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Activation of pruritogenic TGR5, MRGPR A3 and MRGPRC11 on colon-innervating afferents induces visceral hypersensitivity

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Abstract

Itch induces scratching that removes irritants from the skin, whereas pain initiates withdrawal or avoidance of tissue damage. Whilst pain arises from both the skin and viscera, we investigated whether pruritogenic irritant mechanisms also function within visceral pathways. We show that subsets of colon-innervating sensory neurons in mice express, either individually or in combination, the pruritogenic receptors Tgr5 and the Mas-gene-related G protein-coupled receptors, Mrgpra3 and Mrgpra11. Agonists of these receptors activated subsets of colonic sensory neurons and evoked colonic afferent mechanical hypersensitivity via a TRPA1-dependent mechanism. In vivo intra-colonic administration of individual TGR5, MRGPR A3 or MRGPRC11 agonists induced pronounced visceral hypersensitivity to colorectal distension. Co-administration of these agonists as an ‘itch cocktail’ augmented hypersensitivity to colorectal distension and changed mouse behaviour. These irritant mechanisms were maintained and enhanced in a model of chronic visceral hypersensitivity relevant to irritable bowel syndrome. Neurons from human dorsal root ganglia also expressed TGR5 as well as the human ortholog MRGPRX1 and showed increased responsiveness to pruritogenic agonists in pathological states. These data support the existence of an irritant-sensing system in the colon that is a visceral representation of the itch pathways found in skin, thereby contributing to sensory disturbances accompanying common intestinal disorders.
INTRODUCTION

Itch, like pain, is a protective mechanism necessary for survival (1). Itch induces protective scratching that removes harmful irritants from the skin, whilst pain initiates withdrawal from and avoidance of noxious stimulants. Itch and pain are detected by primary sensory dorsal root ganglion (DRG) neurons that project from peripheral tissues into the dorsal horn of the spinal cord, where they release transmitters that excite spinal neurons (2). In the skin, histamine-dependent mechanisms contribute to itch; however, several distinct histamine-independent itch mechanisms have also been described. One involves the Mas-gene-related G protein-coupled receptor family, which includes MRGPR3A and MRGPRC11 (2-5). Another mechanism involves the bile acid receptor TGR5, also known as GPR130 or GpBAR1 (6).

MRGPR3A and MRGPRC11 are expressed by subsets of sensory DRG neurons innervating the skin (7,8). Activation of MRGPR3A by the anti-malarial drug chloroquine (CQ), or MRGPRC11 activation by the endogenous pruritogen, bovine adrenal medulla 8-22 peptide (BAM8-22), induces itch (3,9). Mice lacking a cluster of Mrgpr genes (Mrgpr cluster−/−) display significant deficits in itch induced by either CQ or BAM8-22, but crucially not itch induced by histamine (8). TGR5 is also expressed by a sub-population of peptidergic DRG neurons and activation of TGR5 by bile acids, such as deoxycholic acid (DCA) or oleanolic acid (OA), induces neuronal excitability and also induces itch in mice (6,10). These effects are lost in Tgr5−/− mice and are exacerbated in mice over-expressing TGR5 (Tgr5-Tg), potentially explaining why pruritus is observed in patients with cholestatic liver disease, where circulating bile acids are increased by 20-fold (6). However, it remains unclear if both TGR5 and Mrgpr mechanisms co-exist within the same DRG neuronal populations or whether they exist in, and therefore recruit, distinct populations of DRG neurons.

In the colon, afferent sensitisation occurs via a variety of processes (11), including histamine-dependent mechanisms (12); however, other pathways are also likely involved. For example increased fecal levels of bile acids have been implicated as the cause of diarrhea in a subset of patients with irritable bowel syndrome (IBS) (13), whilst abdominal pain and cramping are known side-effects of chloroquine treatment (14). Therefore, as pain arises from both the skin and viscera, we wondered whether pruritogenic irritant mechanisms identified within the skin have analogous pathways within the viscera. This is important as chronic abdominal pain or discomfort associated with altered bowel habits are key symptoms of IBS, a prevalent functional gastrointestinal disorder affecting ~11% of the global population (15). These symptoms significantly affect patient quality of
life and are notoriously difficult to treat. Although the pathophysiology of IBS is not completely understood, hallmarks of IBS include hypersensitivity to mechanical events within the intestine in the absence of overt pathology to the intestinal mucosa, resulting in allodynia and hyperalgesia (15). Whilst sensitisation and neuroplasticity of colonic afferent pathways has been implicated in the development and maintenance of chronic abdominal pain in IBS (15-17), the underlying mechanisms contributing to afferent sensitisation remain incompletely understood (18). We hypothesized that MRGPR A3-, MRGPR C11- and TGR5-dependent mechanisms could be important, but as of yet unidentified, mechanisms in this process.

The aim of this study was to determine if colonic afferents express TGR5, MRGPR C11 and MRGPR A3, and if so, whether they are present in distinct or overlapping subsets of colon-innervating DRG neurons. We also aimed to determine if agonists for TGR5, MRGPR C11 and MRGPR A3 induce changes in colonic sensory signalling in vitro and ex vivo and whether this translated to altered visceral sensitivity and behaviour in vivo. We determined if such mechanisms were present, or indeed augmented, in a model of chronic visceral hypersensitivity (CVH) relevant to IBS. Crucially, we aimed to translate these findings to humans by using colonic biopsies and DRG sensory neurons from human donors to confirm expression profiles and functional mechanisms.

We demonstrate that Tgr5, Mgrpr A3 and Mgrpr C11 are all expressed by colon-innervating DRG neurons, in both distinct and overlapping subsets of sensory DRG neurons, and their activation causes fundamental signalling changes within colonic afferent pathways in healthy and disease states. In human DRG neurons, TGR5 and MRGPRX1 also display both distinct and overlapping molecular and functional expression profiles, with increased responsiveness to pruritogens in sensitized states.
RESULTS

Agonists for TGR5, MRGPRA3 and MRGPRC11 evoke mechanical hypersensitivity in colonic afferents

In order to determine if pruritogenic receptors have a functional role in colonic sensory function, we made ex vivo recordings of colonic afferents from mice. Application of the TGR5 agonists deoxycholic acid (DCA), oleanolic acid (OA) and 3-(2-chlorophenyl)-N-(4-chlorophenyl)-N,5-dimethyl-4-isoxazolecarboxamide (CCDC) all evoked mechanical hypersensitivity in colonic afferent endings from healthy mice (Figure 1A,1B,1C). Closer examination of individual afferent responses showed that some afferents were unaffected by TGR5 activation, whereas others displayed pronounced mechanical hypersensitivity (Figure 1A,1B,1C), suggesting TGR5 is expressed by specific sub-populations of colonic afferents. Notably, the effects of CCDC were exacerbated in colonic afferents from mice over-expressing TGR5 (Tgr5-Tg, Figure 1D) and lost in afferents from Tgr5 null mutant (Tgr5<sup>−/−</sup>) mice (Figure 1E). As TGR5 activates transient receptor potential ankyrin 1 (TRPA1) to induce itch (10), TRPA1 mediates nociceptive responses (19,20) and we have previously shown that TRPA1 is a key integrator for the induction of mechanical hypersensitivity in colonic afferents by a variety of mediators (21-23), we applied CCDC to colonic afferents from Trpa1<sup>−/−</sup> mice. Correspondingly, we found that CCDC failed to induce mechanical hypersensitivity in afferents from Trpa1<sup>−/−</sup> mice (Figure 1F), suggesting a key integration between TGR5 and TRPA1 exists in colonic afferents. In terms of MRGPR signalling, chloroquine (CQ), an agonist of MRGPRA3, also evoked mechanical hypersensitivity in colonic afferents from healthy mice (Figure 1G). Similarly, the MRGPRC11 agonist BAM8-22 (Figure 1H) and the combined MRGPRC11/MRGPRA4 agonist neuropeptide FF (NPFF) (Figure 1I) also evoked colonic afferent mechanical hypersensitivity. As observed with DCA, OA and CCDC, closer examination of individual afferent responses showed that some afferents were unaffected by CQ, BAM8-22 or NPFF, whereas others displayed pronounced mechanical hypersensitivity (Figure 1G,1H,1I), suggesting MRGPRA3 and MRGPRC11 expression on specific sub-populations of colon-innervating afferents.

To determine the mechanisms by which TGR5 and Mrgpr agonists induce colonic afferent hypersensitivity, we confirmed expression of Tgr5, Mrgpra3 and Mrgprc11 mRNA using qRT-PCR and single-cell RT-PCR studies of colonic DRG neurons. Analysis of colonic mucosa from healthy mice by qRT-PCR revealed that Tgr5, Mrgpra3 and Mrgprc11 mRNA were all expressed in low abundance, particularly when compared with a known epithelial target such a Gucy2c (guanylate cyclase-C; Figure 2A). To determine if Tgr5, Mrgpra3 and Mrgprc11 were expressed by colonic afferent DRG
neurons, we performed single-cell RT-PCR from retrogradely traced colon-innervating DRG neurons. We also compared expression profiles to *Trpv1* and *Trpa1*, key channels involved in colonic afferent function (11,22,24). Of 97 individual neurons, 19% expressed *Tgr5*, 27% expressed *Mrgpra3* and 40% expressed *Mrgprc11* (Figure 2B). In comparison, *Trpv1* and *Trpa1* were expressed by 72% and 56% of colon-innervating DRG neurons respectively (Figure 2B). These findings indicate that these pruritogenic receptors are expressed on sensory neurons innervating the colon, correlating well with our observation that sub-populations of afferents display mechanical hypersensitivity following application of the respective TGR5 and Mrgpr agonists. Interestingly, we found that *Tgr5*, *Mrgpra3* and *Mrgprc11* were expressed either within the same colon-innervating DRG neuron or within separate subtypes of neurons (Figure 2C). For example, of the *Tgr5*-expressing population of colonic DRG neurons, 39% also expressed *Mrgpra3* and 39% *Mrgprc11* (Figure 2D). Of the *Mrgpra3* expressing population, 27% co-expressed *Tgr5*, whilst 58% co-expressed *Mrgprc11* (Figure 2E). Moreover, of the *Mrgprc11*-expressing neurons, 18% co-expressed *Tgr5* with 38% co-expressing *Mrgpra3* (Figure 2F). Overall, *Tgr5*, *Mrgpra3* and *Mrgprc11* were heavily co-expressed with both *Trpv1* (69-90%) and *Trpa1* (50-83%; Figure 2C,2D,2E,2F). Therefore, *Tgr5*, *Mrgpra3* and *Mrgprc11* are expressed by both distinct and overlapping subsets of colon-innervating DRG neurons, the majority of which co-express *Trpa1*, *Trpv1* or both channels (Figure 2C-2F).

**Agonists for TGR5, MRGPR3 and MRGPRC11 activate multiple populations of isolated colon-innervating sensory neurons**

In order to confirm the results of our single cell RT-PCR at a functional level, and to investigate the roles of pruritogenic irritants in activating colon-innervating DRG neurons, we measured intracellular calcium ([Ca$^{2+}$]), using Fura-2 AM in response to application of TGR5 and MRGPR agonists (Figure 3A-3G). In previous studies we have shown that DCA-evoked Ca$^{2+}$ transients in DRG neurons are generated by a TGR5-dependent process (6,10). Here we show in colon-innervating DRG neurons from healthy mice, the TGR5 agonists DCA (Figure 3B), tauroliothocholic acid (TLCA; Figure 3C), and CCDC (Figure 3D) all caused a robust increase in [Ca$^{2+}$] in subpopulations of colon-innervating DRG neurons. Overall, 21.5±4.4% of colonic DRG neurons responded to DCA, 27.1±7.2% responded to TLCA, whilst 28.6±3.1% responded to CCDC (Figure 3B-3D,3G). Furthermore, the MRGPR3 agonist CQ activated 20.7±5.1% of colon-innervating DRG neurons (Figure 3E,3G), with the MRGPRC11 agonist BAM8-22 activating 24.7±3.8% of neurons (Figure 3F,3G).
To further characterize these sub-populations, we quantified the proportion of colon-innervating DRG neurons that responded to TGR5 (CCDC,DCA,TLCA), MRGPRA3 (CQ) or MRGPRC11 (BAM8-22), along with TRPA1 (allyl isothiocyanate; AITC) and TRPV1 (capsaicin) agonists (Figure 3A-3F). Overall, 6-11% of colonic DRG neurons responded to the TGR5 agonists (either DCA,TLCA or CCDC), AITC and capsaicin, suggesting functional co-expression of TGR5, TRPA1 and TRPV1 (Supplementary Figure 1). Furthermore, 7-8% of colon-innervating DRG neurons responded to the TGR5 agonists and AITC, but not capsaicin (suggesting co-expression of TGR5 and TRPA1), with only ~2-6% of neurons responding to the TGR5 agonists and capsaicin alone (co-expression of TGR5 and TRPV1, Supplementary Figure 1). Similarly, ~9% of colon-innervating DRG neurons responded to CQ, AITC and capsaicin (MRGPRA3, TRPA1, TRPV1 co-expression), with 4% responding to CQ and AITC but not capsaicin, and 7% responding to CQ and capsaicin but not AITC (Supplementary Figure 1). Finally, ~10% of colon-innervating DRG neurons responded to BAM8-22, AITC and capsaicin (MRGPRC11, TRPA1, TRPV1 co-expression), with 8% responding to BAM8-22 and AITC but not capsaicin, and 6% responding to BAM8-22 and capsaicin but not AITC (Supplementary Figure 1). These results support a functional role for TGR5, MRGPRA3 and MRGPRC11 in overlapping and distinct populations of TRPA1 and/or TRPV1 expressing DRG neurons.

**In vivo intra-colonic administration of pruritogenic agonists increases signalling within the dorsal horn of the spinal cord**

To determine how activation and sensitisation of colonic afferents by TGR5 and MRGPR agonists results in altered signalling within colonic pathways in vivo, we identified activated dorsal horn neurons by phosphorylated-MAP-kinase-ERK-1/2-immunoreactivity (pERK-IR) in response to colorectal distension (CRD) (25-32). In healthy vehicle treated mice, CRD at a pressure of 40mmHg resulted in activation of dorsal horn (DH) neurons within the thoracolumbar (T10-L1) regions of the spinal cord (Figure 4A,4B). Pre-treatment of healthy mice with the TGR5 agonist CCDC enhanced CRD-evoked activation of DH neurons (Figure 4A,4B). However, intra-colonic administration of CCDC alone in healthy mice did not cause activation of DH neurons within the spinal cord (Supplementary Figure 2). Overall these findings indicate that intra-colonic CCDC induced colonic afferent mechanical hypersensitivity in vivo, which translated to increased neuronal activation within the spinal cord. Consistent with this action of TGR5, intra-colonic pre-treatment with CCDC in mice over-expressing TGR5 (Tgr5-Tg) significantly increased the number of pERK-IR neurons following CRD, compared with CRD plus vehicle in Tgr5-Tg mice (Figure 4C,4D). In contrast, intra-colonic pre-treatment with CCDC in Tgr5−/− mice did not alter the number of pERK-IR neurons following CRD compared with vehicle plus
CRD Tgr5−/− mice (Figure 4E,4F), suggesting that TGR5 does indeed mediate the effects of CCDC. Finally, Trpa1−/− mice pre-treated with intra-colonic CCDC followed by CRD displayed no increase in the number of pERK-IR neurons compared with Trpa1+/− mice with vehicle plus CRD, confirming that TRPA1 is crucial for TGR5-mediated mechanical hypersensitivity (Figure 4G,4H). We also observed that intra-colonic administration of CQ resulted in pronounced activation of neurons within the dorsal horn of the spinal cord, consistent with in vivo activation of MrgprA3 in colonic sensory afferent pathways (Supplementary Figure 3).

In vivo intra-colonic administration of pruritogenic agonists increases mechanically-evoked responses to colorectal distension and alters animal behaviour.

We next assessed whether TGR5 and MRGPR- induced activation of colonic afferents resulted in alterations in visceral sensitivity evoked by CRD in vivo. We measured the visceromotor response (VMR) to increasing CRD pressures by recording electromyographic (EMG) activity from electrodes surgically implanted into the abdominal muscles (30,33-35). In healthy mice, intra-colonic administration of CCDC significantly enhanced VMRs to CRD at all distension pressures, indicating visceral hypersensitivity (Figure 5A,5B, Supplementary Figure 4). In comparison, intra-colonic CCDC in Tgr5−/− mice failed to induce the elevated VMR to CRD observed in Tgr5+/+ mice administered intra-colonic CCDC (Figure 5C,5D, Supplementary Figure 4). Intra-colonic administration of the MRGPRA3 agonist CQ significantly enhanced the VMR to CRD in healthy mice, particularly at pressures ≥40mmHg (Figure 5E,5F, Supplementary Figure 4). However, CQ did not alter the VMR to CRD in Mrgpr cluster−/− mice (Figure 5G,5H, Supplementary Figure 4), confirming the role of MRGPRs in the actions of CQ in colonic pathways. Intra-colonic administration of the MRGPRC11 agonist BAM8-22 in healthy mice also significantly enhanced the VMR to CRD, although this increase was most apparent at higher, noxious distension pressures of ≥60mmHg (Figure 5I,5J, Supplementary Figure 4). In contrast, BAM8-22 did not alter the VMR to CRD in Mrgpr cluster−/− mice (Figure 5K,5L, Supplementary Figure 4). Notably, these CCDC, CQ and BAM8-22-induced changes in VMR to CRD were not due to changes in colonic compliance (Supplementary Figure 5A-5F), suggesting that the actions observed occurred via activation of receptors on afferent endings. Overall, these data show that application of the individual agonists for TGR5, MRGPRA3 and MRGPRC11 can each induce visceral hypersensitivity to CRD in healthy mice.

Since TGR5, MRGPRA3 and MRGPRC11 are expressed in distinct and overlapping populations of colon- innervating DRG neurons, we determined if co-administration of these agonists, as an ‘itch
cocktail’, also exacerbated visceral hypersensitivity. Concurrent intra-colonic administration of CCDC, CQ and BAM8-22 evoked pronounced increases in the VMR to CRD at all distension pressures and significantly increased the total VMR (Figure 5M,5N, Supplementary Figure 4). In contrast, Trpa1−/− mice intra-colonically administered the ‘itch cocktail’ did not show altered VMRs to CRD relative to vehicle administered Trpa1−/− mice (Figure 5O,5P, Supplementary Figure 4), confirming TRPA1 contributes to TGR5, MRGPR3 and MRGPRC11-induced mechanical hypersensitivity in colonic afferent pathways.

To determine if concurrent activation of TGR5, MRGPR3 and MRGPRC11 has effects beyond mechanically evoked visceral sensitisation, we also recorded animal behaviour in response to intra-colonic administration of the itch cocktail. Healthy mice co-administered CCDC, CQ and BAM8-22 covered significantly less distance in their enclosure (Figure 6A-6C), had a slower average velocity of travel (Figure 6D), displayed reduced locomotor activity (Figure 6E) and displayed more grooming behaviour (Figure 6F) compared with vehicle-administered mice. These behavioural changes were not observed when TGR5, MRGPR3 or MRGPRC11 agonists were applied individually (Supplementary Figure 6), suggesting full recruitment of these irritant pathways is required to induce profound behavioural changes in these mice. Notably, mice intra-colonically administered CCDC, CQ or BAM8-22, either individually or in combination, did not display increased scratching behaviour (Supplementary Figure 7), suggesting the agonists were localised to the colon and did not reach the systemic circulation. Overall, our results demonstrate crucial individual and combined roles for TGR5, MRGPR3 and MRGPRC11 in the sensitisation of colonic afferent pathways and the resultant changes in spinal cord processing, responsiveness to CRD and animal behaviour.

TGR5, MRGPR3 and MRGPRC11 also contribute to the sensitisation of colonic afferent pathways during chronic visceral hypersensitivity (CVH).

In order to determine if the roles of TGR5, MRGPR3 and MRGPRC11 in evoking visceral hypersensitivity extends into disease states, we used a CVH mouse model of IBS. CVH was induced by administration of intra-colonic trinitrobenzenesulphonic acid (TNBS), which has been shown to induce colitis (36,37). Whilst colonic inflammation spontaneously heals over a 7-day period, these mice subsequently develop chronic mechanical hypersensitivity of colonic afferents in the post-inflammatory state (25-27,30,34,36,38), display neuroplasticity within spinal cord pathways (16,30,31) and exhibit visceral hypersensitivity to CRD (30,34).
Colonic afferents from CVH mice displayed mechanical hypersensitivity relative to afferents from healthy mice (Supplementary Figure 8), as described previously (25-27,30,34,36,38). Interestingly, application of the TGR5 agonists DCA, OA or CCDC further amplified mechanical hypersensitivity in CVH colonic afferents, above their already elevated baseline levels (Figure 7A,7B,7C). We also observed that a sub-population of CVH afferents displayed action potential firing to application of the TGR5 agonists in the absence of mechanical stimuli, which rarely occurred in healthy colonic afferents (Supplementary Figure 9). Individual application of the MRGPRa3 agonist CQ, the MRGPRc11 agonist BAM8-22 or the MRGPRc11/MRGPRa4 agonist NPFF also further amplified mechanical hypersensitivity in CVH colonic afferents (Figure 7D,7E,7F). This was also associated with action potential firing to application of the individual Mrgpr agonists, which rarely occurred in healthy colonic afferents (Supplementary Figure 9).

Single cell RT-PCR from CVH mice showed that 20% of colon-innervating DRG neurons expressed Tgr5, whilst 39% expressed Mrgpra3, 74% expressed Mrgprc11, with 65% expressing Trpv1 and 74% Trpa1 (Figure 8A,8B). Compared with healthy colon-innervating DRG neurons, this represented a significant increase in the proportion of DRG neurons expressing Mrgprc11 or Trpa1 in CVH states (Supplementary Figure 10). There were also significant changes in the co-expression profiles of CVH colon-innervating DRG neurons (Figure 8C,8D,8E), with significantly more Mrgpra3 expressing CVH DRG neurons now co-expressing Mrgprc11 and Trpa1 (Supplementary Figure 10), and significantly fewer Mrgprc11 neurons co-expressing Trpv1 (Supplementary Figure 10).

We also found that intra-colonic administration of CCDC alone in CVH mice resulted in pERK-IR within DH neurons of the spinal cord (Supplementary Figure 2). Furthermore, CVH mice pre-treated with CCDC displayed significantly more pERK-IR DH neurons within the spinal cord following 40mmHg CRD compared with CVH mice with vehicle plus CRD (Figure 9A,9B). These findings indicate that in vivo intra-colonic CCDC activates colonic afferents and also induces mechanical hypersensitivity in CVH mice. In terms of behavioural responses, CVH mice intra-colonically administered the ‘itch cocktail’ of concurrent CCDC, CQ and BAM8-22 displayed significantly reduced movement in terms of the distance travelled within the central observational area of the enclosure (Figure 9C,9D), a significantly decreased distance from the walls of the enclosure (Figure 9E), and a significantly increased time spent grooming (Figure 9F). However, these CVH mice did not display increased scratching behaviour in response to the intra-colonic itch cocktail (Supplementary Figure 7). Overall, our results demonstrate that TGR5, MRGPRa3 and MRGPRc11 each contribute to the...
sensitisation of colonic afferent pathways in CVH states. There is an increase in MRGPRC11- and TRPA1-dependent mechanisms in CVH and that agonists for TGR5, MRGPR3A and MRGPRC11 profoundly alter the behaviour of CVH mice.

**Human DRG neurons express TGR5 and MRGPRX1 and respond to pruritogenic agonists**

To further investigate the translatability of our findings, we determined the mRNA expression profiles of TGR5 and MRGPRX1 (the human ortholog of murine Mrgpr3a and Mrgrpc11) in human tissue and also tested the responsiveness of human DRG neurons to TGR5, MRGPRX1, TRPV1 and TRPA1 agonists. Firstly, using colonic biopsies from 15 human healthy subjects, we found that TGR5 had low expression compared with a known epithelial target Gucy2c, whilst MRGPRX1 was absent (Figure 10A), which is consistent with our findings in mouse colonic mucosa (see Figure 2A). qRT-PCR of T9-L1 whole thoracolumbar DRG from 4 human donors showed expression of TGR5, with greater abundance of MRGPRX1, and in particular TRPA1 and TRPV1 (Figure 10B). Single cell RT-PCR from 53 individual human DRG neurons, of predominately smaller diameter, demonstrated that 38% expressed TGR5, 79% expressed MRGPRX1, 92% TRPV1 and 58% TRPA1 (Figure 10C,10D). Consistent with our observations of mouse DRG, we found that TGR5 and MRGPRX1 were expressed in both distinct and overlapping populations of human DRG neurons, which heavily co-expressed TRPV1 or TRPA1 (Figure 10D). Specifically, of the TGR5 expressing human DRG neurons, 78% co-expressed MRGPRX1, 97% co-expressed TRPV1 and 56% TRPA1 (Figure 10E). Of the MRGPRX1 expressing population, 37% co-expressed TGR5, 99% TRPV1 and 60% TRPA1 (Figure 10F). Of the TRPV1 expressing population, 40% co-expressed TGR5, 85% MRGPRX1 and 62% TRPA1 (Figure 10G), whilst of the TRPA1-expressing population 31% co-expressed TGR5, 82% MRGPRX1 and 98% TRPV1 (Figure 10H).

Using Ca\(^{2+}\) imaging of dissociated and cultured human DRG neurons, we found that sub-populations of neurons were activated by the application of CCDC (14%;Figure 11A,11G,11H), BAM8-22 (34%;Figure 11B,11G,11H), CQ (10%;Figure 11C,11G,11H) and NPFF (5%;Figure 11D,11G,11H), as indicated by robust increases in [Ca\(^{2+}\)] (Figure 11A-11D). Many of these neurons also responded to capsaicin (62%;Figure 11E,11G,11H) or AITC (27%;Figure 11F,11G,11H). In order to simulate a pathological state, we transiently incubated neurons in culture with inflammatory mediators (histamine, PGE II, serotonin, bradykinin) for 2 hours prior to the Ca\(^{2+}\) imaging experiments. Human DRG neurons from these cultures displayed greater amplitudes of response to the application of CCDC (Figure 11A,11G), CQ (Figure 11C,11G), capsaicin (Figure 11E,11G) and AITC (Figure 11F,11G).
Overall, 30% of neurons from the inflammatory mediator cultures responded to CCDC, 9% to CQ, 39% to BAM8-22, 11% to NPFF, with 84% responding to capsaicin and 30% to AITC (Figure 11I). Overall, significantly more neurons from the inflammatory mediator cultures responded to capsaicin than in the normal untreated cultures (Figure 11H-K, Supplementary Figure 11). Overall, these findings in human DRG neurons largely resemble our findings in mouse colon-innervating DRG neurons and suggest that TGR5 and MRGPRX1 play important roles in pruritogenic signalling from human DRG neurons in a variety of conditions.
DISCUSSION

Irritable Bowel Syndrome affects ~11% of the global population and therapeutic treatments are currently lacking (15). Persistent hypersensitivity of sensory pathways innervating the colon is linked to the initiation, development and maintenance of chronic discomfort and abdominal pain in IBS patients (15,16,39). Therefore, determining the mechanisms contributing to these processes is crucial. In the current study we show that activation of TGR5, MRGPR3 or MRGPRC11, commonly considered as itch receptors, either individually or collectively cause fundamental signalling changes within colonic afferent pathways in healthy states. Crucially, we also show that these mechanisms persist, and in the case of MRGPRC11 are augmented in CVH states. Therefore, this study provides novel insights on how the activation of pruritogenic receptors initiates colonic hypersensitivity and, importantly, how these receptors contribute to chronic hypersensitivity. Accordingly, this information may afford novel therapeutic strategies by directly targeting these receptors for the treatment of chronic discomfort and abdominal pain in IBS.

In the current study, we found that mRNA for the pruritogenic receptors Tgr5, Mrgpra3 and Mrgpc11 were all expressed in a remarkably large population (19%, 27%, and 40% respectively) of mouse colon-innervating DRG neurons in healthy states. Correspondingly, agonists for MRGPR3 (CQ), MRGPRC11 (BAM8-22) and TGR5 (DCA,TLCA,CCDC) activated ~20%-35% of isolated colon-innervating DRG neurons from healthy mice. Moreover, the individual agonists for MRGPR3, MRGPRC11 or TGR5 each induced mechanical hypersensitivity in sub-populations of colonic afferents from healthy mice. The ex vivo and in vivo sensitising effects of CCDC were exacerbated in Tgr5-Tg over-expressing mice and lost in Tgr5−/− mice, thereby confirming the role of TGR5 in these processes. Furthermore, mechanical hypersensitivity induced by either CQ or BAM8-22 was lost in Mrgpr cluster−/− mice, confirming the roles of MRGPRs in this process. In vivo activation of either TGR5, MRGPR3 or MRGPRC11 caused pronounced visceral hypersensitivity to CRD. These findings demonstrate clear and crucial individual roles for MRGPR3, MRGPRC11 and TGR5 in activating colonic afferent neurons and inducing mechanical hypersensitivity.

The sensitising effects of TGR5, MRGPR3 or MRGPRC11 agonists on colonic afferents likely occurs via neuronal mechanisms. This is because MRGPR3 and MRGPRC11 (40) are absent from colonic tissues, but are expressed on mouse and human DRG neurons. Whilst TGR5 is expressed on colonic afferents, it is also expressed on colonic epithelial cells and on enteric neurons (41,42).
However, we did not observe any changes in muscle compliance in our studies, suggesting the actions we observed were via direct actions on afferents rather than by secondary mechanisms. Indeed, very recent findings show that bile acid sensitise afferents in the proximal colon via 5-HT₃-dependent mechanisms, whilst these actions are 5-HT₃-independent more distally (43). Although not specifically investigated in the current study, TGR5 activation stimulates release of gastrin-releasing peptide (GRP) within the spinal cord (6), whilst Mrgpr activation results in the release of both GRP (44) and natriuretic polypeptide B (4) within the spinal cord to induce scratching (45). These mechanisms may also contribute to the transmission of visceral irritant signalling from the periphery to the spinal cord and is subject to further investigation.

Importantly, we show for the first time that MRGPRs and TGR5 are expressed in both distinct and overlapping populations of neurons. Our single cell RT-PCR analysis reveals that 62% of colon-innervating DRG neurons from healthy mice express at least one of the Tgr5, Mrgprc11 or Mrgpra3 receptors. This is an important finding as these different molecular and functional expression profiles would therefore allow individual, overlapping and additive signals to occur in response to a variety of pruritogenic irritants. To test this in vivo we administered CCDC, CQ or BAM8-22 individually to activate either TGR5, MRGPRA3 or MRGPRC11 on colonic afferents, respectively. In each scenario mechanical hypersensitivity was evident in response to CRD, with CQ and CCDC evoking visceral hypersensitivity across a wide range of distension pressures. In the case of BAM8-22, visceral hypersensitivity to CRD was observed at more noxious distension pressures. This is consistent with very recent findings showing that BAM8-22 evoked elevated pain responses to CRD in healthy mice (40). Whilst intra-colonic administration of the individual agonists for TGR5, MRGPRA3 and MRGPRC11 evoked hypersensitivity to CRD, they did not fundamentally affect spontaneous animal behaviour. When we administered an ‘itch cocktail’, consisting of a combination of CCDC, CQ and BAM8-22 to concurrently activate TGR5, MRGPRA3 and MRGPRC11 on colonic afferents, this resulted in pronounced mechanical hypersensitivity to CRD across a wide range of distension pressures. Moreover, by recruiting the full complement of afferents within these irritant pathways we also observed profound changes in spontaneous animal behaviour evoked by visceral hypersensitivity, evident by a reduction in locomotor activity and increased grooming.

We found that the ‘itch cocktail’-induced mechanical hypersensitivity to CRD in vivo was not evoked in Trpa1⁻/⁻ mice. Also, we did not observe afferent hypersensitivity, nor increased numbers of pERK-IR in the DH of the spinal cord in response to CCDC and CRD in Trpa1⁻/⁻ mice. These results are
consistent with the coupling mechanisms described in the skin, whereby TRPA1 has been identified as the downstream target of both MRGPR3 and MRGPR11 (9). These previous studies demonstrated that neither TGR5 (10), MRGPR3 nor MRGPR11 (9) agonists directly activate TRPA1. However, Trpa1⁻/⁻ mice display little to no scratching in response to CQ and BAM8-22 (9). Interestingly, the functional coupling between MRGPR3 and TRPA1 is attenuated by disrupting Gβγ intracellular signalling, while coupling between MRGPR11 and TRPA1 requires PLC signalling (9). Similarly, TGR5 also activates TRPA1 to induce itch in mice, with TGR5 activating and sensitizing TRPA1 via a Gβγ- and PKC-mediated mechanisms (10). Although previous studies identify high co-expression of TRPV1 with MRGPR3 and MRGPR11 (9), as also shown in the current study, there appears to be little to no interaction between these targets. CQ- and BAM8-22-evoked Ca²⁺ signalling and neuronal sensitization is profoundly diminished in neurons from Trpa1⁻/⁻ but not Trpv1⁻/⁻ mice (9). Although Trpv1 co-expresses with Tgr5, deletion or antagonism of TRPV1 has no effect on TGR5-induced itch (10). Comparably, in the current study, although we observed Trpv1 co-expression in Tgr5 (78%), Mrgrpa3 (69%), or Mrgrpc11 (90%) expressing colon-innervating DRG neurons, mechanical hypersensitivity was completely lost in studies using Trpa1⁻/⁻ mice. Accordingly, colonic afferents, like cutaneous afferents, appear to utilize coupling between TGR5, MRGPR3 or MRGPR11 and TRPA1 in order to mediate their sensitising actions. These findings further highlight TRPA1 as a crucial integrator of sensory signals in colonic afferents by inducing mechanical hypersensitivity in response to bradykinin (22), TNF-α (23), proteases (46) and now to bile acids, CQ and BAM8-22. Conversely, histamine-dependent mechanisms in the colon contribute to afferent sensitisation via TRPV1-dependent (12) and TRPV4-dependent (47) mechanisms, potentially suggesting divergent mechanisms between histamine-dependent and histamine-independent afferent sensitisation.

Our observations raise the question of why functional itch receptors are found in colonic sensory pathways. There are several possible roles for such irritant-sensing pathways in the colon. Firstly, bile acids are normally present in the colonic lumen; they are secreted into the intestinal lumen during feeding, are absorbed in the ileum, and are modified by the colonic microbiome (48). Also, TGR5 in enteric neurons of the colon contributes to bile acid-dependent stimulation of peristalsis (41). Secondly, BAM8-22 is a proteolytically cleaved product of proenkephalin A, an endogenous ligand found throughout peripheral tissues, including the gastrointestinal tract (49),(50). Thirdly, while a well-recognised side effect of the use of CQ in the treatment of malaria is itch, less recognised symptoms of CQ treatment include abdominal cramping and pain (51). Therefore, whilst itch induces protective scratching that removes harmful irritants from the skin, identification of TGR5,
Mrgrpa3 and Mrgrp11 in colonic afferents may represent an analogous system in the viscera. This would provide protective mechanisms for detecting harmful irritants within the colon and ultimately expelling them from the body via activation of sensory afferents and recruitment of defecatory mechanisms (41). Accordingly, increased levels of bile acids are implicated in diarrhea-predominant IBS (52). Based on our current findings, bile acids also contribute to visceral hypersensitivity and the development of abdominal discomfort and pain via activation of TGR5 expressed on colonic afferents. In keeping with such a role, in vivo intra-colonic administration of CCDC-evoked mechanical hypersensitivity and increased the number of activated neurons within the DH of the spinal cord following CRD. Similarly, in vivo intra-colonic CQ administration resulted in the subsequent activation of DH neurons within the spinal cord and evoked mechanical hypersensitivity to CRD.

We also demonstrate that TGR5-, MRGPR3- and MRGPRC11-dependent mechanisms extend beyond sensitisation of colonic pathways in healthy states. Crucially, by using a CVH model, we show that colonic afferents from CVH mice display mechanical hypersensitivity compared with afferents from healthy mice. Application of CCDC, CQ or BAM8-22 further enhanced CVH afferent responses to mechanical stimuli, significantly increasing responses above their already elevated levels. Thus, activation of TGR5, MRGPR3 or MRGPRC11 in CVH states can further exacerbate visceral hypersensitivity, leading to hyperalgesia. Correspondingly, we also showed that afferents from CVH mice were more likely to fire action potentials in response to pruritogens and displayed increased numbers of pERK-IR DH neurons in response to intra-colonic CCDC application in the absence of CRD. Notably, significantly more colon-innervating DRG neurons from CVH mice express Mrgrp11 and Trpa1, with a significant increase in the proportion of Mrgrpa3 expressing neurons now also co-expressing Mrgrp11 and Trpa1. Our single cell RT-PCR analysis reveals that 83% of colon-innervating DRG neurons from CVH mice express at least one of the Tgr5, Mrgrp11 or Mrgrpa3 receptors, compared with only in 62% in healthy states. This suggests alterations in the molecular and functional phenotypes of these neuronal sub-populations in CVH mice, allowing more afferents to be activated by pruritogens compared with healthy states. Correspondingly, we found that using an intra-colonic ‘itch cocktail’ of CCDC, CQ and BAM8-22 to concurrently activate TGR5, MRGPR3 and MRGPRC11 on colonic afferents in CVH mice caused decreases in locomotion and increased grooming and thigmotaxis, indicative of anxiety like behaviour. Interestingly, in addition to altered intestinal motility and chronic pain, IBS patients also suffer from psychiatric conditions including depression and anxiety (15).
Finally, we show that these TGR5 and Mrgpr mechanisms are also present in human DRG neurons. Whilst MRGPRs have been previously detected in human DRG (7,49), it is unclear as to their co-expression profiles with TGR5, TRPA1 and TRPV1. Although we could not specifically identify colon-innervating DRG neurons in humans, we could investigate DRG at spinal levels known to innervate the colon (T9-L1), in order to test the concept molecularly and functionally, that TGR5, MRGPRX1, TRPV1 and TRPA1 co-expressing neurons exist in human DRG. This is important as CQ induces itch in humans (53), whilst BAM8-22 produces itch and nociceptive sensations in humans independent of histamine release (54), whilst TGR5 is linked to cholestatic pruritus in humans (55).

As per our findings in mouse DRG, we found with single cell RT-PCR and calcium imaging studies that TGR5 and MRGPRX1 were expressed in both distinct and overlapping populations of human DRG neurons, which largely co-expressed TRPV1 and/or TRPA1. Whilst there are some discrepancies in absolute percentages between Ca^{2+} imaging and single cell RT-PCR studies, this could be attributed to translational efficiency of mRNA to protein and surface expression of the receptors at the time of recording. Simulating a pathological state by incubating neurons with inflammatory mediators, significantly increased [Ca^{2+}]_{i} responses were observed in human DRG neurons to CCDC, CQ, capsaicin and AITC, compared with normal culture conditions. This suggests, like in our mouse studies, that these neuronal responses to pruritogenic irritants can be readily ‘tuned’ to induce hypersensitive responses in pathological conditions.

Overall, our findings shed new light on the mechanisms contributing to colonic afferent hypersensitivity in healthy and disease-relevant states. We identify novel mechanisms by which MRGPRA3, MRGPRC11 and the bile acid receptor TGR5 contribute to the induction of visceral hypersensitivity and altered behaviour in response to known pruritogens. Our findings add to the recent discovery of a novel endogenous mediator, 5-oxoETE, which activates afferents via MRGPRD to evoke visceral hypersensitivity (56). Our findings demonstrate that the role of TGR5 and MRGPRA3 and MRGPRC11 extend beyond itch sensation in the skin, adding to recent work demonstrating that MRGPRC11 expressed on vagal sensory neurons contributes to bronchoconstriction and airway hyper-responsiveness (57). Our findings also demonstrate translatable of these TGR5 and MRGPR mechanisms and their co-expression with TRPV1 and TRPA1 to human DRG neurons. Accordingly, targeting the TGR5- and MRGPR-dependent mechanisms may prove useful in treating visceral hypersensitivity associated with common intestinal disorders.
METHODS:
For extensive descriptions of the methodology please see the Supplementary Material.

Animals:
Male C57BL/6J mice aged 13-17 weeks were used for studies and acquired from an in-house C57BL/6J breeding programme (Jax strain #000664; originally purchased from The Jackson Laboratory (breeding barn MP14; Bar Harbor, ME, USA) within SAHMRI’s specific and opportunistic pathogen-free animal care facility. Some experiments also utilised male mice lacking Tgr5 (Tgr5−/−)(6), Trpa1 (Trpa1−/−)(22), Mrgpr cluster−/− (8) or mice over expressing Tgr5 (Tgr5-Tg)(6) from in-house breeding colonies at SAHMRI, Adelaide, Australia. Tgr5−/− and Tgr5-Tg mice were gifts originally provided by Johan Auwerx and Kristina Schoonjans, Ecole Polytechnique de Lausanne, Lausanne, Switzerland. Mrgpr cluster−/− mice were gifts from Xinzhong Dong, Johns Hopkins University, Baltimore, Maryland, USA. Trpa1−/− mice were gifts originally from David Corey, Harvard University, Cambridge, Massachusetts, USA.

Mouse model of CVH:
Mice were administered intra-colonic trinitrobenzenesulphonic acid (TNBS) and developed colitis (25-27,36,38) which healed over 7-days. These mice subsequently developed chronic colonic afferent hypersensitivity (25-27,36,38).

Ex vivo single fibre colonic nociceptor recordings:
Recordings were made from healthy, CVH or Tgr5−/−, Tgr5-Tg, or Trpa1−/− mice using standard protocols (25-27,34,38). Mechanosensitivity was determined before and after a 5 minute application of oleanolic acid (OA;100µM), Deoxycholic acid (DCA;100µM), 3-(2-chlorophenyl)-N-(4-chlorophenyl)-N,5-dimethyl-4-isoxazolcarboxamide (CCDC;100µM), BAM8-22 (20µM), chloroquine (CQ;10µM) or neuropeptide FF (NPFF;5µM).

qRT-PCR for pruritogenic receptors in mouse colonic epithelial cells:
The epithelial layer was removed from the colon and RNA extracted. qRT-PCR was performed using commercially available hydrolysis TaqMan® probes for Tgr5, Mrgrpα3, MrgrpC11 and Gucy2c (GC-C, Supplementary Table 1). Relative abundance was calculated using the delta Cq method (25).

Retrograde tracing to label the cell bodies of colon innervating afferents:
Dicarbocyanine dye,1,1-dioctadecyl-3,3,3,3-tetramethindocarbocyanine methanesulfonate (DiI, 2% in ethanol; Invitrogen, Carlsbad, CA) or cholera toxin subunit B conjugated to AlexaFluor-555 (CTB-555) was injected at three sites sub-serosally within the distal colon. Animals were left to recover for 7-10 days or 4-days respectively to identify cell bodies within the DRG (25,31,34).

Single cell RT-PCR of colon-innervating DRG neurons from healthy and CVH mice:
Individual retrogradely traced colon-innervating DRG neurons (97 from seven healthy mice and 46 from four CVH mice) were picked, RNA isolated and mRNA expression determined in each neuron for tgr5, MrgprA3, MrgprC11, trpv1 and trpa1 using probes indicated within the Supplementary methods(25).

[Ca\textsuperscript{2+}]\textsubscript{i} assays of colon-innervating DRG neurons from healthy mice:

Neurons were enzymatically dissociated, plated onto coverslip and cultured overnight. Neurons were loaded with Fura-2-AM (2µM) and fluorescence was measured at 340nm and 380nm excitation and 530nm emission(10). Neurons were tested with deoxycholic acid (DCA; 100 µM), 3-(2-chlorophenyl)-N-(4-chlorophenyl)-N,5-dimethyl-4-isoxazolecarboxamide (CCDC;100µM), tauroliothocholic acid (TLCA;100µM), chloroquine (CQ;10µM) or BAM8-22 (20µM), then allyl isothiocyanate (AITC;100 µM), capsaicin (1µM), and KCl (50mM).

Visualisation of pERK neurons within the dorsal horn of the spinal cord following colorectal distension (CRD):

C57BL/6J healthy (32), CVH (25-27,30) or Trpa1\textsuperscript{-/-} (21,22), Tgr5\textsuperscript{-/-} (6,41) or Tgr5-Tg(6,41) mice were briefly anaesthetized with isoflurane anaesthetic and a 100µL enema of CCDC (100µM) or CQ (10µM), or saline (vehicle) administered intra-colonically via a catheter. Subsequently, a 4cm balloon catheter was inserted into the perianal canal and 40mmHg CRD was performed (10 seconds on, 5 second deflation, repeated 5-times). In separate experiments, an enema of CQ (10µM) was applied for 5 minutes. After anaesthetic overdose, mice were fixed by trancardial perfusion of 4% paraformaldehyde. The spinal cord was then removed and cryoprotected. Frozen sections were cut and incubated with monoclonal-rabbit anti-phosphorylated-MAP-kinase-ERK-1/2 (pERK, #4370 Cell Signalling Technology,MA,USA;RIID:AB_2315112) and visualized with AlexaFluor-488 (25-27,30-32).

In vivo visceromotor response (VMR) to CRD:

Visceral sensitivity to CRD (20,40,50,60,70,80mmHg, each 20 second duration, applied at 4 minute-intervals) was assessed using abdominal electromyography (EMG) in fully awake healthy (30,33,34), Tgr5\textsuperscript{-/-}, Mrgpr cluster\textsuperscript{-/-} or Trpa1\textsuperscript{-/-} mice, following intra-colonic administration (100µL) of either CCDC (100µM), CQ (10µM), BAM8-22 (20µM) or an intra-colonic ‘itch cocktail’ consisting of a combination of CCDC (100µM), BAM8-22 (20µM) and CQ (10µM). Colonic compliance was assessed by applying graded volumes (40-200µL,20 second duration)(33,34).

In vivo assessment of animal behaviour:

Behavioural testing was evaluated using a behavioural spectrometer (Behavior Sequencer, Behavioural Instruments, NJ and BiObserve, DE,USA)(58). Healthy or CVH mice were briefly anaesthetized with isoflurane and a 100µL enema of an ‘itch cocktail’, consisting of CCDC (100µM), BAM8-22 (20µM) and CQ (10µM), was administered intra-colonically via a lubricated catheter. A 100µl saline enema was used as control. Mice were individually placed in the centre of the...
behavioural spectrometer, and their behaviour was filmed, tracked and evaluated, analyzed by a computerized video tracking system (Viewer³, BiObserve, DE) for a total of 20 minutes.

Human tissue:
Human DRG were acquired from 5 organ donors with whole ganglia processed for downstream qRT-PCR or dissociated for single-cell RT-PCR analysis or Ca²⁺ imaging(25,30). Human colonic biopsies from 15 healthy subjects were acquired from UCLA recruited primarily by community advertisement.

mRNA analysis of pruritogenic targets from human tissue:
RNA was extracted from colonic biopsies from 15 subjects and whole bilateral DRG from 4 donors. qRT-PCR was performed using EXPRESS One-Step Superscript® qRT-PCR Kit reagents (Life Technologies) with commercially available TaqMan® probes for TGR5, MRGPRX1, GUCY2C, TRPA1, and TRPV1 (Supplementary Table 1). Relative abundance was estimated using delta Cq method (25).

Single cell RT-PCR of human DRG neurons:
A total of 53 human DRG neurons from 4 adult organ donors were individually picked. Ambion® Single Cell-to-CT™ Kit (Life Technologies, Sydney, Australia) was used on an Applied Biosystems® 7500 Real-Time PCR System, with the TaqMan® primers (see Supplementary Table 1) to determine mRNA expression in each neuron for TGR5, MRGPRX1 (human ortholog of mouse Mrgpra3 and Mrgprc11), TRPV1 and TRPA1.

[Ca²⁺]ᵢ assays of human DRG neurons in response to pruritogens:
Human DRG were dissociated, neurons plated on coverslips and cultured. Some coverslips were cultured in ‘normal media’, whilst others, in order to mimic a pathological state, were pre-incubated with an ‘inflammatory soup’ containing 10µM each of Histamine (Sigma), PGEII (Tocris, UK), Serotonin (Tocris, UK) and Bradykinin (Sigma, Sydney, Australia), 2 hour prior to the experiments at 37˚C. For the Ca²⁺ imaging experiments, neurons were loaded with 3µM Fluo 8-AM and responses to CCDC (100µM), CQ (1µM), BAM8-22 (2µM), NPFF (2µM), capsaiacin (100nM) and AITC (50µM) were determined.

Statistics:
Data are expressed as Mean ± SEM or the % of neurons/afferents. Figures were prepared in GraphPad Prism 8 Software (San Diego, CA, USA). N equals the number of animals, whilst n equals the number of neurons/afferents. A P value less than 0.05 was considered significant. Differences were indicated significant at levels of *P<0.05,**P<0.01,***P<0.001,****P<0.0001. VMR to CRD data were statistically analysed by generalised estimating equations followed by LSD post-hoc test using SPSS 23.0 (IMB, USA). All other data were analysed using GraphPad Prism 8 (San Diego, CA, USA) and analysed if the data were normally distributed using Kolmogorov-Smirnov or Shapiro-Wilk tests. These data were then analysed using either 1) one-way ANOVA, with post hoc analysis
conducted by making all possible comparisons among the treatment groups with the Tukey’s tests, 2) two-way ANOVA, with Bonferroni post hoc analysis conducted by making all possible comparisons among the treatment groups, 3) paired or 4) unpaired 2-tailed t-tests or 5) $\chi^2$ analysis. The specific tests used to analyse each data set is indicated within the individual figure legends.

Study approval:

All animal experiments were approved and conformed to regulatory standards and the ARRIVE guidelines. The Animal Ethics Committees of the South Australian Health and Medical Research Institute (SAHMRI), Flinders University, The University of Adelaide and Monash University approved all experiments involving animals. All animal experiments conformed to the relevant regulatory standards and the ARRIVE guidelines.

All human tissues used for the study were obtained by legal consent from organ donors in the US. For DRG studies, the DRG were acquired from 5 organ donors with ethical consent. AnaBios Corporation’s procurement network includes only US based Organ Procurement Organizations and Hospitals. Policies for donor screening and consent are the ones established by the United Network for Organ Sharing (UNOS). Organizations supplying human tissues to AnaBios follow the standards and procedures established by the US Centres for Disease Control (CDC) and are inspected biannually by the Department of Health and Human Services (DHHS). Tissue distribution is governed by internal Institutional Review Board (IRB) procedures and compliance with HIPAA regulations regarding patient privacy. All transfers of donor organs to AnaBios are fully traceable and periodically reviewed by US Federal authorities. For human colonic biopsies study approval was obtained from the University of California, Los Angeles (UCLA, IRB#12-001731) IRBs and all subjects signed a written informed consent form prior to starting the study.

Author contributions:

J.C, L.G and S.M.B designed, performed and analysed the colonic afferent recordings. A.M.H, J.M, T.OD and S.M.B designed, performed and analysed the pERK dorsal horn studies. J.C, J.M, G.S and S.M.B designed, performed and analysed the VMR to CRD studies. G.S and S.M.B designed and performed the behavioural studies. TM.L, S.G.C, N.W.B and S.M.B designed, performed and analysed the mouse single cell RT-PCR experiments. TM.L, D.P.P, N.W.B and S.M.B designed, performed and analysed the mouse $\text{Ca}^{2+}$ imaging experiments. S.G.C and S.M.B designed, performed and analysed the human DRG neuron single cell PCR and whole human DRG qRT-PCR expression studies. L.C collected and provided human colonic biopsies. X.D provided Mrgpr cluster-$\text{m}$ mice. A.L and S.M.B designed, performed and analysed the mouse colonic mucosal and the human biopsy qRT-PCR expression studies. P.M, A.G and S.M.B designed, performed and analysed the human DRG $\text{Ca}^{2+}$ imaging studies. All authors contributed to the discussion and interpretation of the results. S.M.B wrote the manuscript with contributions and suggestions from all authors.
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Figure 1: Agonists for TGR5, MRGPR3 and MRGPRC11 all evoke mechanical hypersensitivity in colonic afferents.

A) Application of the TGR5 agonist deoxycholic acid (DCA;100µM) to the colonic mucosa for 5 minutes resulted in subsequent mechanical hypersensitivity of colonic nociceptors from healthy mice.
Lower panel shows representative recordings from a single colonic afferent nerve fibre responding to a 2g von Frey hair (vfh) before and after incubation with DCA. B) Application of the TGR5 agonist oleanolic acid (OA; 100µM for 5 minutes) also caused mechanical hypersensitivity in nociceptors from healthy mice (**P<0.01,N=8). C) The potent synthetic TGR5 agonist CCDC (100µM for 5 minutes) also evoked mechanical hypersensitivity of colonic nociceptors from healthy mice (*P<0.05,N=7). D) CCDC (100µM) induced mechanical hypersensitivity was enhanced in colonic nociceptors from mice over-expressing TGR5 (Tgr5-Tg, ****P<0.0001,N=10), but E) was not observed in colonic nociceptors from Tgr5-/- mice (P>0.05, N=10). F) Furthermore, CCDC (100µM) induced mechanical hypersensitivity was not observed in colonic nociceptors from Trpa1-/- mice (P>0.05,N=7), suggesting a key interaction between TGR5 and TRPA1 in the mechanical hypersensitivity evoked by TGR5 activation. G) Application of the MRGPR A3 agonist chloroquine (CQ; 10µM for 5 minutes) resulted in subsequent mechanical hypersensitivity of colonic nociceptors from healthy mice (****P<0.01,N=8). H) The MRGPR C11 agonist BAM8-22 (20µM for 5 minutes) also caused mechanical hypersensitivity in nociceptors from healthy mice (*P<0.05,N=8). I) Application of the combined MRGPR C11/MRGPR A4 agonist neuropeptide FF (NPFF;5µM for 5 minutes) also evoked mechanical hypersensitivity of colonic nociceptors from healthy mice (****P<0.01,N=8). Data represent Mean±SEM. P values determined by paired t-tests (A-I).
Figure 2: *Tgr5*, *Mrgprc11* and *Mrgpra3* are expressed in both distinct and overlapping subpopulations of colon-innervating DRG neurons

**A)** qRT-PCR analysis showing low mRNA abundance for *Tgr5*, *Mrgpra3* and *Mrgprc11* in the colonic mucosa compared with a known epithelial target *gucy2c* (guanylate cyclase-C, ****P<0.0001,N=7, each dot represents data from an individual mouse). **B)** Single-cell RT-PCR of 97 retrogradely traced colon-innervating DRG neurons (from N=5 mice) reveals that sub-populations express transcripts encoding *Tgr5* (19%), *Mrgpra3* (27%), *Mrgprc11* (40%), *Trpv1* (72%) and *Trpa1* (56%). **C)** Donut plot showing expression and co-expression of genes encoding *Tgr5*, *Mrgpra3*, *Mrgprc11*, *Trpv1* and *Trpa1* in 97 individual retrogradely traced colon-innervating DRG neurons. Each colour represents an individual gene with expression marked by bold shading. *Tgr5* is represented in the outer ring with *Trpa1* in the inner ring. Individual neurons are arranged radially, such that co-expression of genes in a single neuron can be easily identified running from outside to inside. Some neurons express all targets, whilst other neurons express combinations of targets. **D-F)** Group data showing that **D)** *Tgr5*, **E)** *Mrgpra3* and **F)** *Mrgprc11* are expressed individually within subpopulations of colon-innervating DRG neurons and also co-express together in other sub-populations. For example, of the *Tgr5* expressing colon-innervating DRG neurons from healthy mice, 39% co-express *Mrgpra3* and 39% co-express *Mrgprc11*. Furthermore, *Tgr5*, *Mrgpra3* and *Mrgprc11* also co-express with *Trpv1* (69-90%) and *Trpa1* (50-83%). Data in (A) represent Mean±SEM, with P values determined by one-way ANOVAs with Tukey’s multiple comparison tests.
Figure 3: Colon-innervating DRG neurons respond to pruritogenic agonists for TGR5, MRGPR3 and MRGPRC11.

**A)** Representative Ca\(^{2+}\) responses to the application of the TRPA1 agonist allyl isothiocyanate (AITC; 100\(\mu\)M), the TGR5 agonist CCDC (100\(\mu\)M), and the TRPV1 agonist capsaicin (1\(\mu\)M) in three Dil-labelled DRG neurons retrogradely labelled from the mouse colon. Right panels show Fura-2AM image of all cells within the field of view and the three Dil labelled colon-innervating DRG neurons recorded from the left panel. Scale bar 20\(\mu\)m. **B-F)** Representative traces of Ca\(^{2+}\) responses in Dil-labelled colon-innervating DRG neurons to sequential application of AITC (100\(\mu\)M), the TGR5 agonists **B**) deoxycholic acid (DCA; 100\(\mu\)M), **C**) tauroliothocholic acid (TLCA; 100\(\mu\)M) and **D**) CCDC (100\(\mu\)M), or the **E**) MRGPR3 agonist chloroquine (CQ: 10\(\mu\)M), the **F**) MRGPRC11 agonist BAM8-22 (2\(\mu\)M), followed by capsaicin (1\(\mu\)M) and KCl (50mM; not shown). DCA, TLCA, CCDC, CQ and BAM8-22, all activated sub-populations of colon-innervating DRG neurons with varying functional co-expression with TRPA1 (AITC) and/or TRPV1 (capsaicin). **G**) Group data showing the percentage of colon-innervating DRG neurons responding to DCA (61 neurons tested), TLCA (93 neurons tested), CCDC (93 neurons tested), CQ (94 neurons tested) and BAM8-22 (110 neurons tested). Each dot represents data from individual coverslips from a total of 6 mice. Data presented are Mean±SEM.
Figure 4: In vivo intra-colonic administration of the TGR5 agonist CCDC enhances colorectal distension-induced signalling within the dorsal horn of the spinal cord

A) Colorectal distension (CRD) at a pressure of 40mmHg in healthy mice results in activation of dorsal horn (DH) neurons within the thoracolumbar (T10-L1) spinal cord, as indicated by phosphorylated-MAP-kinase-ERK-1/2-immunoreactivity (pERK-IR, yellow arrows). pERK-IR neurons within the thoracolumbar DH, activated in response to 40mmHg CRD, were predominantly located in laminae I–IV. 

B) Group data showing that mice pre-treated with intra-colonic CCDC (100µM) displayed significantly more pERK-IR DH neurons within the thoracolumbar spinal cord following 40mmHg CRD.
compared with 40mmHg CRD alone (*P<0.05, Dots indicate individual counts in spinal cord sections from CRD mice:N=7 vs. CCDC + CRD mice:N=7). **C-D** Similarly, intra-colonic pre-treatment with CCDC in mice over-expressing TGR5 (Tgr5-Tg) increased the number of pERK-IR activated neurons following 40mmHg CRD, compared with 40mmHg CRD alone (*P<0.05, Tgr5-Tg CRD mice:N=4 vs. Tgr5-Tg CCDC + CRD mice:N=4). **E-F** In contrast, intra-colonic pre-treatment with CCDC in Tgr5−/− mice did not result in an increase in pERK-IR activated neurons following 40mmHg CRD, compared with 40mmHg CRD alone (P>0.05, Tgr5−/− CRD mice:N=4 vs. Tgr5−/− CCDC + CRD mice:N=4). **G-H** Trpa1−/− mice administered an intra-colonic pre-treatment with CCDC did not display increased numbers of pERK-IR neurons following 40mmHg CRD, compared with CRD 40mmHg alone (P>0.05, CRD mice:N=4 vs. CCDC + CRD mice:N=4). Data presented are Mean±SEM. P values determined by unpaired t-tests **(B,D,F,H)**. Dots represent data from individual sections of spinal cord from N=4-7 mice. Scale bars in **A,C,E,G** equal 100µm.
Figure 5: In vivo intra-colonic administration of TGR5, MRGPR A3 and MRGPR C11 agonists, alone or in combination, induces visceral hypersensitivity to colorectal distension.

A) Intra-colonic administration of CCDC (100µM) resulted in significantly enhanced visceromotor responses (VMRs) to colorectal distension (CRD) in healthy mice, with significant increases observed across all distension pressures (*P<0.05, **P<0.01, ****P<0.0001, vehicle:N=12; CCDC:N=10).

B)
Group data expressed as the total area under the curve (AUC) of the VMR to CRD shows significantly elevated responses following intra-colonic CCDC (*\(P<0.01\), Vehicle:N=12; CCDC:N=10). Each dot represents the total AUC from an individual animal. C) Tgr5\(^{-/-}\) mice administered intra-colonic CCDC (100\(\mu\)M) showed significantly reduced VMRs compared with Tgr5\(^{+/+}\) littermates administered intra-colonic CCDC (*\(P<0.05\), Tgr5\(^{+/+}\):N=15; Tgr5\(^{-/-}\):N=10). D) Significantly reduced total VMRs in Tgr5\(^{-/-}\) mice administered CCDC compared with Tgr5\(^{+/+}\) (*\(P<0.05\), Tgr5\(^{+/+}\):N=15; Tgr5\(^{-/-}\):N=10). E) Healthy mice administered intra-colonic chloroquine (CQ;10\(\mu\)M) have significantly elevated VMRs, particularly at 40-80mmHg distension (**\(P<0.01\), ***\(P<0.001\), Vehicle:N=12; CQ:N=7). F) Intra-colonic CQ significantly enhanced total VMRs compared with vehicle (**\(P<0.001\) Vehicle:N=12; CQ:N=7). G) Mrgpr cluster\(^{/-}\) mice intra-colonically administered 10\(\mu\)M CQ did not show altered VMRs nor altered H) total VMR relative to Mrgpr cluster\(^{/-}\) mice administered vehicle (ns, \(P>0.05\), Mrgpr cluster\(^{/-/+}\) vehicle:N=9; Mrgpr cluster\(^{/-/+}\) + CQ:N=7). I) Mice administered intra-colonic BAM8-22 (20\(\mu\)M) have significantly elevated VMRs, particularly at noxious distension pressures of 70-80mmHg (*\(P<0.05\), **\(P<0.01\), Vehicle:N=12; BAM8-22:N=5). J) Intra-colonic BAM8-22 significantly enhanced total VMRs compared with vehicle (**\(P<0.01\) Vehicle:N=12; BAM822: N=5). K) Mrgpr cluster\(^{/-}\) mice intra-colonically administered 20\(\mu\)M BAM8-22 had unaltered VMRs and unaltered L) total VMRs to CRD relative to Mrgpr cluster\(^{/-}\) mice administered vehicle (ns, \(P>0.05\), Mrgpr cluster\(^{/-/+}\) + vehicle:N=9; Mrgpr cluster\(^{/-/+}\) + BAM8-22:N=9). M) An intra-colonic ‘itch cocktail’ consisting of a combination of CCDC (100\(\mu\)M), BAM8-22 (20\(\mu\)M) and CQ (10\(\mu\)M) significantly enhanced VMRs in healthy mice. This hypersensitivity was evident at 40-50mmHg (****\(P<0.0001\)), 60-70mmHg (**\(P<0.01\)) and 80mmHg (**\(P<0.001\), Vehicle:N=12; Itch cocktail:N=8). N) The itch cocktail also significantly enhanced the total VMR compared with vehicle (**\(P<0.001\) Vehicle:N=12; Itch cocktail:N=8). O) Trpa1\(^{-/-}\) mice intra-colonically administered the itch cocktail did not show altered VMRs relative to vehicle administered Trpa1\(^{-/-}\) mice (ns, \(P>0.05\), Trpa1\(^{-/-/+}\) vehicle:N=7; Trpa1\(^{-/-/+}\) + Itch cocktail:N=7). P) Total VMR was unchanged in Trpa1\(^{-/-}\) mice administered the itch cocktail compared with vehicle (ns, \(P>0.05\), Trpa1\(^{-/-/+}\) vehicle:N=7; Trpa1\(^{-/-/+}\) + Itch cocktail:N=7). Data represent Mean±SEM. \(P\)-values determined by generalized estimating equations, followed by least significant difference post hoc tests (A,C,E,G,I,K,M,O) or by unpaired t-tests (B,D,F,H,J,L,N,P).
Figure 6: In vivo intra-colonic administration of an ‘itch cocktail’ consisting of TGR5, MRGPR A3 and MRGPR C11 agonists alters animal behaviour.

Representative track paths are shown for a A) healthy mouse intra-colonically administered vehicle (saline) and for a B) healthy mouse intra-colonically administered an itch cocktail consisting of a combination of CCDC (100µM), BAM8-22 (20µM) and CQ (10µM). Intracolonic administration of the itch cocktail significantly reduced C) the total track length covered (*P<0.05, Vehicle:N=9, Itch cocktail:N=7), D) the average velocity of travel (*P<0.05, Vehicle:N=9; Itch cocktail:N=7) and E) locomotor activity time compared to vehicle treatment (**P<0.01, Vehicle:N=9, Itch cocktail:N=7). F) Intracolonic administration of the itch cocktail also significantly increased grooming behaviour compared with vehicle (*P<0.05, Vehicle:N=9, Itch cocktail:N=7). Data represent Mean±SEM. Dots represent values from individual mice. P values were by unpaired t-tests (C,D,E,F).
Figure 7: TGR5, MRGPR3 and MRGPRC11 agonists evoke mechanical hypersensitivity in colonic afferents from mice with chronic visceral hypersensitivity (CVH).

A) Application of the TGR5 agonist deoxycholic acid (DCA; 100µM) to the colonic mucosa of CVH mice induces mechanical hypersensitivity of colonic nociceptors (***P<0.01, N=7). Dots represent values from individual CVH afferents before and after DCA application. Lower panels show representative recordings from a single colonic afferent nerve fibre from a CVH mouse responding to a 2g vfh before and after incubation with DCA.

B) Application of the TGR5 agonist oleanolic acid (OA; 100µM for 5 minutes) also caused mechanical hypersensitivity in nociceptors from CVH mice (**P<0.01, N=8).

C) The TGR5 agonist CCDC (100µM for 5 minutes) also evoked mechanical hypersensitivity of colonic nociceptors from CVH mice (*P<0.05, N=7).

D) Colonic nociceptors from CVH mice also displayed mechanical hypersensitivity to the application of the MRGPR3 agonist chloroquine (CQ: 10µM for 5 minutes).
minutes, *P<0.05, N=9), **E** MRGPRC11 agonist BAM8-22 (20µM for 5 minutes, **P<0.01, N=9) and **F** the combined MRGPRC11/MRGPR A4 agonist neuropeptide FF (NPFF; 5µM for 5 minutes, **P<0.01, N=9 mice). Data represent Mean±SEM. P values determined by paired t-tests (A-F).
Figure 8: Distinct and overlapping expression patterns for Tgr5, Mrgpra3 and Mrgpc11 in colon-innervating DRG neurons from CVH mice.

A) Single cell RT-PCR of 46 retrogradely traced colon-innervating DRG neurons from 4 CVH mice reveals that sub-populations express Tgr5 (20%), Mrgpra3 (39%), Mrgpc11 (74%), Trpv1 (65%) and Trpa1 (74%). B) Donut plot showing expression and co-expression of genes encoding Tgr5, Mrgpra3, Mrgpc11, Trpv1 and Trpa1 in 46 individual retrogradely traced colon-innervating DRG neurons from CVH mice. Each colour represents an individual gene with expression marked by bold shading (Tgr5: outer ring, Trpa1: inner ring). Some CVH DRG neurons express all targets, whilst other neurons express combinations of targets. C-E) Group data showing C) Tgr5, D) Mrgpra3 and E) Mrgpc11 are expressed individually within subpopulations of colon-innervating DRG neurons, and also co-expressed together in other sub-populations. For example, of the Tgr5 expressing colon-innervating DRG neurons from CVH mice, 56% co-express Mrgpra3, and 33% co-express Mrgpc11. Tgr5, Mrgpra3 and Mrgpc11 also heavily co-express with Trpv1 and in particular Trpa1 in CVH states.
Figure 9: In vivo intra-colonic administration of pruritogenic agonists in CVH mice increases dorsal horn neuron activation in response to colorectal distension and alters animal behaviour.

A) CRD at a pressure of 40mmHg in CVH mice results in activation of DH neurons within the thoracolumbar (T10-L1) spinal cord, as indicated by pERK-IR (yellow arrows). CVH mice pre-treated with intra-colonic CCDC (100µM) display more DH neurons in the spinal cord following 40mmHg CRD. Scale bars equal 100µm. B) Group data showing that CVH mice pre-treated with intra-colonic CCDC (100µM) displayed significantly more pERK-IR DH neurons within the spinal cord following 40mmHg CRD compared with 40mmHg CRD alone (**P<0.0001, dots indicate individual counts in spinal cord sections from N=6:CVH CRD, N=6:CVH CCDC+CRD). C) Representative track paths are shown for individual CVH mice administered either intra-colonic vehicle (saline) or an itch cocktail consisting of a combination of CCDC (100µM), BAM8-22 (20µM) and CQ (10µM). D-F) Intracolonic administration of the itch cocktail to CVH mice significantly reduces D) their track length covered within the central area of the observation enclosure (**P<0.01, CVH + vehicle:N=9, CVH + itch cocktail:N=7) and E) reduces their distance from the walls of the enclosure (*P<0.05, CVH + vehicle:N=9, CVH + itch cocktail:N=7) compared to vehicle-treated CVH mice. F) Intracolonic administration of the itch cocktail to CVH mice also significantly increased grooming behaviour compared to CVH vehicle treated mice (*P<0.05, CVH + vehicle:N=9, CVH + itch cocktail:N=7). Data represent Mean±SEM. Dots represent values from individual mice. P values determined by unpaired t-tests (B,D,E,F).
Figure 10: Human DRG neurons co-express TRG5, MRGPRX1, TRPV1 and TRPA1

A) qRT-PCR analysis from colonic biopsies from healthy human subjects show low levels of mRNA expression for TRG5 and absent MRGPRX1 (human ortholog of the mouse Mrgpra3 and Mrgprc11) compared with a known epithelial target GUCY2C (GC-C, ****P<0.0001, N=15 subjects. Dots represent averaged values from each patient sample). B) qRT-PCR expression analysis of whole human thoracolumbar (TL; T9-L1) DRG from 4 human donors. Analysis reveals abundant expression of MRGPRX1 and TRPV1 and TRPA1, plus expression of the bile acid receptor TRG5. Dots represent averaged values from each donor at each DRG level. C) Single-cell RT-PCR analysis showing the percentage of individual human DRG neurons expressing the TRG5, MRGPRX1 in addition to TRPV1 and TRPA1. Data show that of the 85-individual human thoracolumbar DRG neurons examined, 38% express TRG5, 79% express MRGPRX1, 92% expressed TRPV1, with 58% expressing TRPA1. D) Polar
plot analysis showing co-expression profiles of 53-individual human TL DRG neurons using single cell RT-PCR for TGR5, MRGPRX1, TRPV1 and TRPA1. 

**E)** Of the 38% of human TL DRG neurons expressing TGR5, 78% co-express MRGPRX1, 95% co-express TRPV1, with 56% co-expressing TRPA1. 

**F)** Of the 79% of human DRG neurons expressing MRGPRX1, 37% co-express TGR5, 99% co-express TRPV1, with 60% co-expressing TRPA1. 

**G)** TRPV1 expressing human DRG neurons also express TGR5 (40%), MRGPRX1 (85%), and TRPA1 (62%). 

**H)** TRPA1 expressing human DRG neurons also express TGR5 (31%), MRGPRX1 (82%), and TRPV1 (98%). 

Data in (**A,B**) represent Mean±SEM. P values determined by one-way ANOVAs with Tukey’s multiple comparison tests (**A**).
Figure 11: Human DRG neurons respond to pruritogenic agonists for TGR5, in addition to the MRGPRX1 agonists chloroquine and BAM8-22.

A-F) Human DRG neurons were cultured in control media, and in order to simulate a pathological state, a subset of cultures incubated with ‘inflammatory mediators’. This consisted of histamine (10µM), PGE II (10µM), serotonin (10µM) and bradykinin (10µM) being incubated with the neurons for 2 hours before Ca²⁺ imaging experiments commenced. Human DRG neurons from this cohort are referred to as ‘inflammatory mediators’. Grouped data of Ca²⁺ responses in control (n=74) and inflammatory mediator (n=44) cultured human DRG neurons to application of the A) TGR5 agonist CCDC (100µM), B-D) MRGPRX1 agonists B) BAM8-22 (2µM), C) CQ (1µM), D) NPFF (2µM), E) TRPV1 agonist capsaicin (100nM), F) TRPA1 agonist AITC (50µM). Two-way ANOVAs indicate responses to
CQ (*P<0.05), capsaicin (**P<0.01) and AITC (**P<0.01) are all significantly increased in neurons that had been exposed to inflammatory mediators. **P<0.01), CQ (****P<0.0001), capsaicin (****P<0.0001) and AITC (***P<0.001) were all significantly increased in human DRG neurons incubated with inflammatory mediators. Peak response of neurons to CCDC (**P<0.01), CQ (****P<0.0001), capsaicin (****P<0.0001) and AITC (***P<0.001) were all significantly increased in human DRG neurons incubated with inflammatory mediators. Human DRG neurons from inflammatory mediator cultures responding to CCDC, CQ, BAM8-22, NPFF, capsaicin and AITC. Donut plot analysis showing the functional co-expression profiles as determined by Ca^2+ imaging of 74-individual human DRG neurons from control cultures and 32-individual human DRG neurons from inflammatory mediator cultures in response to CCDC, CQ, BAM8-22, NPFF, capsaicin and AITC. Data presented are Mean±SEM. P values determined by two-way ANOVAs and Bonferroni post-hoc tests (indicated within panels) (A-F), unpaired t-tests (G).