Quantitative assessment and characterization of visceral nociception and hyperalgesia in mice

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Kamp, Elizabeth H., R. Carter W. Jones, III, Shelly R. Tillman, and G. F. Gebhart. Quantitative assessment and characterization of visceral nociception and hyperalgesia in mice. Am J Physiol Gastrointest Liver Physiol 284: G434–G444, 2003. First published November 20, 2002; 10.1152/ajpgi.00324.2002.—Colorectal distension (CRD) is a well-characterized model of visceral nociception, which we adapted to the mouse. CRD reproducibly evoked contractions of the abdominal musculature (visceromotor response [VMR]), which was graded to stimulus intensity. The magnitude of VMR was greater in male C57BL6 and female 129S6 mice than in male 129S6 and B6.129 mice. In 129S6, C57BL6, and B6.129 mouse strains, VMR was reduced dose dependently by morphine (1–10 mg/kg) and by the &kappa;-opioid agonist U-69593 (0.2–2 mg/kg), although U-69593 was significantly less potent in C57BL6 mice. In additional experiments, the VMR was recorded from adult male 129S6 mice before and after intracolonic administration of various irritants. Only 30% ethanol significantly enhanced responses to CRD. The colon hyperalgesia persisted for 14 days and was associated with a significant shift of the morphine dose–response function to the left. We believe this will be a useful model for study of visceral nociception and hyperalgesia, including studies of transgenic mice with mutations relevant to pain.

PAIN IS THE MOST COMMON REASON patients seek care from a physician, and the majority of visits are for pain of visceral origin. Current knowledge about mechanisms of pain has been derived from studies of somatic, principally cutaneous pain. Visceral pain has been less well studied, in part, because of more difficult access to visceral structures. Additionally, stimuli that are painful when applied to the skin, such as burning, crushing, and cutting, evoke no pain when applied to most visceral structures. Furthermore, there is growing evidence that the mechanisms of visceral and cutaneous pain are different (8, 19). Certainly, the characteristics of these pains are different; visceral pain is diffuse, poorly localized, and referred to overlying structures (see Refs. 7 and 46 for reviews).

Balloon distension of hollow organs, such as the colon, stomach, or urinary bladder produces pain in humans and quantifiable behavioral and autonomic responses in nonhuman animals. These responses have been most extensively characterized for colorectal distension (CRD) in the rat. When applied to rats at pressures comparable to those that produce pain in humans, CRD is aversive and produces tachycardia, pressor effects, contraction of the abdominal musculature, activation of afferent (sensory) fibers in the pelvic nerve, and activation of second-order neurons in the dorsal horn of the spinal cord (45, 46, 52).

The availability of genetically modified mice has increased interest in the mouse as a nonhuman experimental animal. Numerous mice have been generated with mutations relevant to the study of pain; most have been tested in cutaneous models of nociception only. Some transgenic mice have been tested using the intraperitoneal acetic acid model of visceral nociception (writhing test); however, a limited understanding of the underlying structures and sensory neurons activated in this model confounds interpretation of results.

Therefore, we adapted the CRD model to the mouse. CRD is a well-characterized model of visceral nociception, produces an easily quantifiable response (contraction of the abdominal musculature, termed the visceromotor response [VMR]) and allows animals to be tested multiple times and over a range of stimulus intensities. Our second objective was to develop a murine model of sustained inflammation and colon hyperalgesia. Some of these data have been previously published in abstract form (24, 25).

MATERIALS AND METHODS

Animals. Adult male (20–30 g) 129S6/SvEvTac mice (129S6; Taconic, Germantown, NY) were used in all experiments except as noted. In some experiments, adult female 129S6/SvEvTac, male C57BL6, and male B6.129.F1 (B6.129) mice (all from Taconic) were used. Mice were housed singly with free access to food and water. Female mice were tested without regard to phase of the estrous cycle. Because we were concerned that surgery and/or chemically induced colon inflammation would negatively impact feeding and animal health, we also provided a chow paste (prepared by soaking powdered standard chow in water overnight). Mice ate this paste preferentially over dry chow and lost less weight after

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surgery and colon inflammation. Experimental procedures adhered to the International Association for the Study of Pain Research Guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Surgical preparation. Mice were anesthetized with 75 mg/kg ip pentobarbital sodium (Abbott Laboratories, North Chicago, IL) or a 7:1 combination of ketamine (87.5–175 mg/kg) and xylazine (12.5–25 mg/kg ip) (Abbott Laboratories and Phoenix Pharmaceutical, St. Joseph, MO, respectively). Mice anesthetized with ketamine/xylazine were pretreated with 40 μg/kg ip atropine 10–60 min before ketamine/xylazine (Fujisawa, Deerfield, IL). Electrodes (Teflon-coated stainless steel wire, 5- to 10-mm tip separation; Cooner Wire Sales, Chatsworth, CA) were sewn into the external oblique abdominal musculature, just above the inguinal ligament, for electromyographic (EMG) recording. The EMG electrodes were subcutaneously guided to the dorsum of the neck and externalized for future access. At the same time, a subcutaneous catheter for drug administration (PE-10, 5 cm) was placed at the dorsum of the neck in some mice. Incisions were closed with 5-0 silk. After recovery, mice were given 0.3 ml of 5% dextrose in normal saline (to replace fluids lost during surgery and to provide some nutrition) and allowed to recover for a minimum of 3 days before testing.

Spinal transection. After generating a baseline stimulus-response function (as described in Visceral nociceptive testing), mice were given 1 ml of 5% dextrose in normal saline (ip) for hemodynamic support. Approximately 1 h later, mice were deeply anesthetized with pentobarbital sodium (as described in Surgical preparation). Under a surgical microscope, the skin was cut with surgical scissors and a cautery was used to remove a piece of muscle overlying the vertebra (~5 × 15 mm). Ice was applied to the exposed vertebra for 5 min to minimize bleeding. A laminectomy was performed with No. 5 Dumont Biologie forceps, and the spinal cord was again iced. With the use of microscissors, the spinal cord and dura were cut twice, ~1 mm apart. The resulting piece of spinal cord was removed and a small (~1 × 1 mm) square of Gelfoam (Upjohn, Kalamazoo, MI) soaked in epinephrine (1:1,000; American Regin Laboratories, Shirley, NY) was inserted into the gap. An ~5 × 15-mm piece of epinephrine-soaked Gelfoam was also laid atop the spinal cord in the cavity left by the laminectomy and was loosely secured to the back muscles with 6-0 silk. The skin was closed in layers with 5-0 silk. Mice were very closely monitored after surgery. Approximately 8 h after surgery, their bladders were expressed and mice were given an additional 1 ml of 5% dextrose in saline (ip) to provide some nutrition. All steps except cutting the spinal cord and insertion of Gelfoam into the resulting gap were performed in sham-operated mice. After testing, the level of each spinal cord transection was verified; all were at spinal level T-9/10. Colon afferents in the mouse enter in dorsal roots caudal to T-9/10 (50).

CRD balloons. Distension balloons were prepared by stretching a small square (~3 × 3 cm) of thin (~15 μm) polyethylene plastic over a polyvinyl chloride rod (9-mm diameter), thereby removing all compliance from the plastic and creating a balloon. These balloons, 20-mm in length, were tied with 6-0 silk to PE-24 thin-wall tubing (Cole-Parmer Instrument, Vernon Hills, IL) 15 mm from the tip of the tubing and 20 mm from the closed end of the balloon (5-mm allowance for inflation). Before securing the balloon to the tubing, several holes were punched in the distal 15 mm of the tubing with a 27-gauge needle to allow the balloon to inflate even if the catheter tip was occluded by the plastic balloon. To facilitate insertion and protect the delicate balloon from damage, the balloon and tubing were covered by a 6-cm-long sheath prepared from PE-240 tubing (2-mm diameter). One wall of the sheath was cut lengthwise to accommodate the girth of the balloon and silk suture.

On the day of testing, mice were briefly anesthetized with halothane (1–5% in 100% O2 at 2 l/min; ≥5 min; Halocarbon Laboratories, River Edge, NJ). The sheath was lubricated with Surgilube (E. Fougera, Melville, NY) and inserted intranally until the silk tie was 5 mm inside the rectum (total insertion distance, 25 mm). The sheath was removed and the tubing was taped to the base of the tail to prevent displacement. Mice were placed in restraint devices (see Restraint devices) while still sedated and were allowed to recover/acclimate for a minimum of 30 min before testing. The lack of effect of halothane on responses to CRD 30 min after termination of the anesthesia was determined in preliminary experiments.

Restraint devices. Restraint devices were constructed from a plastic 60-ml syringe (Becton Dickinson, Franklin Lakes, NJ) With the plunger removed. The needle attachment port was sawed off and the remaining tube was cut at the 40-ml mark (total length, 7.5 cm). In addition, an opening (~7 × 9 mm) was made in the top of the tube for access to the EMG recording electrodes and subcutaneous catheter. The internal diameter of these tubes is ~25 mm, which holds a 15- to 28-g mouse. For larger mice, similar devices were constructed from translucent plastic (30-, 32-, and 40-mm internal diameters).

After placing the mouse in the tube, the open end was secured with a gauze square and paper tape. The tube was then placed in a dark-colored fabric sheath (to reduce ambient light) containing a small window (~5 × 5 mm) for access to the EMG electrodes and catheter. The behavior of mice before, during, and after distension can be easily monitored by partial retraction of the fabric.

EMG recording. CRD-evoked contraction of the abdominal musculature, termed the VMR, was the behavioral response quantified. The EMG signal was filtered, amplified, and recorded as has been described for rats (10). Briefly, the balloon was connected to a pressure control device (Bioengineering, University of Iowa, Iowa City, IA) that regulated inflation of the balloon. Each distension trial lasted 40 s and EMG activity was quantified during 10 s before distension, 20 s during distension, and 10 s after distension.

Colon inflammation. Under brief halothane anesthesia (as described in CRD balloons), an inflammmogen or irritant was instilled into the lumen of the colon after the last distension in the baseline testing period. Briefly, the distension balloon was removed and 0.1 ml of inflammmogen/irritant (see Visceral nociceptive testing) was instilled into the colon 0.5–2.5 cm proximal to the rectum using a 22-gauge, 24-mm-long stainless steel feeding needle (Fisher Scientific, Fair Lawn, NY) attached to a 1-ml syringe. Mice were then removed from the restraint devices and returned to their cages with their hind limbs elevated (to prevent immediate leakage of colon contents). During recovery from anesthesia and periodically thereafter, mice were closely observed for signs of discomfort (e.g., writhing, immobilization, restlessness) or gastrointestinal dysfunction (e.g., diarrhea, bleeding, weight loss); none was observed.

Solutions of 30% ethanol (vol/vol) and 0.6% acetic acid (vol/vol, from glacial acid-acid; Fisher) were prepared in sterile, preservative-free saline (Abbott), which had been adjusted to pH 7.2 before use. Solutions of 0.003% capsaicin (wt/vol) and 0.25% mustard oil (vol/vol; both from Sigma, St. Louis, MO) were prepared in 5% dextrose in water (wt/vol,
pH 7.2; Baxter Healthcare, Deerfield, IL) with the addition of a few drops of Tween 80 (Fisher).

**Visceral nociceptive testing.** Stimulus-response functions to graded intensities of CRD were generated to evaluate reproducibility and differences due to strain, gender, spinal transection and colon inflammation. In all cases, stimulus-response functions were generated using CRD pressures of 15, 30, 45, and 60 mmHg. Three distensions were performed at each pressure at 4-min intervals. To test the reproducibility of the VMR to CRD, one group of mice was tested 3 days and again 10 days after EMG electrode implantation. For mice undergoing spinal transection (or sham transection), the stimulus-response protocol was performed before and again 18 h after surgery.

In experiments screening strategies for production of hyperalgesia, mice were distended 5 times to 60 mmHg (at 5 min intervals) before and 1, 2, 4, 6, 8, 12, and 24 h after intracolonic instillation of an inflammogen/irritant. If responses to CRD were enhanced, then experiments in different mice were carried out using the full range of CRD intensities (15, 30, 45, and 60 mmHg). Three distensions were performed at each pressure at 4-min intervals (as above). This protocol was performed once before any treatment to establish a baseline. Immediately after the last distension, an inflammogen/irritant was instilled into the colon (as described in Colon inflammation). Graded CRD was repeated at 3 and 24 h and 3, 5, 7, and 14 days after intracolonic treatment.

One group of mice received no intracolonic treatment (anesthesia and gentle perianal stimulation only). The behavioral responses of these mice were only tested before and 1 h after this sham treatment. Twenty-four hours later, the colon was removed for myeloperoxidase (MPO) assay (described in MPO assay) for comparison with mice receiving an inflammogen.

**Drug effects.** On the day of testing, five phasic distensions (45 mmHg, 20 s) at 5 min intervals were given to establish a baseline. Immediately after the fifth distension, drug or vehicle was administered subcutaneously via the chronically placed catheter. CRD (45 mmHg, 20 s) was repeated 5, 10, 15, 20, 25, 30, 40, 50, and 60 min later.

In experiments evaluating the effect of morphine in mice with colon hyperalgesia, baseline responses to CRD (45 mmHg, 20 s) were determined as above, after which 30% ethanol was instilled into the colon (as in Colon inflammation). Twenty-four hours later, five distensions (45 mmHg, 20 s, 5-min intervals) were repeated to quantify the hyperalgesia. Immediately after the fifth distension, morphine (or saline vehicle) was administered subcutaneously via the chronically placed catheter. CRD (45 mmHg, 20 s) was repeated 5, 10, 15, 20, 25, 30, 40, 50, and 60 min later. Each animal received only one dose of any drug and dose-response curves were obtained using multiple mice.

**Drugs.** All drugs were given in a volume of 0.1 ml/kg body wt. The drugs used in this study were the κ-opioid receptor agonist U-69593 (Sigma), the μ-opioid receptor agonist morphine sulfate (Spectrum Quality Products, Gardena, CA), and the benzodiazepine diazepam (Sigma). Morphine was prepared in sterile, preservative-free saline (Abbott). U-69593 was dissolved in 4.5% 2-hydroxypropyl-β-cyclodextrin (wt/vol; Sigma) in sterile, preservative-free saline. Diazepam was dissolved in 50% propylene glycol (vol/vol; Fisher) in sterile, preservative-free saline.

**MPO assay.** The MPO assay was used to quantify colon inflammation. The procedure was performed as described by Krawisz et al. (30). Briefly, mice were killed by an overdose of pentobarbital sodium and the distal colon was removed via laparotomy. The fresh tissue was suspended in hexadecyltrimethylammonium bromide (a detergent; Sigma), minced with scissors, homogenized/sonicated, and freeze-thawed three times. The tissue suspensions were centrifuged, and the supernatant was assayed for MPO activity spectrophotometrically by measuring the change in absorbance at 460 nm. The color change was accomplished by mixing an aliquot of the supernatant with phosphate buffer containing 0.005% hydrogen peroxide (vol/vol) and o-dianisidine hydrochloride (a pH sensitive indicator). The greater the conversion of hydrogen peroxide into acid (by MPO), the more intense the color.

Mice received either no treatment (naive) or intracolonic administration of 0.1 ml of saline (vehicle) or 30% ethanol. Colon was removed for this assay 3 or 24 h or 3, 5, 7, or 14 days after intracolonic treatment (as in behavioral testing above). In a separate group of mice, the effect of repetitive distension on MPO activity was tested.

**Histology.** To remove tissue for histologic analysis, mice were terminally anesthetized with pentobarbital sodium (200 mg/kg, ip; Abbott) and 3 cm of distal colon was removed. The colon was rinsed with cold zinc-formalin fixative (Labco, Louisville, KY). With the use of conventional techniques, fixed tissue was paraffin embedded, cut in longitudinal sections with a microtome, and stained with hematoxylin and eosin.

**Data analysis.** EMG activity was collected at 250 Hz using the Cambridge Electronic Design data collection system (model 1401 plus) running the Spike2 software (version 3.18, both from Cambridge Electronic Design, Cambridge, UK). The number of EMG spikes >300 mV were counted in 10-s bins off-line by a Spike2 script. The first 10 s of the distension period were used for analysis, because responses were most robust and reproducible during the first half of the distension period. The overall effect of any treatment was determined by taking the area under the curve (AUC) of the stimulus-response function or time course of drug effect.

For stimulus-response functions, the response to CRD is represented as the mean number of spikes (during the 10-s data analysis window) or percentage of control in which the mean of responses to 60 mmHg is defined as 100%. For mice in which multiple stimulus-response functions were determined, the mean of the responses to 60 mmHg for the first stimulus-response function was defined as 100%. The AUC was calculated as the sum of responses plotted against pressure using the trapezoidal rule where $AUC = \sum_{x} y_{x}$, where $x$ refers to the coordinate values on the x and y axes (pressure, response) for each pressure tested (15–60 mmHg).

For time courses of drug effects, the visceromotor response is represented as %control in which the mean of the five baseline (predrug) responses was defined as 100%. Converting responses to %control allowed normalization of data between mice and mouse strains, facilitating comparison of drug effects in the different strains tested. The AUC was calculated as the sum of the change in the postdrug response from the mean vehicle response (for that strain and time point) plotted against time (AUC = deviation in response × 60 min). This method effectively quantifies the area between the effect of drug and vehicle across the entire time course. By normalizing the data in this way, any enhanced responses to a given distensive stimulus (seen in mice receiving vehicle) were eliminated as a confounding factor to data analysis.

Statistical tests were performed as indicated in the text or figure legends (SigmaStat; Jandel Scientific, San Rafael, CA). In all cases, statistical significance is indicated when $P < 0.05$. For all ANOVAs, the Bonferroni correction for multiple
comparisons was used when $P < 0.05$ for the ANOVA of the factor of interest.

**RESULTS**

The VMR to CRD models visceral pain. In mice, like rats and humans, CRD produced robust contractions of the abdominal musculature graded to stimulus intensity (Fig. 1A). The lowest stimulus intensity (15 mmHg) generally did not produce a response significantly greater than background activity. At 30 mmHg CRD, the VMR was typically twice the magnitude of background activity; pressures of 45 and 60 mmHg reliably produced robust responses (see Fig. 1A, inset for an example of response to 60 mmHg CRD). To test the reproducibility of the response to CRD, a group of six mice was tested 3 and 10 days after EMG electrode placement. As seen in Fig. 1A, the responses of these mice to graded CRD are virtually identical when tested 1 wk apart, revealing that neither postsurgical sensitization at 3 days nor possible damage to the colon by repetitive CRD contribute to the response measure. The mean background activity (determined in the 10 s immediately before each distension), shown by the shaded bar in Fig. 1A, also was not different on days 3 and 10 ($P = 0.795$, paired $t$-test) nor did it differ with repetitive-graded CRD.

To determine whether the VMR to CRD in mice, as in rats (45), is a spinobulbospinal pseudaffective reflex (54), mice were tested before and 18 h after T-9/10 spinal cord transection. Responses to graded CRD were abolished in both male and female 129S6 mice after spinal cord transection. For example, responses to 60 mmHg CRD in spinal transected mice were significantly reduced to 1.9 ± 0.7% (male) and 6.7 ± 3.1% (female) of the respective pretransection response to 60 mmHg CRD (defined as 100%; $P < 0.005$ vs. sham for both groups, two-way, repeated-measures ANOVA; no responses at any distending pressure were significantly different from background activity).

**Strain and gender differences.** Because one goal of these studies was to develop a model of visceral nociception suitable for testing transgenic mice, we chose to test basal nociceptive sensitivity of the two most commonly used background strains (129S6 and C57BL6) and the resulting F1 hybrid strain (B6.129). Background activity (recorded in the 10 s before each distension) did not differ between strains and gender ($F_{3,27} = 0.35$, $P = 0.789$; one-way ANOVA) and data were pooled for presentation (shaded bar in Fig. 1B). Figure 1B shows the stimulus-response functions of male 129S6, female 129S6, C57BL6, and B6.129 mice. The AUC presentation (Fig. 1B, inset) reveals that responses of male C57BL6 and female 129S6 mice are significantly greater than responses of male 129S6 and B6.129 mice. Accordingly, male and female 129S6 mice differed in responses to CRD; responses in males was significantly less across the range of distending pressures tested. This is consistent with reports that male C57BL6 mice are more sensitive to both visceral and cutaneous noxious stimuli than male 129S6 mice (41, reviewed in Ref. 34).

**Opioid effects.** To further establish CRD as a model of visceral nociception in mice, we tested the effects of opioids on responses to distension. Doses of 3 and 10 mg/kg morphine significantly reduced responses of male 129S6 mice to noxious CRD (Fig. 2A). In female 129S6 mice, 3 mg/kg morphine reduced responses to a similar extent as in male 129S6 mice (Fig. 2B). Therefore, other doses were not tested. This outcome parallels results from a study (cutaneous nociception) showing equipotent and equieffective morphine antinociception in male and female 129S6 mice (27). Similarly, morphine significantly reduced responses to noxious CRD in C57BL6 and B6.129 mice (Fig. 2B). The effect of morphine did not differ among
strains of mice (F_{3,92} = 2.6, P = 0.057; two-way ANOVA for factor strain).

In addition to differences in responses to noxious stimuli, there have been reports of strain and gender differences in the antinociceptive effects of κ-opioid receptor agonists (20, 47, 62). Also, it has been suggested that κ-opioid receptors are more strongly linked with visceral pain than cutaneous pain (3, 55). For these reasons, we tested the effect of the κ-opioid receptor agonist U-69593 in male and female 129S6 and male C57BL6 and B6.129 mice. Figure 3A shows the time course of action of U-69593 in male 129S6 mice. The AUC presentation in Fig. 3B compares dose-dependent effects of morphine (AUC analysis of the time courses of effect; see MATERIALS AND METHODS). There were no differences in the effect of morphine among mouse strains (two-way ANOVA with the Bonferroni correction for multiple comparisons).

Because opioids can have anxiolytic as well as antinociceptive properties, we confirmed that reduction of VMRs to CRD by opioids was due to an antinociceptive effect. To that end, the effect of an anxiolytic dose of diazepam was tested on responses to CRD in male 129S6 mice. At a dose reported to reduce anxiety without producing motor effects (0.3 mg/kg), diazepam had no significant effect (vs. vehicle) on VMRs to CRD (to 88.5 ± 7.8 and 86.5 ± 17.1% of baseline, respectively, 15 min after vehicle or diazepam; P = 0.909, unpaired t-test). This outcome also suggests that stress-produced anxiety or fear do not contribute to the response measure.

Visceral hyperalgesia. All further experiments were carried out in adult male 129S6 mice. Intracolonic instillation of 0.6% acetic acid has been reported to enhance pressor responses to CRD in anesthetized mice 1 h after treatment (32). Therefore, we tested mice before (data not shown) and 1 h after anesthesia alone or intracolonic instillation of 0.6% acetic acid or saline (Fig. 4A). However, responses to CRD were not...
enhanced by acetic acid treatment (F_{3,60} = 1.227, \(P = 0.308\); two-way ANOVA for factor treatment), and we subsequently screened potential colonic inflammogens/irritants over longer time courses. Mice were tested before and at 1, 2, 4, 6, 8, 12, and 24 h after intracolonic instillation of one of the following: 5% dextrose in water (not shown), saline, 0.25% mustard oil, 0.003% capsaicin, or 30% ethanol. The only intracolonic treatment to significantly enhance responses to CRD was 30% ethanol (Fig. 4B).

Accordingly, we further characterized the hyperalgesia produced by 30% ethanol. Stimulus-response functions were recorded before and at 3 and 24 h and 3, 5, 7, and 14 days after intracolonic instillation of saline or 30% ethanol in saline (Fig. 5, A and B, respectively, page 17). Responses of ethanol-treated mice were significantly enhanced at all time points tested (vs. baseline and saline, two-way ANOVA for factor time and treatment). EMG recordings from the same mouse before and 24 h after instillation of 30% ethanol showed typical enhancement of the response to 60 mmHg CRD (Fig. 5B, inset). In untreated mice, EMG activity typically increases immediately (<0.5 s) after the onset of CRD and lasts 2–5 s, followed by a period of enhanced activity that persists until termination of the stimulus (also see Fig. 1A, inset). As illustrated, EMG recordings from ethanol-treated mice show significantly enhanced

Fig. 4. Effects of intracolonic treatment on the VMR to CRD. A: VMR to graded CRD (15–60 mmHg, 20 s each) was recorded before (not shown) and 1 h after anesthesia only or intracolonic instillation of 0.6% acetic acid or saline. No intracolonic treatment significantly enhanced responses to graded CRD over anesthesia only (2-way ANOVA, F_{3,60} = 1.227, \(P = 0.308\); n = 3–6 per treatment group). B: VMR to CRD (60 mmHg, 20 s) was recorded before and 1–24 h after intracolonic instillation of 30% ethanol, 0.25% mustard oil, or 0.003% capsaicin. Only 30% ethanol enhanced responses to CRD.

Fig. 5. Effect of intracolonic instillation of 30% ethanol (EtOH) on the VMR to graded CRD. A and B: VMR to graded CRD (15–60 mmHg, 20 s each) was recorded before (baseline) and 3 h to 14 days after intracolonic instillation of either saline (A) or 30% ethanol (B). Saline-treatment did not significantly affect responses to graded CRD (vs. baseline, 2-way, repeated-measures ANOVA). In ethanol-treated mice, responses were significantly enhanced (vs. baseline) at all times from 3 h to 14 d after ethanol treatment (2-way, repeated-measures ANOVA). B, inset: representative EMG records of the VMR to 60 mmHg CRD recorded from the same mouse before and 24 h after intracolonic instillation of 30% ethanol. C: quantification of colon inflammation by myeloperoxidase (MPO) activity 3 h to 14 days after saline or ethanol treatment. MPO activity was significantly increased from 3 h to 7 days after ethanol treatment (vs. saline treatment; \(n = 6–12\)). The mean MPO activity of naive (untreated) mice is illustrated for comparison (dotted line). ANOVA for factors treatment and time was significant (\(P < 0.001\)); \(*P < 0.05\) for saline vs. ethanol treatment at that time point.
background activity (recorded in the 10 s before distension) and CRD-evoked VMRs. Activity ceases abruptly on termination of the distending stimulus and is less than the predistension background activity. This is consistent with response characteristics of pelvic nerve afferent fibers on termination of CRD in the rat (52).

Colon inflammation. MPO activity, an index of inflammation, was assayed in the colons of mice after no treatment (naive), 1 h after repetitive CRD (anesthesia only), and intracolonic instillation of acetic acid or saline. The colons of naive mice contained 0.7 ± 0.1 units MPO activity (per g colon wet weight). Neither treatment with saline or acetic acid (0.6%) significantly affected MPO activity (0.4 ± 0.05 and 0.4 ± 0.2 U/g, \( P = 0.82 \) and 0.69, respectively, one-way ANOVA) nor did repetitive CRD (anesthesia only, 1 h; 1.4 ± 0.5 U/g, \( P = 0.102 \)).

The colons of separate groups of mice were tested for MPO activity after intracolonic saline or 30% ethanol treatment at the same time points tested for behavioral hyperalgesia (3 and 24 h, and 3, 5, 7, and 14 days). Colon MPO activity was significantly enhanced (vs. saline) at all time points tested (except 14 days) after ethanol treatment (Fig. 5C), paralleling the effect of ethanol on responses to CRD.

In different mice, colons were removed for histology. The colons of ethanol-treated mice (24 h) exhibited focal disruption of crypts characterized by destruction of the architecture of the tubular intestinal glands, irregular and ectopic placement of goblet cells, and interruption of the normal, simple columnar epithelium. Additionally, there were irregular crypt depth, mucosal edema (especially in the lamina propria) with neutrophil infiltration, and atrophy of the muscularis externa (Fig. 6).

**Morphine effects.** Twenty-four hours after intracolonic instillation of 30% ethanol, responses to 45 mmHg CRD were significantly enhanced to 232 ± 52% of the preethanol treatment baseline (defined as 100% vs. pretreatment; one-way repeated-measures ANOVA; Fig. 7A). In contrast, responses of intracolonic saline-treated mice (24 h) were 108 ± 10% of baseline (\( P > 0.05 \) vs. 24 h ethanol-treatment, unpaired \( t \)-test; data not shown). Morphine dose-dependently reduced responses to 45 mmHg CRD in ethanol-treated mice (Fig. 7B). When compared with the dose-response function for morphine determined in naive mice (data from Fig. 2B), ethanol treatment significantly shifted the dose-response relationship (Fig. 7B, two-way ANOVA for factor treatment).

**DISCUSSION**

The present study demonstrates that VMRs to CRD in mice are easily quantified, reproducible, reliable, graded to stimulus intensity, and supraspinally mediated. Although these conclusions are limited to the strains tested, they are likely to be applicable to other mouse strains because the 129S6 and C57BL6 strains of mice are among the least sensitive strains to visceral/somatic noxious chemical stimuli (writhing tests) (42). In addition, the VMR to CRD was reduced dose-dependently by opioid receptor agonists (morphine and U-69593), but not by an anxiolytic (diazepam). The potencies of morphine in this model are comparable to those reported for cutaneous tests in the 129S6 and C57BL6 mouse strains (13, 41). Accordingly, CRD in the mouse represents a reliable model of acute visceral nociception. Furthermore, colon inflammation produced by intracolonic (intraluminal) instillation of 30% ethanol produced a robust and persistent hyperalgesia to CRD. As has been reported in the rat (15, 53), the potency of opioids (morphine in the present experiments) was increased in the presence of colon inflammation.

**Advantages of CRD.** Other models of acute visceral nociception exist for the mouse; however, CRD presents several advantages over other models. First, CRD is a stimulus restricted to the viscus. The most commonly used model of visceral nociception in the mouse is intraperitoneal injection of a chemical irri-
Fig. 7. Potency of morphine is increased in male 129S6 mice with visceral hyperalgesia. A: VMR to CRD (45 mmHg, 20 s) was recorded before and 24 h after intracolonic instillation of 30% ethanol, after which morphine (1, 3, or 10 mg/kg sc) or vehicle (saline; n = 7–8 per treatment group) was given and responses to CRD were recorded over the next 60 min. AUC for each dose (relative to vehicle-treated mice) was given and responses to CRD were recorded. B: comparison of dose-dependent effects of morphine in EtOH-treated mice and male 129S6 mice with no intracolonic pretreatment (naive, taken from Fig. 2B). Morphine was significantly more potent in EtOH-treated mice (P < 0.001, two-way ANOVA for factor treatment).

Intensities can be tested in a single mouse. An alternative model of acute visceral pain in the mouse employs the intracolonic instillation of capsaicin or mustard oil to produce spontaneous pain-related behaviors (abdominal licking, stretching, and muscle contractions) that are counted over time (20 min); stimulus intensity is varied by adjusting the concentration of the irritant solution (33). Like intraperitoneal injection of chemical irritants, mice are tested only once and humanly killed to limit the duration of visceral nociception. Accordingly, the number of mice required may be large, which may be problematic when limited numbers of transgenic mice are available. Lastly, CRD can be done in unanesthetized animals. Beyond introduction of potential confounding antinociceptive effects, anesthesia precludes the study and observation of animal behavior.

Potential disadvantages of the model, including stress contributed by confining the mouse during distension, halothane anesthesia or tissue damage due to balloon insertion were not evident from observation of the mouse or inspection of the data. Responses to distension were stable and reproducible over time and not affected by diazepam. It is our impression that the brief halothane anesthesia used for balloon insertion or the administration of intracolonic treatments reduced potential stress and variability in responses to distension.

Considerably more women than men suffer from organic and functional bowel disorders. Several authors have reported that women are more sensitive to pain than men (49, 61, 64), although this is not a universal finding (36, 44) and may depend on stimulus modality (35, 51). Therefore, we tested whether female mice, without consideration of estrous cycle phase, had a lower threshold or greater response magnitude to CRD than male mice. Male and female 129S6 mice did not differ in thresholds for response to CRD or background (resting) activity, but female mice responded significantly more robustly to CRD over the range of pressures tested.

Several investigators have reported that k-opioid receptor agonists are more efficacious and/or potent in females than males in both rodents (2) and humans (16, 17). Thus we anticipated that antinociception produced by the k-opioid receptor agonist U-69593 would be greater in female mice. Some authors suggest that the gender differences reported are an artifact of differences in basal nociceptive sensitivity, but even when predrug responses were normalized (to remove this possible confound), U-69593 produced a more robust antinociception in female than male 129S6 mice.

The k-opioid receptor has been considered especially relevant to visceral pain, primarily because k-opioid receptor knockout mice write more than their wild-type littermates after intraperitoneal injection of acetic acid (3, 55) and k-opioid receptor agonists uniformly reduce visceral nociception (writhing or CRD) (1, 4, 6, 53, 58, 60, 62). In contrast, studies of k-opioid receptor agonist effects in cutaneous models of nociception report an antinociceptive effect (2, 26, 55, 60), no effect...
(1, 62), or even a hyperalgesic effect (47). In studies (1, 60, 62) that have directly compared the antinociceptive effect of kappa agonists in models of visceral and cutaneous nociception, ED_{50} values were not different. Although we did not test the effect of U-69593 in a cutaneous pain model, the effective dose range studied here is similar to that reported by others (26, 40).

**Colon inflammation.** Exposure of the gastrointestinal tract to ethanol has been used in a variety of animal species to produce inflammation and ulceration of the stomach, duodenum, ileum, or colon (5, 12, 14, 37, 43). When neutrophil infiltration into the colon was examined at various times after intracolonic instillation of 30% ethanol, MPO activity was maximal at 24 h and returned to baseline at 2 wk. Histologic examination of colons 24 h after intracolonic ethanol revealed frank damage evidenced by superficial erosions, mucosal edema, and infiltration of immune cells (supported by the MPO activity assay). Mice receiving saline were indistinguishable from untreated animals, both histologically and in the MPO activity assay.

**Visceral hyperalgesia.** Visceral hyperalgesia was reported first in humans with irritable bowel syndrome (IBS) (48) and has since been reported for all functional bowel disorders (see Ref. 39 for review). Similarly, inflammatory bowel disorders and colitis are often associated with discomfort and pain (56, 57). To study potential mechanisms of these human conditions, models of colon inflammation in the mouse have been developed, but none have been evaluated for hyperalgesia. In rats, visceral afferents sensitize in conditions that cause behavioral hyperalgesia. For example, intracolonic administration of zymosan to the rat produces hyperalgesia (10), sensitization of pelvic nerve colon afferents (11), and activation of silent fibers (18). Centrally, spinal N-methyl-D-aspartate (10, 21, 28, 65), neurokinin (22, 23, 31), opioid (45) and CCK-B receptors (15), and nitric oxide (9) have been shown to modulate behavioral or electrophysiological responses to noxious colon distension.

In summary, the VMR to CRD is a reproducible, supraspinally mediated contraction of the abdominal musculature that is graded to stimulus intensity and attenuated by opioids (morphine and U-69593). Responses to CRD were significantly increased for at least 2 wk after intracolonic instillation of 30% ethanol. This visceral (colon) hyperalgesia was accompanied by a parallel increase in MPO activity. This model is clearly useful for pharmacologic studies because responses to CRD can be readily quantified, can be repeatedly measured in the same animal to determine time course of drug action, and are determined in unanesthetized subjects. The 129S6 and C57BL6 mouse strains were chosen for study because they are the most common background strains for the creation of knockout mice. In addition, we tested the F1 cross, the B6.129 mouse strain, and to our knowledge, this is the first experimental report on these mice in the pain literature. These mice were constructed as a control for the “hitchhiking donor gene confound” because their genotype is closer to that of knockout mice than even those of the progenitor strains. Preliminary studies show that this model of visceral nociception will be useful for testing knockout mice.

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