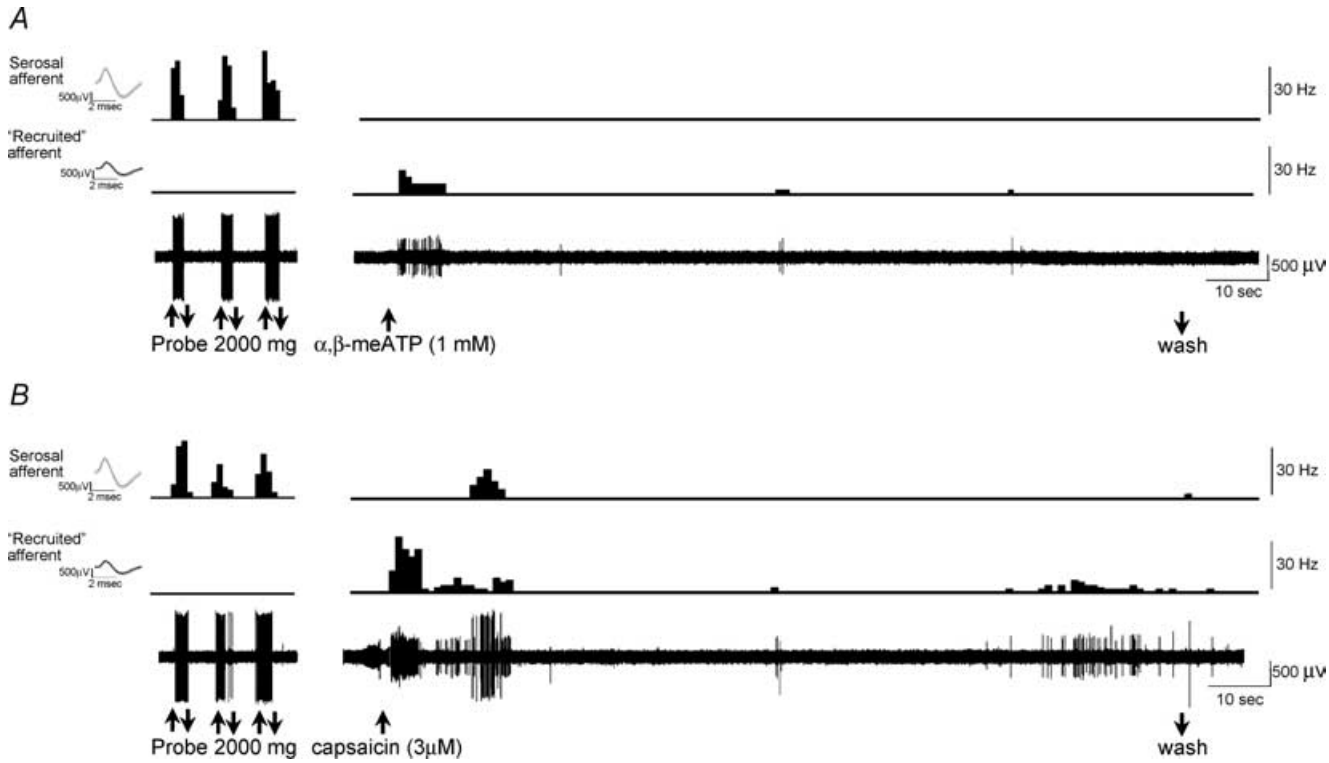

Figure 5. Recruitment of a mechanically insensitive splanchnic afferent by α,β-meATP and capsaicin
A, a mechanically sensitive LSN (large-amplitude) afferent that was unresponsive to α,β-meATP. However, the addition of α,β-meATP recruited a mechanically insensitive (small-amplitude) afferent. *B*, both the mechanically sensitive (large-amplitude) and mechanically insensitive (small-amplitude) afferent responded to capsaicin. Insets: the average spike shape of the LSN serosal afferent and the recruited mechanically insensitive afferent. Upper traces show spike rate and lower traces show raw electrophysiological data. Scale bars apply throughout.

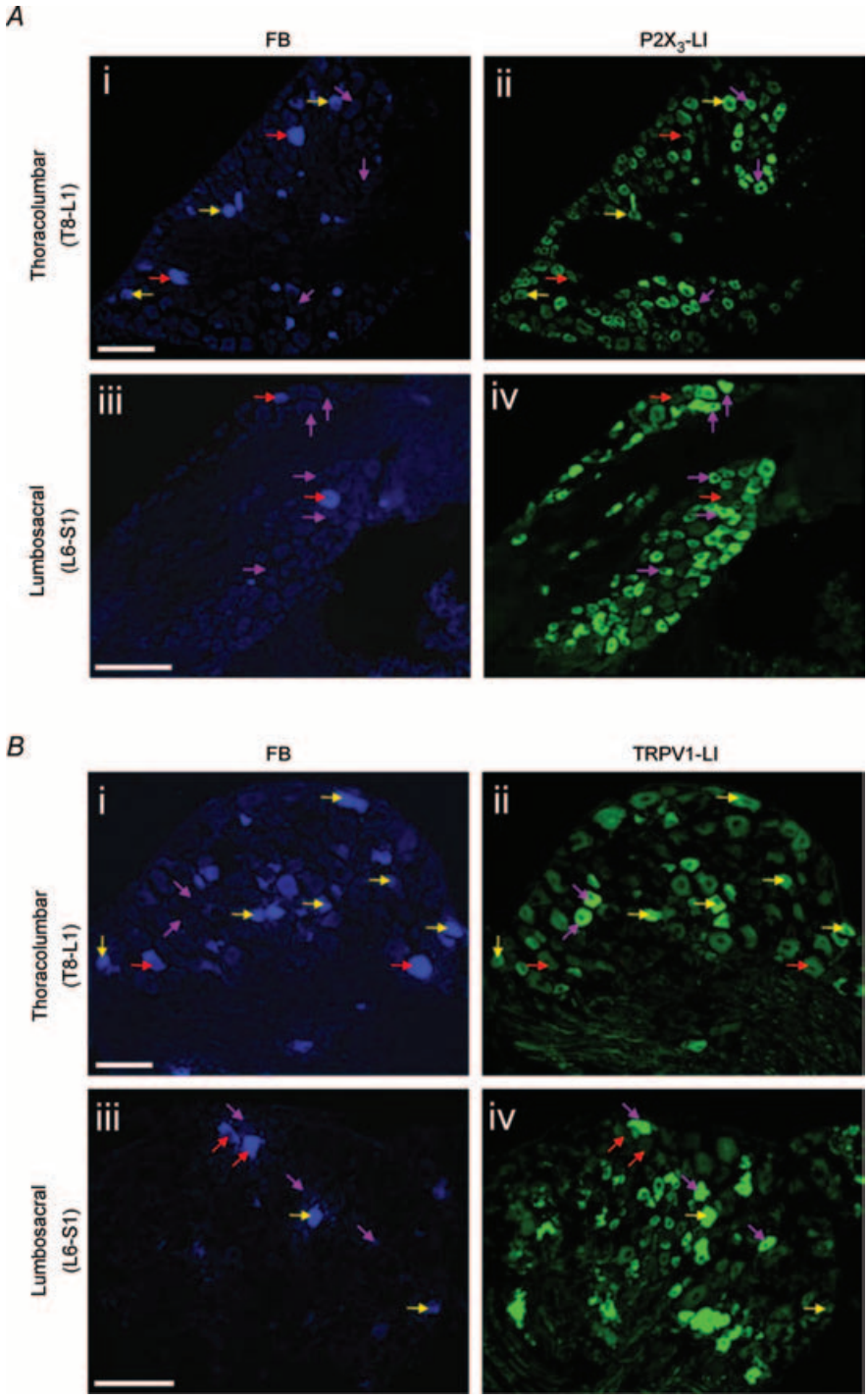

Figure 6. $P2X_3$ and TRPV1 receptor labelling in retrogradely labelled mouse colonic thoracolumbar (LSN; T8–L1) and lumbosacral (PN; L6–S1) afferent neurones
A, representative retrogradely labelled (with Fast Blue; FB) thoracolumbar (*i*) and lumbosacral (*iii*) dorsal root ganglia (DRG) sections that have also been immunostained for $P2X_3$-LI (*ii* and *iv*). More thoracolumbar FB-labelled colonic neurones exhibited $P2X_3$-LI (yellow arrows) than lumbosacral neurones. Red arrows indicate FB-labelled neurones that did not exhibit $P2X_3$-LI. Purple arrows indicate non-FB-labelled neurones that exhibited $P2X_3$-LI. DRG sections from thoracolumbar and lumbosacral levels illustrated are from DRG levels T13 and L6, respectively. Scale bars are 100 μm on all panels. *B*, representative retrogradely labelled (FB) thoracolumbar (*i*) and lumbosacral (*iii*) DRG sections that have also been immunostained for TRPV1-LI (*ii* and *iv*). The majority of thoracolumbar colonic, cells exhibited TRPV1-LI (yellow arrows). Red arrows indicate FB-labelled neurones that did not exhibit TRPV1-LI. Purple arrows indicate non-FB-labelled neurones that exhibited TRPV1-LI. DRG sections illustrated are from DRG levels T11 and S1, respectively. Scale bars are 100 μm on all panels.

Table 2. Comparison of the total proportion of LSN and PN (serosal and mechanically insensitive) afferents responding to α,β-meATP (1 mM) and capsaicin (3 μM), and the proportion of thoracolumbar and lumbosacral colonic neurones expressing $P2X_3$ and TRPV1-LI

	% of colonic afferents responding to agonist		% of colonic neurones expressing marker	
Marker	Splanchnic nerve	Pelvic nerve	Thoracolumbar DRG (T8–L1)	Lumbosacral DRG (L6–S1)
$P2X_3$	40	7	36	19
TRPV1	61	47	82	50

More LSN afferents in total responded to α,β-meATP and capsaicin while more thoracolumbar colonic neurones expressed $P2X_3$-LI and TRPV1-LI.

of discharge lasting 87 ± 26 s after a latency of 16 ± 4 s (Fig. 3*C* and *D*). A comparison of the pattern of responses to capsaicin revealed significant differences between the two pathways. PN afferents displayed significantly longer latencies of response ($P < 0.01$, *t* test, Fig. 3*C*) and significantly longer durations of response ($P < 0.05$, *t* test, Fig. 3*D*). Only one mechanically insensitive PN afferent was recruited during the addition of capsaicin (3 μM), giving a total of 8 of 17 (47%) PN afferents responsive to capsaicin (3 μM; Fig. 2*D*). In contrast to LSN serosal afferents, PN serosal afferents did not display mechanical desensitization (Fig. 4*B*). Overall these data reveal three patterns of serosal afferent chemosensitivity in the PN pathway, in contrast to four different patterns of serosal afferent chemosensitivity in the LSN pathway (Table 1). Moreover, these data reveal only one pattern of recruited (mechanically insensitive) afferent chemosensitivity in the PN pathway in contrast to three different patterns of recruited afferent chemosensitivity in the LSN pathway (Table 1).

Immunohistochemistry

We also investigated the labelling of $P2X_3$ and TRPV1 in primary afferent neurones retrogradely labelled from the mouse descending colon. Data were analysed separately for thoracolumbar (T8–L1) and lumbosacral (L6–S1) DRG, representing innervation from the LSN and PN afferent pathways, respectively (Ness & Gebhart, 1988; Robinson *et al.* 2004). Approximately one-third of all FB-labelled cells displayed $P2X_3$-like immunoreactivity (LI), however, a greater proportion of retrogradely labelled thoracolumbar neurones displayed $P2X_3$-LI (36%) than lumbosacral (19%) colonic neurones (Fig. 6*A*, Table 2). The majority (80%) of all FB labelled cells displayed TRPV1-LI; however, further analysis revealed that a greater proportion of retrogradely labelled thoracolumbar neurones displayed TRPV1-LI (82%) than lumbosacral (50%) colonic neurones (Fig. 6*B*, Table 2).

Discussion

This study has five major findings relating to transmission of sensory information from the colon. First, we have revealed that LSN and PN colonic afferents transmit different patterns of information to the central nervous system, not only in terms of signalling mechanical events but also in terms of signalling chemical activation. Second, we have found a population of afferents in both pathways that are recruited by chemical stimuli and remain mechanically insensitive, and therefore serve a novel role. Third, in LSN mechanically sensitive afferents, activation of TRPV1 receptors by capsaicin may subsequently attenuate responsiveness to mechanical stimuli. Fourth, from their markedly different response profiles to capsaicin, we suggest that LSN and PN afferents may differ in their mechanisms of downstream signalling via TRPV1. Fifth, we found no evidence for a role of the P2X receptor in mechanical transduction, in contrast to studies of afferents innervating different targets in other species.

Differences between splanchnic and pelvic pathways

Our findings concerning the mechanosensitivity of colonic afferents corroborate those we obtained previously in part of a comprehensive study of several different classes in the LSN and PN (Brierley *et al.* 2004). We have shown that PN serosal afferents differ from LSN afferents in several ways: they are more distally located, adapt more slowly to mechanical stimuli, and respond across a wider stimulus range. In the present study we focused specifically on one class of afferents, the serosal afferents. We chose this class because they are the most abundant in both the LSN and PN, and may thus provide a meaningful comparison of chemosensitivity between pathways; also they are probably linked to signalling of pain. Based on our previous findings we proposed that serosal afferents signal transient, sharp pain at the onset of spasm or distension. Distension of this nature would result from rapid transit of

contents or experimental balloon inflation, during which acute intense mechanical stimulation might be achieved. Consequently, PN serosal afferents would provide a signal from more distal regions of the colon and rectum than LSN serosal afferents, and generate a more intense and sustained afferent barrage in response to acute mechanical events. We can now add an extra dimension to this profile of PN and LSN serosal afferents, showing that LSN afferents are more likely to express $P2X_3$ and TRPV1 receptors than PN afferents. This is also reflected in the proportions of sensory neurones retrogradely labelled from the colon in thoracolumbar and lumbosacral DRG that express these ion channel receptors anatomically. In the whole animal these different profiles of receptor expression would mean that the distal colon and rectal region is less chemosensitive to vanilloid or purinergic stimuli than more proximal regions of the mid- to distal colon. It would also mean that pathways activated in the lumbosacral spinal cord are less likely to receive colorectal chemosensory input than those in the thoracolumbar cord. Because $P2X_3$ and TRPV1 receptors are likely to be continually activated endogenously (see below), this adds further complexity to the stream of afferent information reaching the spinal cord from the colon and rectum.

Mechanically insensitive chemosensitive afferents

Mechanically insensitive fibres were recruited by α,β-meATP, when added to a ring surrounding the serosal mechanoreceptive field, and these were found to be exclusive to the LSN pathway. Most of these mechano-insensitive afferents also responded to capsaicin. Another subgroup of LSN mechano-insensitive afferents were recruited selectively by capsaicin. In total, 12 mechanically insensitive chemosensitive afferents were observed in the LSN pathway compared with only one in the PN pathway. Mechanically insensitive afferents showed similar overall distribution to mechanically sensitive afferents. Importantly, they are distinct from the previously described 'silent nociceptors' in skin (Cervero, 1994; Michaelis *et al.* 1996) and 'chemospecific afferents' in the colon (Lynn & Blackshaw, 1999; Gebhart, 2000) because they remained insensitive to mechanical stimuli after they had been recruited by chemical stimuli; they provide evidence for a novel class of truly chemospecific colonic afferents that are primarily confined to the LSN pathway.

TRPV1 signalling and mechanosensitivity

Capsaicin evoked a powerful excitation of discharge in both LSN and PN afferents, comparable to that observed in similar gastrointestinal preparations (Blackshaw *et al.* 2000; Rong *et al.* 2004). An interesting observation from the present study was the ability of capsaicin to cause marked mechanical desensitization. Notably this occurred only in LSN afferents that gave a response to capsaicin, whereas capsaicin-responsive PN afferents and capsaicin-unresponsive afferents in both pathways displayed no mechanical desensitization. Mechanical desensitization after capsaicin has been observed previously *in vitro* using gastroesophageal (Blackshaw *et al.* 2000) and jejunal (Rong *et al.* 2004) preparations. However, in the gastroesophageal preparation, mechanical desensitization was also observed in capsaicin-unresponsive afferents in addition to capsaicin-responsive afferents. Desensitization and subsequent degeneration of primary afferents by capsaicin is thought to follow from uncontrolled cation influx into afferent endings, resulting in depolarization block and subsequent osmotic damage (Holzer, 1998). Based on this mechanism one would expect that stronger activation by capsaicin would result in greater desensitization. However, we found that PN serosal afferents showed far less desensitization than LSN afferents, even though they showed significantly more prolonged capsaicin responses than LSN afferents. This suggests there are distinct mechanisms linking activation with desensitization, that these mechanisms are specific to the LSN pathway, and that they are independent of the degree of activation by capsaicin. Pelvic afferents also differed from LSN afferents in their latencies of response to capsaicin, even though their endings were distributed similarly. Although the differences in duration and latency of response could possibly be explained by microanatomical factors that may have influenced the rate of diffusion of the stimulus to the ending, there remains the possibility that they result from differences in downstream signal transduction following activation of TRPV1. It was not possible to determine other aspects of the pharmacology of TRPV1 activation in each pathway because of the inherent problem of desensitization associated with this receptor, but our findings do suggest there may be different coupling of TRPV1 to mechanisms of initiation or termination of neuronal excitation in the two pathways.

Purinergic signalling and mechanosensitivity

α,β-meATP evoked a reproducible and concentration-dependent excitation of LSN serosal afferents that was reversed by the P2X antagonist PPADS and was comparable to that observed in similar gastrointestinal preparations (Page *et al.* 2002; Wynn *et al.* 2003; Zagorodnyuk *et al.* 2003). The latency and duration of response to α,β-meATP in the current study are similar to those observed previously in the mouse stomach and oesophagus using an almost identical *in vitro* electrophysiological preparation (Page *et al.* 2002).

However, the latency of response to α,β-meATP in the current study is significantly shorter (5.3 ± 1.6 s) than that observed in the guinea-pig oesophagus (Zagorodnyuk *et al.* 2003) (18.5 ± 3 s) and rat colon (Wynn *et al.* 2003) (13.7 ± 0.85 s), which may relate to the relative thickness of these preparations and therefore drug accessibility. We did not observe chemical or mechanical sensitization or desensitization following α,β-meATP treatment in either LSN or PN afferents, unlike our findings with capsaicin, indicating a difference in the coupling of P2X receptors and TRPV1 receptors with mechanisms influencing long-term excitability.

Endogenous activation via $P2X_3$ and TRPV1?

Two of the important current questions concerning primary afferent purinergic and vanilloid receptors are whether they are endogenously activated, and whether endogenous activation contributes to mechanical transduction. The mechanical desensitization we observed after capsaicin in LSN afferents is not inconsistent with a role for TRPV1 in splanchnic mechanotransduction (but interestingly not in pelvic mechanotransduction). A recent study of mechanosensitivity in TRPV1+/+ and −/− mice showed more directly that mechanotransduction was reduced in the knockout compared to the wild type, and the TRPV1 antagonist capsazepine was effective in reducing mechanical responses in the wild type (Rong *et al.* 2004). They also showed that the response was totally lost in TRPV1−/− mice. The presence and release of TRPV1 ligands by mechanical and other stimuli is a complex issue, because there are numerous candidates, including heat, low pH and endogenous vanilloids. Temperature changes in the colon are unlikely to be sufficient to activate TRPV1 in health, but in diseased states such as IBS, where there is increased exposure to mediators like serotonin (Houghton *et al.* 2003), TRPV1 can become sensitive to temperatures in the physiological range (Sugiura *et al.* 2004). Changes in pH are established consequences of both inflammation and luminal metabolism of colonic flora, and are therefore likely contributors to endogenous activation of TRPV1. Endogenous vanilloids are present in the colon, particularly in disease states (Manara *et al.* 2002; Pinto *et al.* 2002; Ligresti *et al.* 2003; Storr *et al.* 2004), and are the subject of continued investigation in our laboratories in the context of postinflammatory changes in colonic afferent fibre sensitivity. Notably TRPV1 receptors have been shown to be upregulated in colonic nerve fibres of patients with inflammatory bowel disease (Yiangou *et al.* 2001*b*), while administration of TRPV1 antagonists can attenuate disease severity in dextran sulphate sodium-induced colitis in mice (Kimball *et al.* 2004).

We saw no change in mechanosensitivity after treatment with α,β-meATP or the P2X antagonist PPADS, which is evidence against a direct role for P2X receptors in mechanotransduction. Other recent studies suggest, however, that ATP is present in the colon and is released by distension (Wynn *et al.* 2003; Wynn *et al.* 2004) and that responses to distension are inhibited by P2X receptor antagonists. Other studies of urinary bladder afferents in $P2X_3$+/+ and −/− mice agree that these receptors play an important direct role in mechanotransduction (Cockayne *et al.* 2000; Souslova *et al.* 2000). How therefore can the difference between these results be explained? The most plausible explanation relates to the different stimuli applied to activate these endings, and the location of the afferent nerve endings themselves. Receptive fields of afferents in previous studies were sensitive to distension of their host organ, placing them in close proximity to sources of endogenous ATP, released in response to the distension stimulus (Wynn *et al.* 2003). In the present study, however, endings were located on the outermost layer of the colon, which is further from the epithelium and myenteric neurones, both of which are major potential sources of endogenous ligand. There are other possible explanations, but ultimately the present data would argue that the role of ATP in mechanotransduction may be restricted to specific subpopulations of visceral afferents. It is also possible that the purinergic system may play a greater role in disease in the afferents we observed, as ATP is released in inflammatory conditions from a number of cell types, and notably, $P2X_3$ receptors have been shown to be upregulated in colonic nerve fibres of patients with inflammatory bowel disease (Yiangou *et al.* 2001*a*).

Correlation of chemosensitivity of colonic afferents with receptor expression on their cell bodies

We have demonstrated that more thoracolumbar colonic sensory neurones express $P2X_3$-LI (36%) than lumbosacral colonic sensory neurones (19%). In addition, 82% of thoracolumbar colonic sensory neurones express TRPV1-LI, more than the 50% of lumbosacral colonic sensory neurones expressing TRPV1-LI. The data from our electrophysiological and anatomical studies differ in the exact proportions of neurones expressing each type of receptor, but agree on the general trend of greater receptor expression in the LSN pathway (Table 2). The small differences between these findings could be due to a number of technical factors, but also a basic physiological feature of our approach – the fact that retrograde tracing of colonic afferent pathways does not discriminate between functional classes of primary afferents innervating the colonic wall (muscular, mucosal or serosal (Brierley *et al.* 2004)), whereas our electrophysiological analysis was restricted to serosal afferents. This was because serosal afferents constitute the only major population of afferents common to both pathways, and

therefore allow direct comparison between the two. Thus, other functional classes of afferents may have made slightly different contributions to the total of retrogradely labelled populations that were TRPV1 and $P2X_3$-LI. Nonetheless it appears that activation of TRPV1 and $P2X_3$ is more likely to be signalled via the thoracolumbar LSN pathway than the lumbosacral PN pathway. Therefore our electrophysiological observations of serosal afferents are broadly representative of the whole population of afferents in each pathway.

In conclusion we have made a direct comparison of the chemosensitivity of LSN and PN colonic afferents and the corresponding receptor expression and chemical coding of LSN and PN colonic neurones in thoracolumbar and lumbosacral DRG. With this approach we have demonstrated that LSN and PN pathways from the colon display different mechanosensitivity, chemosensitivity and receptor expression. As these pathways relay information that may relate to symptoms in functional gastrointestinal disease, these results may have implications in the efficacy of therapies targeting receptor modulation.

References

Blackshaw LA & Gebhart GF (2002). The pharmacology of gastrointestinal nociceptive pathways. *Curr Opin Pharmacol* **2**, 642–649.

Blackshaw LA, Page AJ & Partosoedarso ER (2000). Acute effects of capsaicin on gastrointestinal vagal afferents. *Neuroscience* **96**, 407–416.

Blumberg H, Haupt P, Janig W & Kohler W (1983). Encoding of visceral noxious stimuli in the discharge patterns of visceral afferent fibres from the colon. *Pflugers Arch* **398**, 33–40.

Bradbury EJ, Burnstock G & McMahon SB (1998). The expression of P2X3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. *Mol Cell Neurosci* **12**, 256–268.

Brierley SM, Jones RCW III, Gebhart GF & Blackshaw LA (2004). Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice. *Gastroenterology* **127**, 166–178.

Camilleri M (2001). Management of the irritable bowel syndrome. *Gastroenterology* **120**, 652–668.

Caterina MJ & Julius D (2001). The vanilloid receptor: a molecular gateway to the pain pathway. *Annu Rev Neurosci* **24**, 487–517.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR. *et al.* (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **288**, 306–313.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD & Julius D (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**, 816–824.

Cervero F (1994). Sensory innervation of the viscera: peripheral basis of visceral pain. *Physiol Rev* **74**, 95–138.

Chen CC, Akopian AN, Sivilottit L, Colquhoun D, Burnstock G & Wood JN (1995). A P2X purinceptor expressed by a subset of sensory neurons. *Nature* **377**, 428–431.

Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S. *et al.* (2000). Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature* **407**, 1011–1015.

Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P. *et al.* (2000). Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* **405**, 183–187.

Gebhart GF (2000). J.J. Bonica Lecture – 2000: Physiology, pathophysiology, and pharmacology of visceral pain. *Reg Anesth Pain Med* **25**, 632–638.

Geppetti P & Trevisani M (2004). Activation and sensitisation of the vanilloid receptor: role in gastrointestinal inflammation and function. *Br J Pharmacol* **141**, 1313–1320.

Gralnek IM, Hays RD, Kilbourne A, Naliboff B & Mayer EA (2000). The impact of irritable bowel syndrome on health-related quality of life. *Gastroenterology* **119**, 654–660.

Guo A, Vulchanova L, Wang J, Li X & Elde R (1999). Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. *Eur J Neurosci* **11**, 946–958.

Haupt P, Janig W & Kohler W (1983). Response pattern of visceral afferent fibres, supplying the colon, upon chemical and mechanical stimuli. *Pflugers Arch* **398**, 41–47.

Hicks GA, Coldwell JR, Schindler M, Ward PA, Jenkins D, Lynn PA. *et al.* (2002). Excitation of rat colonic afferent fibres by 5-HT(3) receptors. *J Physiol* **544**, 861–869.

Holzer P (1998). Neural injury, repair, and adaptation in the GI tract. II. The elusive action of capsaicin on the vagus nerve. *Am J Physiol* **275**, G8–G13.

Houghton L, Atkinson W, Whitaker R, Whorwell P & Rimmer M (2003). Increased platelet depleted plasma 5-hydroxytryptamine concentration following meal ingestion in symptomatic female subjects with diarrhoea predominant irritable bowel syndrome. *Gut* **52**, 663–670.

Janig W & Koltzenburg M (1991). Receptive properties of sacral primary afferent neurons supplying the colon. *J Neurophysiol* **65**, 1067–1077.

Kidd EJ, Miller K, Sansum AJ & Humphrey PPA (1998). Evidence for P2X3 receptors in the developing rat brain. *Neuroscience* 87.

Kimball ES, Wallace NH, Schneider CR, D'Andrea MR & Hornby PJ (2004). Vanilloid receptor 1 antagonists attenuate disease severity in dextran sulphate sodium-induced colitis in mice. *Neurogastroenterol Motil* **16**, 1–8.

Kirkup AJ, Booth CE, Chessell IP, Humphrey PP & Grundy D (1999). Excitatory effect of P2X receptor activation on mesenteric afferent nerves in the anaesthetised rat. *J Physiol* **520**, 551–563.

Kirkup AJ, Brunsden AM & Grundy D (2001). Receptors and transmission in the brain-gut axis: potential for novel therapies. I. Receptors on visceral afferents. *Am J Physiol Gastrointest Liver Physiol* **280**, G787–G794.

Ligresti A, Bisogno T, Matias I, De Petrocellis L, Cascio M, Cosenza V. *et al.* (2003). Possible endocannabinoid control of colorectal cancer growth. *Gastroenterology* **125**, 677–687.

Lynn PA & Blackshaw LA (1999). In vitro recordings of afferent fibres with receptive fields in the serosa, muscle and mucosa of rat colon. *J Physiol* **518**, 271–282.

Manara L, Croci T, Guagnini F, Rinaldi-Carmona M, Maffrand J, Le Fur G. *et al.* (2002). Functional assessment of neuronal cannabinoid receptors in the muscular layers of human ileum and colon. *Dig Liver Dis* **34**, 262–269.

Mayer EA (2001). Some of the challenges in drug development for irritable bowel syndrome. *Gut* **48**, 585–586.

Mayer EA & Gebhart GF (1993). Functional Bowel Disorders & the visceral hyperalgesia hypothesis. In *Basic and Clinical Aspects of Chronic Abdominal Pain*, ed Mayer EA & Raybould HE, pp. 3–28. Elsevier, New York.

Michaelis M, Habler H & Jaenig W (1996). Silent afferents: a separate class of primary afferents? *Clin Exp Pharmacol Physiol* **23**, 99–105.

Naliboff BD, Munakata J, Fullerton S, Gracely RH, Kodner A, Harraf F & Mayer EA (1997). Evidence for two distinct perceptual alterations in irritable bowel syndrome. *Gut* **41**, 505–512.

Ness TJ & Gebhart GF (1988). Characterization of neurons responsive to noxious colorectal distension in the T13–L2 spinal cord of the rat. *J Neurophysiol* **60**, 1419–1438.

North RA (2002). Molecular Physiology of P2X Receptors. *Physiol Rev* **82**, 1013–1067.

North RA (2004). P2X3 receptors and peripheral pain mechanisms. *J Physiol* **554**, 301–308.

Page AJ, Martin CM & Blackshaw LA (2002). Vagal mechanoreceptors and chemoreceptors in mouse stomach and esophagus. *J Neurophysiol* **87**, 2095–2103.

Pinto L, Izzo A, Cascio M, Bisogno T, Hospodar-Scott K, Brown D. *et al.* (2002). Endocannabinoids as physiological regulators of colonic propulsion in mice. *Gastroenterology* **123**, 227–234.

Ritchie J (1973). Pain from distention of the pelvic colon by inflating a balloon in the irritable bowel syndrome. *Gut* **6**, 105–112.

Robinson DR, McNaughton PA, Evans ML & Hicks GA (2004). Characterization of the primary spinal afferent innervation of the mouse colon using retrograde labelling. *Neurogastroenterol Motil* **16**, 113–124.

Rong W, Hillsley K, Davis JB, Hicks GA, Winchester WJ & Grundy D (2004). Jejunal afferent nerve sensitivity in wild type and TRPV1 knockout mice. *J Physiol* **560**, 867–881.

Sengupta JN & Gebhart GF (1994). Characterization of mechanosensitive pelvic nerve afferent fibers innervating the colon of the rat. *J Neurophysiol* **71**, 2046–2060.

Souslova V, Cesare P, Ding Y, Akopian AN, Stanfa L, Suzuki R. *et al.* (2000). Warm-coding deficits and aberrant inflammatory pain in mice lacking P2X3 receptors. *Nature* **407**, 1015–1017.

Storr M, Sibaev A, Marsicano G, Lutz B, Schusdziarra V, Timmermans J-P & Allescher HD (2004). Cannabinoid receptor type 1 modulates excitatory and inhibitory neurotransmission in mouse colon. *Am J Physiol Gastrointest Liver Physiol* **286**, G110–G117.

Sugiura T, Bielefeldt K & Gebhart GF (2004). TRPV1 function in mouse colon sensory neurons is enhanced by metabotropic 5-hydroxytryptamine receptor activation. *J Neurosc* **24**, 9521–9530.

Vulchanova L, Riedl MS, Shuster SJ, Stone LS, Hargreaves KM, Buell G. *et al.* (1998). P2X3 is expressed by DRG neurons that terminate in inner lamina II. *Eur J Neurosci* **10**, 3470–3478.

Wynn G, Ma B, Ruan HZ & Burnstock G (2004). Purinergic component of mechanosensory transduction is increased in a rat model of colitis. *Am J Physiol Gastrointest Liver Physiol* **287**, G647–G657.

Wynn G, Rong W, Xiang Z & Burnstock G (2003). Purinergic mechanisms contribute to mechanosensory transduction in the rat colorectum. *Gastroenterology* **125**, 1398–1409.

Yiangou Y, Facer P, Baecker PA, Ford AP, Knowles CH, Chan CL. *et al.* (2001*a*). ATP-gated ion channel P2X(3) is increased in human inflammatory bowel disease. *Neurogastroenterol Motil* **13**, 365–369.

Yiangou Y, Facer P, Dyer NHC, Chan CLH, Knowles C, Williams NS & Anand P (2001*b*). Vanilloid receptor 1 immunoreactivity in inflamed human bowel. *Lancet* **357**, 1338–1339.

Zagorodnyuk VP, Chen BN, Costa M & Brookes SJ (2003). Mechanotransduction by intraganglionic laminar endings of vagal tension receptors in the guinea-pig oesophagus. *J Physiol* **553**, 575–587.

Acknowledgements

Stuart M. Brierley was supported by an Australian Postgraduate Award. L. Ashley Blackshaw was supported by a National Health and Medical Research Council of Australia Senior Research Fellowship. Work in Adelaide was supported by NHMRC Australia grant numbers 104814 and 298942 to L. Ashley Blackshaw. R. Carter W. Jones, III and G. F. Gebhart were supported by National Institutes of Health Awards F30 NS 46941, NS 19912 and NS 35790. David R. Robinson was supported by a GlaxoSmithKline PhD Studentship.

Author's present address

Gareth A. Hicks: Novartis Pharmaceuticals Corporation, One Health Plaza, East Hanover, NJ 07936, USA.