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

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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.

Materials and Methods

Animals

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

Immunohistochemistry

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

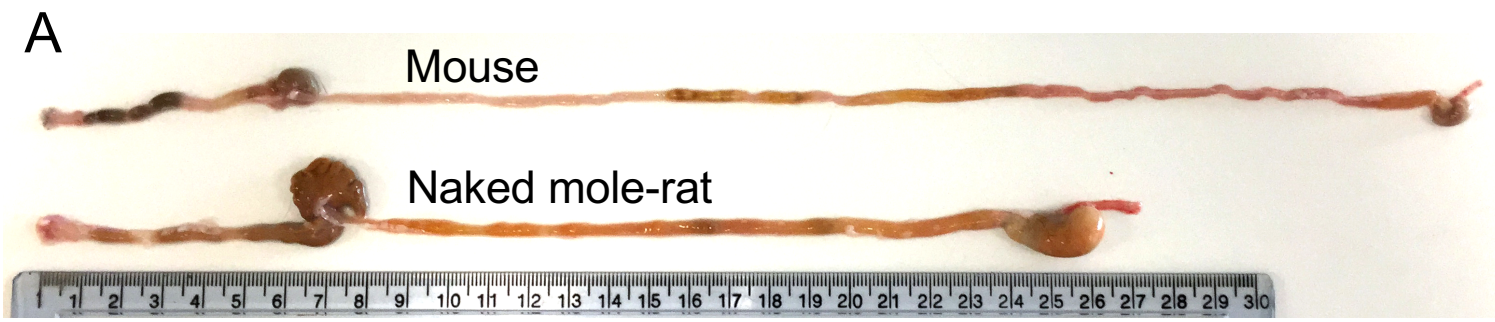
We first compared the gross anatomy of the NMR and mouse gastrointestinal tract. As the NMR is greatly long lived compared to the mouse, with a life expectancy of >30-years, we chose animals of an equivalent phase of life and body size (e.g. young adult: for mouse, 6-8-weeks, snout to anus length 92 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).

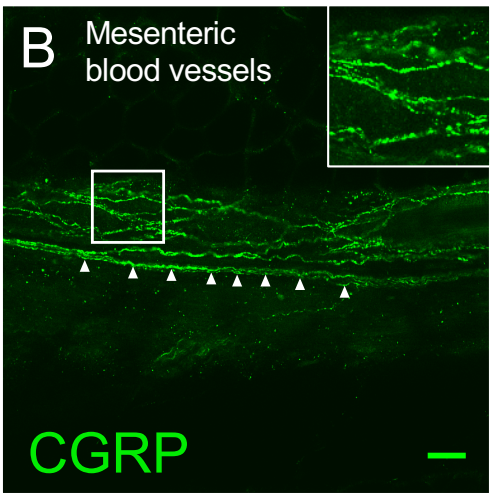
We next confirmed the presence of extrinsic sensory fibres innervating different layers of the NMR colorectum using immunohistochemistry. Equivalent staining in the mouse are widely available within the literature and we did not seek to duplicate them here [25]. Using antibodies raised against calcitonin 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).

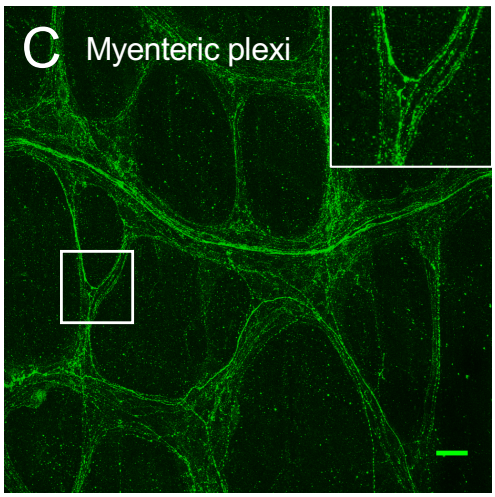
Colonic afferent mechanosensitivity does not differ in the NMR compared to mouse

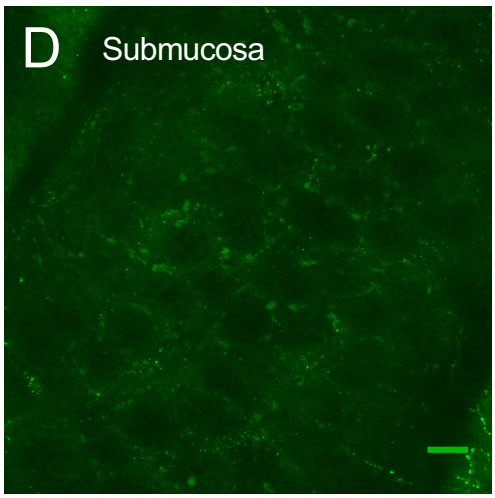
In order to understand whether the peripheral terminals of sensory neurones innervating the gastrointestinal tract of the NMR possessed altered acid and inflammatory sensitivity compared to mouse, we made *ex vivo* multi-unit electrophysiological recordings of lumbar splanchnic nerve activity using a 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

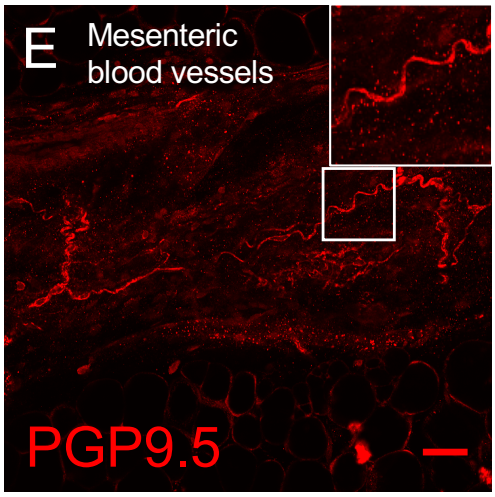
Figure 1

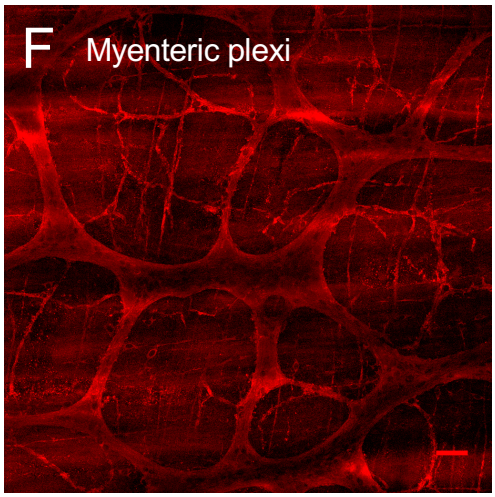


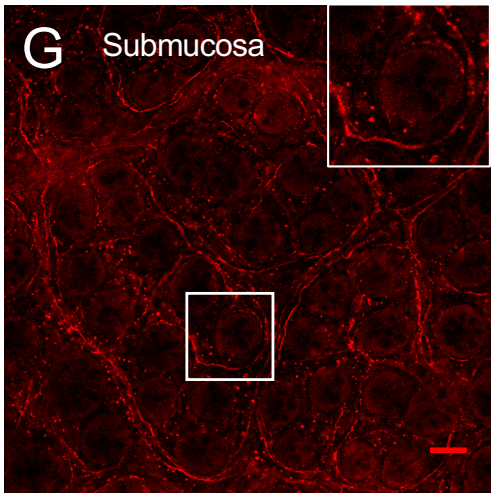


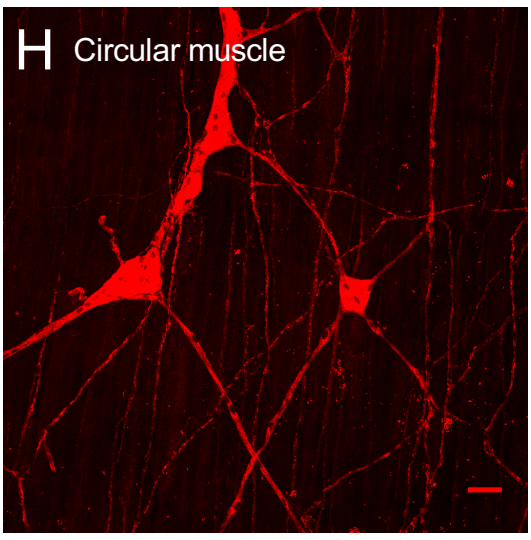


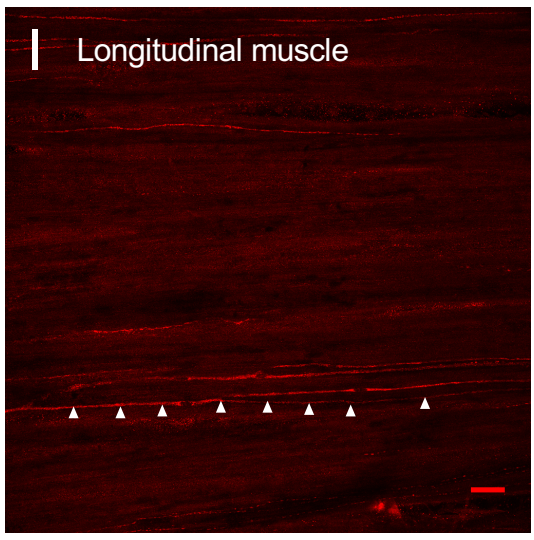








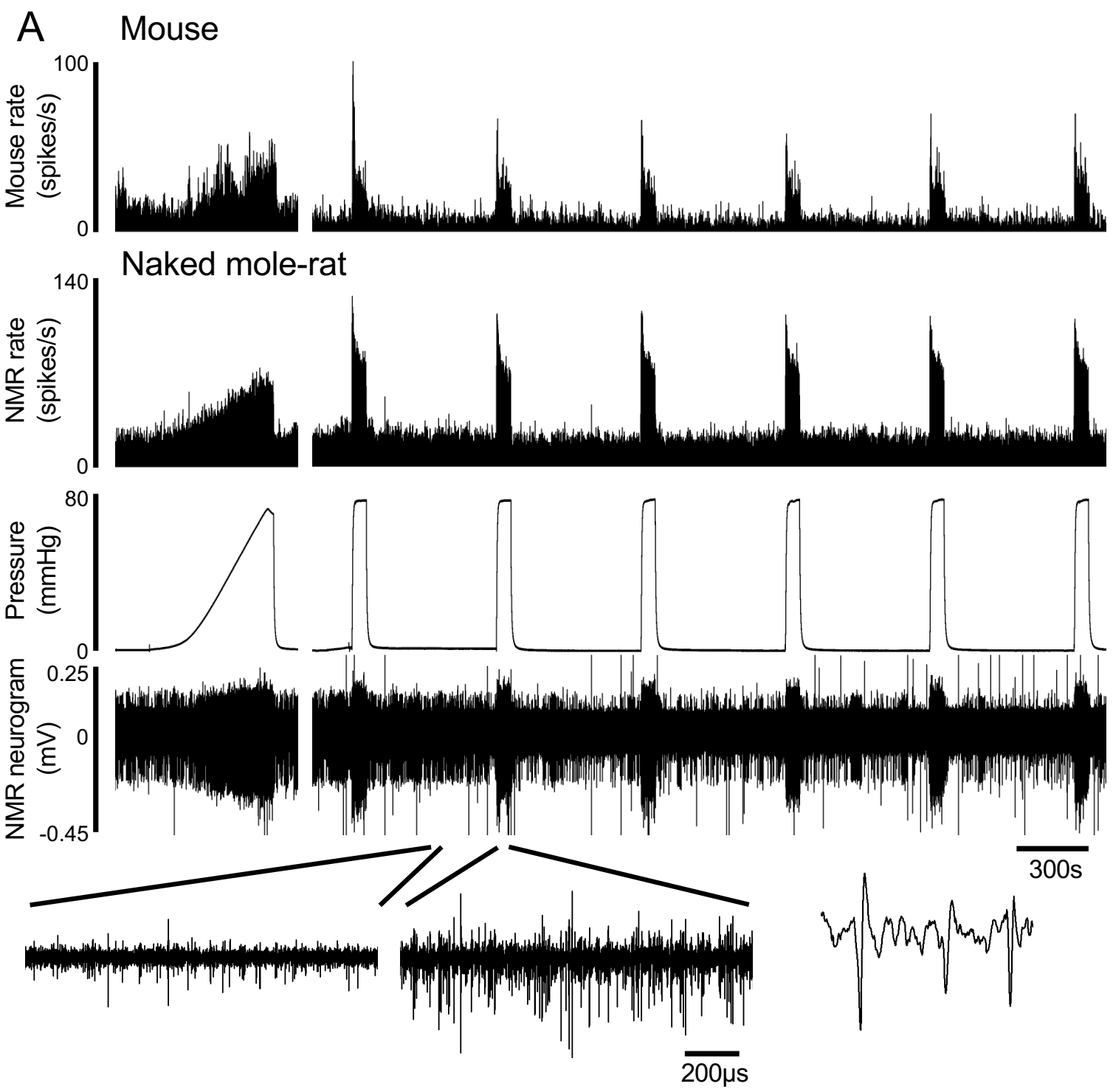


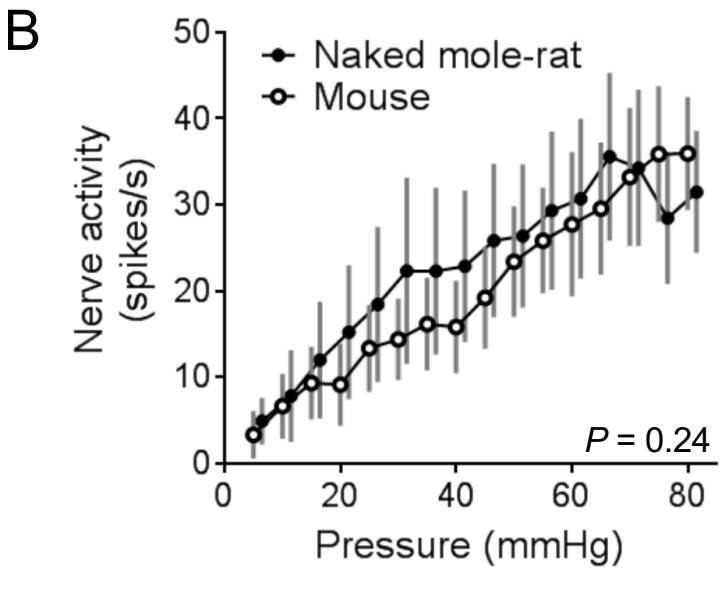


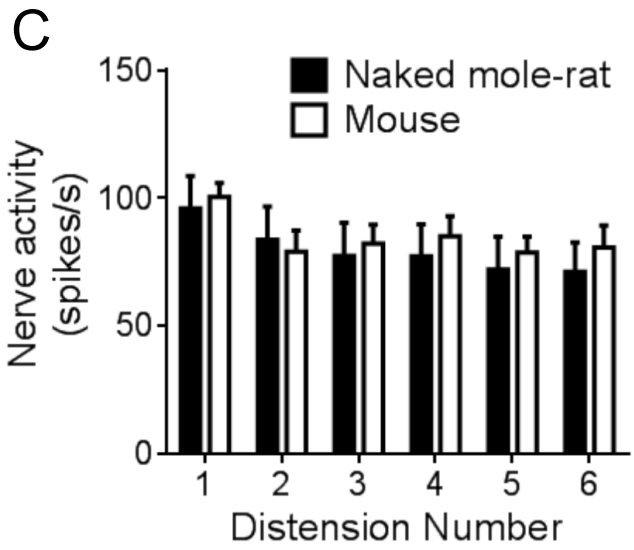
conscious sensation transduced [27]. The colorectum, once dissected free from the animal, was cannulated and both luminally perfused and bath superfused with Krebs buffer, thus allowing mechanical distension of the bowel or application of chemical stimuli, respectively.

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





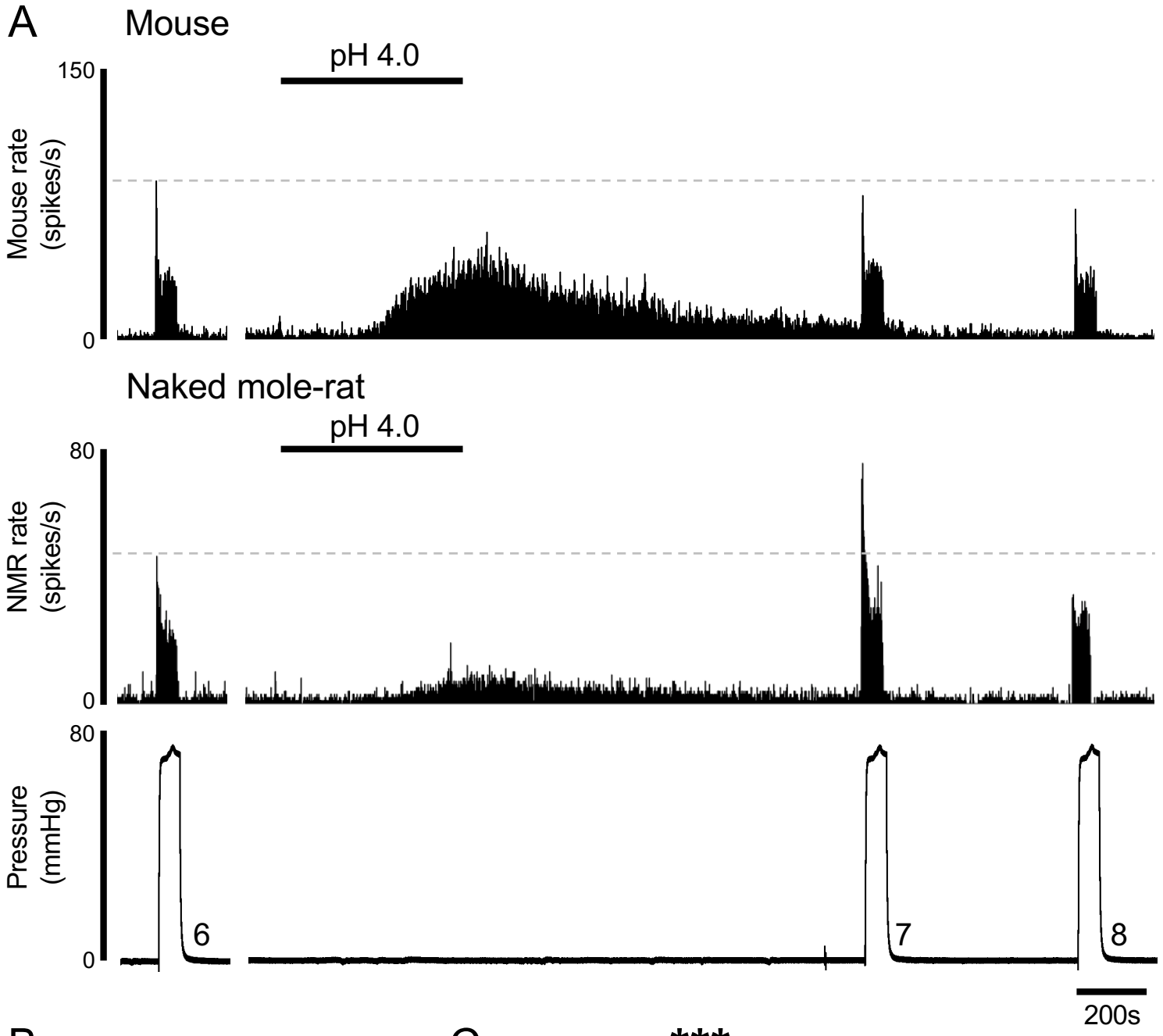


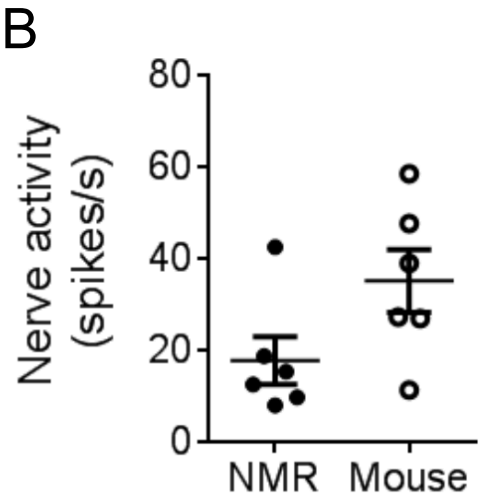
Extracellular acid evokes mechanical hypersensitivity in NMR but not mouse

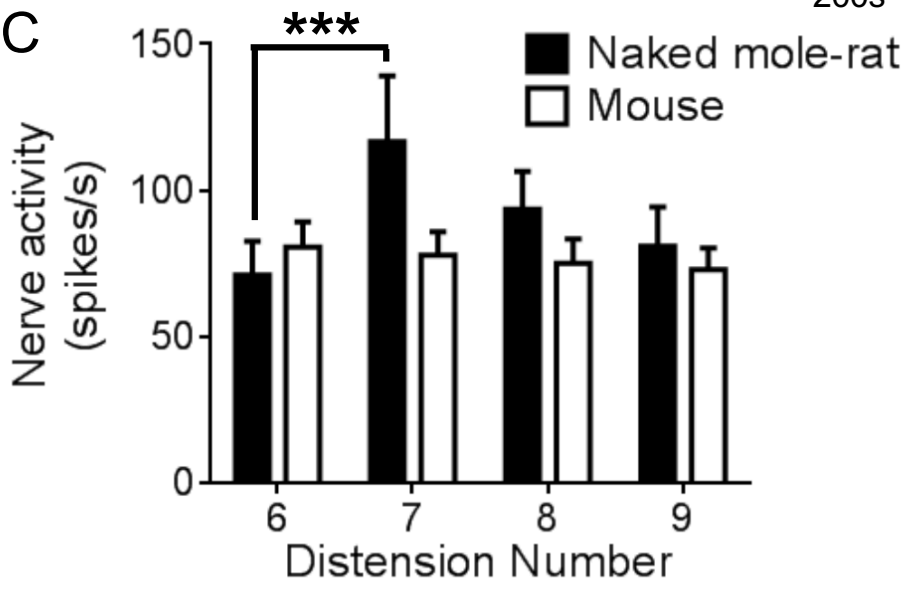
Next, we investigated the effect of extracellular acid on visceral afferent firing and mechanical hypersensitivity to phasic distension (Fig. 3A). We chose a pH 4.0 stimulus to broadly activate acid-sensitive ion channels [31] and a stimulus that is capable of evoking pain both in humans and rodents 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

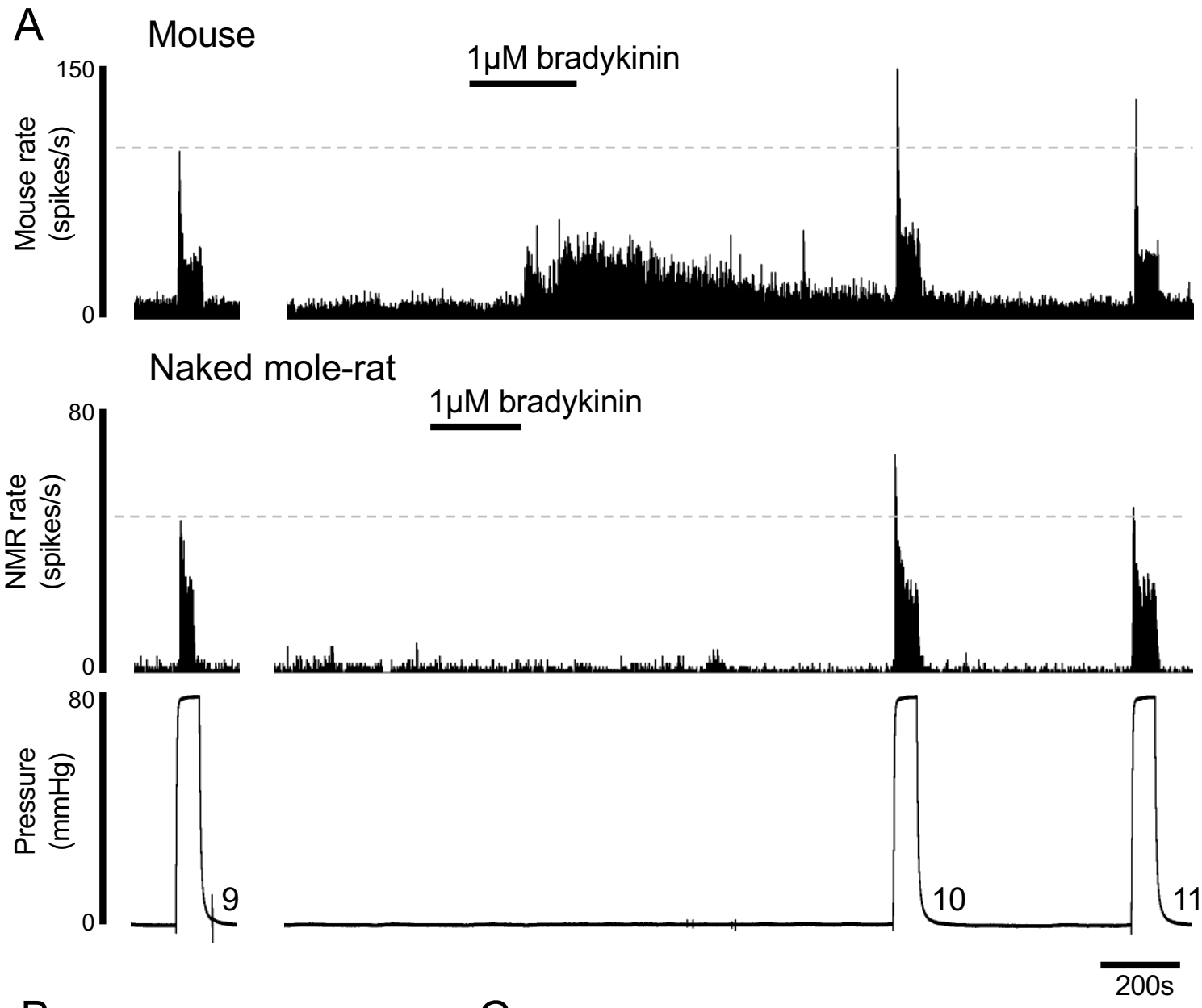

A
Mouse
pH 4.0
150
Mouse rate (spikes/s)
0
Naked mole-rat
pH 4.0
80
NMR rate (spikes/s)
0
80
Pressure (mmHg)
0
6
7
8
200s

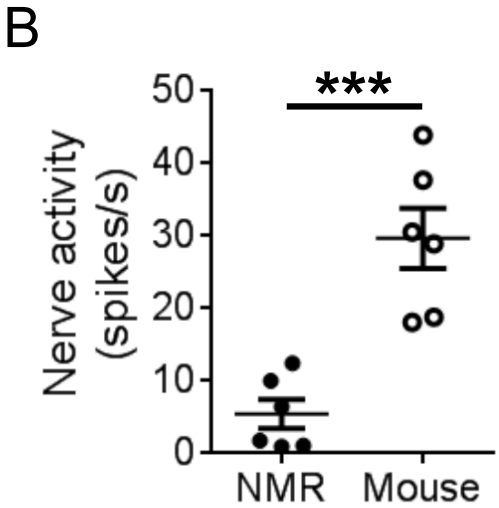

B
Nerve activity (spikes/s)
80
60
40
20
0
NMR
Mouse

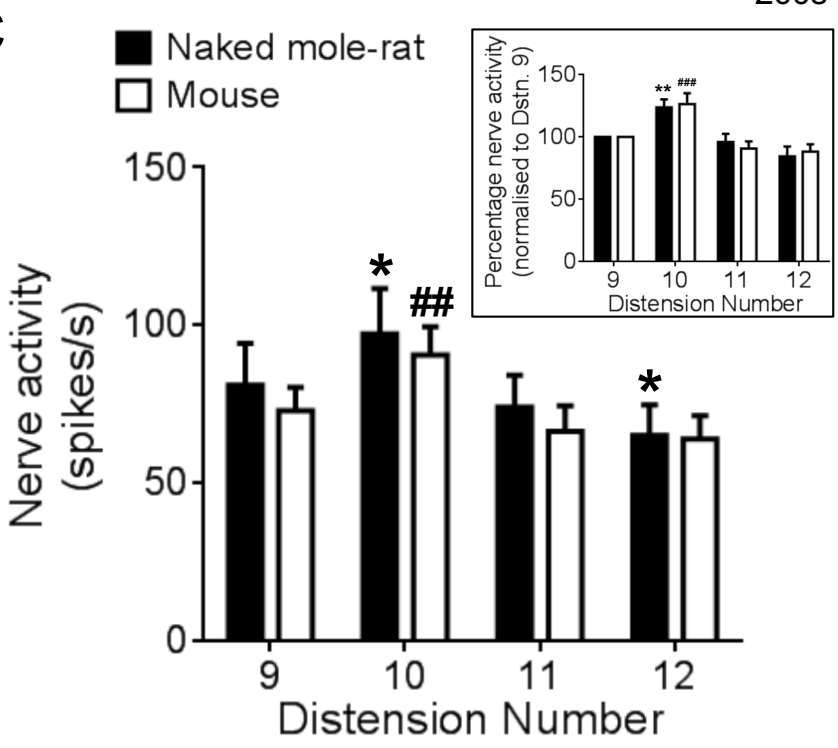

C
***
Naked mole-rat
Mouse
Nerve activity (spikes/s)
150
100
50
0
6
7
8
9
Distension Number

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

Discussion

Acid sensing in the gastrointestinal tract is necessary to maintain gut homeostasis by providing feedback for gastric and intestinal acid regulation, and secondly for detecting tissue acidosis caused by inflammation, infection and ischaemia during disease. Acidity is monitored by a range of cells including epithelial cells, and both intrinsic and extrinsic sensory neurones innervating the GI tract. Whilst the low pH (reaching pH 1) of the stomach is required for the physiological breakdown of ingested food and eradication of pathogens, HCl released by gastric parietal cells is not the sole source of acid in the gut [31]. Indeed, the luminal pH of the digestive system has two distinct peaks in acidity: the stomach and the proximal large bowel [6,7]. Release of HCO_3^- and lactic acid from the mucosa, the microbial conversion of carbohydrates to short chain fatty acids (SCFAs: acetic, butyric and propionic acid), and lastly digestive bile acids, all contribute to luminal acidity in the large bowel [31]. The activation of acid-sensing cells drives local homeostatic changes in mucosal defence, such as changes in blood flow, secretion and repair mechanisms, and autonomic and primary afferents coordinate together to facilitate changes at an organ level (e.g. motility and secretion) and can change organism behaviour through sensation (e.g. discomfort, pain and hyperalgesia). When these systems become dysregulated then significant pathology can ensure, of which pain is often the principal symptom.

Acid-evoked pain in the gastrointestinal tract is mediated through the activities of a number of different molecular mechanisms. In dorsal root ganglia (DRG) neurones, protons can activate fast-inactivating, largely Na^+ permeable ASICs at pH levels just below physiological range (e.g. pH 7.0) [36]. In retrogradely labelled colon-projecting DRG neurones isolated from the thoracolumbar vertebrae, *in situ* hybridisation against ASIC subtypes indicates that ASIC2 is expressed at the greatest frequency, with both ASIC1 and ASIC3 present in about a quarter of neurones [37]. A number of non-selective cation transient receptor potential (TRP) channels are also sensitive to protons, for example, TRPV1 is activated by pH levels below 6.2 [38], TRPV4 can be opened by pH 6.0 and below [39], and TRPC4 and TRPC5 are active between pH 7.4 and pH 7.0 [40]. Immunohistochemical analysis suggests that both TRPV1 and TRPV4 are expressed by afferents innervating the distal colon of mouse [41,42]. Whilst present in DRG neurones, evidence for the expression of TRPC4 and TRPC5

at the protein level in a colon-specific population is lacking, however single-cell RNA sequencing efforts in this population suggest significant expression of TRPC4, but not TRPC5, mRNA, colocalising with TRPV1 expressing neurones [15]. The vast majority of colonic sensory neurones possess inward sustained currents in response to low pH indicative of TRP channel activation [14], with afferent firing observed in response to pH 6.5 [43]. Members of the two-pore domain K^+ (K2P) channels including TASKs, TRESK, TREK and TRAAK, which contribute to setting resting membrane potential and therefore have an important role in regulating neuronal excitability, are also sensitive to extracellular acid [44]. The expression of TREK-1, TREK-2 and TRAAK are observed in thoracolumbar and lumbosacral sensory neurones innervating the GI tract and are modulated by colitis [45]. Many P2X purinoceptors are also modulated by extracellular pH affecting the potency of ATP gating [46], and $P2X_2$ and $P2X_3$ subunits, are widely expressed by colonic sensory neurones [47,48]. Lastly, there exist 4 proton-sensing G protein-coupled receptors (Gpr65, Gpr68, Gpr4 and Gpr132), which are activated by extracellular acidosis, however only mRNA for Gpr68 has been detected in colonic sensory neurones of mice [15].

Whilst many of these receptors are directly modulated by protons, the vast majority integrate additional mechano- and chemo-sensitive functions, such that acid sensitises the channel. For example, genetic ablation of ASIC3 or TRPV1 in mice causes a reduction in the visceral motor reflex (VMR) to colorectal distension and stretch sensitisation to acidic inflammatory soup; effects that are lost in $ASIC3^{-/-}$ mice [33]. $TRPV4^{-/-}$ mice possess reduced VMR to colorectal distension and significant reductions in mechanosensitivity [41]. During colitis, reductions in expression levels of K2P channels are observed [45]. Specifically, TREK-2-like currents evoked by pH 6.3 and osmotic membrane stretch are attenuated, which likely contribute to the increased colonic mechanosensitivity that develops in bowel disorders such as inflammatory bowel disease [45]. Pharmacological block of $P2X_3$-containing purinoceptors results in reductions in visceral pain behaviours in animals models and activation with selective agonist α,β-methylene ATP can sensitise responses to mechanical stimuli [49]. Interestingly, Gpr68, the acid-sensitive GPCR with the highest expression in gut sensory neurones, has also been described as a novel mechanotransducer [50] providing a further potential point of molecular integration for acid-induced mechanosensitisation. We observed no overt difference in the mechanosensitivity of

NMR colonic afferents compared to mouse at baseline conditions, suggesting that visceral mechanotransduction is not significantly altered in the NMR. By contrast we did see greatly differing responses to both, the direct exposure of extracellular acid and to induced mechanical hypersensitivity, implicating altered integration of acid sensors, mechanotransducers and modulators of spontaneous afferent firing.

NMR acid sensing differs significantly to other mammals. For example, in hippocampal and cortex neurones, the peak current density of NMR ASIC-like responses is reduced compared to mouse brain neurones [51], a likely adaptation to the NMR relying on fructose-fuelled glycolysis to sustain life during severe hypoxia that would generate lactic acid as a by-product [52]. In the peripheral nervous system, subcutaneous injection of acid (pH 3.5), capsaicin or histamine does not cause the nocifensive or pruriceptive behaviours in NMR that such stimuli characteristically induce in mice [10,11]. This acid insensitivity is a function of altered ASIC responses compared to mouse [18] and a variation in NMR $Na_V1.7$, which renders the channel hypersensitive to proton-mediated block and therefore prevents acid-driven action potential initiation from the skin [17]. We have shown previously that pharmacological inhibition or genetic ablation of $Na_V1.7$ in mouse does not impair colonic afferent firing or alter pain behaviours [24]. Therefore, if $Na_V1.7$ is redundant in colonic afferents compared to those innervating the hindpaw, then it would be predicted that NMR colonic afferents would not be as insensitive to acid as their somatic equivalents. Whilst we observed a trend towards low firing rates in response to application of acid to NMR colonic afferents compared mouse, these did not differ significantly. As shown previously, acid alone was unable to induce mechanical sensitisation in mouse colonic afferents [33], whereas robust sensitisation was observed in NMR colonic afferents, i.e. there is likely differential coupling of molecular acid sensors to mechanotransducers in mouse and NMR.

In NMRs, the absence of thermal hypersensitivity induced by capsaicin and lack of histamine-induced scratching are thought to be due to a lack of cutaneous neuropeptides, such that both behaviours can be “rescued” by intrathecal administration of SP [10,11]. We show here by immunohistochemistry that CGRP is expressed within nerve fibres found encapsulating both blood vessels of the NMR colonic mesentery and myenteric plexi within the smooth muscle layers of the colon

wall, which aligns with previous findings of CGRP-positive fibres innervating NMR mesenteric arteries [26]. By contrast, whilst PGP9.5 staining identified nerve fibres within the submucosa these did not express CGRP, highlighting potential restricted penetration of extrinsic sensory fibres innervating the NMR colorectum.

We also found that the inflammatory mediator bradykinin failed to activate NMR colonic afferents, but that it could induce a robust mechanical hypersensitisation comparable to the effects in mouse. Although we do not confirm bradykinin B2 receptor expression in NMR colonic afferents in this study, such mechanical hypersensitivity suggests that the B2 receptor activity is unimpaired, but that alternate coupling to its molecular transducers (including K_V7 [53], TRPV1 [54], TRPA1 [55], Ca^{2+}-activated Cl^- channels [53,56] and K_{Ca} [57,58]) may explain the altered response profiles compared to mouse.

Understanding how noxious pH is sensed and gastrointestinal homeostasis is maintained in the NMR may help to inform our understanding of other model species and gastrointestinal acid sensing during human disease. One explanation for the altered colonic afferent sensitivities observed may be differences in the microbiome between species. Microbiota can generate metabolites (including organic acids) that have a significant bearing on luminal acidity. And vice versa, variations in luminal pH can greatly impact microbiota diversity and activity [59]. Indeed, NMR gut microbiota is distinct from other rodents, with a greater propensity to produce increased levels of SCFAs and support anaerobic oxidative metabolism, which is probably as a result of their relatively homogeneous diet [60]. Such altered gut metabolism may explain the greater tolerances observed in colonic afferent sensitivities to acid and noxious inflammatory stimuli, alongside other adaptations to habitat. Further studies are required to fully understand how the NMR maintains gut pH homeostasis whilst 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.

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680

Figure Legends

Figure 1. Extrinsic sensory innervation of NMR colorectum

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

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

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

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

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

distension in NMR, but not mouse, was significantly sensitised by acid (***$P < 0.01$, $N = 6$, two-way repeated-measures ANOVA with Sidak's post-hoc).

Figure 4. Colonic afferent excitation to bradykinin is blunted in NMR, but mechanical sensitisation is unaffected

A. Example rate histograms of colonic splanchnic nerve activity from mouse and NMR with accompanying pressure trace showing addition of 1 μM bradykinin (20 mL) and subsequent repeat (x 3) phasic distension. B. Mean increase in peak firing after application of 1 μM bradykinin (***$P < 0.001$, $N = 6$, unpaired t-test). C. Peak firing change to phasic distension after superfusion with 1 μM bradykinin. The response to phasic distension in both NMR and mouse was significantly sensitised by application of bradykinin (*$P < 0.05$ vs. 9th distension in NMR, $^{\#\#}P < 0.01$ vs. 9th distension in mouse, $N = 6$, two-way repeated-measures ANOVA with Sidak's post-hoc). *Insert*, phasic distension responses in both NMR and mouse normalised to the pre-bradykinin distension response (**$P < 0.01$ vs. normalised 9th distension in NMR, $^{\#\#\#}P < 0.001$ vs. normalised 9th distension in mouse, $N = 6$, two-way repeated-measures ANOVA with Sidak's post-hoc).