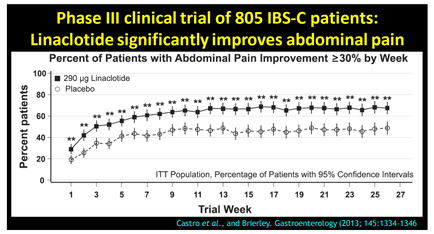
Extracellular cyclic GMP (cGMP), the downstream mediator released in response to linaclotide-induced activation of guanylate cyclase-C (GC-C), reduces excitability of murine and human dorsal root ganglion neurons

[1,2] Joel Castro, [3] Grigori Y. Rychkov, [4] Andrea Ghetti, [1,2] Andrea M. Harrington, [5] Caroline Kurtz, [5] Ada Silos-Santiago and [1,2,3] **Stuart M. Brierley**

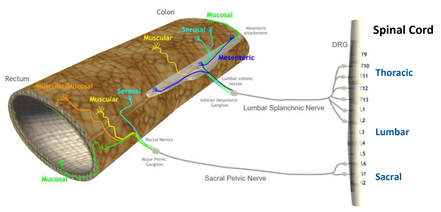
[1] Visceral Pain Group, Discipline of Medicine, SAHMRI, [2]Dept of Gastroenterology & Hepatology, Royal Adelaide Hospital. [3] Discipline of Physiology, University of Adelaide, AUSTRALIA. [4] Anabios Inc, USA. [5] Ironwood Pharmaceuticals Inc, USA.

BACKGROUND

- Linaclotide, an investigational GC-C agonist, improves abdominal pain and bowel symptoms in humans with Irritable Bowel Syndrome with Constipation (IBS-C)[1].

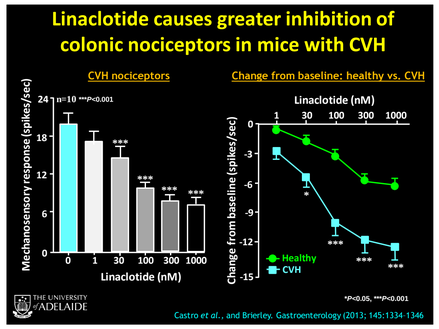


- Pain from the colon and rectum is signalled via two distinct anatomical spinal afferent pathways [2].

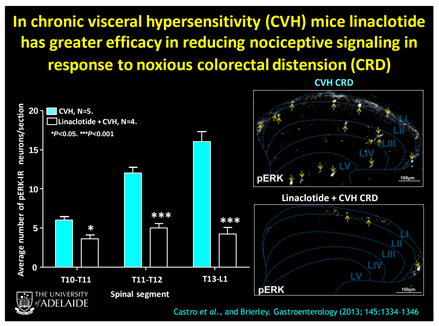


Colonic sensory neurons reside in the thoracolumbar (TL: T10-L1) and lumbosacral (LS: L6-S1) dorsal root ganglia (DRG), with afferents projecting via the splanchnic and pelvic nerves respectively. Five different subclasses of mechanosensitive afferent innervate the colorectum via these pathways. Modified from [3].

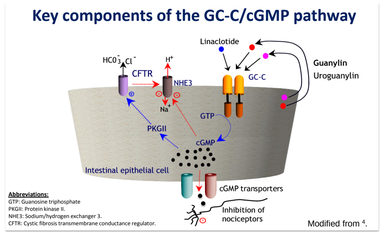
- Linaclotide inhibits colonic nociceptors *in vitro* [1].



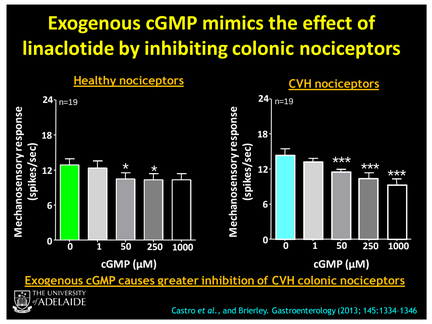
- *In vivo* intra-colonic administration of linaclotide also reduces nociceptive signalling from the colon [1].



- Cyclic GMP (cGMP) is a second messenger produced in intestinal epithelial cells when activated by guanylate-cyclase C (GC-C) agonists [1].



- cGMP also inhibits colonic nociceptors *in vitro*



Therefore, both linaclotide and exogenous extracellular cGMP inhibit the peripheral endings of colonic nociceptors, with greater efficacy during CVH [1].

However, the effects of exogenous cGMP on sensory neuron function remain to be determined in isolation.

AIMS

We investigated the effects of exogenous extracellular cGMP on dorsal root ganglion (DRG) neurons isolated from both mice and humans.

METHODS

Mouse DRG studies

- We performed whole cell patch clamp recordings [5] in current clamp mode from retrogradely traced thoracolumbar (T10-L1) colonic DRG neurons.
- We compared the effect of exogenous extracellular cGMP (100nM-50µM) on the rheobase, or threshold for action potential firing, of DRG neurons from healthy C57BL/6 mice and mice with CVH, 28 days post-TNBS administration [1,6].

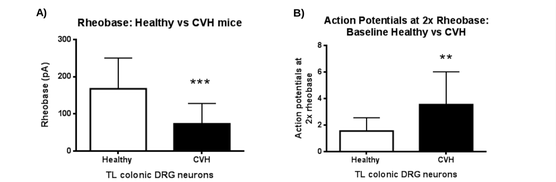
Human DRG studies

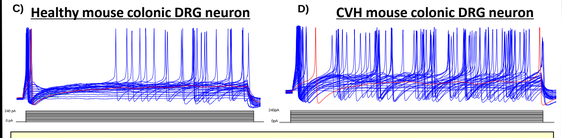
- We performed calcium-imaging studies and compared the effects of cGMP (10µM-300µM) on calcium (Ca^{2+}) influx in response to hypo-osmotic stimuli of DRG neurons from healthy donors.

RESULTS

Mouse colonic DRG studies

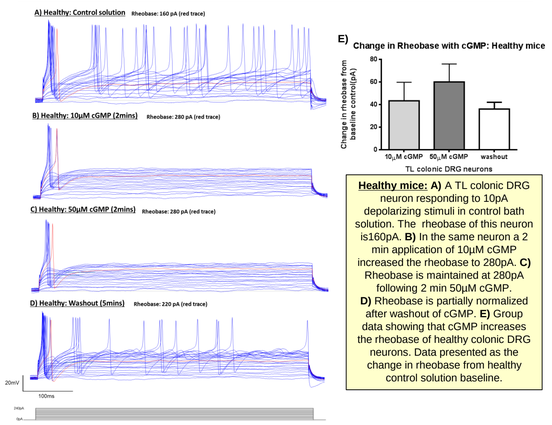
TL colonic DRG neurons from CVH mice display neuronal hyper-excitability





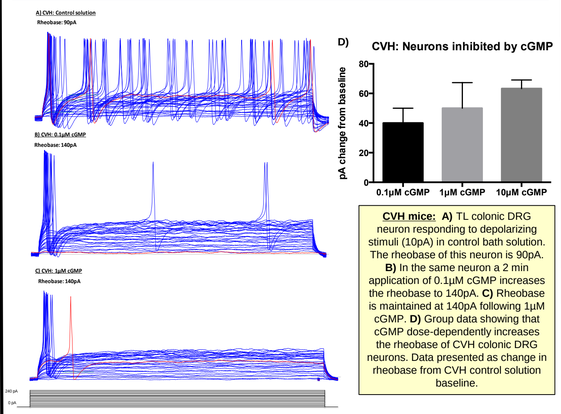
A) TL colonic DRG neurons from CVH mice have a significantly reduced rheobase (***P<0.001, Healthy: n=23 vs. CVH: n=13). **B)** CVH colonic DRG neurons fire more action potentials at 2x rheobase (**P<0.01, Healthy: n=23 vs. CVH: n=13. **C&D)** Current clamp recordings of TL colonic DRG neurons in response to depolarizing stimuli (10pA current steps) from **C)** Healthy and **D)** CVH mice.

Healthy mice: Exogenous extracellular cGMP application inhibits colonic DRG neurons



Healthy mice: A) A TL colonic DRG neuron responding to 10pA depolarizing stimuli in control bath solution. The rheobase of this neuron is160pA. **B)** In the same neuron a 2 min application of 10µM cGMP increased the rheobase to 280pA. **C)** Rheobase is maintained at 280pA following 2 min 50µM cGMP. **D)** Rheobase is partially normalized after washout of cGMP. **E)** Group data showing that cGMP increases the rheobase of healthy colonic DRG neurons. Data presented as the change in rheobase from healthy control solution baseline.

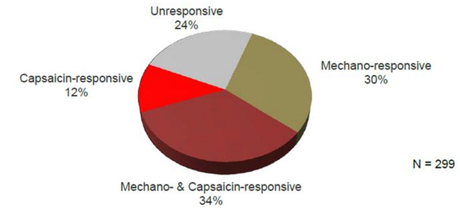
CVH mice: Exogenous extracellular cGMP application causes potent inhibition of colonic DRG neurons



CVH mice: A) TL colonic DRG neuron responding to depolarizing stimuli (10pA) in control bath solution. The rheobase of this neuron is 90pA. **B)** In the same neuron a 2 min application of 0.1µM cGMP increases the rheobase to 140pA. **C)** Rheobase is maintained at 140pA following 1µM cGMP. **D)** Group data showing that cGMP dose-dependently increases the rheobase of CVH colonic DRG neurons. Data presented as change in rheobase from CVH control solution baseline.

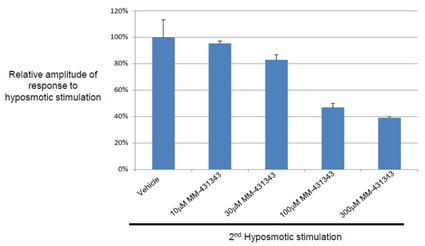
Human DRG studies

Hypo-osmotic stimuli and capsaicin reveal distinct sub-populations of human DRG neurons



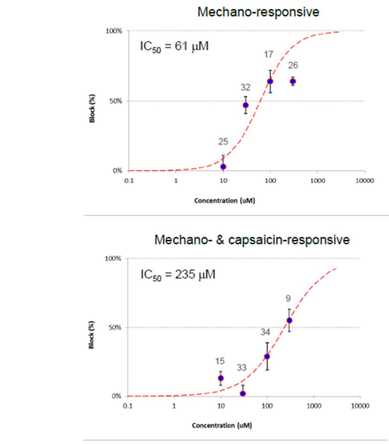
Human DRG neurons were tested with a hypo-osmotic stimuli (70mM NaCl; 5mM KCl, 2mM CaCl2, 1mM MgCl2, 10mM HEPES, 10mM glucose, pH 7.40, 165 mOsm) followed by capsaicin (200nM). Neurons that did not respond to either stimuli were classified as "un-responsive'. Neurons responding to the hypo-osmotic stimuli, but not capsaicin, were termed "mechano-responsive". Neurons responding to capsaicin only were termed "capsaicin-responsive", whilst neurons responding to both stimuli were termed "Mechano- and Capsaicin responsive".

Responsiveness of human DRG neurons to hypo-osmotic stimuli is dose-dependently decreased by exogenous extracellular cGMP application



Human DRG neurons were tested with a hypo-osmotic stimuli (70mM NaCl; 5mM KCl, 2mM CaCl2, 1mM MgCl2, 10mM HEPES, 10mM glucose, pH 7.40, 165 mOsm) followed by increasing doses (10µM-300µM) of cGMP (MM-431343). Each dose of cGMP was applied for 2 minutes. After each dose of cGMP neuronal responsiveness to the hypo-osmotic stimuli was re-tested.

Exogenous extracellular cGMP application causes greater inhibition in a select sub-population of human DRG neurons



Human DRG neurons were split into specific subpopulations based on their responsiveness to hypo-osmotic stimuli and capsaicin. This revealed that mechano-responsive neurons were inhibited at lower concentrations of exogenous extracellular cGMP.

SUMMARY

Mouse DRG studies

- Colonic DRG neurons from CVH mice displayed a significantly reduced rheobase, firing significantly more action potentials compared with healthy mice at baseline.
- In a subpopulation of colonic DRG neurons, cGMP inhibited the neuronal excitability of putative nociceptors, significantly increasing their rheobase and reducing action potential discharge.
- This effect was evident in both healthy and CVH DRG neurons, was most apparent in CVH DRG neurons, and occurred at concentrations as low as 100nM cGMP.

Human DRG studies

- In human DRG neurons, cGMP induced an overall reduction in the number of cells responding to hypo-osmotic stimulation.
- In addition in human DRG neurons cGMP caused, in a concentration-dependent manner, up to 60% inhibition of the Ca^{2+} influx induced by hypo-osmotic stimulation.

CONCLUSIONS

- Exogenous cGMP directly decreases the excitability of sensory DRG neurons isolated from both mice and humans.
- These results complement our previous findings in mice, which demonstrated that cGMP inhibited the peripheral endings of nociceptors within the wall of the colon.
- These current findings also provide further mechanistic insight into how linaclotide, through GC-C agonism and the release of cGMP from mucosal epithelial cells, reduces nociceptive signaling from the colon.

ACKNOWLEDGEMENTS

Supported by Ironwood Pharmaceuticals Inc, Forest Laboratories and NHMRC Australia.

REFERENCES

1) Castro *et al.*, and Brierley 2013, *Gastroenterology.*
2) Brierley *et al., Gastroenterology,* 2004, Jul;127(1):166-78.
3) Brierley. Autonomic Neuroscience. 2010. Special Edition, 153, 1-2, 58-68.
4) Brierley. Current Opinion in Pharmacology. 2012. Special edition. 2012 Dec;12(6):632-40.
5) Brierley *et al., Journal of Physiology* 2011 Jul 15;589(Pt 14):3575-93.
6) de Araujo *et al.,* Brierley & Alewood. *Nature Communications.* 2014;5:3165.