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3 Acid and inflammatory sensitisation of naked mole-rat colonic 4 afferent nerves

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23 Abstract

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

44

45 Keywords

46 Acid, bradykinin, mechanosensation, sensitisation, visceral pain

47 Introduction

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

61

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

70

71 The naked mole-rat (*Heterocephalus glaber*; NMR) has adaptations enabling it to not
72 only survive but prosper in the subterranean (thus likely hypercapnic and hypoxic)
73 environment in which it lives. Many of these adaptations have led to altered sensory
74 processing of external stimuli, for example the NMR shows no pain-related behaviour
75 to subcutaneous injection of acid and capsaicin [10], lacks an itch response to
76 histamine [11] and shows no thermal hyperalgesia in response to a variety of stimuli,
77 including nerve growth factor [10,12]. These adaptations are believed to provide a
78 fitness advantage to living in a subterranean environment, for example, the likely high
79 CO₂ environment of NMR nests would evoke noxious stimulation of C-fibres through

80 acidosis in almost any other rodent [13]. Whilst clearly valuable in supporting its
81 lifestyle in its niche, detection of acid by sensory neurones innervating the gut is
82 required for maintenance of gut homeostasis and for the detection of tissue acidosis.
83 In other rodent species, viscerally-projecting afferent fibres can sense tissue acidosis
84 by specialised ion channels [1] including acid-sensing ion channels (ASICs; which
85 respond to mild acidification), transient receptor potential vanilloid subtype (TRPV1;
86 which are gated by severe acidosis), ionotropic purinoceptor ion channels (e.g. P2X₃)
87 and two-pore domain potassium channels (e.g. TASK, TRESK, TREK and TRAAK
88 subtypes). Additionally, a number of proton-sensing G protein-coupled receptors exist
89 that are also sensitive to mild acidification (e.g. Gpr68, Gpr4, Gpr132 and Gpr65). In
90 the mouse colorectum, TRPV1 and ASIC3 are necessary for proton sensing by the
91 vast majority of colonic afferents [14]. Recent single-cell RNA-sequencing analysis of
92 colonic sensory neurones suggests that TRPV1 and ASIC3 are mainly expressed by
93 discrete populations of mouse colonic afferents, namely the mPEPb and pPEP
94 subtypes for TRPV1 and the mNFb, mPEPa and pNF subtypes for ASIC3; suggesting
95 functional specialism [15]. Of the 4 acid-sensing GPCRs, only Gpr68 is expressed by
96 mouse colonic afferents [15]. Compared to mice, NMRs display a similar expression
97 profile of ASICs throughout the nervous system [16], and of those analysed, with the
98 exception of ASIC3, NMR acid sensors show similar activation profiles to those of mice
99 [12,17,18]. NMR TRPV1 is also expressed in sensory afferents and shows similar
100 proton sensitivity to mouse TRPV1 [17]. The NMR acid-insensitivity is likely due to an
101 amino acid variation in the voltage-gated sodium channel 1.7 subunit (Nav1.7), which
102 results in acid anaesthetising, rather than activating their cutaneous sensory neurones
103 [17]. Considering the unusual cutaneous acid-insensitivity of the NMR, it is of interest
104 to determine how GI sensory surveillance and detection of visceral tissue acidosis
105 occurs in this species, especially considering the growing reputation of the NMR as a
106 model of healthy ageing [19] and the perturbation of GI function that occurs with ageing
107 [20]. In order to investigate this, we examined the sensory innervation of the NMR
108 colon and made electrophysiological recordings in both NMR and mouse from the
109 lumbar splanchnic nerve innervating the colorectum, and applied noxious mechanical
110 and chemical stimuli, including acid and bradykinin, a prototypic inflammatory
111 mediator.
112

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 housed 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 CO₂ 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).

145 Electrophysiology recordings of visceral afferent activity

146 Colonic nerves innervating the colorectum of mouse and NMR were isolated and
147 electrophysiological activity was recorded as previously described [21]. Mice were
148 humanely killed by cervical dislocation of the neck and cessation of circulation. NMRs
149 were humanely killed by CO₂ exposure followed by decapitation. For both species, the
150 colorectum with associated lumbar splanchnic nerve was dissected free from the
151 animal and transferred to a recording chamber superfused with carbogenated Krebs
152 buffer (in mM: 124 NaCl, 4.8 KCl, 1.3 NaH₂PO₄, 2.5 CaCl₂, 1.2 MgSO₄·7H₂O, 11.1
153 glucose and 25 NaHCO₃; 7 ml/min; 32-34 °C). The colorectum was cannulated and
154 perfused with Krebs buffer (100 µl/min) enabling distension of the colon by closure of
155 the out-flow. The Krebs buffer was supplemented with nifedipine (10 µM) and atropine
156 (10 µM) to inhibit smooth muscle activity and with indomethacin (3 µM) to restrict
157 endogenous prostanoid production. Multi-unit electrophysiological activity of the
158 lumbar splanchnic nerve rostral to the inferior mesenteric ganglia was recorded using
159 a borosilicate glass suction electrode. Signals were amplified and bandpass filtered
160 (gain 5K; 100-1300 Hz; Neurolog, Digitimer Ltd, UK) and digitised at 20 kHz
161 (micro1401; Cambridge Electronic Design, UK) before display on a PC using Spike 2
162 software. The signal was digitally filtered online for 50 Hz noise (Humbug, Quest
163 Scientific, Canada) and action potential firing counts were determined using a
164 threshold of twice the background noise (typically 100 µV).

165

166

167 Electrophysiological protocols

168 Tissues were stabilised for 30 min before noxious intraluminal distension pressures
169 were applied by blocking the luminal out-flow of the cannulated mouse or NMR
170 colorectum. The pressures reached are above threshold for all known visceral afferent
171 mechanoreceptors [22] and evoke pain behaviours in rodents *in vivo* [23].
172 Mechanosensitivity and chemosensitivity were investigated using a combined
173 sequential protocol. As such, a slow ramp distension (0-80 mmHg, 4-5 min) and set of
174 6 rapid phasic distensions (0-80 mmHg, 60 s at 9 min intervals) were applied as
175 previously described [24] prior to bath superfusion of pH 4.0 Krebs buffer (50 mL
176 volume) and a set of 3 phasic distensions (0-80 mmHg, 60 s at 9 min) to test for acid-
177 induced acute mechanical hypersensitivity. After a 20 min wash-out period, 1 μ M
178 bradykinin was applied by bath superfusion (20 mL volume) and a further set of 3
179 phasic distensions were performed. Phasic distension protocols were automated
180 using an Octaflow II perfusion system (ALA Scientific, USA) to standardise duration
181 and intervals.

182

183 Data analysis

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

190

191 Drugs

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

196 Results

197 Gastrointestinal neuroanatomy of the NMR

198 We first compared the gross anatomy of the NMR and mouse gastrointestinal tract.

199 As the NMR is greatly long lived compared to the mouse, with a life expectancy of
200 >30-years, we chose animals of an equivalent phase of life and body size (e.g. young
201 adult: for mouse, 6-8-weeks, snout to anus length 92 mm and for NMR, 76-weeks,
202 snout to anus length 113 mm). Compared to the mouse, the NMR colorectum (from
203 caecum to anus) length was broadly equivalent (mouse, 60 mm; NMR, 70mm), whilst
204 the caecum and stomach were greatly enlarged and the small intestine (from the
205 caecum to pyloric sphincter of the stomach) was shorter in length (mouse, 312 mm;
206 NMR, 165 mm; Fig. 1A).

207

208 We next confirmed the presence of extrinsic sensory fibres innervating different layers
209 of the NMR colorectum using immunohistochemistry. Equivalent staining in the mouse
210 are widely available within the literature and we did not seek to duplicate them here
211 [25]. Using antibodies raised against calcitonin gene-related peptide (CGRP) and
212 protein gene product 9.5 (PGP9.5) we stained for neuronal fibres within flat-sheet
213 whole-mount preparations of multiple layers of the NMR colon. Specifically, CGRP-
214 positive extrinsic neuronal varicosities were identified encircling and tracking with
215 blood vessels within the mesentery supplying the distal colon of NMR; such fibres
216 likely contribute to the larger lumbar splanchnic nerve upon which these coalesce (Fig.
217 1B). Although NMR lack CGRP in cutaneous afferent neurones, this finding is in line
218 with the observation that mesenteric arteries in NMR and the common mole-rat
219 (*Cryptomys hottentotus*) express CGRP [26]. Neuronal fibres staining for PGP9.5
220 were also observed within the mesentery of NMR, again localised around blood
221 vessels (Fig. 1E). The mucosa and submucosa were separated from the muscle
222 (circular and longitudinal) layers. CGRP-positive, presumably extrinsic, sensory fibres
223 were observed coursing through the myenteric plexi between these muscle layers
224 (Fig. 1C). PGP9.5 staining revealed the myenteric soma and additional neuronal fibres
225 within this layer of the NMR colon (Fig. 1F). Whilst PGP9.5-positive fibres were
226 observed encircling the base of colonic villi (see Fig. 1G insert), CGRP labelling of
227 these fibres was not seen (Fig. 1D). Interdigitating fibres within both the circular and
228 longitudinal muscle layers are positive for PGP9.5 (Fig. 1H and 1I), with what is likely
229 submucosal ganglia retained on the circular muscle layer after separation of the
230 mucosa from this layer (Fig. 1H).

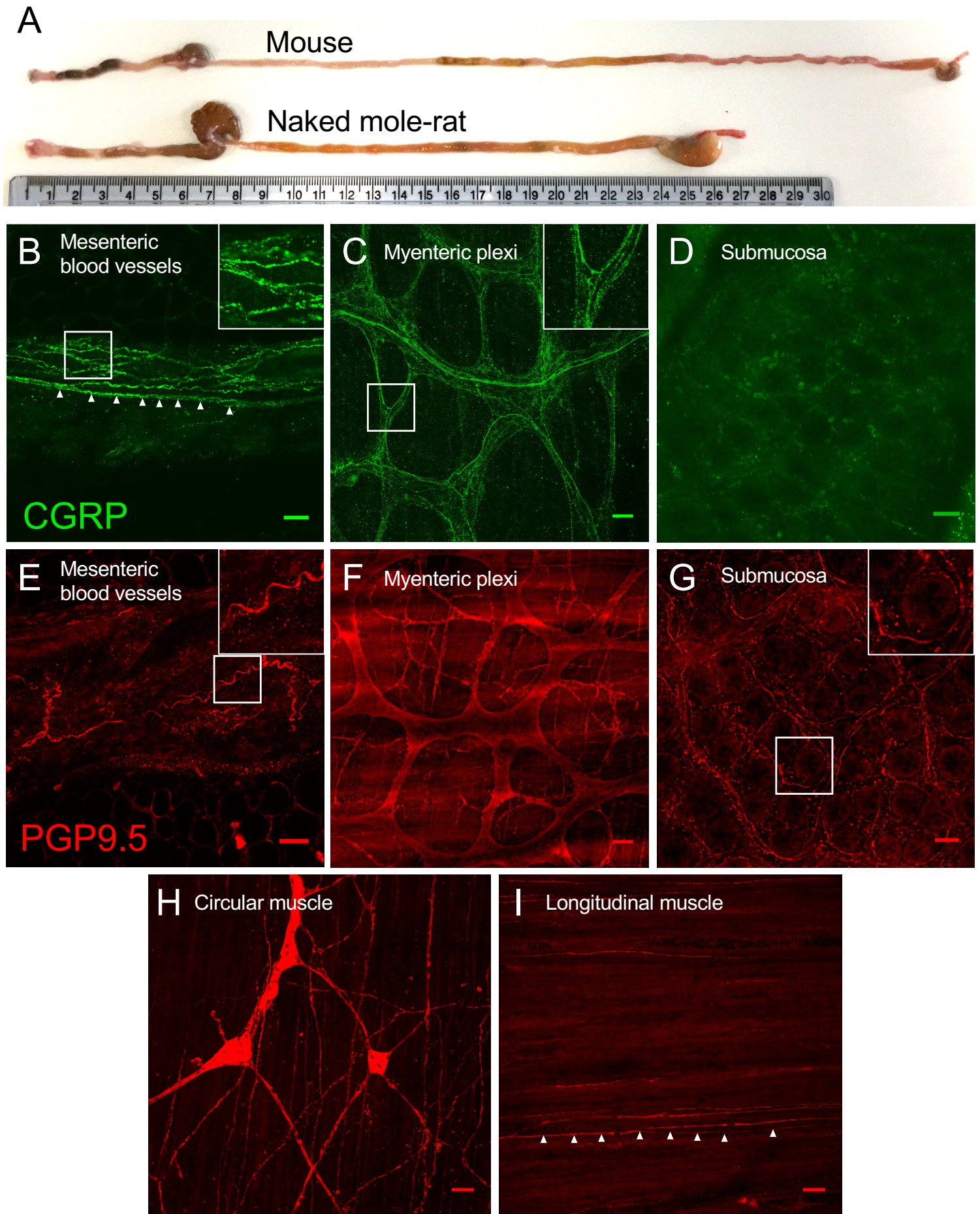
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232 Colonic afferent mechanosensitivity does not differ in the NMR compared
233 to mouse

234

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

Figure 1



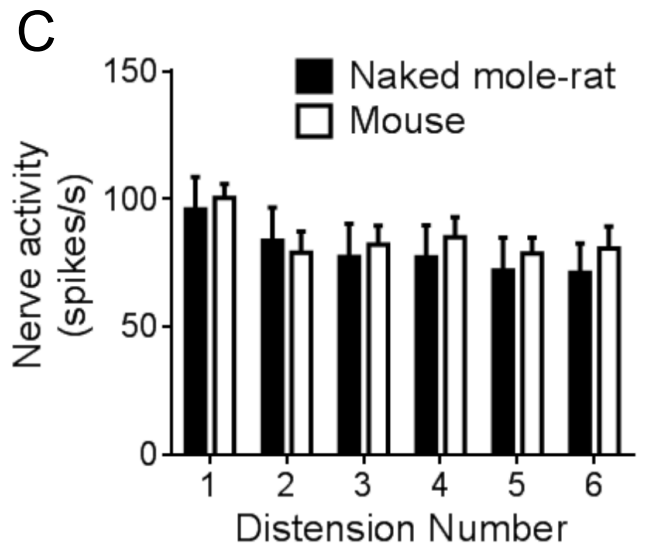
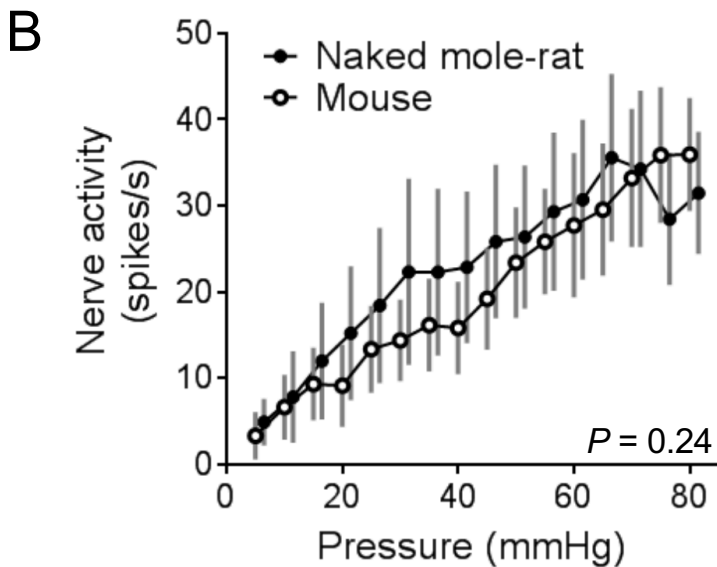
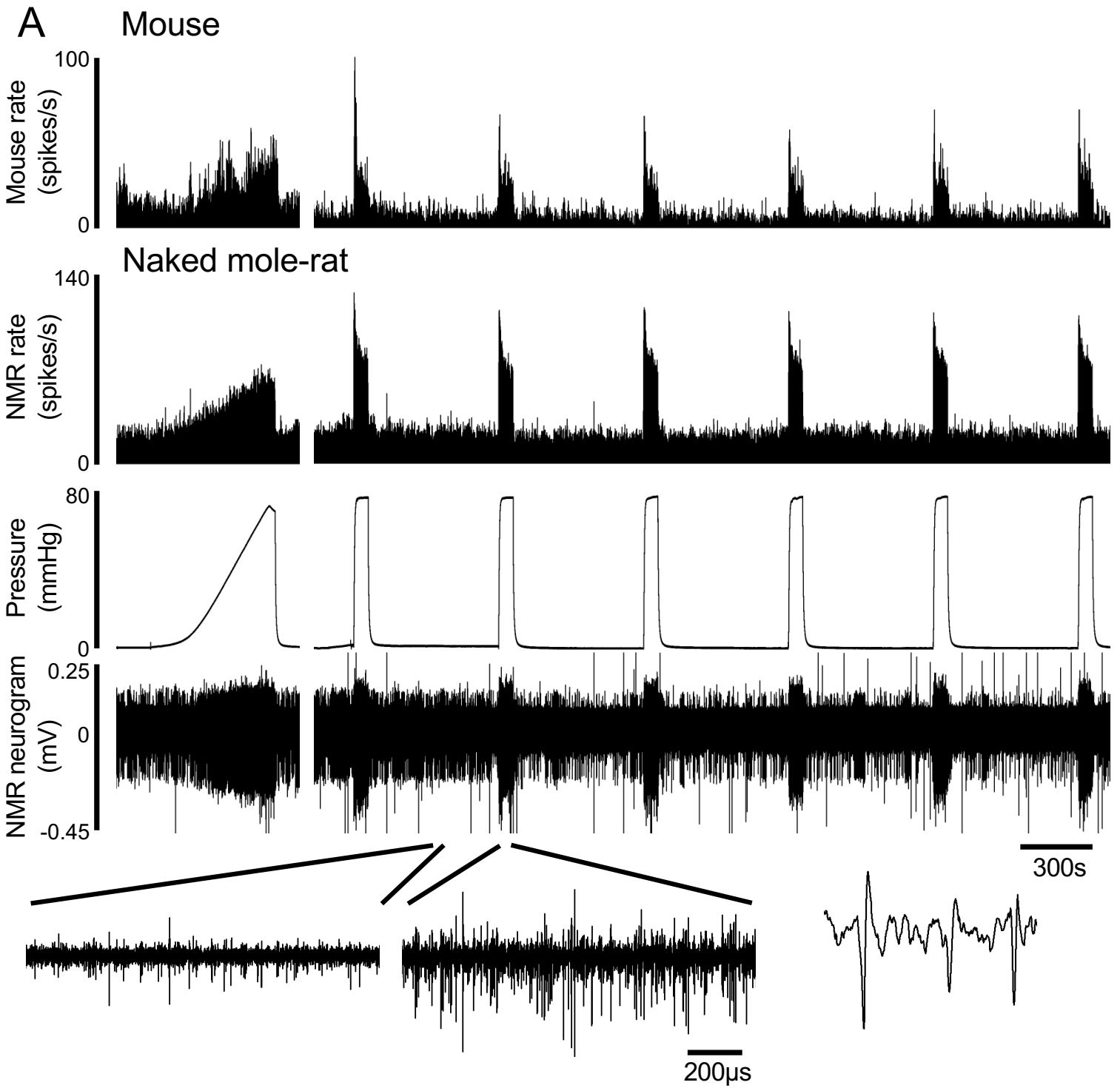
241 conscious sensation transduced [27]. The colorectum, once dissected free from the
242 animal, was cannulated and both lumenally perfused and bath superfused with Krebs
243 buffer, thus allowing mechanical distension of the bowel or application of chemical
244 stimuli, respectively.

245

246 We first investigated mechanosensitivity of visceral afferents in the NMR compared to
247 mouse gastrointestinal tract. There were no significant differences in the baseline
248 spontaneous activity measured between NMR and mouse (3 min average: 9.7 ± 3.5
249 spikes/s vs. 9.3 ± 1.7 spikes/s, respectively, $N = 6$, $P = 0.91$, unpaired t-test). We
250 applied known innocuous and noxious mechanical stimuli, firstly by way of a ramp
251 distension (0 to 80 mmHg) and using repeat phasic distension (Fig. 2A). Colorectal
252 distension by insertion via the anus of an inflatable balloon in humans evokes
253 sensations of urge, discomfort and pain at increasing distension pressures [28].
254 Importantly, not only the absolute pressure applied, but also the dynamic quality of the
255 stimuli (such as rate of change in pressure and duration of application) also
256 significantly impact the extent of afferent activation [29]. By using a slow ramp
257 distension, we were able to assess visceral afferent responses across a range of
258 physiologically-relevant distension pressures typically exposed to the rodent gut
259 [29,30]. We observed no difference in the nerve firing recorded during ramp distension
260 in NMR (e.g. at 80 mmHg, 31.5 ± 7.1 spikes/s) compared to mouse (e.g. at 80 mmHg,
261 35.9 ± 6.5 spikes/s; Fig. 2B, $P = 0.24$, $N = 5-6$, two-way ANOVA). We next applied
262 repeat phasic distension of the colon to noxious (0-80 mmHg) pressures. As previously
263 reported in mouse, we observed a rapid increase in nerve activity to initial phasic
264 distension (100.4 ± 5.4 spikes/s) and significant adaptation during the 60 s distension
265 (Fig. 2C; [21,24]). Following subsequent repeat distensions at 9 min intervals,
266 tachyphylaxis occurred with a decrease in peak firing of 19.7 % by the sixth distension
267 compared to the first. In NMR, afferent discharge reached an equivalent peak firing
268 compared to mouse during the first distension and the degree of desensitisation during
269 subsequent distensions was similar (25.8 % by the sixth distension, $P = 0.74$, $N = 6$,
270 two-way repeated-measures ANOVA; Fig. 2C). Similar afferent responses to
271 mechanical stimuli in NMR compared to mouse suggest that there is no intrinsic
272 difference in the way sensory nerves transduce physiological and noxious mechanical
273 stimuli.

274

Figure 2



275 Extracellular acid evokes mechanical hypersensitivity in NMR but not
276 mouse

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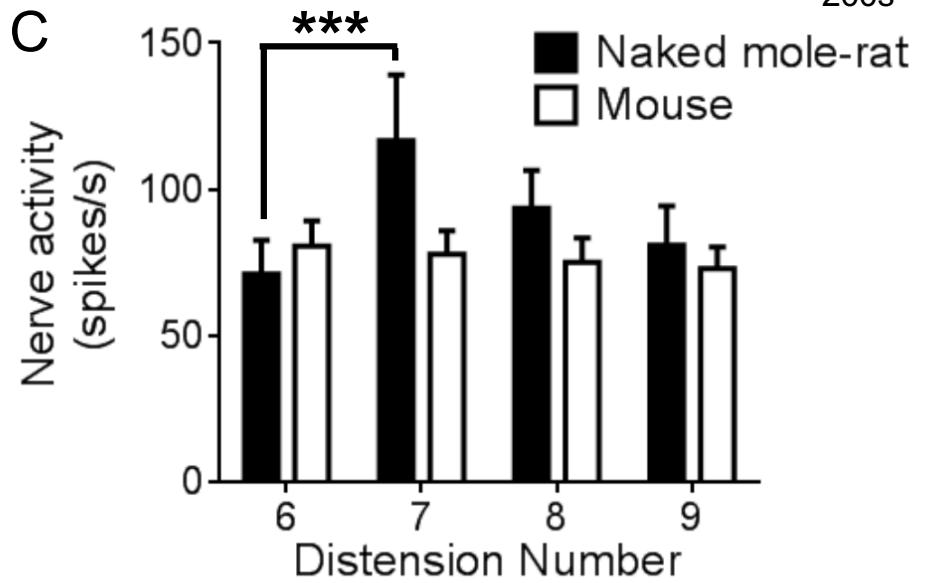
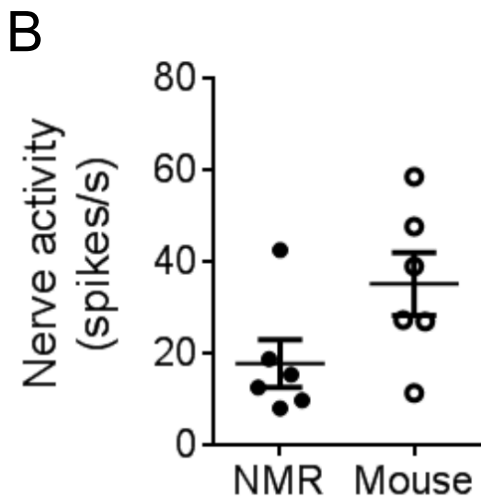
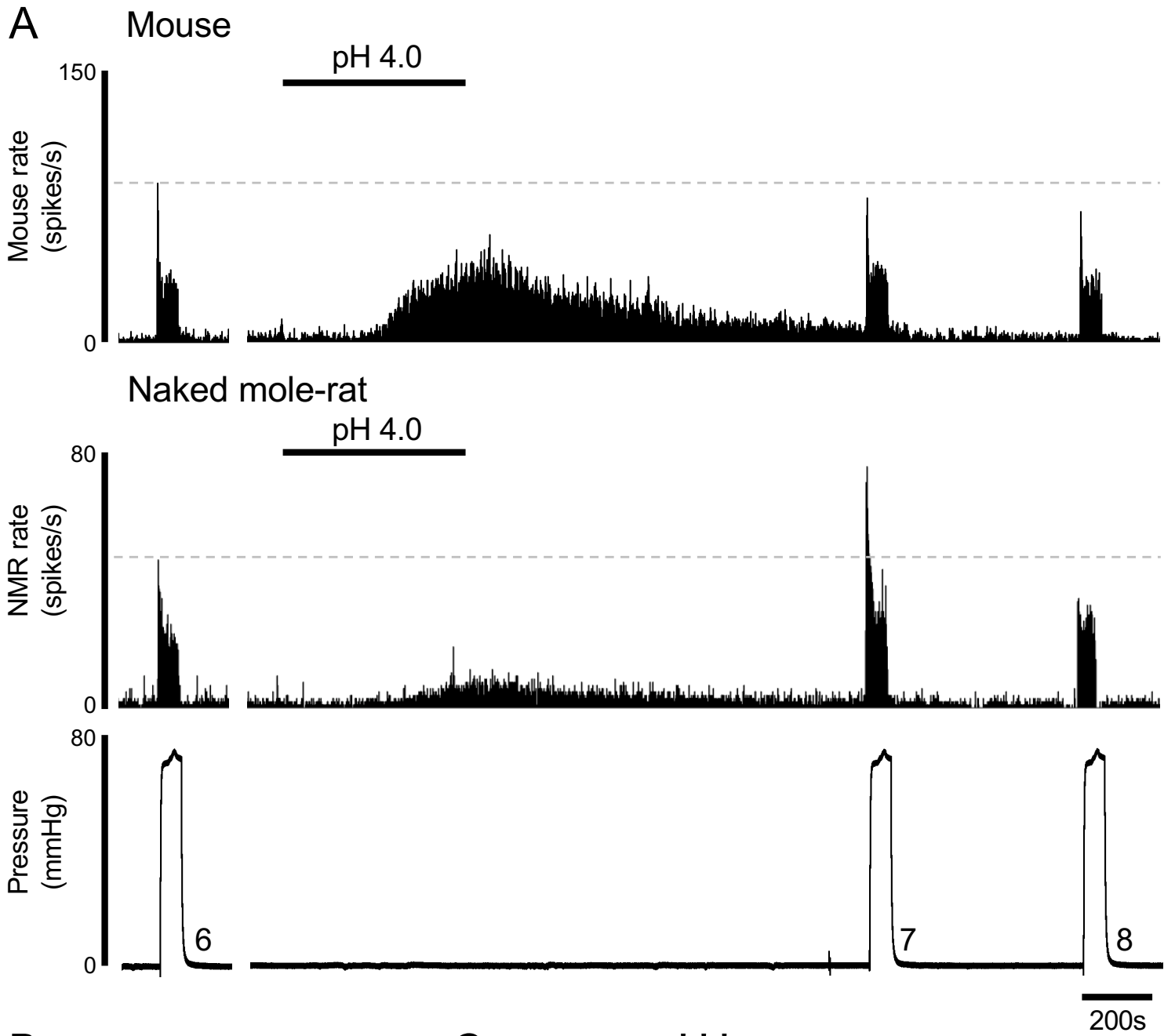
278 Next, we investigated the effect of extracellular acid on visceral afferent firing and
279 mechanical hypersensitivity to phasic distension (Fig. 3A). We chose a pH 4.0 stimulus
280 to broadly activate acid-sensitive ion channels [31] and a stimulus that is capable of
281 evoking pain both in humans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

303

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

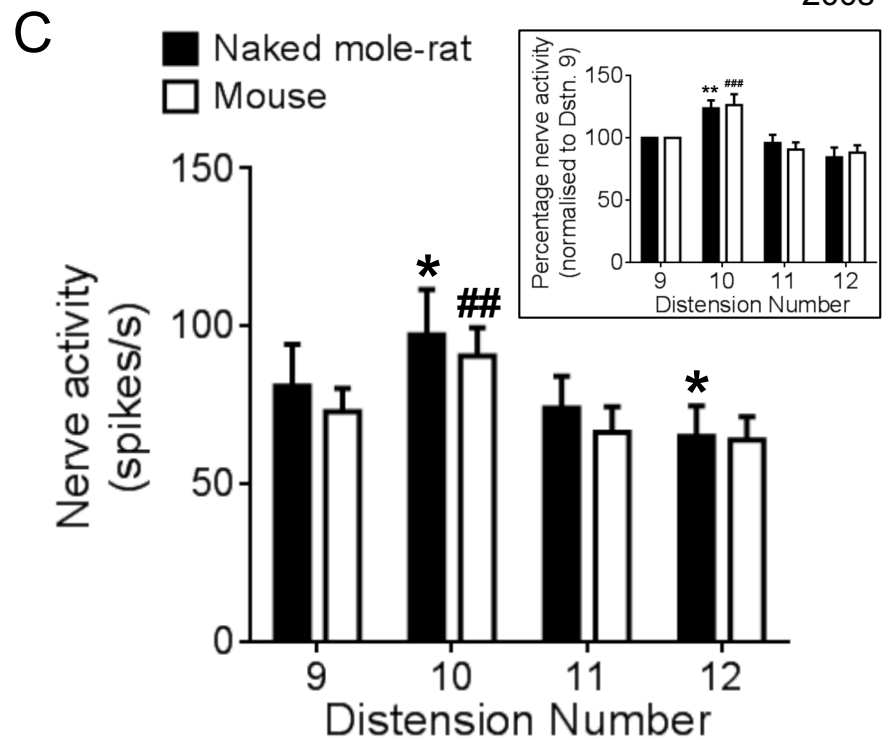
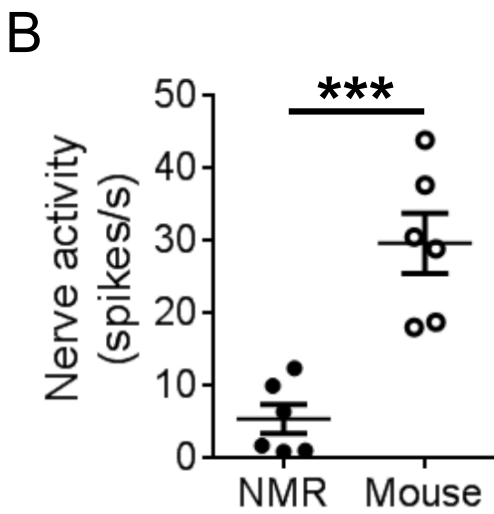
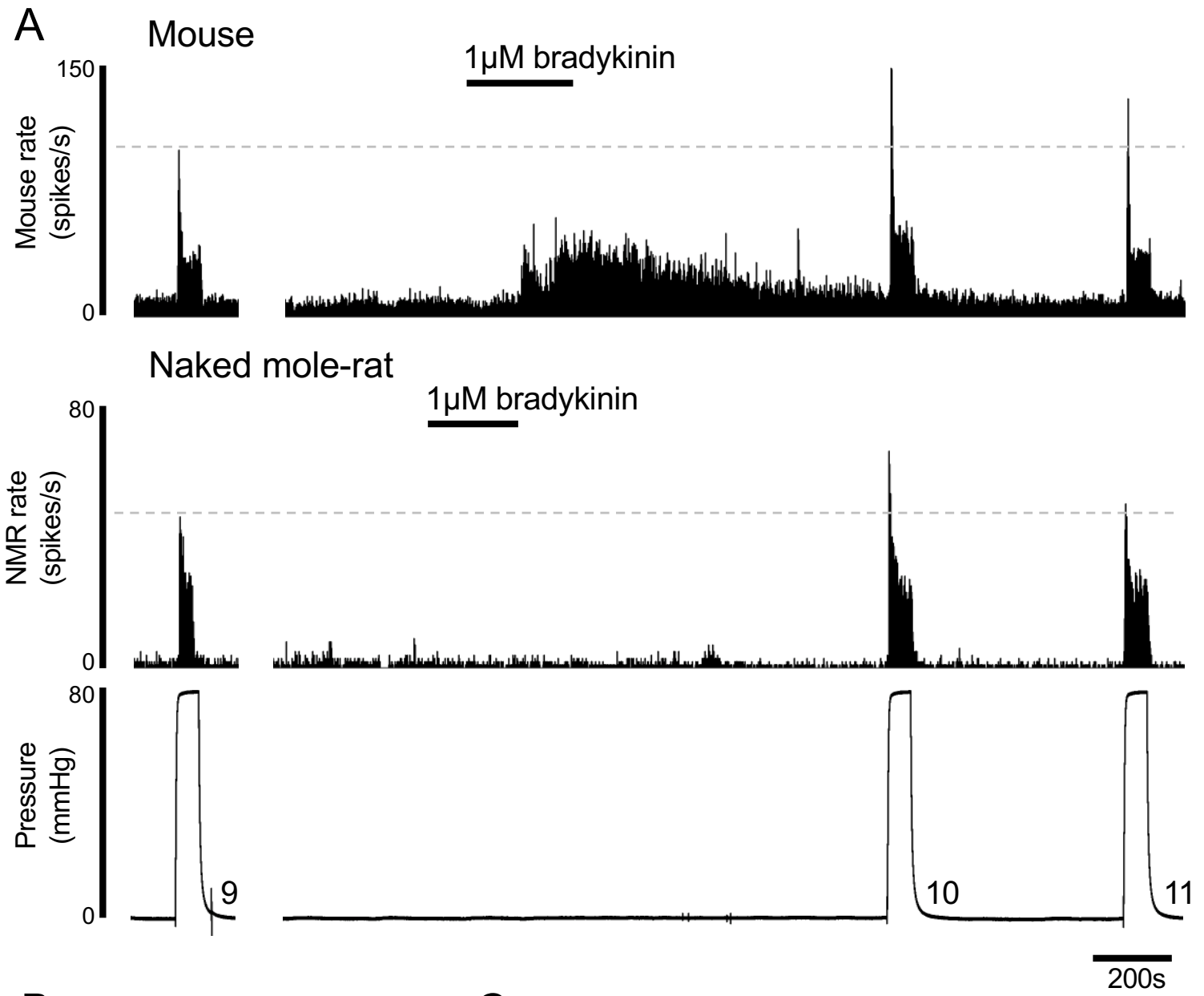
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Figure 3



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 μ M) 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 $\sim 25\%$ 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



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 ensue, 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 P2X₂ and P2X₃
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 P2X₃-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 Nav1.7, 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 Nav1.7 in mouse does not impair colonic afferent firing or alter pain
408 behaviours [24]. Therefore, if Nav1.7 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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458

459 Author Contributions

460

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

465

466 Declaration of Conflicting Interests

467

468 The authors have no conflicting interests to declare.

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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. Wholemout 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 μ m.

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).