Acid and inflammatory sensitisation of naked mole-rat colonic afferent nerves

Authors

James R.F. Hockley[1$], Toni S. Taylor[1], Gerard Callejo[1], Zoe M. Husson[1#], Ewan St. J. Smith[1*]

Affiliations

[1]Department of Pharmacology, University of Cambridge, Cambridge, Cambridgeshire, United Kingdom

[$]Current Address: Adaptive Immunology Research Unit, GlaxoSmithKline, Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire, United Kingdom

[#]Current Address: INSERM U1215, Université Bordeaux, Bordeaux, France

*Corresponding author

Email: es336@cam.ac.uk

Abstract

Acid sensing in the gastrointestinal tract is required for gut homeostasis and the detection of tissue acidosis caused by ischaemia, inflammation and infection. In the colorectum, activation of colonic afferents by low pH contributes to visceral hypersensitivity and abdominal pain in human disease including during inflammatory bowel disease. The naked mole-rat (*Heterocephalus glaber;* NMR) shows no pain-related behaviour to subcutaneous acid injection and cutaneous afferents are insensitive to acid, an adaptation thought to be a consequence of the subterranean, likely hypercapnic, environment in which it lives. As such we sought to investigate whether NMR interoception within the gastrointestinal tract differed from other rodents, specifically the mouse. Here we show the presence of calcitonin gene regulated peptide (CGRP) expressing extrinsic nerve fibres innervating both mesenteric blood vessels and the myenteric plexi of the smooth muscle layers of the NMR colorectum. Using *ex vivo* colonic-nerve electrophysiological recordings we show differential sensitivity of NMR, compared to mouse, colonic afferents to acid and the prototypic inflammatory mediator bradykinin, but not direct mechanical stimuli. In NMR, but not mouse, we observed mechanical hypersensitivity to acid, whilst both species sensitised to bradykinin. Collectively, these findings suggest that NMR colonic afferents are capable of detecting acidic stimuli however their intracellular coupling to downstream molecular effectors of neuronal excitability and mechanotransduction likely differs between species.

Keywords

Acid, bradykinin, mechanosensation, sensitisation, visceral pain

Introduction

The gastrointestinal tract coordinates the digestion of food, absorption of nutrients and evacuation of waste with acidification of the stomach contents a critical component of this process. Through compartmentalisation, sensory surveillance and specialised mucosal defence mechanisms, not only is the breakdown of food and elimination of ingested pathogens achieved through acidification in the foregut, but also the delicate gut microbiota-host symbiosis of the hindgut maintained. It is clear that when gastric acid regulation is lost then significant pathogenesis can occur, including acid-related diseases such as gastro-eosophageal reflux disease, gastroduodenal ulceration, dyspepsia and gastritis [1]. Recent associations between gut microbiota and a diverse range of human disease, from depression, autism, schizophrenia, Alzheimer's disease and Parkinson's disease, to diabetes and obesity [2], also highlight the significant impact that alterations in the gut luminal environment, including pH on which this microbiota rely, can have on human behaviour, mood and physiology.

Sensory neurones innervating the gastrointestinal tract are central to the feedback regulation of gastric acid secretion and can additionally detect tissue acidosis caused by inflammation, ischaemia and microbial activity [3,4], often resulting in visceral hypersensitivity and abdominal pain [5]. Whilst luminal pH varies along the length of the healthy human gut (with lower pH found in the stomach and colon [6,7]), both surgical intervention and disease (e.g. chronic pancreatitis and inflammatory bowel disease [6,8,9]; a significant symptom of which is abdominal pain) can also result in abnormal acidification of the gut.

The naked mole-rat (*Heterocephalus glaber;* NMR) has adaptations enabling it to not only survive but prosper in the subterranean (thus likely hypercapnic and hypoxic) environment in which it lives. Many of these adaptations have led to altered sensory processing of external stimuli, for example the NMR shows no pain-related behaviour to subcutaneous injection of acid and capsaicin [10], lacks an itch response to histamine [11] and shows no thermal hyperalgesia in response to a variety of stimuli, including nerve growth factor [10,12]. These adaptations are believed to provide a fitness advantage to living in a subterranean environment, for example, the likely high CO_2 environment of NMR nests would evoke noxious stimulation of C-fibres through

acidosis in almost any other rodent [13]. Whilst clearly valuable in supporting its lifestyle in its niche, detection of acid by sensory neurones innervating the gut is required for maintenance of gut homeostasis and for the detection of tissue acidosis. In other rodent species, viscerally-projecting afferent fibres can sense tissue acidosis by specialised ion channels [1] including acid-sensing ion channels (ASICs; which respond to mild acidification), transient receptor potential vanilloid subtype (TRPV1; which are gated by severe acidosis), ionotropic purinoceptor ion channels (e.g. $P2X_3$) and two-pore domain potassium channels (e.g. TASK, TRESK, TREK and TRAAK subtypes). Additionally, a number of proton-sensing G protein-coupled receptors exist that are also sensitive to mild acidification (e.g. Gpr68, Gpr4, Gpr132 and Gpr65). In the mouse colorectum, TRPV1 and ASIC3 are necessary for proton sensing by the vast majority of colonic afferents [14]. Recent single-cell RNA-sequencing analysis of colonic sensory neurones suggests that TRPV1 and ASIC3 are mainly expressed by discrete populations of mouse colonic afferents, namely the mPEPb and pPEP subtypes for TRPV1 and the mNFb, mPEPa and pNF subtypes for ASIC3; suggesting functional specialism [15]. Of the 4 acid-sensing GPCRs, only Gpr68 is expressed by mouse colonic afferents [15]. Compared to mice, NMRs display a similar expression profile of ASICs throughout the nervous system [16], and of those analysed, with the exception of ASIC3, NMR acid sensors show similar activation profiles to those of mice [12,17,18]. NMR TRPV1 is also expressed in sensory afferents and shows similar proton sensitivity to mouse TRPV1 [17]. The NMR acid-insensitivity is likely due to an amino acid variation in the voltage-gated sodium channel 1.7 subunit ($Na_V1.7$), which results in acid anaesthetising, rather than activating their cutaneous sensory neurones [17]. Considering the unusual cutaneous acid-insensitivity of the NMR, it is of interest to determine how GI sensory surveillance and detection of visceral tissue acidosis occurs in this species, especially considering the growing reputation of the NMR as a model of healthy ageing [19] and the perturbation of GI function that occurs with ageing [20]. In order to investigate this, we examined the sensory innervation of the NMR colon and made electrophysiological recordings in both NMR and mouse from the lumbar splanchnic nerve innervating the colorectum, and applied noxious mechanical and chemical stimuli, including acid and bradykinin, a prototypic inflammatory mediator.

113 # Materials and Methods

114 ## Animals

115 Experiments were performed in C57BL6/J mice (6-41 wks; 3F, 3M) and NMR (25-146
116 wks; 2F, 5M). Mice were conventionally house with nesting material and a red plastic
117 shelter in temperature-controlled rooms (21 °C) with a 12 h light/dark cycle and access
118 to food and water *ad libitum*. NMRs were bred in-house and maintained in an inter-
119 connected network of cages in a humidified temperature-controlled room (28 °C) with
120 red lighting (08:00-16:00) and had access to food *ad libitum*. In addition, a heat cable
121 provided extra warmth under 2-3 cages/colony. Experiments were conducted under
122 the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 under
123 Project Licenses (70/7705 & P7EBFC1B1) granted to E. St. J. Smith by the Home
124 Office and approved by the University of Cambridge Animal Welfare Ethical Review
125 Body.
126

127 ## Immunohistochemistry

128 NMR were humanely killed by CO_2 exposure followed by decapitation. The colorectum
129 was dissected free before opening along the mesenteric border and pinning flat in a
130 Sylgard lined dissection tray. After fixing in Zamboni's fixative (2 % paraformaldehyde
131 / 15 % picric acid in 0.1 M phosphate buffer; pH 7.4) overnight, the mesentery and
132 mucosa were dissected free from the muscle layers. Small 1.5 cm x 1.5 cm sections
133 were subsequently washed in 100 % DMSO (3 x 10 min) and phosphate buffered
134 saline (PBS; 3 x 10 min). Tissues were blocked with antibody diluent (10 % donkey
135 serum, 1 % bovine serum albumin (BSA) in 0.2 % Triton X-100) for 1 h, then primary
136 antibodies were applied overnight at 4 °C. The following day, tissues were washed
137 (PBS, 3 x 10 min), donkey anti-rabbit IgG-AF488 (1:500, Life Technologies A21206)
138 antibody applied for 2 h, washed (PBS; 3 x 10 min), mounted and coverslipped.
139 Primary antibodies used were rabbit anti-calcitonin gene-related peptide (1:5000,
140 Sigma C8198) and rabbit anti-protein gene product 9.5 (1:500, Abcam ab10404). No
141 labelling was observed in control sections where primary antibody was excluded.
142 Tissues were imaged using a Leica SP5 confocal microscope and z-stack
143 reconstructions of nerve fibres within different layers of the NMR gut produced with
144 ImageJ (v1.51a, NIH).

Electrophysiology recordings of visceral afferent activity

Colonic nerves innervating the colorectum of mouse and NMR were isolated and electrophysiological activity was recorded as previously described [21]. Mice were humanely killed by cervical dislocation of the neck and cessation of circulation. NMRs were humanely killed by CO_2 exposure followed by decapitation. For both species, the colorectum with associated lumbar splanchnic nerve was dissected free from the animal and transferred to a recording chamber superfused with carbogenated Krebs buffer (in mM: 124 NaCl, 4.8 KCl, 1.3 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4.7H_2O$, 11.1 glucose and 25 $NaHCO_3$; 7 ml/min; 32-34 °C). The colorectum was cannulated and perfused with Krebs buffer (100 µl/min) enabling distension of the colon by closure of the out-flow. The Krebs buffer was supplemented with nifedipine (10 µM) and atropine (10 µM) to inhibit smooth muscle activity and with indomethacin (3 µM) to restrict endogenous prostanoid production. Multi-unit electrophysiological activity of the lumbar splanchnic nerve rostral to the inferior mesenteric ganglia was recorded using a borosilicate glass suction electrode. Signals were amplified and bandpass filtered (gain 5K; 100-1300 Hz; Neurolog, Digitimer Ltd, UK) and digitised at 20 kHz (micro1401; Cambridge Electronic Design, UK) before display on a PC using Spike 2 software. The signal was digitally filtered online for 50 Hz noise (Humbug, Quest Scientific, Canada) and action potential firing counts were determined using a threshold of twice the background noise (typically 100 µV).

Electrophysiological protocols

Tissues were stabilised for 30 min before noxious intraluminal distension pressures were applied by blocking the luminal out-flow of the cannulated mouse or NMR colorectum. The pressures reached are above threshold for all known visceral afferent mechanoreceptors [22] and evoke pain behaviours in rodents *in vivo* [23]. Mechanosensitivity and chemosensitivity were investigated using a combined sequential protocol. As such, a slow ramp distension (0-80 mmHg, 4-5 min) and set of 6 rapid phasic distensions (0-80 mmHg, 60 s at 9 min intervals) were applied as previously described [24] prior to bath superfusion of pH 4.0 Krebs buffer (50 mL volume) and a set of 3 phasic distensions (0-80 mmHg, 60 s at 9 min) to test for acid-induced acute mechanical hypersensitivity. After a 20 min wash-out period, 1 μM bradykinin was applied by bath superfusion (20 mL volume) and a further set of 3 phasic distensions were performed. Phasic distension protocols were automated using an Octaflow II perfusion system (ALA Scientific, USA) to standardise duration and intervals.

Data analysis

Peak changes in firing rates of electrophysiological nerve recordings were determined by subtracting baseline firing (3 min before distension or drug application) from increases in nerve activity following distension or chemical stimuli. Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Sidak's post hoc test in Prism 6 (GraphPad Inc., USA). Statistical significance was set at $P < 0.05$. Data are displayed as means ± SEM.

Drugs

Stock concentrations of bradykinin (10 mM; water), nifedipine (100 mM; DMSO), atropine (100 mM; ethanol) and indomethacin (30 mM; DMSO) were dissolved as described, diluted to working concentration in Krebs buffer on the day of experiment as described above and were all purchased from Sigma-Aldrich.

Results

Gastrointestinal neuroanatomy of the NMR

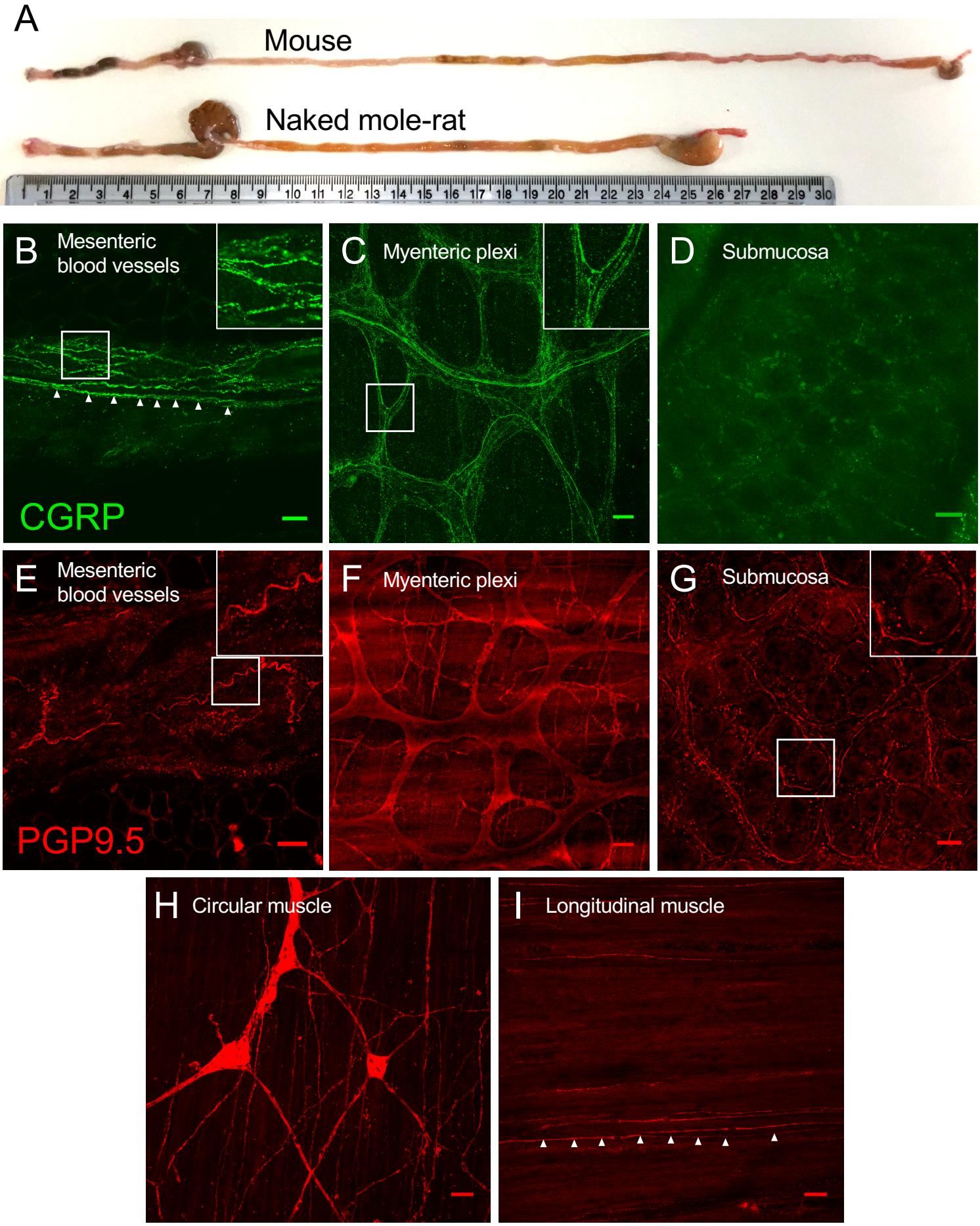
We first compared the gross anatomy of the NMR and mouse gastrointestinal tract. As the NMR is greatly long lived compared to the mouse, with a life expectancy of >30-years, we chose animals of an equivalent phase of life and body size (e.g. young adult: for mouse, 6-8-weeks, snout to anus length 92 mm and for NMR, 76-weeks, snout to anus length 113 mm). Compared to the mouse, the NMR colorectum (from caecum to anus) length was broadly equivalent (mouse, 60 mm; NMR, 70mm), whilst the caecum and stomach were greatly enlarged and the small intestine (from the caecum to pyloric sphincter of the stomach) was shorter in length (mouse, 312 mm; NMR, 165 mm; Fig. 1A).

We next confirmed the presence of extrinsic sensory fibres innervating different layers of the NMR colorectum using immunohistochemistry. Equivalent staining in the mouse are widely available within the literature and we did not seek to duplicate them here [25]. Using antibodies raised against calcitonin gene-related peptide (CGRP) and protein gene product 9.5 (PGP9.5) we stained for neuronal fibres within flat-sheet whole-mount preparations of multiple layers of the NMR colon. Specifically, CGRP-positive extrinsic neuronal varicosities were identified encircling and tracking with blood vessels within the mesentery supplying the distal colon of NMR; such fibres likely contribute to the larger lumbar splanchnic nerve upon which these coalesce (Fig. 1B). Although NMR lack CGRP in cutaneous afferent neurones, this finding is in line with the observation that mesenteric arteries in NMR and the common mole-rat (*Cryptomus hottentotus*) express CGRP [26]. Neuronal fibres staining for PGP9.5 were also observed within the mesentery of NMR, again localised around blood vessels (Fig. 1E). The mucosa and submucosa were separated from the muscle (circular and longitudinal) layers. CGRP-positive, presumably extrinsic, sensory fibres were observed coursing through the myenteric plexi between these muscle layers (Fig. 1C). PGP9.5 staining revealed the myenteric soma and additional neuronal fibres within this layer of the NMR colon (Fig. 1F). Whilst PGP9.5-positive fibres were observed encircling the base of colonic villi (see Fig. 1G insert), CGRP labelling of these fibres was not seen (Fig. 1D). Interdigitating fibres within both the circular and longitudinal muscle layers are positive for PGP9.5 (Fig. 1H and 1I), with what is likely submucosal ganglia retained on the circular muscle layer after separation of the mucosa from this layer (Fig. 1H).

Colonic afferent mechanosensitivity does not differ in the NMR compared to mouse

In order to understand whether the peripheral terminals of sensory neurones innervating the gastrointestinal tract of the NMR possessed altered acid and inflammatory sensitivity compared to mouse, we made *ex vivo* multi-unit electrophysiological recordings of lumbar splanchnic nerve activity using a suction electrode from the colorectum of both NMR and mouse. The lumbar splanchnic nerve innervates the colorectum and is a pathway through which pain is the predominant

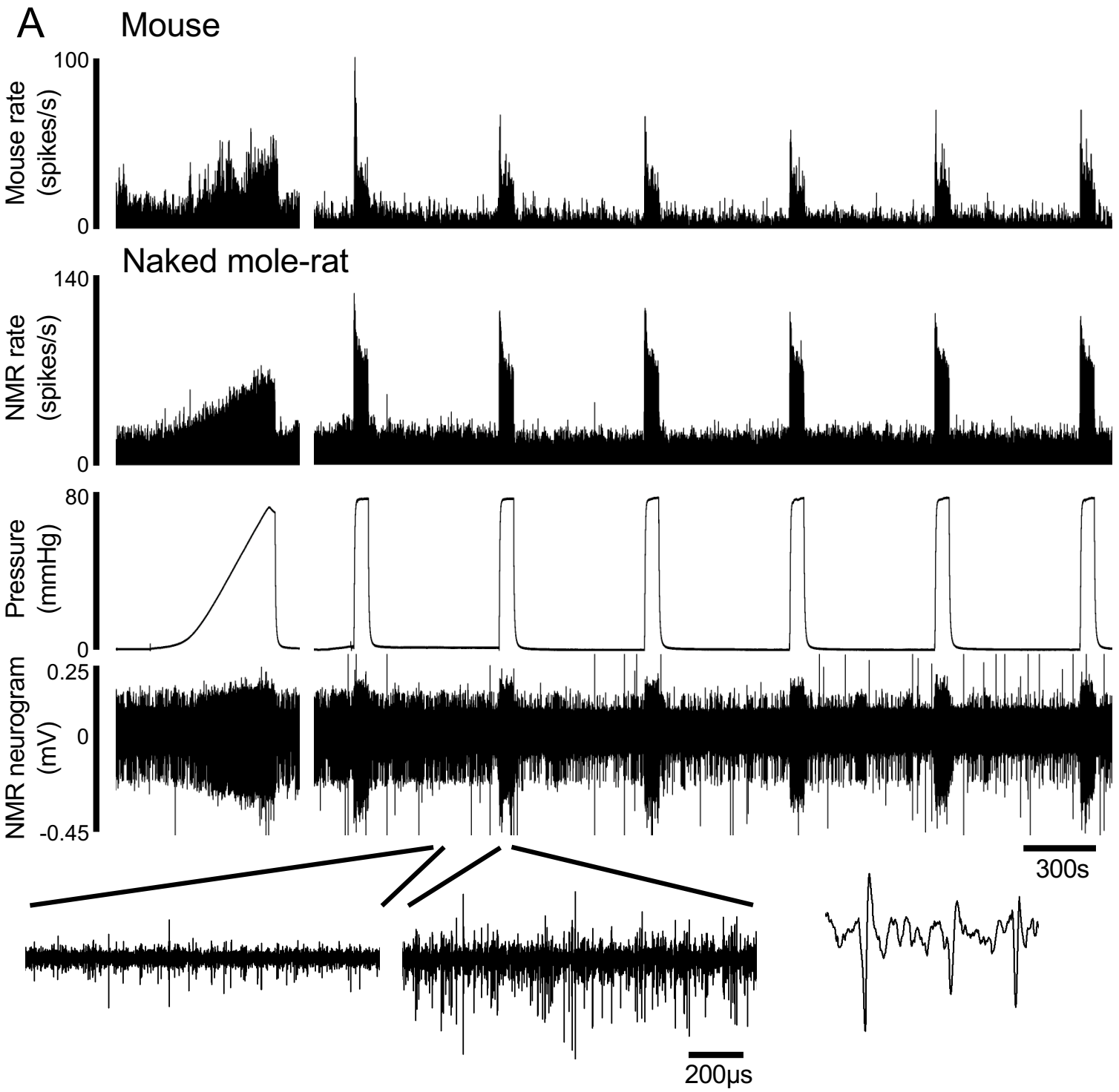
Figure 1

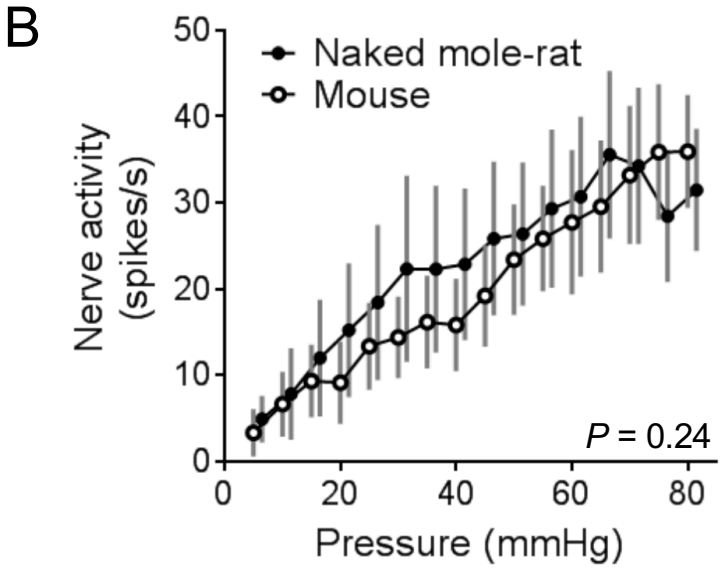

A
Mouse
Naked mole-rat
B
Mesenteric blood vessels
CGRP
C
Myenteric plexi
D
Submucosa
E
Mesenteric blood vessels
PGP9.5
F
Myenteric plexi
G
Submucosa
H
Circular muscle
I
Longitudinal muscle

conscious sensation transduced [27]. The colorectum, once dissected free from the animal, was cannulated and both luminally perfused and bath superfused with Krebs buffer, thus allowing mechanical distension of the bowel or application of chemical stimuli, respectively.

We first investigated mechanosensitivity of visceral afferents in the NMR compared to mouse gastrointestinal tract. There were no significant differences in the baseline spontaneous activity measured between NMR and mouse (3 min average: 9.7 ± 3.5 spikes/s vs. 9.3 ± 1.7 spikes/s, respectively, N = 6, P = 0.91, unpaired t-test). We applied known innocuous and noxious mechanical stimuli, firstly by way of a ramp distension (0 to 80 mmHg) and using repeat phasic distension (Fig. 2A). Colorectal distension by insertion via the anus of an inflatable balloon in humans evokes sensations of urge, discomfort and pain at increasing distension pressures [28]. Importantly, not only the absolute pressure applied, but also the dynamic quality of the stimuli (such as rate of change in pressure and duration of application) also significantly impact the extent of afferent activation [29]. By using a slow ramp distension, we were able to assess visceral afferent responses across a range of physiologically-relevant distension pressures typically exposed to the rodent gut [29,30]. We observed no difference in the nerve firing recorded during ramp distension in NMR (e.g. at 80 mmHg, 31.5 ± 7.1 spikes/s) compared to mouse (e.g. at 80 mmHg, 35.9 ± 6.5 spikes/s; Fig. 2B, P = 0.24, N = 5-6, two-way ANOVA). We next applied repeat phasic distension of the colon to noxious (0-80 mmHg) pressures. As previously reported in mouse, we observed a rapid increase in nerve activity to initial phasic distension (100.4 ± 5.4 spikes/s) and significant adaptation during the 60 s distension (Fig. 2C; [21,24]). Following subsequent repeat distensions at 9 min intervals, tachyphylaxis occurred with a decrease in peak firing of 19.7 % by the sixth distension compared to the first. In NMR, afferent discharge reached an equivalent peak firing compared to mouse during the first distension and the degree of desensitisation during subsequent distensions was similar (25.8 % by the sixth distension, P = 0.74, N = 6, two-way repeated-measures ANOVA; Fig. 2C). Similar afferent responses to mechanical stimuli in NMR compared to mouse suggest that there is no intrinsic difference in the way sensory nerves transduce physiological and noxious mechanical stimuli.

Figure 2


A
Mouse
Mouse rate (spikes/s)
100
0
Naked mole-rat
NMR rate (spikes/s)
140
0
Pressure (mmHg)
80
0
NMR neurogram (mV)
0.25
0
-0.45
300s
200µs


B
Naked mole-rat
Mouse
Nerve activity (spikes/s)
50
40
30
20
10
0
0
20
40
60
80
Pressure (mmHg)
P = 0.24

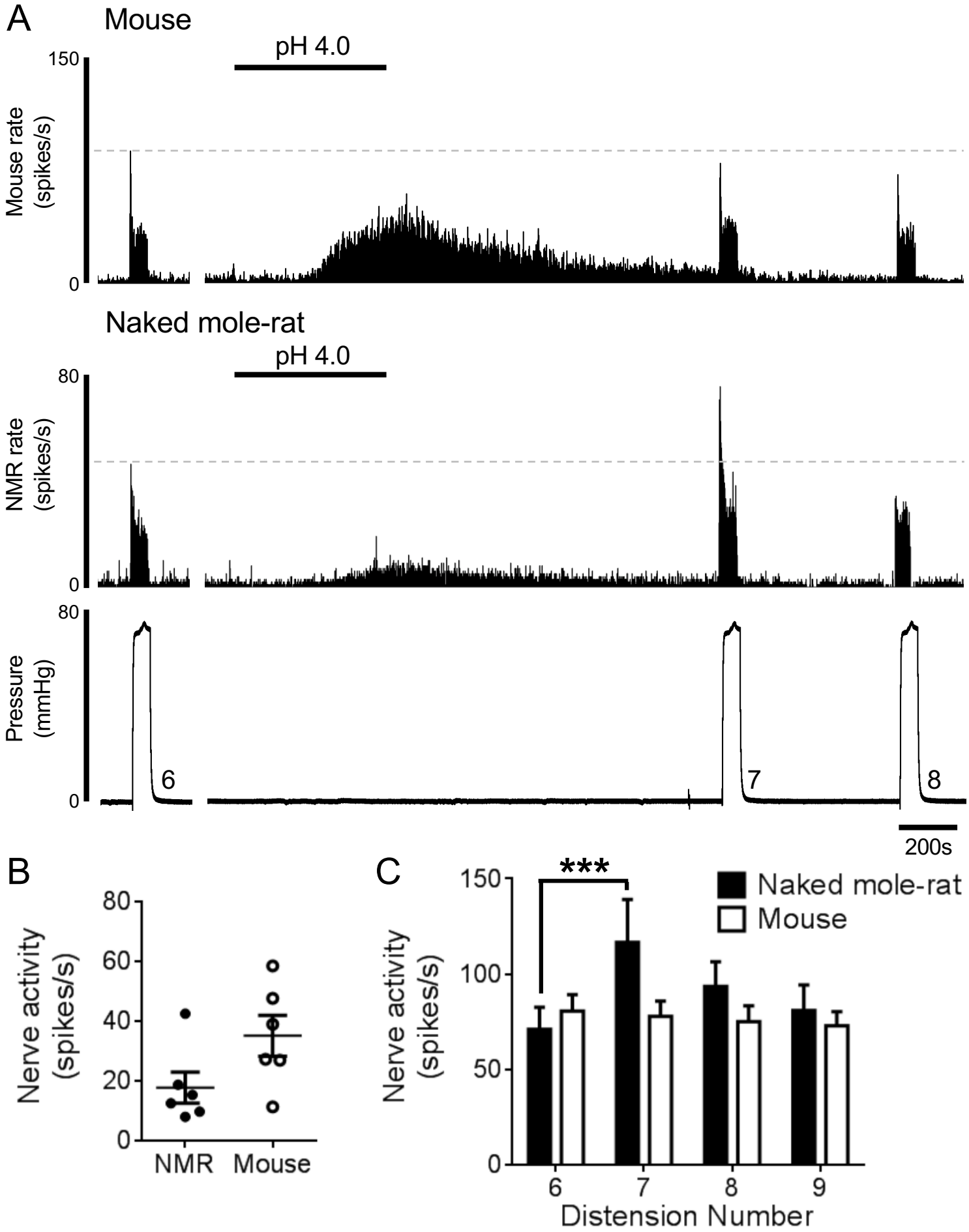
C
Naked mole-rat
Mouse
Nerve activity (spikes/s)
150
100
50
0
1
2
3
4
5
6
Distension Number

Extracellular acid evokes mechanical hypersensitivity in NMR but not mouse

Next, we investigated the effect of extracellular acid on visceral afferent firing and mechanical hypersensitivity to phasic distension (Fig. 3A). We chose a pH 4.0 stimulus to broadly activate acid-sensitive ion channels [31] and a stimulus that is capable of evoking pain both in humans and rodents when injected subcutaneously [10,32]. The vast majority of colonic sensory neurones possess inward sustained currents in response to low pH [14]. Bath superfusion of pH 4.0 to mouse colon directly excited visceral afferents evoking a peak firing increase of 35.1 ± 6.9 spikes/s returning to baseline firing rates after 1666 ± 131 s. Direct excitation of NMR visceral afferents as a result of acid did not significantly differ compared to mouse (17.8 ± 5.2 spikes/s and duration of 1755 ± 89 s, $P = 0.07$ and $P = 0.59$, respectively, $N = 6$, unpaired t-test; Fig. 3B). Immediately after returning to baseline, a set of three phasic distensions (60s, at 9 min intervals) was applied to test whether extracellular acid induced mechanical sensitisation. In agreement with previous studies in mouse, application of acid did not altered firing rates in response to any of the three subsequent phasic distensions when compared to the response prior to acid application (Fig. 3C; [33]). By contrast, extracellular acid caused significant mechanical sensitisation in the NMR, such that the response to phasic distension immediately after acid application was 63.8 % greater than before ($P < 0.01$, $N = 6$, 2-way ANOVA with Sidak's post hoc test). This mechanical sensitisation was lost by the second post-acid phasic distention and by the third phasic distension afferent firing had recovered to baseline levels and was comparable to mouse (Fig. 3C). That low pH conditions, such as that observed during inflammation, can evoke robust mechanical hypersensitivity in NMR, but not mouse, suggests fundamental differences in the mechanism by which acid-sensitive receptors are coupled to mechanotransducers in the peripheral terminals of colonic sensory neurones.

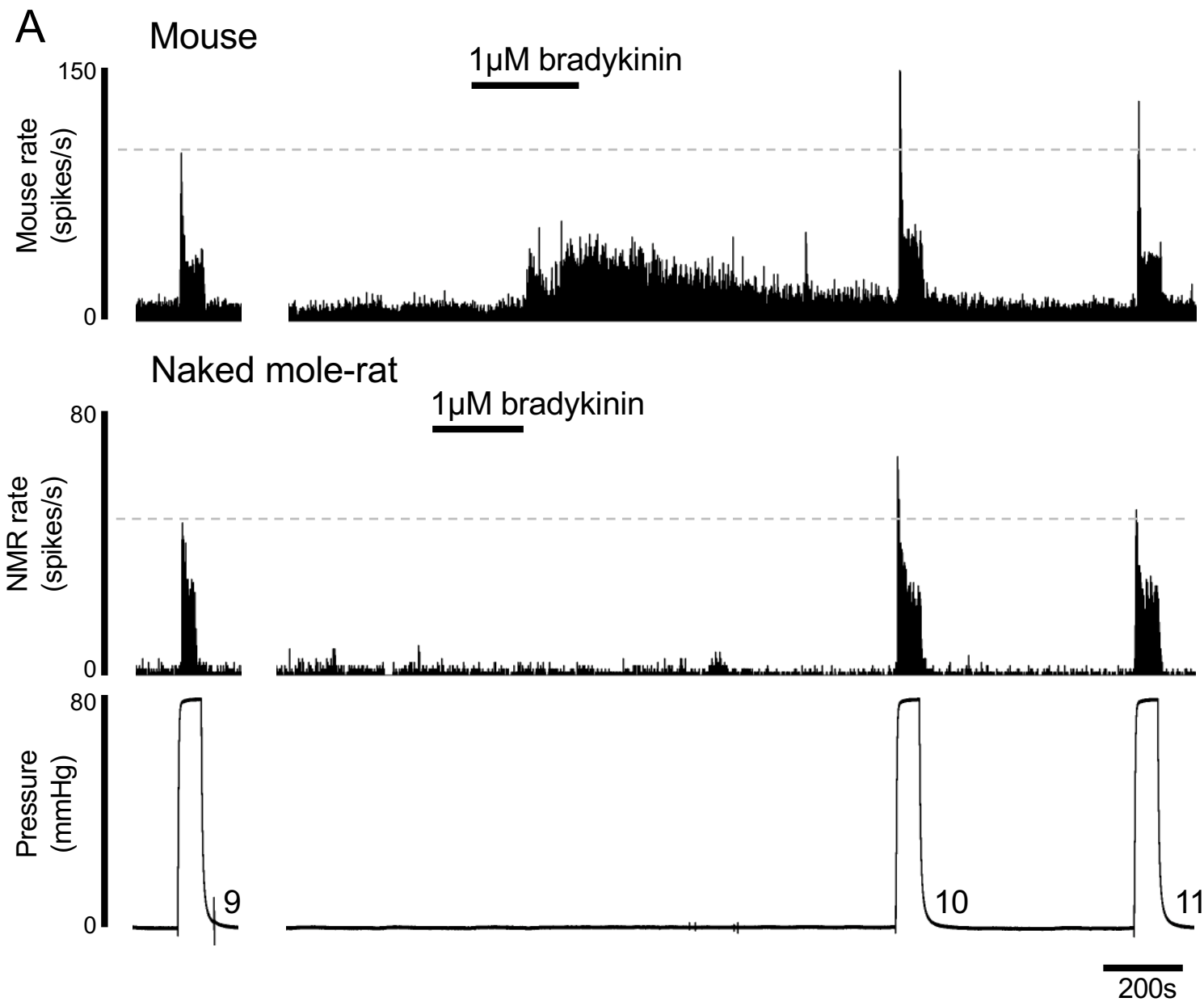
Afferent excitation to bradykinin is blunted in NMR, but mechanical sensitisation is unaffected

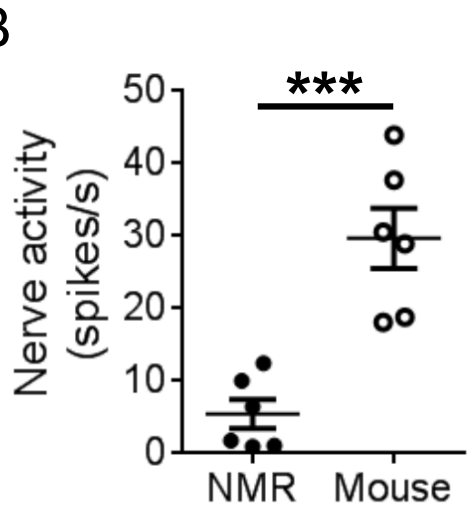
Figure 3

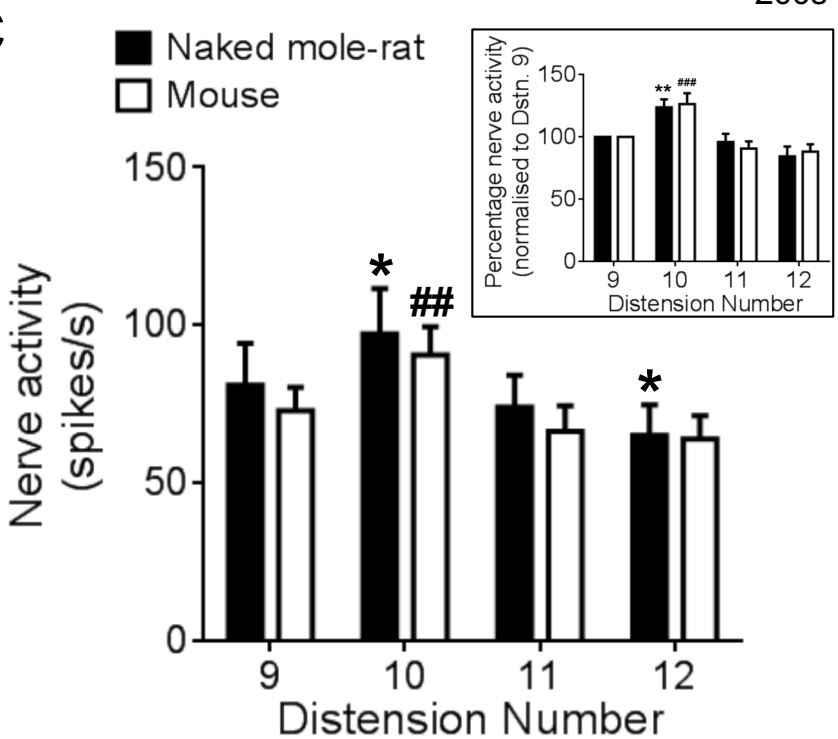

A
Mouse
pH 4.0
150
Mouse rate (spikes/s)
0
Naked mole-rat
pH 4.0
80
NMR rate (spikes/s)
0
80
Pressure (mmHg)
0
6
7
8
200s
B
80
60
40
20
0
Nerve activity (spikes/s)
NMR
Mouse
C
***
150
100
50
0
Nerve activity (spikes/s)
Naked mole-rat
Mouse
6
7
8
9
Distension Number

307 Given that inflammatory pain responses in NMR are blunted to some inflammatory
308 stimuli [10], we investigated the ability for the prototypical inflammatory mediator,
309 bradykinin, to not only activate, but evoke mechanical hypersensitivity in NMR visceral
310 afferent fibres. Application of bradykinin (1 μM) by bath superfusion to mouse colonic
311 afferents led to an increase in peak firing of 29.6 ± 4.2 spikes/s in agreement with
312 previous studies in mouse and human colonic tissues (Fig. 4A; [34,35]). In NMR this
313 was not the case, with peak firing only increased by 5.3 ± 2.0 spikes/s following
314 addition of bradykinin ($P < 0.01$, $N = 6$, unpaired t-test; Fig. 4B). However, in both
315 mouse and NMR, a robust mechanical hypersensitivity to phasic distension was
316 observed immediately after bradykinin application, such that the response to 80 mmHg
317 phasic distension was potentiated by ~25 % in both species (Fig. 4C). This may
318 suggest that the bradykinin receptor B2 in NMR colonic sensory neurones couples
319 differentially with known modulators of neuronal excitability (e.g. TRP channels, Ca^{2+}-
320 dependent potassium channels, Ca^{2+}-activated chloride channels and K_V7) thus
321 limiting the ability for bradykinin to directly drive action potential firing, however it can
322 still couple effectively to mechanotransducers facilitating their sensitisation.

Figure 4


A
Mouse
1μM bradykinin
150
Mouse rate (spikes/s)
0
Naked mole-rat
1μM bradykinin
80
NMR rate (spikes/s)
0
80
Pressure (mmHg)
0
9
10
11
200s


B
***
50
40
30
20
10
0
Nerve activity (spikes/s)
NMR
Mouse


C
Naked mole-rat
Mouse
150
100
50
0
Nerve activity (spikes/s)
*
##
*
9
10
11
12
Distension Number
Percentage nerve activity (normalised to Dstn. 9)
150
100
50
0
**
###
9
10
11
12
Distension Number

323 # Discussion

324 Acid sensing in the gastrointestinal tract is necessary to maintain gut homeostasis by
325 providing feedback for gastric and intestinal acid regulation, and secondly for detecting
326 tissue acidosis caused by inflammation, infection and ischaemia during disease.
327 Acidity is monitored by a range of cells including epithelial cells, and both intrinsic and
328 extrinsic sensory neurones innervating the GI tract. Whilst the low pH (reaching pH 1)
329 of the stomach is required for the physiological breakdown of ingested food and
330 eradication of pathogens, HCl released by gastric parietal cells is not the sole source
331 of acid in the gut [31]. Indeed, the luminal pH of the digestive system has two distinct
332 peaks in acidity: the stomach and the proximal large bowel [6,7]. Release of HCO_3^-
333 and lactic acid from the mucosa, the microbial conversion of carbohydrates to short
334 chain fatty acids (SCFAs: acetic, butyric and propionic acid), and lastly digestive bile
335 acids, all contribute to luminal acidity in the large bowel [31]. The activation of acid-
336 sensing cells drives local homeostatic changes in mucosal defence, such as changes
337 in blood flow, secretion and repair mechanisms, and autonomic and primary afferents
338 coordinate together to facilitate changes at an organ level (e.g. motility and secretion)
339 and can change organism behaviour through sensation (e.g. discomfort, pain and
340 hyperalgesia). When these systems become dysregulated then significant pathology
341 can ensure, of which pain is often the principal symptom.
342
343 Acid-evoked pain in the gastrointestinal tract is mediated through the activities of a
344 number of different molecular mechanisms. In dorsal root ganglia (DRG) neurones,
345 protons can activate fast-inactivating, largely Na^+ permeable ASICs at pH levels just
346 below physiological range (e.g. pH 7.0) [36]. In retrogradely labelled colon-projecting
347 DRG neurones isolated from the thoracolumbar vertebrae, *in situ* hybridisation against
348 ASIC subtypes indicates that ASIC2 is expressed at the greatest frequency, with both
349 ASIC1 and ASIC3 present in about a quarter of neurones [37]. A number of non-
350 selective cation transient receptor potential (TRP) channels are also sensitive to
351 protons, for example, TRPV1 is activated by pH levels below 6.2 [38], TRPV4 can be
352 opened by pH 6.0 and below [39], and TRPC4 and TRPC5 are active between pH 7.4
353 and pH 7.0 [40]. Immunohistochemical analysis suggests that both TRPV1 and
354 TRPV4 are expressed by afferents innervating the distal colon of mouse [41,42].
355 Whilst present in DRG neurones, evidence for the expression of TRPC4 and TRPC5

356 at the protein level in a colon-specific population is lacking, however single-cell RNA
357 sequencing efforts in this population suggest significant expression of TRPC4, but not
358 TRPC5, mRNA, colocalising with TRPV1 expressing neurones [15]. The vast majority
359 of colonic sensory neurones possess inward sustained currents in response to low pH
360 indicative of TRP channel activation [14], with afferent firing observed in response to
361 pH 6.5 [43]. Members of the two-pore domain K^+ (K2P) channels including TASKs,
362 TRESK, TREK and TRAAK, which contribute to setting resting membrane potential
363 and therefore have an important role in regulating neuronal excitability, are also
364 sensitive to extracellular acid [44]. The expression of TREK-1, TREK-2 and TRAAK
365 are observed in thoracolumbar and lumbosacral sensory neurones innervating the GI
366 tract and are modulated by colitis [45]. Many P2X purinoceptors are also modulated
367 by extracellular pH affecting the potency of ATP gating [46], and $P2X_2$ and $P2X_3$
368 subunits, are widely expressed by colonic sensory neurones [47,48]. Lastly, there exist
369 4 proton-sensing G protein-coupled receptors (Gpr65, Gpr68, Gpr4 and Gpr132),
370 which are activated by extracellular acidosis, however only mRNA for Gpr68 has been
371 detected in colonic sensory neurones of mice [15].
372
373 Whilst many of these receptors are directly modulated by protons, the vast majority
374 integrate additional mechano- and chemo-sensitive functions, such that acid
375 sensitises the channel. For example, genetic ablation of ASIC3 or TRPV1 in mice
376 causes a reduction in the visceral motor reflex (VMR) to colorectal distension and
377 stretch sensitisation to acidic inflammatory soup; effects that are lost in $ASIC3^{-/-}$ mice
378 [33]. $TRPV4^{-/-}$ mice possess reduced VMR to colorectal distension and significant
379 reductions in mechanosensitivity [41]. During colitis, reductions in expression levels of
380 K2P channels are observed [45]. Specifically, TREK-2-like currents evoked by pH 6.3
381 and osmotic membrane stretch are attenuated, which likely contribute to the increased
382 colonic mechanosensitivity that develops in bowel disorders such as inflammatory
383 bowel disease [45]. Pharmacological block of $P2X_3$-containing purinoceptors results
384 in reductions in visceral pain behaviours in animals models and activation with
385 selective agonist α,β-methylene ATP can sensitise responses to mechanical stimuli
386 [49]. Interestingly, Gpr68, the acid-sensitive GPCR with the highest expression in gut
387 sensory neurones, has also been described as a novel mechanotransducer [50]
388 providing a further potential point of molecular integration for acid-induced
389 mechanosensitisation. We observed no overt difference in the mechanosensitivity of

390 NMR colonic afferents compared to mouse at baseline conditions, suggesting that
391 visceral mechanotransduction is not significantly altered in the NMR. By contrast we
392 did see greatly differing responses to both, the direct exposure of extracellular acid
393 and to induced mechanical hypersensitivity, implicating altered integration of acid
394 sensors, mechanotransducers and modulators of spontaneous afferent firing.
395
396 NMR acid sensing differs significantly to other mammals. For example, in hippocampal
397 and cortex neurones, the peak current density of NMR ASIC-like responses is reduced
398 compared to mouse brain neurones [51], a likely adaptation to the NMR relying on
399 fructose-fuelled glycolysis to sustain life during severe hypoxia that would generate
400 lactic acid as a by-product [52]. In the peripheral nervous system, subcutaneous
401 injection of acid (pH 3.5), capsaicin or histamine does not cause the nocifensive or
402 pruriceptive behaviours in NMR that such stimuli characteristically induce in mice
403 [10,11]. This acid insensitivity is a function of altered ASIC responses compared to
404 mouse [18] and a variation in NMR $Na_V1.7$, which renders the channel hypersensitive
405 to proton-mediated block and therefore prevents acid-driven action potential initiation
406 from the skin [17]. We have shown previously that pharmacological inhibition or
407 genetic ablation of $Na_V1.7$ in mouse does not impair colonic afferent firing or alter pain
408 behaviours [24]. Therefore, if $Na_V1.7$ is redundant in colonic afferents compared to
409 those innervating the hindpaw, then it would be predicted that NMR colonic afferents
410 would not be as insensitive to acid as their somatic equivalents. Whilst we observed a
411 trend towards low firing rates in response to application of acid to NMR colonic
412 afferents compared mouse, these did not differ significantly. As shown previously, acid
413 alone was unable to induce mechanical sensitisation in mouse colonic afferents [33],
414 whereas robust sensitisation was observed in NMR colonic afferents, i.e. there is likely
415 differential coupling of molecular acid sensors to mechanotransducers in mouse and
416 NMR.
417
418 In NMRs, the absence of thermal hypersensitivity induced by capsaicin and lack of
419 histamine-induced scratching are thought to be due to a lack of cutaneous
420 neuropeptides, such that both behaviours can be “rescued” by intrathecal
421 administration of SP [10,11]. We show here by immunohistochemistry that CGRP is
422 expressed within nerve fibres found encapsulating both blood vessels of the NMR
423 colonic mesentery and myenteric plexi within the smooth muscle layers of the colon

424 wall, which aligns with previous findings of CGRP-positive fibres innervating NMR
425 mesenteric arteries [26]. By contrast, whilst PGP9.5 staining identified nerve fibres
426 within the submucosa these did not express CGRP, highlighting potential restricted
427 penetration of extrinsic sensory fibres innervating the NMR colorectum.
428
429 We also found that the inflammatory mediator bradykinin failed to activate NMR
430 colonic afferents, but that it could induce a robust mechanical hypersensitisation
431 comparable to the effects in mouse. Although we do not confirm bradykinin B2
432 receptor expression in NMR colonic afferents in this study, such mechanical
433 hypersensitivity suggests that the B2 receptor activity is unimpaired, but that alternate
434 coupling to its molecular transducers (including K_V7 [53], TRPV1 [54], TRPA1 [55],
435 Ca^{2+}-activated Cl^- channels [53,56] and K_{Ca} [57,58]) may explain the altered response
436 profiles compared to mouse.
437
438 Understanding how noxious pH is sensed and gastrointestinal homeostasis is
439 maintained in the NMR may help to inform our understanding of other model species
440 and gastrointestinal acid sensing during human disease. One explanation for the
441 altered colonic afferent sensitivities observed may be differences in the microbiome
442 between species. Microbiota can generate metabolites (including organic acids) that
443 have a significant bearing on luminal acidity. And vice versa, variations in luminal pH
444 can greatly impact microbiota diversity and activity [59]. Indeed, NMR gut microbiota
445 is distinct from other rodents, with a greater propensity to produce increased levels of
446 SCFAs and support anaerobic oxidative metabolism, which is probably as a result of
447 their relatively homogeneous diet [60]. Such altered gut metabolism may explain the
448 greater tolerances observed in colonic afferent sensitivities to acid and noxious
449 inflammatory stimuli, alongside other adaptations to habitat. Further studies are
450 required to fully understand how the NMR maintains gut pH homeostasis whilst
451 balancing adaptations enabling a fitness advantage through acid insensitivity.

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Author Contributions

JRFH designed the research studies, conducted the experiments, acquired and analysed the data and wrote the manuscript. TST, GC and ZH acquired and analysed the data. EStJS designed the research studies and wrote the manuscript. All authors approve the final version of the manuscript.

Declaration of Conflicting Interests

The authors have no conflicting interests to declare.

469 # References

470 1. Holzer P. Acid sensing by visceral afferent neurones. Acta Physiol (Oxf) [Internet].
471 2011;201:63–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20456281
472 2. Fülling C, Dinan TG, Cryan JF. Gut Microbe to Brain Signaling: What Happens in
473 Vagus…. Neuron [Internet]. 2019;101:998–1002. Available from:
474 http://www.ncbi.nlm.nih.gov/pubmed/30897366
475 3. Sengupta JN, Su X, Gebhart GF. Kappa, but not mu or delta, opioids attenuate
476 responses to distention of afferent fibers innervating the rat colon. Gastroenterology
477 [Internet]. 1996;111:968–80. Available from:
478 https://www.ncbi.nlm.nih.gov/pubmed/8831591
479 4. Su X, Gebhart GF. Mechanosensitive pelvic nerve afferent fibers innervating the
480 colon of the rat are polymodal in character. J Neurophysiol [Internet]. 1998;80:2632–
481 44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9819269
482 5. Brookes SJ, Spencer NJ, Costa M, Zagorodnyuk VP. Extrinsic primary afferent
483 signalling in the gut. Nat Rev Gastroenterol Hepatol [Internet]. 2013;10:286–96.
484 Available from: http://www.ncbi.nlm.nih.gov/pubmed/23438947
485 6. Nugent SG, Kumar D, Rampton DS, Evans DF. Intestinal luminal pH in
486 inflammatory bowel disease: possible determinants and implications for therapy with
487 aminosalicylates and other drugs. Gut [Internet]. 2001;48:571–7. Available from:
488 http://www.ncbi.nlm.nih.gov/pubmed/11247905
489 7. Fallingborg J. Intraluminal pH of the human gastrointestinal tract. Dan Med Bull
490 [Internet]. 1999;46:183–96. Available from:
491 https://www.ncbi.nlm.nih.gov/pubmed/10421978
492 8. Andersen JR, Bendtsen F, Ovesen L, Pedersen NT, Rune SJ, Tage-Jensen U.
493 Pancreatic insufficiency. Duodenal and jejunal pH, bile acid activity, and micellar lipid
494 solubilization. Int J Pancreatol [Internet]. 1990;6:263–70. Available from:
495 http://www.ncbi.nlm.nih.gov/pubmed/2212745
496 9. Fallingborg J, Christensen LA, Jacobsen BA, Rasmussen SN. Very low
497 intraluminal colonic pH in patients with active ulcerative colitis. Dig Dis Sci [Internet].
498 1993;38:1989–93. Available from: https://www.ncbi.nlm.nih.gov/pubmed/8223071
499 10. Park TJ, Lu Y, Jüttner R, Smith ES, Hu J, Brand A, et al. Selective inflammatory
500 pain insensitivity in the African naked mole-rat (Heterocephalus glaber). PLoS Biol
501 [Internet]. 2008;6:e13. Available from:

502 http://www.ncbi.nlm.nih.gov/pubmed/18232734
503 11. Smith ESJ, Blass GRC, Lewin GR, Park TJ. Absence of histamine-induced itch
504 in the African naked mole-rat and “rescue” by Substance P. Mol Pain. 2010;6:29.
505 12. Omerbašić D, Smith ESJ, Moroni M, Homfeld J, Eigenbrod O, Bennett NC, et al.
506 Hypofunctional TrkA Accounts for the Absence of Pain Sensitization in the African
507 Naked Mole-Rat. Cell Rep [Internet]. 2016;17:748–58. Available from:
508 https://www.ncbi.nlm.nih.gov/pubmed/27732851
509 13. Steen KH, Reeh PW. Sustained graded pain and hyperalgesia from harmless
510 experimental tissue acidosis in human skin. Neurosci Lett [Internet]. 1993;154:113–
511 6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8361622
512 14. Sugiura T, Bielefeldt K, Gebhart GF. Mouse colon sensory neurons detect
513 extracellular acidosis via TRPV1. Am J Physiol Cell Physiol. 2007;292:C1768–74.
514 15. Hockley JRFF, Taylor TS, Callejo G, Wilbrey AL, Gutteridge A, Bach K, et al.
515 Single-cell RNAseq reveals seven classes of colonic sensory neuron. Gut [Internet].
516 2019;68:633–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29483303
517 16. Schuhmacher L-N, Smith ESJ. Expression of acid-sensing ion channels and
518 selection of reference genes in mouse and naked mole rat. Mol Brain [Internet].
519 2016;9:97. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27964758
520 17. Smith ES, Omerbašić D, Lechner SG, Anirudhan G, Lapatsina L, Lewin GR. The
521 molecular basis of acid insensitivity in the African naked mole-rat. Science (80-)
522 [Internet]. 2011;334:1557–60. Available from:
523 http://www.ncbi.nlm.nih.gov/pubmed/22174253
524 18. Schuhmacher L-N, Callejo G, Srivats S, Smith ESJ. Naked mole-rat acid-sensing
525 ion channel 3 forms nonfunctional homomers, but functional heteromers. J Biol
526 Chem [Internet]. 2018;293:1756–66. Available from:
527 http://www.jbc.org/lookup/doi/10.1074/jbc.M117.807859
528 19. Schuhmacher L-N, Husson, Zoé SESJ. The naked mole-rat as an animal model
529 in biomedical research: current perspectives. Open Access Anim Physiol [Internet].
530 2015;Volume 7:137. Available from: https://www.dovepress.com/the-naked-mole-rat-
531 as-an-animal-model-in-biomedical-research-current-p-peer-reviewed-fulltext-article-
532 OAAP
533 20. Firth M, Prather CM. Gastrointestinal motility problems in the elderly patient.
534 Gastroenterology [Internet]. 2002;122:1688–700. Available from:
535 http://www.ncbi.nlm.nih.gov/pubmed/12016432

536 21. Hockley JRF, Boundouki G, Cibert-Goton V, McGuire C, Yip PK, Chan C, et al.
537 Multiple roles for Na(V)1.9 in the activation of visceral afferents by noxious
538 inflammatory, mechanical, and human disease-derived stimuli. Pain [Internet].
539 2014;155:1962–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24972070
540 22. Hughes PA, Brierley SM, Martin CM, Brookes SJH, Linden DR, Blackshaw LA, et
541 al. Post-inflammatory colonic afferent sensitisation: different subtypes, different
542 pathways and different time courses. Gut [Internet]. 2009;58:1333–41. Available
543 from: http://www.ncbi.nlm.nih.gov/pubmed/19324867
544 23. Ness TJ, Gebhart GF. Colorectal distension as a noxious visceral stimulus:
545 physiologic and pharmacologic characterization of pseudaffective reflexes in the rat.
546 Brain Res [Internet]. 1988;450:153–69. Available from:
547 http://www.ncbi.nlm.nih.gov/pubmed/3401708
548 24. Hockley JR, González-Cano R, McMurray S, Tejada-Giraldez MA, McGuire C,
549 Torres A, et al. Visceral and somatic pain modalities reveal NaV 1.7-independent
550 visceral nociceptive pathways. J Physiol [Internet]. 2017;595:2661–79. Available
551 from: https://www.ncbi.nlm.nih.gov/pubmed/28105664
552 25. Spencer NJ, Zagorodnyuk V, Brookes SJ, Hibberd T. Spinal afferent nerve
553 endings in visceral organs: recent advances. Am J Physiol Gastrointest Liver Physiol
554 [Internet]. 2016;311:G1056–63. Available from:
555 http://www.ncbi.nlm.nih.gov/pubmed/27856418
556 26. Park TJ, Comer C, Carol A, Lu Y, Hong HS, Rice FL. Somatosensory
557 organization and behavior in naked mole-rats: II. Peripheral structures, innervation,
558 and selective lack of neuropeptides associated with thermoregulation and pain. J
559 Comp Neurol. 2003;465:104–20.
560 27. Grundy L, Erickson A, Brierley SM. Visceral Pain. Annu Rev Physiol [Internet].
561 2019;81:261–84. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30379615
562 28. Cervero F. Neurophysiology of gastrointestinal pain. Baillieres Clin Gastroenterol
563 [Internet]. 1988;2:183–99. Available from:
564 http://www.ncbi.nlm.nih.gov/pubmed/2838108
565 29. Booth CE, Shaw J, Hicks GA, Kirkup AJ, Winchester W, Grundy D. Influence of
566 the pattern of jejunal distension on mesenteric afferent sensitivity in the
567 anaesthetized rat. Neurogastroenterol Motil [Internet]. 2008;20:149–58. Available
568 from: http://www.ncbi.nlm.nih.gov/pubmed/17931340
569 30. Christianson JA, Gebhart GF. Assessment of colon sensitivity by luminal

570 distension in mice. Nat Protoc [Internet]. 2007;2:2624–31. Available from:
571 http://www.ncbi.nlm.nih.gov/pubmed/17948005
572 31. Holzer P. Acid-sensing ion channels in gastrointestinal function.
573 Neuropharmacology [Internet]. Elsevier Ltd; 2015;94:72–9. Available from:
574 http://dx.doi.org/10.1016/j.neuropharm.2014.12.009
575 32. Schwarz MG, Namer B, Reeh PW, Fischer MJM. TRPA1 and TRPV1
576 Antagonists Do Not Inhibit Human Acidosis-Induced Pain. J Pain [Internet].
577 2017;18:526–34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28062311
578 33. Jones RC, Xu L, Gebhart GF. The mechanosensitivity of mouse colon afferent
579 fibers and their sensitization by inflammatory mediators require transient receptor
580 potential vanilloid 1 and acid-sensing ion channel 3. J Neurosci [Internet].
581 2005;25:10981–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16306411
582 34. Peiris M, Hockley JR, Reed DE, Smith ESJ, Bulmer DC, Blackshaw LA.
583 Peripheral KV7 channels regulate visceral sensory function in mouse and human
584 colon. Mol Pain [Internet]. 2017;13:1744806917709371. Available from:
585 https://www.ncbi.nlm.nih.gov/pubmed/28566000
586 35. McGuire C, Boundouki G, Hockley JR, Reed D, Cibert-Goton V, Peiris M, et al.
587 Ex vivo study of human visceral nociceptors. Gut [Internet]. 2016; Available from:
588 https://www.ncbi.nlm.nih.gov/pubmed/27654583
589 36. Soto E, Ortega-Ramírez A, Vega R. Protons as Messengers of Intercellular
590 Communication in the Nervous System. Front Cell Neurosci [Internet]. 2018;12:342.
591 Available from: http://www.ncbi.nlm.nih.gov/pubmed/30364044
592 37. Hughes PA, Brierley SM, Young RL, Blackshaw LA. Localization and
593 comparative analysis of acid-sensing ion channel (ASIC1, 2, and 3) mRNA
594 expression in mouse colonic sensory neurons within thoracolumbar dorsal root
595 ganglia. J Comp Neurol [Internet]. 2007;500:863–75. Available from:
596 http://www.ncbi.nlm.nih.gov/pubmed/17177258
597 38. Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, et al.
598 The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron
599 [Internet]. 1998;21:531–43. Available from:
600 http://www.ncbi.nlm.nih.gov/pubmed/9768840
601 39. Suzuki M, Mizuno A, Kodaira K, Imai M. Impaired pressure sensation in mice
602 lacking TRPV4. J Biol Chem [Internet]. 2003;278:22664–8. Available from:
603 http://www.ncbi.nlm.nih.gov/pubmed/12692122

604 40. Semtner M, Schaefer M, Pinkenburg O, Plant TD. Potentiation of TRPC5 by
605 protons. J Biol Chem [Internet]. 2007;282:33868–78. Available from:
606 http://www.ncbi.nlm.nih.gov/pubmed/17884814
607 41. Brierley SM, Page AJ, Hughes PA, Adam B, Liebregts T, Cooper NJ, et al.
608 Selective role for TRPV4 ion channels in visceral sensory pathways.
609 Gastroenterology [Internet]. 2008;134:2059–69. Available from:
610 http://www.ncbi.nlm.nih.gov/pubmed/18343379http://www.ncbi.nlm.nih.gov/pmc/articl
611 es/PMC2504007/pdf/nihms58638.pdf
612 42. Chen BN, Olsson C, Sharrad DF, Brookes SJH. Sensory innervation of the
613 guinea pig colon and rectum compared using retrograde tracing and
614 immunohistochemistry. Neurogastroenterol Motil [Internet]. 2016;28:1306–16.
615 Available from: http://www.ncbi.nlm.nih.gov/pubmed/27038370
616 43. Wynn G, Burnstock G. Adenosine 5'-triphosphate and its relationship with other
617 mediators that activate pelvic nerve afferent neurons in the rat colorectum.
618 Purinergic Signal [Internet]. 2006;2:517–26. Available from:
619 http://www.ncbi.nlm.nih.gov/pubmed/18404489http://www.ncbi.nlm.nih.gov/pmc/articl
620 es/PMC2104004/pdf/11302_2005_Article_5305.pdf
621 44. Holzer P. Acid-sensitive ion channels and receptors. Handb Exp Pharmacol
622 [Internet]. 2009;283–332. Available from:
623 http://www.ncbi.nlm.nih.gov/pubmed/19655111
624 45. La JH, Gebhart GF. Colitis decreases mechanosensitive K2P channel
625 expression and function in mouse colon sensory neurons. Am J Physiol Gastrointest
626 Liver Physiol [Internet]. 2011;301:G165-74. Available from:
627 https://www.ncbi.nlm.nih.gov/pubmed/21512155
628 46. North RA. Molecular physiology of P2X receptors. Physiol Rev [Internet].
629 2002;82:1013–67. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12270951
630 47. Shinoda M, La J-HH, Bielefeldt K, Gebhart GF. Altered purinergic signaling in
631 colorectal dorsal root ganglion neurons contributes to colorectal hypersensitivity. J
632 Neurophysiol [Internet]. 2010;104:3113–23. Available from:
633 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3007642&tool=pmcentrez
634 &rendertype=abstract
635 48. Burnstock G. Purine-mediated signalling in pain and visceral perception. Trends
636 Pharmacol Sci. 2001;22:182–8.
637 49. Xu G-YY, Shenoy M, Winston JH, Mittal S, Pasricha PJ. P2X receptor-mediated

638 visceral hyperalgesia in a rat model of chronic visceral hypersensitivity. Gut
639 [Internet]. 2008;57:1230–7. Available from:
640 http://www.ncbi.nlm.nih.gov/pubmed/18270243
641 50. Xu J, Mathur J, Vessières E, Hammack S, Nonomura K, Favre J, et al. GPR68
642 Senses Flow and Is Essential for Vascular Physiology. Cell [Internet]. 2018;173:762–
643 775.e16. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29677517
644 51. Husson Z, Smith ESJ. Naked mole-rat cortical neurons are resistant to acid-
645 induced cell death. Mol Brain [Internet]. 2018;11:26. Available from:
646 http://www.ncbi.nlm.nih.gov/pubmed/29739425
647 52. Park TJ, Reznick J, Peterson BL, Blass G, Omerbašić D, Bennett NC, et al.
648 Fructose-driven glycolysis supports anoxia resistance in the naked mole-rat. Science
649 [Internet]. 2017;356:307–11. Available from:
650 http://www.ncbi.nlm.nih.gov/pubmed/28428423
651 53. Liu B, Linley JE, Du X, Zhang X, Ooi L, Zhang H, et al. The acute nociceptive
652 signals induced by bradykinin in rat sensory neurons are mediated by inhibition of M-
653 type K+ channels and activation of Ca2+-activated Cl- channels. J Clin Invest
654 [Internet]. 2010;120:1240–52. Available from:
655 https://www.ncbi.nlm.nih.gov/pubmed/20335661
656 54. Lee SY, Lee JH, Kang KK, Hwang SY, Choi KD, Oh U. Sensitization of vanilloid
657 receptor involves an increase in the phosphorylated form of the channel. Arch Pharm
658 Res [Internet]. 2005;28:405–12. Available from:
659 https://www.ncbi.nlm.nih.gov/pubmed/15918513
660 55. Wang S, Dai Y, Fukuoka T, Yamanaka H, Kobayashi K, Obata K, et al.
661 Phospholipase C and protein kinase A mediate bradykinin sensitization of TRPA1: a
662 molecular mechanism of inflammatory pain. Brain [Internet]. 2008;131:1241–51.
663 Available from: https://www.ncbi.nlm.nih.gov/pubmed/18356188
664 56. Oh EJ, Weinreich D. Bradykinin decreases K(+) and increases Cl(-)
665 conductances in vagal afferent neurones of the guinea pig. J Physiol [Internet].
666 2004;558:513–26. Available from: https://www.ncbi.nlm.nih.gov/pubmed/15169850
667 57. Weinreich D. Bradykinin inhibits a slow spike afterhyperpolarization in visceral
668 sensory neurons. Eur J Pharmacol [Internet]. 1986;132:61–3. Available from:
669 https://www.ncbi.nlm.nih.gov/pubmed/3816966
670 58. Undem BJ, Weinreich D. Electrophysiological properties and chemosensitivity of
671 guinea pig nodose ganglion neurons in vitro. J Aut Nerv Syst [Internet]. 1993;44:17–

672 33. Available from: https://www.ncbi.nlm.nih.gov/pubmed/8104970
673 59. Tana C, Umesaki Y, Imaoka A, Handa T, Kanazawa M, Fukudo S. Altered
674 profiles of intestinal microbiota and organic acids may be the origin of symptoms in
675 irritable bowel syndrome. Neurogastroenterol Motil [Internet]. 2009; Available from:
676 http://doi.wiley.com/10.1111/j.1365-2982.2009.01427.x
677 60. Debebe T, Biagi E, Soverini M, Holtze S, Hildebrandt TB, Birkemeyer C, et al.
678 Unraveling the gut microbiome of the long-lived naked mole-rat. Sci Rep [Internet].
679 2017;7:9590. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28852094
680

681 # Figure Legends

682 **Figure 1. Extrinsic sensory innervation of NMR colorectum**

683 A. Comparison of mouse and NMR gastrointestinal tracts from anus (*left*) to
684 oesophagus (*right*), with a 30 cm ruler providing scale. Wholemount immunostaining
685 for CGRP in the mesentery (B; *insert*, nerve fibres encircling a mesenteric blood
686 vessel. *Arrows*, example nerve fibre on the blood vessel margin), myenteric plexi (C;
687 *insert*, extrinsic nerve fibres infiltrating myenteric ganglia) and submucosa (D) of NMR.
688 Equivalent nerve fibre staining was also observed with PGP9.5 in the mesentery (E;
689 *insert*, nerve fibre surrounding mesenteric blood vessel), myenteric plexi (F),
690 submucosa (G; *insert*, nerve fibre surrounding the base of a mucosal villi), circular
691 muscle (H) and longitudinal muscle (I; *arrows*, nerve fibre innervating longitudinal
692 muscle). Scale bar in each panel, 50 μm.

693

694 **Figure 2. Colonic afferent responses to noxious ramp and repeat phasic**
695 **distension in mouse and NMR**

696 A. Example rate histograms of colonic lumbar splanchnic nerve activity from mouse
697 and NMR with intraluminal pressure trace and neurogram trace following ramp
698 distension (0 to 80 mmHg) and repeat phasic distension (0-80 mmHg, 60 s, 9 min
699 intervals). Below, expanded neurogram traces showing NMR before and after phasic
700 distension and an example trace showing three action potentials. B. Mean firing rates
701 to ramp distension at 5 mmHg increments in mouse and NMR (P = 0.67, N = 5-6, two-
702 way repeated-measures ANOVA). C. Average change in peak firing rate during repeat
703 phasic distension in mouse and NMR (P = 0.74, two-way repeated-measures
704 ANOVA).

705

706 **Figure 3. Extracellular acid evokes mechanical hypersensitivity in NMR, but not**
707 **mouse**

708 A. Example rate histograms of colonic splanchnic nerve activity from mouse and NMR
709 with accompanying pressure trace showing bath superfusion of pH 4.0 Krebs buffer
710 (50 mL) and subsequent repeat (x 3) phasic distension. B. Mean increase in peak
711 firing after application of pH 4.0 (P = 0.07, N = 6, unpaired t-test). C. Peak firing change
712 to phasic distension after superfusion with pH 4.0 solution. The response to phasic

713 distension in NMR, but not mouse, was significantly sensitised by acid (***$P < 0.01$, N
714 = 6, two-way repeated-measures ANOVA with Sidak's post-hoc).
715
716 **Figure 4. Colonic afferent excitation to bradykinin is blunted in NMR, but**
717 **mechanical sensitisation is unaffected**
718
719 A. Example rate histograms of colonic splanchnic nerve activity from mouse and NMR
720 with accompanying pressure trace showing addition of 1 μM bradykinin (20 mL) and
721 subsequent repeat (x 3) phasic distension. B. Mean increase in peak firing after
722 application of 1 μM bradykinin (***$P < 0.001$, N = 6, unpaired t-test). C. Peak firing
723 change to phasic distension after superfusion with 1 μM bradykinin. The response to
724 phasic distension in both NMR and mouse was significantly sensitised by application
725 of bradykinin (*$P < 0.05$ vs. 9th distension in NMR, $^{\#\#}P < 0.01$ vs. 9th distension in
726 mouse, N = 6, two-way repeated-measures ANOVA with Sidak's post-hoc). *Insert*,
727 phasic distension responses in both NMR and mouse normalised to the pre-bradykinin
728 distension response (**$P < 0.01$ vs. normalised 9th distension in NMR, $^{\#\#\#}P < 0.001$ vs.
729 normalised 9th distension in mouse, N = 6, two-way repeated-measures ANOVA with
730 Sidak's post-hoc).