Letter for Gut: Resubmission. **Word Count**: 693.

Increased kappa-opioid receptor expression and function during chronic visceral hypersensitivity

Patrick A. Hughes [1,2], Joel Castro [1,2], Andrea M. Harrington [1,2], Nicole Isaccs [1], Melissa Moretta [1], Gareth A. Hicks [4], David M. Urso [4] and Stuart M. Brierley [1,2,3]

[1] Nerve-Gut Research Laboratory, Discipline of Medicine, Faculty of Health Sciences, The University of Adelaide, Adelaide, South Australia, AUSTRALIA, 5000.

[2] Department of Gastroenterology and Hepatology, Hanson Institute, Royal Adelaide Hospital, Adelaide, South Australia, AUSTRALIA, 5000.

[3] Discipline of Physiology, Faculty of Health Sciences, The University of Adelaide, Adelaide, South Australia, AUSTRALIA, 5000.

[4] Tioga Pharmaceuticals Inc., San Diego, CA, 92121, USA.

Corresponding Author: **Stuart M. Brierley**: Nerve-Gut Research Laboratory, Level 1 Hanson Institute, Frome Road, Adelaide, South Australia, AUSTRALIA 5000.

Email: stuart.brierley@adelaide.edu.au Phone: +61 8 8222 2077, Fax: +61 8 8222 5934

Grant support: This work was funded by Tioga Pharmaceuticals Inc. P.A.H is an NHMRC Australian Biomedical Fellow. A.M.H is an Australian Research Council Discovery Early Career Research Fellow. S.M.B is an NHMRC R.D Wright Biomedical Fellow.

Keywords: IBS, abdominal pain, nociception, sensitization, pharmacotherapy.

Abbreviations:

CVH: Chronic Visceral Hypersensitivity.

C-IBS: Constipation-predominant Irritable Bowel Syndrome.

D-IBS: Diarrhea-predominant Irritable Bowel Syndrome.

DRG: Dorsal Root Ganglia.

KOR: Kappa-Opioid Receptor.

MOR: Mu-Opioid Receptor

PBMC: Peripheral Blood Mononuclear Cell.

TNBS: Trinitrobenzene Sulphonic Acid.

Competing interests: S.M.B and P.A.H received funding from Tioga Pharmaceuticals Inc. to conduct the study. G.A.H and D.M.U are employees of Tioga Pharmaceuticals Inc. J.C, A.M.H, Ni and M.M have nothing to declare.

Article:

In a recent article in Gut, Hughes *et al.,*[1] identified distinct patterns of immune dysfunction in Irritable Bowel Syndrome (IBS) patients compared with healthy subjects. In particular, they showed peripheral-blood-mononuclear-cell (PBMC) supernatants from healthy subjects inhibited colonic afferents in a mu-opioid-receptor (MOR)-mediated manner. These findings correlated with β-endorphin from T-lymphocytes providing an important MOR-mediated anti-nociceptive influence in the healthy gut[2]. Intriguingly these inhibitory effects were lost with PBMC supernatants from constipation-predominant-IBS (C-IBS) patients suggesting a loss of MOR-mediated inhibition, coupled with increased excitatory mediators (TNF-α, IL-1β, IL-6), contributes to abdominal pain[1].

We evaluated if this alteration was MOR specific, or whether it extended to other members of the opioid receptor family. As clinical studies have shown varying outcomes on visceral pain perception with kappa-opioid-receptor (KOR) agonists[3,4,5], we postulated KOR expression and function are altered during visceral hypersensitivity. Therefore, we determined if the peripherally restricted selective KOR agonist, asimadoline, was able to modify colonic nociceptor function in health and during inflammatory and post-inflammatory chronic visceral mechanical hypersensitivity (CVH)[6].

We performed *in vitro* afferent recordings from mouse splanchnic high-threshold nociceptors, which respond to focal compression and noxious stretch/distension[1,6]. They also display pronounced mechanical hypersensitivity and lowered activation thresholds in models of acute and chronic visceral pain[6]. We assessed nociceptor mechanosensitivity before and after increasing doses of asimadoline, which has a 500-fold selectivity for KOR versus MOR and delta-opioid-receptor subtypes and is 500-1000-fold less potent at sodium channels[5].

We found healthy nociceptor mechanosensitivity was not effected by asimadoline at any of the doses tested (Figure 1A), which were in the range of KOR affinity and not high enough to block sodium channels[5]. In contrast, when we performed recordings from mice with colonic inflammation we found that asimadoline dose-dependently inhibited colonic nociceptors (Figure 1B). Furthermore, asimadoline also dose-dependently inhibited colonic nociceptors from CVH mice (Figure 1C), an effect that was prevented by the prior application of a KOR antagonist (Figure 1D). Overall, these data indicate KOR expression is functionally up-regulated during inflammation and CVH. Correspondingly, retrogradely-traced colonic DRG neurons from CVH mice displayed KOR-immunoreactivity (Figure 1E) and significant up-regulation of KOR mRNA (Figure 1F).

Our findings are consistent with recent somatosensory studies showing KOR mRNA is maintained in a dormant state, but is subjected to transcriptional and post-transcriptional influences. Induction of functional KOR occurs via sensitizing agents, such as bradykinin, activating the phospholipase-C pathway and membrane integrins or via neuronal depolarization stimulating KOR mRNA, axonal transport and protein translation[7,8].

Although previous studies of acute TNBS-induced inflammation show greater potency of KOR agonists *in vivo*[9], our findings are the first to demonstrate increased KOR agonist efficacy during CVH. Notably, we used doses in the range of KOR affinity and were able to block the inhibitory effects of asimadoline with a KOR antagonist. As bouts of gastroenteritis can contribute to IBS onset and prolonged low-grade intestinal inflammation is apparent in IBS patients[10], our findings provide further information on the potential therapeutic benefit of KOR agonists for abdominal pain relief. In healthy volunteer barostat studies asimadoline did not significantly reduce pain perception scores, even at noxious levels of distension[3].

This suggests KOR activation does not significantly alter noxious sensation in health, which is consistent with our lack of effect of asimadoline on healthy colonic nociceptors. However, in IBS patients a single dose of asimadoline significantly decreased pain perception across a range of colonic distension pressures[4], whilst in a Phase IIb study of 596 patients asimadoline's efficacy against IBS symptoms was greatest in D-IBS patients, with at least moderate pain at baseline[5]. This suggests a preferential efficacy of asimadoline in the relief of abdominal pain in patients with visceral hypersensitivity, which is consistent with our findings of increased KOR expression and function during CVH. However, in a confirmatory clinical trial in D-IBS patients, efficacy was not observed with asimadoline.

These findings add another layer of complexity to KOR signaling, whereby KOR expression is up-regulated during inflammation and CVH. Correspondingly, asimadoline inhibits nociceptors during inflammation and CVH, but not in health, via peripheral KOR on colonic afferent endings. Overall, KOR appears to be a silent receptor system that is activated 'on demand' in response to neuronal sensitization and remains active during CVH.

REFERENCES:

1 Hughes PA, Harrington AM, Castro J, Liebregts T, Adam B, Grasby DJ*, et al.* Sensory neuro-immune interactions differ between Irritable Bowel Syndrome subtypes. Gut 2013;**Oct 62**:1456-65.

2 Verma-Gandhu M, Bercik P, Motomura Y, Verdu EF, Khan WI, Blennerhassett PA*, et al.* CD4+ T-cell modulation of visceral nociception in mice. Gastroenterology 2006;**130**:1721-8.

3 Delgado-Aros S, Chial HJ, Camilleri M, Szarka LA, Weber FT, Jacob J*, et al.* Effects of a kappa-opioid agonist, asimadoline, on satiation and GI motor and sensory functions in humans. American journal of physiology Gastrointestinal and liver physiology 2003;**284**:G558-66.

4 Delvaux M, Beck A, Jacob J, Bouzamondo H, Weber FT, Frexinos J. Effect of asimadoline, a kappa opioid agonist, on pain induced by colonic distension in patients with irritable bowel syndrome. Alimentary pharmacology & therapeutics 2004;**20**:237-46.

5 Mangel AW, Hicks GA. Asimadoline and its potential for the treatment of diarrhea-predominant irritable bowel syndrome: a review. Clinical and experimental gastroenterology 2012;**5**:1-10.

6 Hughes PA, Brierley SM, Martin CM, Brookes SJ, Linden DR, Blackshaw LA. Post-inflammatory colonic afferent sensitisation: different subtypes, different pathways and different time courses. Gut 2009;**58**:1333-41.

7 Berg KA, Rowan MP, Sanchez TA, Silva M, Patwardhan AM, Milam SB*, et al.* Regulation of kappa-opioid receptor signaling in peripheral sensory neurons in vitro and in vivo. The Journal of pharmacology and experimental therapeutics 2011;**338**:92-9.

8 Bi J, Tsai NP, Lin YP, Loh HH, Wei LN. Axonal mRNA transport and localized translational regulation of kappa-opioid receptor in primary neurons of dorsal root ganglia. Proceedings of the National Academy of Sciences of the United States of America 2006;**103**:19919-24.

9 Sengupta JN, Snider A, Su X, Gebhart GF. Effects of kappa opioids in the inflamed rat colon. Pain 1999;**79**:175-85.

10 Hughes PA, Zola H, Penttila IA, Blackshaw LA, Andrews JM, Krumbiegel D. Immune Activation in Irritable Bowel Syndrome: Can Neuroimmune Interactions Explain Symptoms? American Journal of Gastroenterology 2013;**Jul;108**:1066-74.

Figure Legend: (95 words)

Figure 1:A) Asimadoline had no effect on healthy splanchnic colonic serosal nociceptor mechanosensitivity (*P*>0.05,n=6), but **B)** caused dose-dependent inhibition of colonic nociceptors from mice with inflammatory hypersensitivity (7-days-post-TNBS-administration;***P*<0.001,n=10) and **C)** Chronic Visceral Hypersensitivity (CVH;28-days-post-TNBS-administration;**P*<0.05;***P*<0.001,n=10).**D)**The KOR antagonist Nor-BNI (100nM) did not alter CVH nociceptor mechanosensitivity (*NS*,*P*>0.05,n=8), but did block asimadoline-induced inhibition (*P*>0.05,n=8).**E)i)**Retrogradely traced colonic neurons within the thoracolumbar (T10-L1) DRG.**ii)**KOR-immunoreactivity (KOR-IR) using immunohistochemistry. Yellow arrows: CVH colonic neurons expressing KOR-IR. White arrows: CVH colonic neurons lacking KOR-IR. Scale bar:50µm. **F)**Quantitative-RT-PCR showing significant up-regulation of KOR mRNA in retrogradely labeled colonic thoracolumbar DRG neurons from CVH mice (***P*<0.001).