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Lactobacillus reuteri ingestion prevents hyperexcitability of colonic DRG neurons induced by noxious stimuli

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[1]*Institute of Physiology, School of Medicine, Shandong University, Shandong, China;* [2]*Brain-Body Institute, St Joseph's Healthcare, Hamilton, Ontario; Departments of* [3]*Medicine,* [4]*Pathology and Molecular Medicine, and* [5]*Psychiatry and Behavioral Neurosciences, McMaster University, Hamilton, Ontario, Canada*

Ma X, Mao Y-K, Wang B, Huizinga JD, Bienenstock J, Kunze W. *Lactobacillus reuteri* ingestion prevents hyperexcitability of colonic DRG neurons induced by noxious stimuli. *Am J Physiol Gastrointest Liver Physiol* 296: G868–G875, 2009. First published January 29, 2009; doi:10.1152/ajpgi.90511.2008.—*Lactobacillus* species ingestion can decrease autonomic responses and spinal fiber discharge to nociceptive colorectal distension (CRD), even in the absence of inflammation. The present study aimed to determine whether dorsal root ganglion (DRG) somas could be a locus where the antinociceptive probiotic may have an effect. Healthy rats were fed with *Lactobacillus reuteri* or vehicle control for 9 days whereupon they were anesthetized, and intermittent distal colonic CRD at 80 mmHg distension was either performed for 1 h or not. The animals were immediately euthanized and patch-clamp recordings taken after isolation and overnight culture from those DRG that projected to the distal colon. CRD decreased the threshold for action potential generation and increased the number of spikes discharged during a standard depolarizing test stimulus, and this effect was blocked by prior probiotic ingestion. The increase in excitability was paralleled by an increase in DRG capacitance, which was not altered by *Lactobacillus reuteri* ingestion. CRD did not increase tissue weight or myeloperoxidase activity. We suggest that the effects of CRD may have been caused by activity-dependent neurotransmission between DRG somas. CRD evoked increases in action potential upstroke speed, which suggests that it may also have led to augmentation of sodium channel conductances. Probiotic ingestion may have interfered with this hypothetical mechanism since it blocked the effect of CRD on the action potential.

colorectal distension; dorsal root ganglion; probiotics; sensory neurons

PROBIOTICS ARE HEALTH-PROMOTING commensal bacteria that can have therapeutic or preventative roles when ingested. Probiotics are most frequently of the *Lactobacillus* or *Bifidobacterium* species, and clinically beneficial effects of probiotics have been described in travelers' diarrhea, irritable bowel syndrome, and inflammatory bowel disease (13, 29, 35, 44, 46). In addition, probiotics can modulate postinflammatory gut hypersensitivity and alter neuropeptide content (20, 43). Because of their therapeutic potential and their presumed consumer safety, the actions of probiotics on the physiological mechanisms underlying peripheral and centrally directed nociceptive signals are increasingly being investigated.

Ingestion of probiotic commensals can decrease colorectal distension (CRD)-evoked pain in healthy animals, that is, in the absence of overt inflammation in the intestine. Experimental modulation of intestinal motility (32) and visceral pain (18, 32, 43) by *Lactobacillus* species have been convincingly demonstrated for the murine colon. *Lactobacillus reuteri* (*L. reuteri*) ingestion can alter the excitability of myenteric intrinsic sensory neurons that were recorded from colon segments taken from healthy rats. Remarkably, in anesthetized healthy rats, intraduodenal injection of a *Lactobacillus* species alters spike discharge activity in cervical vagal and renal nerve trunks (42) within 20–30 min. This might also be relevant for our experiments since nodose neurons innervate the murine distal colon (2, 47). It thus seems probable that probiotics can signal directly to the nervous system without first recruiting immune cells as intermediaries. This is a suggestion that was also made by Kamiya et al. (18), who observed that prior feeding with *L. reuteri* probiotics modulated spinal dorsal root fiber extracellularly recorded spike responses to CRD. These authors argued that the locus of action of *L. reuteri* was either at the level of the intestine or between the organ and its connections with the spinal cord (18). Recent work has shown that CRD, although often used as a test stimulus to detect inflammatory hypersensitivity, can in itself cause sensitization of the pain pathway (22, 28, 34). In addition, Lu et al. (22) have shown that 1-h intermittent CRD in healthy anesthetized rats causes a change in spinal substance P (SP) content (and other neuropeptides) and mRNA in dorsal root ganglia (DRG) somas, which persisted for up to 24 h. These results (22) suggest that DRG somas could thus be a locus at which the effect of prior *L. reuteri* ingestion was expressed. To test this hypothesis, we have administered intermittent CRD for 1 h to healthy rats and investigated whether physiological adaptation has been retained in DRG soma and whether prior ingestion of *L. reuteri* for 9 days can moderate any effects of CRD.

MATERIALS AND METHODS

All experiments were performed using male Sprague-Dawley rats (Charles River Breeding Laboratories) weighing 340–450 g and were approved by the Animal Care Committee of McMaster University.

Experimental design. DRG neurons projecting to the distal colon are identified by injecting a lipophilic tracer 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen, Burlington, ON) into the wall of the colon 14 days before CRD is

applied. The tracer is injected over the same region of the distal colon that receives the balloon used for CRD. After dissociation and isolation of DRG neurons, membrane measurements were made from those whose fluorescence indicated that they projected to the distal colon.

Animals were fed with *L. reuteri* in broth (vehicle) or with the vehicle alone. At the end of the feeding period, animals were randomly assigned to have either CRD performed or not (noCRD). There were four treatment groups designed to isolate the effects of distension alone and the effect of the probiotic on either resting activity or that evoked by distension; these were designated broth-CRD, broth-noCRD, *L. reuteri*-CRD, and *L. reuteri*-noCRD. To aid in the recognition of these specific comparisons, they were often identified in the present manuscript by the use of dagger symbols. The effects of CRD in the absence of *L. reuteri* were tested for by comparing data taken from broth-CRD with that from broth-noCRD treatment groups (†). The influence of *L. reuteri* on CRD-evoked effects were tested for by comparing the broth-CRD with *L. reuteri*-CRD groups (††). Finally, effects of *L. reuteri* on DRG neurons from unstressed (nondistended) colon were examined by comparing the broth-noCRD group with the *L. reuteri*-noCRD one (†††).

Probiotic ingestion and labeling of colon projecting DRG neurons. Animals were anesthetized 14 days before CRD with the use of ketamine hydrochloride (75 mg/kg) and xylazine (10 mg/kg ip), and surgery was performed under aseptic conditions. The abdomen was opened by midline laparotomy, and the distal colon was gently exposed. The distal colon wall was injected with DiI (2.5% in methanol, total volume 20 μl). Eight to twelve injections with a Hamilton syringe and 30-gauge needle were made circumferentially within a ~2-cm-long segment of the distal colon, 2 cm from the anus. The colon was replaced in the abdomen and the wound sutured with 4–0 silk. Animals recovered from the anesthetic on a heated blanket to maintain normal temperature; thereafter animals were monitored postoperatively. For 9 days before CRD, the rats were gavaged daily with *L. reuteri* in Man-Rogosa-Sharpe liquid medium (MRS; Difco Laboratories, Detroit, MI) broth vehicle or with MRS broth vehicle control. Daily feeding was with 10^9 *L. reuteri* in 0.2-ml MRS broth vehicle. Bacteria were grown from frozen stocks ($-80°C$) and prepared for ingestion as described previously (18), and bacterial counts were determined using a colorimeter (18).

CRD and preparation of neurons. Rats were deeply anesthetized for CRD with a mixture of ketamine hydrochloride (75 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Supplemental anesthesia was given as required. A 5-cm-long plastic balloon attached to a plastic catheter was inserted intrarectally into the distal colon. The tip was 6 cm from the anus. The catheter was connected to a barostat system composed of a flow meter and pressure control program (Barostat; G & J Electronics, Toronto, Canada). Repeated distensions (80 mmHg 30 s on and 120 s off) were performed for 1 h. In previous experiments (18), a minimal intraluminal balloon pressure of 80 mmHg was required for there to be statistically significant pseudoaffective responses, and thus we used this pressure in the present report.

After the CRD, the rats were immediately euthanized. The spinal column was completely removed from the body, transferred to a beaker containing ice-cold sterile Krebs solution, and bisected longitudinally. DRG were exposed by dissection from both sides and collected from all L-S spinal levels.

Whole DRG were twice washed with Leibovitz's L-15 medium (GIBCO, Gaithersburg, MD; www.invitrogen.com) and then incubated for 50 min in collagenase type 1 (1 mg/ml; Sigma, St. Louis, MO) and 0.5 ml Trypsin (0.25%, GIBCO) in 20 ml L-15 at 37°C. After further addition of 5 ml L-15 containing 10% heat-inactivated FBS (GIBCO), the ganglia were centrifuged for 5 min at 1,000 revolutions/min and then washed twice with growth medium (L-15, containing 10% FBS, 1% penicillin streptomycin, 2 mM glutamine, and 0.2% glucose). The DRG were placed in 2 ml of growth medium and triturated ~10 times (using a fire-polished Pasteur pipette). The

ganglia were then centrifuged for 10 s at 500 revolutions/min and the supernatant transferred to a sterile centrifuge tube. The ganglia were resuspended in 2 ml of growth medium and triturated as above. This process was repeated until the volume of supernatant transferred was ~10 ml. The collected supernatant was centrifuged for 5 min at 1,000 revolutions/min, and the final pellet, which contained the dissociated neurons, was resuspended in 3 ml of growth medium with 50 ng/ml glial cell line-derived neurotrophic factor. The neurons were then plated onto two or three 35-mm-diameter Petri dishes previously coated with poly-L-lysine. An additional 1.5 ml of growth medium was added to each dish after 30 min. The cells were then incubated at 37°C with 5% CO_2 and 95% O_2 and were used for experiments after 24–30-h incubation.

Measures of inflammation. The presence of reactive acute inflammation that may have been caused by CRD, and any effects that probiotic ingestion may have had on this, was assayed using the criteria of Moore et al. (27) by measurement of the wet weight of the tissue and assessment of macroscopic mucosal damage (the presence of erosions, adhesions, hyperemia, and petechial hemorrhage). We also assayed for the expression of myeloperoxidase (MPO). Tissue wet weight was used as a measure of edema and wall hypertrophy (27). MPO is a marker of neutrophil infiltration and is commonly used as an index of acute inflammatory reaction (26). MPO activity was determined according to the method of McCafferty et al. (26) and expressed as enzyme activity units per gram wet weight of tissue (U/g). The distal colon was removed, blotted dry, and weighed from broth-fed rats with CRD or not and *L. reuteri*-fed rats with CRD. Then the tissue samples were sonicated and centrifuged and the supernatant snap frozen with liquid nitrogen. Actual MPO determination was made by measuring absorbance at 450 nm using a Titertek plate scanner (Flow Laboratories, Toronto, ON, Canada).

Electrophysiological recordings. The recording dishes with DRG neurons adhering to the bottom were retrieved after 24–30-h incubation. The cells were gently washed with Krebs saline of the following composition (in mM): 118.1 NaCl, 4.8 KCl, 25.0 NaHCO3, 1.0 NaH2PO4, 1.2 MgSO4, 11.1 glucose, and 2.5 CaCl2, mounted on the stage of an inverted microscope (Nikon TE2000-S; Mississauga, ON, Canada; www.nikon.ca) equipped with epifluorescence. DiI-labeled neurons were visualized under UV illumination through a Texas red filter. Fluorescent and bright-field images of the neurons were visualized using a CCD camera (Rolero-XR, www.qimaging.com) attached to the side port of the microscope, and frames were captured on a personal computer. Images were analyzed offline and the area of colon projecting somas measured using ImageJ software (NIH).

During the experiment, the tissue was continuously superfused (4 ml/min) with Krebs saline gassed with 95% O_2-5% CO_2 and warmed to 32–34°C. Patch pipettes were pulled on a Flaming-Brown-P97 (Sutter Instruments, Novato, CA; www.sutter.com) electrode puller to produce micropipettes with resistances 4–8 MΩ. Signals were recorded using an Axon Instruments Multiclamp 700A amplifier (Axon Instruments, Foster City, CA; www.moleculardevices.com) and a Digidata 1322A (Axon Instruments) digitizer A/D converter. Analog data were low pass, 4-point Bessel filtered at 2-5 kHz and then digitized at 5 or 20 kHz, stored on a computer, and analyzed offline using Clampex 9 software (Axon Instruments).

The patch pipette filling solution used was one that aids the preservation of the postaction potential slow afterhyperpolarization (sAHP) (23). Its composition was (in mM): 110–115.00 KMeSO4, 9.00 NaCl, 0.09 CaCl2, 1.00 MgCl2, 10.00 HEPES, 0.20 Na3GTP, and 0.20 BAPTA.K4 with 14 mM KOH to bring the pH to 7.3.

In some previous studies of DRG neuron somas innervating the large or small intestine, only small soma (<30 μm diameter) (3, 27) or <40 pF membrane capacitance (31) were selected for recording on the assumption that only these carry nociceptive impulses. However, in patching neurons we did not select for size since all rat colon DRG neurons have TTX-sensitive and -insensitive sodium currents and thus may be sensitized (15); furthermore, any changes in size could

produce selection biases between treated and untreated sample groups. It is also relevant that a recent report (41) has shown that a majority (70%) of DRG neurons projecting to colon or small intestine have medium or large somas. With the amplifier in voltage clamp mode, gigaohm seal cell-attached recordings were made from neurons that showed DiI fluorescence on brief exposure to UV light. Whole cell mode was entered by applying mild suction, and input resistance and membrane capacitance were measured using PClamp membrane test protocol facility. Then the amplifier was switched to voltage recording mode to measure action potential and passive membrane characteristics. Throughout the experiment, access resistance and cell membrane resistance, capacitance, and membrane time constants were periodically monitored by software-programmed switching to the PClamp membrane test protocol, which injects square-wave pulses oscillating about the holding potential. Action potential (AP) parameter values were read from the spike statistics module in PClamp software. Neurons with membrane polarization ≤40 mV or whose action potential lacked an overshoot were excluded from analysis.

Statistics. Descriptive statistics are given as means ± SE and number of neurons (n) or animals (N). One-way ANOVA was used to compare the measured parameters between the different treatments described above. When a statistically significant ($P ≤ 0.05$) treatment was identified, post hoc tests were used to establish where the differences lie. Variable specific summary statistics, one-way ANOVA, and Bonferroni tests for selected pairs of treatment groups (see *Experimental design* above) were calculated using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com).

RESULTS

As described in the MATERIALS AND METHODS, neuron membrane parameter measurements were broken down according to treatment groups (broth-CRD, broth-noCRD, *L. reuteri*-CRD, and *L. reuteri*-noCRD). To aid in identifying the three comparison groups used for post hoc analysis, double-headed connecting arrows were drawn over treatment groups in Figs. 2 and 4 with single, double, and triple daggers denoting each of the group comparisons in the sequence mentioned above. Pairwise differences that significantly contributed to the ANOVA group effect are indicated by bolded, italicized daggers.

Inflammation. The presence of acute inflammation was assessed only for the broth-CRD, broth-noCRD, and *L. reuteri*-CRD groups since we sought to discover whether CRD could induce overt inflammation. The wet weight for distal colon was 0.074 ± 0.012 g/cm length for broth-CRD animals ($N = 5$), and this was not increased for colon segments from broth-noCRD ($N = 5$) or *L. reuteri*-CRD ($N = 6$) (ANOVA, $P = 0.9$) (Fig. 1) groups. We also failed to find evidence of inflammation-related mucosal damage (27) because none of the tissue segments examined for weight showed any of the macroscopic lesions listed in the METHODS. MPO for broth and CRD was 58.6 ± 26.7 ($n = 5$) U/g, and neither distension nor *L. reuteri* significantly altered this amount ($N = 5$) or *L. reuteri* ($N = 6$) (ANOVA, $P = 0.6$) (Fig. 1).

DRG soma size. Measurements were made from 190 DiI-filled neurons. 44 ($N = 14$) were broth-CRD, 68 ($N = 21$) were broth-noCRD, 28 ($N = 10$) were *L. reuteri*-CRD, and 50 ($N = 16$) were *L. reuteri*-noCRD. Neurons projecting to the distal colon varied in size (15), with the cross-sectional area being 1,040 ± 57 μm² for control neurons taken from animals not subject to CRD (broth-noCRD). One-way ANOVA revealed significant differences in cell soma sizes between the four
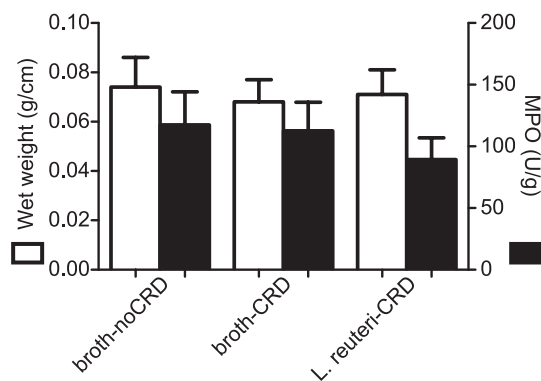

Fig. 1. Absence of indictors of inflammation in gut tissue. Bar graphs illustrate colon tissue wet weight (open bars) and myeloperoxidase (MPO) scores (solid bars); means ± SE between distal colon segments exposed to 3 treatments as indicated on the ordinate. Neither wet weight ($P = 0.9$, ANOVA, $n = 5$) nor MPO ($P = 0.6$, ANOVA, $n = 6$) were affected by the treatments. CRD, colorectal distension; *L. reuteri*, *Lactobacillus reuteri*.

treatment groups ($P < 0.0001$). Post hoc Bonferroni's multiple comparison tests indicated that CRD significantly ($P < 0.001$) increased the cell size to 1,514 ± 100 μm². There was no statistical difference ($P > 0.05$) in the sizes of cells taken from broth-CRD vs. *L. reuteri*-CRD groups. Thus, even if the probiotic moderated the effect of distension, it could not significantly reverse it. *L. reuteri* on its own did not alter cell size. In addition, whole cell membrane capacitance (C_m), which is proportional to the total amount of plasma membrane, was measured in real time using the membrane test facility in Clampex 9. Dot plots of C_m for each treatment group are given in Fig. 2. Membrane capacitance measurements shown in Fig. 2 show that capacitance mirrored changes in cell size with treatments. C_m for cells from the control (broth-noCRD) group was 50 ± 3 pF, and this was increased to 76 ± 6 pF by CRD. One-way ANOVA showed that the effects of treatments on C_m across groups was significant at $P < 0.0001$. According to post hoc tests, CRD significantly ($P < 0.05$) increased C_m and *L. reuteri* ingestion did not prevent this increase.

Excitability of DRG soma. The excitability of DRG neuron somas was measured by testing for the rheobase and repetitive firing ability. Rheobase is the threshold intensity for firing a single AP using a long-duration (1 s) current pulse. The current required to move the membrane potential from rest to a given threshold is larger for big cells compared with smaller cells; thus rheobase would be expected to be larger for bigger cells even if there were no change in membrane properties. We normalized the measured current values to unit membrane area or capacitance to prevent this effect from obscuring the effect of treatments on intrinsic membrane excitability. The ability to fire repeatedly in trains of APs was tested by injecting a standard 1-s current pulse that was exactly twice the intensity of the rheobase for each neuron tested (Fig. 3). For this test, the number of APs that the neuron was able to fire was counted. Figure 3, *A* and *B*, shows responses from a broth-CRD neuron. In Fig. 3*A* several traces from juxtathreshold responses are superimposed to illustrate spike initiation near rheobase; Fig. 3*B* demonstrates a robust tonic discharge from this neuron when stimulation intensity was raised to 2× rheobase. Figure 3*C* depicts just juxtathreshold responses of a neuron from the *L. reuteri*-CRD treatment group; this neuron fired only at the
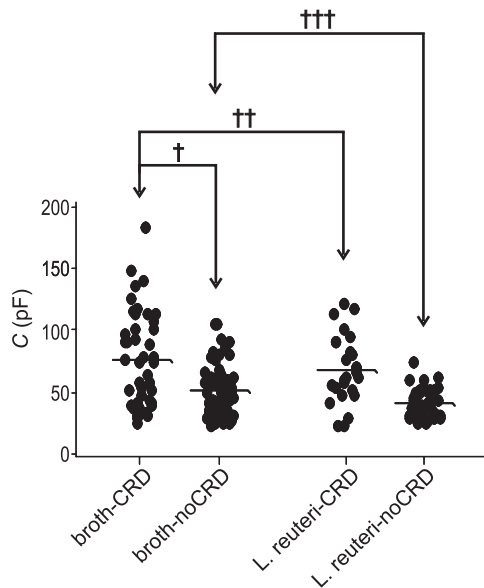
Fig. 2. Estimates of cell size by whole cell capacitance. Measurements for each soma are given in dot plots; horizontal bars in each plot indicate location of the mean. Comparisons made with post hoc tests are indicated by dagger symbols and bridging arrows; †effects of CRD alone; ††effects of *L. reuteri* on CRD; †††effects of *L. reuteri* on dorsal root ganglion (DRG) soma taken from the normal (undistended) gut. The treatments had statistically significant effects on capacitance; ANOVA, *P* < 0.0001. Post hoc Bonferroni comparisons revealed that CRD significantly increased capacitance (†) but that *L. reuteri* did not decrease this effect (††) nor did *L. reuteri* affect the capacitance of soma taken from normal gut (†††).

Fig. 4. Summary bar graphs (means ± SE) of the effects of treatments on rheobase (*A*) and repetitive firing (*B*). Effects of CRD (†), *L. reuteri* ingestion on CRD effects (††), and *L. reuteri* ingestion on normal gut (†††) were measured by post hoc Bonferroni comparisons indicated by arrowed lines connecting the relevant pairs of measurements. Numbers within each bar give the number of neurons recorded (*n*) for each treatment group. *A* and *B*: post hoc comparisons revealed that CRD on its own significantly ($P < 0.05$) reduced rheobase and increased the number of APs fired at 2× rheobase (†), and this effect was blocked by prior *L. reuteri* ingestion (††). *L. reuteri* ingestion did not have a statistically significant effect on these measures of excitability when neurons only taken from undistended tissues were compared ($P > 0.05$) (†††).

beginning of 2× threshold current pulse. Overall, the treatments significantly affected rheobase (ANOVA, *P* <0.0001). Under control conditions (broth-noCRD), rheobase was 17.5 ± 1.7 pA/pF (current normalized to whole cell membrane capacitance). Post hoc multiple comparisons (Fig. 4) revealed where specific significant differences between the parameters lay. CRD on its own (broth-CRD) significantly decreased rheobase by about 50% 9.4 ± 1.5 pA/pF († in Fig. 4*A*). In addition, neurons in the broth-CRD group had a significantly lower rheobase than those from the *L. reuteri*-CRD group (16.0 ± 2.2
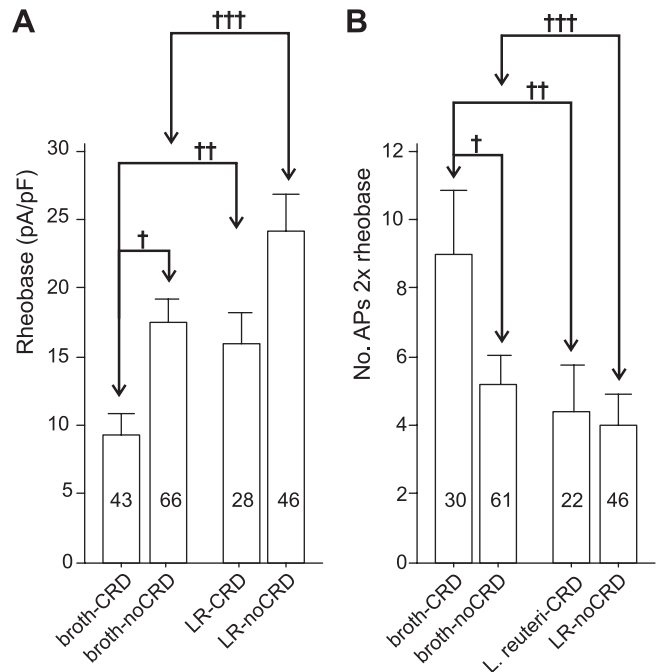
pA/pF) (†† in Fig. 4*A*). Hence prior *L. reuteri* feeding moderated the decrease in firing threshold that CRD elicits. However, *L. reuteri* had no discernible effect on rheobase control values since there was no significant difference between the broth-noCRD and *L. reuteri*-noCRD (24.1 ± 2.7 pA/pF) groups (†††).
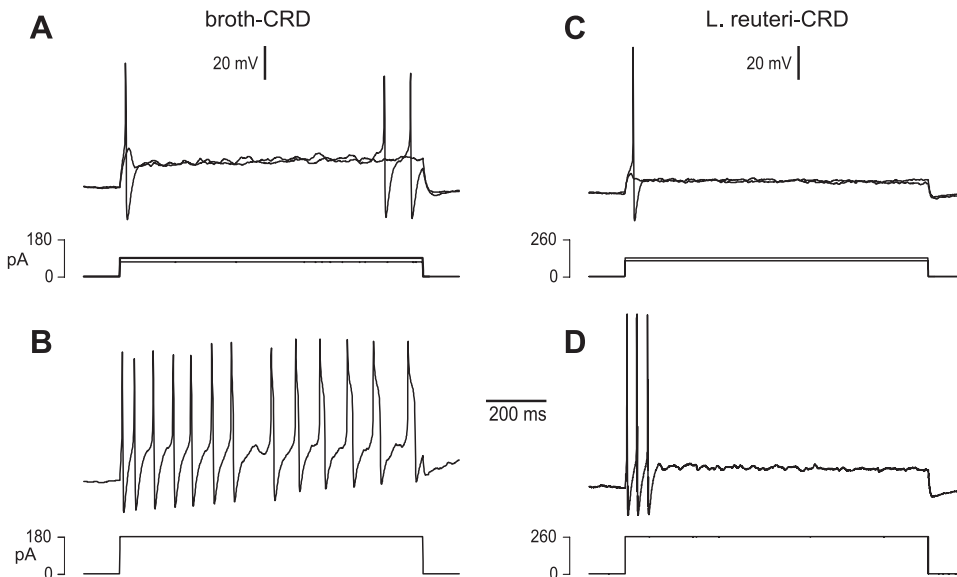
Fig. 3. Traces of intracellular recordings illustrating the effect of *L. reuteri* ingestion on 2 measures (rheobase, *A* and *C*; repetitive firing, *B* and *D*) of DRG soma excitability. *Top*: intracellular voltage recording. *Bottom*: stimulus current injected via the patch-clamp electrode. *A*: juxta-rheobase recording neuron subject to distension and fed vehicle control. *B*: repetitive tonic discharge when the same neuron is injected with current at 2× rheobase intensity. Note the spike broadening during the second half of the duration of the volley. *C*: juxta-rheobase recordings from neuron taken from gut subjected to CRD using *L. reuteri*-fed animals. *D*: traces from the same neuron as in *C*, stimulation at 2× rheobase intensity yielded action potential (AP) discharge only at the beginning of stimulus current pulse.

The number of APs discharged at $2\times$ rheobase intensity was also altered by the treatments (ANOVA, $P = 0.0008$) in a manner similar to the effect on rheobase. CRD significantly increased the number of APs from 5.2 ± 0.92 (broth-noCRD) to 9.0 ± 1.9 (broth-CRD) (Fig. 4*B*, †). However, *L. reuteri* ingestion abrogated the effect of CRD so that neurons in the *L. reuteri*-CRD group only fired 4.4 ± 1.4 APs (Fig. 4*B*, ††), which was a statistically significant decrease ($P < 0.05$). *L. reuteri* did not affect the control values for the number of APs fired (†††).

Passive membrane and AP parameters. We next examined the specific passive membrane and AP parameters in an attempt to reveal which electrophysiological characteristics of DRG soma (27) may have produced the CRD-evoked excitability increase and how *L. reuteri* ingestion could have blocked this effect. AP parameters that were measured for each neuron are illustrated in Fig. 5. These parameters are given in Table 1 and include some, such as leak current (g_{leak}) and AP maximal depolarization (upstroke) rate of rise ($AP_{dV/dt}$), that have been shown to alter when gut spinal sensory neurons are excited by peripheral inflammatory mediators or neurotransmitter release within DRG (15, 16, 27). These measurements thus allow for comparison between neuron responses to the mainly inflammatory stimuli as reported previously (15, 27) and the mainly nociceptive one applied here. AP amplitude (AP_{amp}), half width ($AP_{1/2}$), and dV/dt were measured using the first spike evoked after a stable recording was achieved 5 min after switching to a whole cell mode (Fig. 5*A*). This minimized variability in measurement introduced by the ordinary spike broadening effect of high-frequency repetitive discharge (36); see for example Fig. 3*B* (*top*) where repetitive discharge lead to spike broadening.

The membrane potential (V_m) for control group (broth-noCRD) neurons was -52 ± 2 mV, and this was not altered by
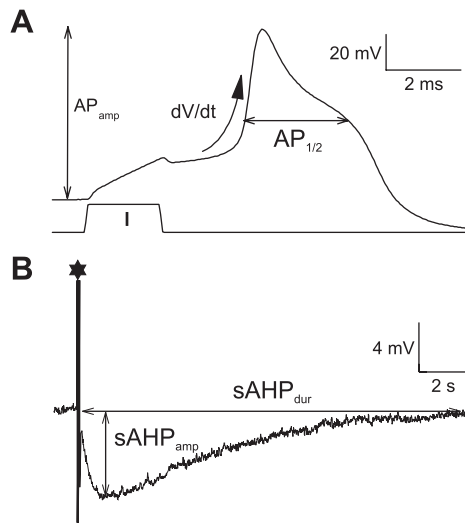

Fig. 5. Traces showing AP taken from control neuron at fast (*A*) and slow (*B*) time base. *A*, *top*: voltage recording. *Bottom*: current being passed through the patch-clamp electrode. A single AP was evoked by a brief to polarizing current pulse (*I*, *bottom*). Total AP amplitude (AP_{amp}), maximal rate of the depolarization (dV/dt), and AP width at half height ($AP_{1/2}$) were measured as shown. *B*: Filled star indicates location of stimulus current pulse and action potential that evoked the slow afterhyperpolarization potential (sAHP) shown in the voltage trace. The amplitude and duration of the sAHP were measured as indicated.

the treatments ($P = 0.42$, ANOVA); consistent with this observation, control group leak (background) conductance (g_{leak}) was also not significantly altered by distension or *L. reuteri* ($P = 0.98$) (Table 1). About half of the DRG neurons identified as projecting to the colon had prominent sAHPs following a single AP (44/68) for the control (broth-noCRD) group (Table 1); this proportion was not altered by the treatments used ($P = 0.44$). sAHP amplitude ($sAHP_{amp}$) and duration ($sAHP_{dur}$) were -3.1 ± 0.4 mV and 7.7 ± 0.9 s, respectively, for the control group, and this also was not significantly altered by any of the treatments (Table 1). AP_{amp} and $AP_{1/2}$ were 85 ± 3 mV and 2.4 ± 0.2 s, respectively; neither of these parameters was significantly changed by CRD or *L. reuteri* ingestion (Table 1). The rate of rise of the $AP_{dV/dt}$ was 100 ± 7 V/s, and, in contrast to the other membrane AP parameters, this was significantly affected by the treatments ($P < 0.0001$). Post hoc analysis showed that this effect was attributable to differences between both the broth-CRD vs. broth-noCRD (†) and the broth-CRD vs. *L. reuteri*-CRD (††) treatment groups (Table 1). *L. reuteri* appeared to have no effect on dV/dt in the absence of CRD (†††).

DISCUSSION

Main conclusion. The present work extends previous observations that ingestion of probiotic bacteria can moderate pain transmission and neurally based reflexes in the absence of an overt inflammatory model (18, 25, 32, 42). CRD in healthy animals, and in the apparent absence of inflammation, increased the excitability of DRG neurons that were isolated and recovered in culture hours afterward. Previous feeding for 9 days with *L. reuteri* prevented this excitability increase. We had earlier shown that *L. reuteri* ingestion decreased CRD-evoked pseudoaffective responses (bradycardia) and single unit dorsal root fiber discharge in the anesthetized rat (18). As predicted in our hypothesis, the effects of CRD and their modulation by *L. reuteri* were discernible in DRG somas 12 h or more after they were removed for culture; moreover, animals were euthanized immediately after the 1-h duration distension protocol ceased.

The changes in DRG somas evoked by CRD were persistent because, during the time of recording, the soma was removed from the activating stimulus (4, 27). The effects on DRG soma excitability described here are consistent with a role of the soma in the behavioral sensitization elicited by CRD and its modulation by the probiotic. However, the extent to which changes in the soma compared with peripheral or central terminals and synapses are responsible for the modulation of behavioral sensitization cannot be deduced from the present study. Our results add to an increasing list (9, 17, 21) of animal models where sensory pathway sensitization is induced in the absence of overt induction of chronic inflammation by chemical irritants or infection.

Although we did not use an explicit inflammatory model, it might be argued that CRD caused tissue damage and local inflammation, which may have, at the end of an hour distension, increased the excitability of the DRG tissue receptors, and prior *L. reuteri* ingestion may have prevented such inflammation by actions on immune cells and their mediators (37). In addition, it is well known that inflammatory cells and their mediators are present in the gut even in the absence of

Table 1. *Passive and active membrane parameters of dorsal root ganglion neurons*

	Broth- CRD††	Broth-noCRD†	*L. reuteri*- CRD††	*L. reuteri*-noCRD†††
V_m, mv	-50 ± 3 (44)	-52 ± 2 (68)	-49 ± 4 (28)	-55 ± 2 (50)
g_{leak}, nS/pF	0.137 ± 0.016 (43)	0.129 ± 0.048 (66)	0.121 ± 0.022 (28)	0.121 ± 0.013 (50)
$sAHP_{no}$	22/44	44/68	17/29	27/50
$sAHP_{amp}$, mV	-3.7 ± 0.5 (22)	-3.1 ± 0.4 (24)	-2.8 ± 0.7 (12)	-4.5 ± 0.8 (23)
$sAMP_{dur}$, s	7.7 ± 1.0 (22)	7.7 ± 0.9 (24)	5.5 ± 1.0 (12)	6.0 ± 0.9 (23)
AP_{amp}, mV	89 ± 2 (39)	85 ± 3 (55)	80 ± 4 (29)	81 ± 2 (26)
$AP_{1/2}$, ms	2.3 ± 0.4 (39)	2.4 ± 0.2 (53)	2.5 ± 0.3 (28)	2.1 ± 0.1 (26)
$AP_{dV/dt}$, V/s	174 ± 12 (39)	100 ± 7 (55)	115 ± 11 (29)	98 ± 8 (26)

Values are means $\pm$ SE. Values in parentheses are n = number of neurons. Probability of group effects were calculated with one-way ANOVA, except $sAHP_{no}$ where proportions were compared. Membrane potential (V_m), $P = 0.42$; group leak conductance (g_{leak}), $P = 0.98$; slow afterhyperpolarization potential ($sAHP_{no}$), $P(X^2) = 0.44$; sAHP amplitude ($sAHP_{amp}$), $P = 0.25$; sAHP duration ($sAHP_{dur}$), $P = 0.29$; action potential amplitude (AP_{amp}), $P = 0.18$; AP half width ($AP_{1/2}$), $P = 0.84$; AP depolarization speed ($AP_{dV/dt}$), $P < 0.0001$. Broth colorectal distension (CRD) vs. broth without CRD (noCRD) (†), $P < 0.05$, broth-CRD vs. *Lactobacillus reuteri* (*L. reuteri*)-CRD (††), $P < 0.05$.

pathology (45). However, since *L. reuteri* did not have an effect on DRG excitability in control undistended animals, it is unlikely that the probiotic had any effect on the healthy colon. Furthermore, the lack of effect of CRD on MPO levels and colon wet weight argues that significant acute inflammation may not have been established within the hour before the animals were euthanized. This deduction is consistent with the results published by others who found no evidence of an increase in MPO levels or histological damage or inflammatory infiltrate after 1-h repetitive CRD at 60 mmHg in rat (22) or mouse (24) colon. Similarly, Wang et al. (48) reported that repeated CRD in healthy rats at 78–100 mmHg activated certain hypothalamic brain nuclei 1 h after CRD cessation; no inflammation was reported that might have evoked the brain activity.

However, the failure of MPO to increase during the CRD period does not necessarily preclude the possibility that distension could have induced proinflammatory cytokines, such as IL-1β, to be released. The cytokines may have mediated the observed increase in excitability. Also, probiotic ingestion might have activated DRG microglia, which could have moderated the excitatory effects of CRD.

Potential mechanisms underlying the actions of CRD and L. reuteri ingestion. There were some differences from chronic inflammation. Chronic inflammation in visceral organs is well established to increase the excitability of DRG neurons (4, 27, 51, 52). For somatic pain pathways, repeated painful stimuli, as well as peripheral inflammation, can elicit sensitization of pain afferent signaling at various levels (51). For these pathways, sensitization involves transcriptional or posttranslational changes at presynaptic and postsynaptic elements in the spinal cord dorsal horn (51). Similarly, for the intestine, repeated painful stimuli, in the absence of extra inflammation, can cause sensitization of pseudoaffective (nociceptive) or motor reflexes (1, 11, 22). Less is known about the cellular and molecular processes involved in the sensitization of visceral pain pathways in the absence of pathological inflammation compared with its presence.

We found that prior *L. reuteri* ingestion abrogated the heightened excitability induced by acute CRD; however, the DRG soma hyperexcitability we observed was accompanied by membrane changes that differed in significant ways from those seen after chronic trinitrobenzene sulfonic acid inflammation (4, 27). We found no evidence that outward currents were decreased to enhance cell excitability. For example, the whole

cell leak conductance (g_{leak}) or the post-AP sAHP (14) were unaltered by CRD; in contrast, chronic trinitrobenzene sulfonic acid inflammation significantly reduces g_{leak} and several potassium currents, such as the transient outward and delayed rectifiers (4). On the other hand, we found that the $AP_{dV/dt}$ was increased by CRD, an effect that is also produced by chronic inflammation (27). Finally, acute CRD increased cell size as also does chronic inflammation (27, 52).

The increase in AP depolarization speed (dV/dt) may have been due to an increase in an inward conductance such as gNa^+ or gCa^{2+}; in the case of chronic experimental ileitis (3, 27), gastritis (5), of paw inflammation (6), the expression or activation and inactivation kinetics of TTX-R currents were altered. An increase in channel expression, or a left shift in the activation curve in TTX-R currents, could also explain the increased excitability and decreased threshold we recorded since these currents not only modify the readiness with which an action potential is evoked but also influence subthreshold membrane oscillations near threshold (7, 8, 30).

Repeated high-intensity nociceptive stimulation can cause immediate (within minutes) posttranslational sensitization at the DRG-dorsal horn neuron synapses in the spinal cord (51); similarly, tissue damage in the target organ may release a host of factors that can cause early second messenger-related sensitization within the peripheral sensory terminals, thus changing transduction sensitivity (51). On the other hand, changes in transcription at the level of the DRG soma (whether stimulus dependent or evoked by inflammation) are associated with retrograde transport of growth factors such as brain-derived neurotrophic factor or nerve growth factor (NGF) from the periphery to the DRG. Such changes are believed to typically require 2 h or more to occur (51). Similarly, inflammation-dependent DRG neuron hypertrophy appears to require transport of NGF after functional changes in the target organ (39), the process taking several days (27, 52). In the case of intestinal obstruction, soma hypertrophy is usually seen by *day 8* (49). Hence the hypertrophy and increased excitability in our DRG neurons cannot be readily explained by afferent transport of inflammation-related factors. Moreover, the distension may have caused the release of soluble factors such as serotonin, which could have contributed to the changes in DRG somas.

It is possible that the probiotic reduced the CRD-evoked afferent signals to levels where they did not alter soma excitability. Terminals in the colon of DRG neurons express acid-sensing (ASIC3) (10) or transient receptor potential [TRP1

(41) and TRP4 (10)] mechanosensitive ion channels to varying degrees, and any combination of these may have been modified with respect to their mechanosensitive gating characteristics or protein expression.

Action on the enteric nervous system may also have contributed to the probiotic effect. We have recently shown that *L. reuteri* ingestion facilitates the excitability of myenteric intrinsic sensory neurons (19), which would augment the tonic inhibitory tone that the enteric nervous system exerts on the intestinal circular muscle (12, 38, 40, 50) (see also discussion in Ref. 19). Since active smooth muscle contraction is required for afferent nociceptive signaling and pseudoaffective response occurrence in response to CRD (33), the action on enteric neurons may have decreased the intensity of afferent discharge during CRD.

Another potential mechanism derives from the observation that, when they are depolarized, rat lumbar DRG neuron somas can excite their neighbors by Ca^{2+}-dependent SP exocytosis (16). Both the capacitance and soma size increased within minutes of a depolarizing pulse as the membrane from SP-containing vesicles was incorporated in the plasmalemma (16). This process may explain how DRG somas can increase in size when they are subject to intense excitation.

The present report may be consistent with previous evidence that commensal bacteria can signal to sensory neurons and reset their excitability state (18, 42), a state that can persist even after the bacteria are no longer present. These earlier experiments involving dorsal root fiber (18) and vagal nerve recording (42) do not prove that in our experiments the probiotic acted on neurons without first acting on the immune system. Ingestion of the probiotic for 9 days may have altered the immune system in such a way that the neural response to CRD was altered. Thus CRD, acting as an extra stressor, could have revealed the effects of probiotic action, which would be occult in the absence of CRD.

The pathway by which probiotics signal to primary afferents is unknown. Ingestion of *L. acidophilus* by rats has been shown to increase colonic mucosal expression of opioid and cannabinoid receptors (32); however, signal transmission pathways between the epithelial cells expressing these receptors and DRG or other nerve axon terminals have not yet been determined. Ingestion of broth in which *L. reuteri* were first cultured for 48 h and then removed by centrifugation decreased the pseudoaffective responses to CRD (18). This suggests that soluble metabolic products excreted by the probiotic could contribute to the effects described in the present paper. Such products, if they exist, will need to be determined in future experiments that identify the biochemical products secreted by *L. reuteri* and their effects on sensory neurons. In addition, our laboratory is extending the present observations to explore the nature of the inward currents, particularly Na^+ conductances that were modulated by prior *L. reuteri* ingestion.

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