Quantitative assessment and characterization
of visceral nociception and hyperalgesia in the mouse

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Abstract

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) that was graded to stimulus intensity. The magnitude of the 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, the VMR was reduced dose-dependently by morphine (1-10 mg/kg) and by the kappa opioid agonist U69,593 (0.2-2 mg/kg), although U69,593 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.
Introduction

Pain is the most common reason that 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. Further, there is growing evidence that the mechanisms of visceral and cutaneous pain are different (Gebhart and Ness 1991, Cervero and Laird 1999). Certainly, the characteristics of these pains are different; visceral pain is diffuse, poorly localized, and referred to overlying structures (see Ness and Gebhart 1990, and Cervero 1994 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 non-human 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 (Ness and Gebhart 1988, 1990, Sengupta and Gebhart 1994).

The availability of genetically modified mice has increased interest in the mouse as a non-human 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 (i.p.) 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) 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 (Kamp et al. 2001b, Kamp and Gebhart 2001).
Methods

Animals. Adult male 129S6/SvEvTac mice (129S6; 20-30 g, 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 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, The University of Iowa.

Surgical preparation. Mice were anesthetized with pentobarbital sodium 75 mg/kg i.p. (Abbott Laboratories, North Chicago, IL) or a 7:1 combination of ketamine (87.5-175 mg/kg) and xylazine (12.5-25 mg/kg) i.p. (Abbott Laboratories and Phoenix Pharmaceutical Inc., St. Joseph, MO, respectively). Mice anesthetized with ketamine/xylazine were pretreated with atropine (10-60 min before ketamine/xylazine, 40 µg/kg i.p., Fujisawa USA, Inc., Deerfield, IL). Electrodes (Teflon-coated stainless steel wire, 5-10 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 (PE10, 5 cm) was placed at the dorsum of the neck in some mice. The incisions were closed in layers with 5-0 silk. Following surgery, 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 a minimum of three days before testing.
Spinal transection. After generating a baseline stimulus-response function (as described below), mice were given 1 mL of 5% dextrose in normal saline (i.p.) for hemodynamic support. Approximately one hour later, mice were deeply anesthetized with pentobarbital sodium (as above). 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 (approximately 5 x 15 mm). Ice was applied to the exposed vertebra for 5 min to minimize bleeding. A laminectomy was performed with #5 Dumont Biologie forceps and the spinal cord was again iced. Using microscissors, the spinal cord and dura were cut twice approximately 1 mm apart. The resulting piece of spinal cord was removed and a small (~1x1 mm) square of Gelfoam® (The Upjohn Company, Kalamazoo, MI) soaked in epinephrine (1:1000; American Regin Laboratories, Shirley, NY) was inserted into the gap. An ~5 x 15 mm piece of epinephrine-soaked Gelfoam was also laid atop the spinal cord in the cavity left by the laminectomy and 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 hours after surgery, their bladders were expressed and mice were given an additional 1 mL of 5% dextrose in saline (i.p.) 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 T9/10. Colon afferents in the mouse enter in dorsal roots caudal to T9/T10 (Robinson et al. 2001).

CRD balloons. Distension balloons were prepared by stretching a small square (~3 x 3 cm) of thin (~15 µm) polyethylene plastic over a PVC 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 PEFE-24 thin wall tubing (Cole-Palmer Instrument Company, 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). Prior to 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 (2mm dia). One wall of the sheath was cut lengthwise to accommodate the girth of the balloon and silk suture. We gratefully acknowledge the advice and instruction of Alfred Bayati, AstraZeneca, Mölndal, Sweden, in fabrication of the distension balloons.

On the day of testing, mice were briefly anesthetized with halothane (1-5% in 100% O₂ @ 2 L/min; ≤ 5 min; Halocarbon Laboratories, River Edge, NJ). The sheath was lubricated with Surgilube (E. Fougera and Co., Melville, NY) and inserted intra-anally until the silk tie was 5 mm inside the rectum (total insertion distance, 25 mm). The sheath was removed and the tubing taped to the base of the tail to prevent displacement. Mice were placed in restraint devices (see below) while still sedated and allowed to recover/acclimate for a minimum of 30 min prior to 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 and Co., 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 x 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 approximately 25 mm, which holds a 15-28 g mouse. For larger mice, similar devices were constructed from translucent plastic (30, 33, and 40 mm internal diameter).

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 x 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 visceromotor response (VMR), was the behavioral response quantified. The EMG signal was filtered, amplified, and recorded as has been described for rats (e.g., Coutinho et al., 1996). Briefly, the balloon was connected to a pressure control device (Bioengineering, The University of Iowa, Iowa City, IA) that regulated inflation of the balloon. Each distension trial lasted 40 sec and EMG activity was quantified during the 10 sec before distension, the 20 sec during distension, and the 10 sec after distension.

**Colon inflammation.** Under brief halothane anesthesia (as above), an inflammogen 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 inflammogen/irritant (see below) 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 GI dysfunction (e.g., diarrhea, bleeding, weight loss); none were observed.

Solutions of 30% ethanol (v/v) and 0.6% acetic acid (v/v, from glacial acidic acid; Fisher) were prepared in sterile, preservative-free saline (Abbott) that had been adjusted to pH 7.2 prior to use. Solutions of 0.003% capsaicin (w/v) and 0.25% mustard oil (v/v; both from Sigma Chemical Co., St. Louis, MO) were prepared in 5% dextrose in water (w/v, pH 7.2; Baxter Healthcare Corporation, 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 visceromotor response to CRD, one group of mice was tested 3 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 hours after surgery (described above).

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 hr following 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 preformed once prior to any treatment to establish a baseline. Immediately following the last distension, an inflammogen/irritant was instilled into the colon (as described above). Graded CRD was repeated 3 and 24 hr and 3, 5, 7 and 14 days following 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 hr following this sham treatment. Twenty-four hours later, the colon was removed for myeloperoxidase assay (described below) for comparison with mice receiving an inflammogen.
Drug effects. On the day of testing, five phasic distensions (45 mmHg, 20 sec) at 5 min intervals were given to establish a baseline. Immediately following the fifth distension, drug or vehicle was administered subcutaneously (s.c.) via the chronically placed catheter. CRD (45 mmHg, 20 sec) 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 sec) were determined as above, after which 30% ethanol was instilled into the colon (as above). Twenty-four hours later, five distensions (45 mmHg, 20 sec, 5 min intervals) were repeated to quantify the hyperalgesia. Immediately following the fifth distension, morphine (or saline vehicle) was administered s.c. via the chronically placed catheter. CRD (45 mmHg, 20 sec) 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/10 g body weight. The drugs used in this study were the kappa opioid receptor agonist U69,593 (U69; Sigma), the mu opioid receptor agonist morphine sulfate (Spectrum Quality Products, Inc., Gardena, CA) and the benzodiazepine diazepam (Sigma). Morphine was prepared in sterile, preservative-free saline (Abbott). U69 was dissolved in 4.5% 2-hydroxypropyl-β-cyclodextrin (w/v; Sigma) in sterile, preservative-free saline. Diazepam was dissolved in 50% propylene glycol (v/v; Fisher) in sterile, preservative-free saline.

Myeloperoxidase assay. The myeloperoxidase (MPO) assay was used to quantify colon inflammation. The procedure was preformed as developed by Krawisz et al. 1984. Briefly, mice were killed by an overdose of pentobarbital and the distal colon removed via laparotomy. The fresh tissue was suspended in hexadecyltrimethylammonium bromide (HTAB, a detergent; Sigma), minced with scissors, homogenized/sonicated, and freeze-thawed three times. The
tissue suspensions were centrifuged and the supernatant 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 0.0005% hydrogen peroxide (v/v) 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. Colons were removed for this assay 3 or 24 hr or 3, 5, 7 or 14 days following 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, i.p.; Abbott) and 3 cm of distal colon removed. Tissue was rinsed with saline and immediately placed into cold zinc-formalin fixative (Labco, Louisville, KY). Using conventional techniques, fixed tissue was paraffin embedded, cut in longitudinal sections with a microtome, and stained with hematoxylin and eosin (H&E).

Data analysis. EMG activity was collected at 250 Hz using the CED 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 greater than 300 mV were counted in 10 sec bins off-line by a Spike2 script. The first 10 sec of the distension period were used for analysis because responses were most robust and reproducible during the first half of the distention 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 sec data analysis window) or percentage of control (% control)
where 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 \( \text{AUC} = x_{15}y_{15} + (x_{30} - x_{15})y_{30} + (x_{45} - x_{30})y_{45} + (x_{60} - x_{45})y_{60} \) and \( x_n \) and \( y_n \) refer 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 where the mean of the five baseline (pre-drug) 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 post-drug response from the mean vehicle response (for that strain and time point) plotted against time (AUC=\( \Sigma \) change in response x 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 CRD with repetitive distension (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 visceromotor response to CRD models visceral pain. In mice, like rats and humans, CRD produced robust contractions of the abdominal musculature graded to stimulus intensity (figure 1A). The lowest stimulus intensity (15 mmHg) generally did not produce a response that was significantly greater than background activity. At 30 mmHg CRD, the visceromotor response (VMR) was typically twice the magnitude of background activity; pressures of 45 and 60 mmHg reliably produced robust responses (see inset, figure 1A, for 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 figure 1A, the responses of these mice to graded CRD are virtually identical when tested one week apart, revealing that neither post-surgical 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 sec immediately prior to each distension), shown by the shaded box, 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 (Ness and Gebhart 1988), is a spinobulbospinal pseudoadfective reflex (Sherrington 1906), mice were tested before and 18 hr after T9-10 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 pre-transection 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 sec prior to each distension) did not differ between strains or gender \( (F_{3,27}=0.35, p=0.789; \text{one-way ANOVA}) \) and therefore was pooled (shaded bar in figure 1B). Figure 1B shows the stimulus-response functions of male 129S6, female 129S6, C57BL6 and B6.129 mice. The AUC presentation (inset in figure 1B) 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; response magnitude 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 (Mogil and Wilson 1997, reviewed by Lariviere et al. 2001).

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 (figure 2A). In female 129S6 mice, 3 mg/kg morphine reduced responses to a similar extent as in male 129S6 mice (figure 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 (Kest et al. 1999). Similarly, morphine significantly reduced responses to noxious CRD in C57BL6 and B6.129 mice (figure 2B). The
The effect of morphine did not differ between strains of mice (F\textsubscript{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 kappa opioid receptor agonists (Ramabadran 1984, Ikeda et al. 1999, Wilson et al. 2000). Also, it has been suggested that kappa opioid receptors are more strongly linked with visceral pain than cutaneous pain (Black and Trevethick 1998, Simonin et al. 1998). For these reasons, we tested the effect of the kappa opioid receptor agonist U69 in male and female 129S6 and male C57BL6 and B6.129 mice. Figure 3A shows the time-course of action of U69 in male 129S6 mice. The AUC presentation in figure 3B compares dose-dependent effects of U69 in all groups of mice tested. U69 was significantly more potent in female 129S6 mice than in male 129S6 mice (p<0.05, two-way ANOVA). In addition, the effect of U69 in male B6.129 was significantly greater than in male 129S6 and C57BL6 mice (p<0.05, two-way ANOVA). In C57BL6 mice, greater doses of U69 (2 and 20 mg/kg s.c.) were required to produce a significant antinociception; neither motor nor sedative effects were observed at 20 mg/kg U69 (s.c).

Because opioids can have anxiolytic as well as antinociceptive properties, we confirmed that reduction of visceromotor responses 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 visceromotor responses to CRD (to 88.5 ± 7.8% and 86.5 ± 17.1% of baseline, respectively, 15 min after vehicle or diazepam;
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 hr after treatment (Laird et al. 2000). Therefore, we tested mice before (data not shown) and 1 hr following anesthesia alone or intracolonic instillation of 0.6% acetic acid or saline (figure 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 inflammmogens/irritants over longer time-courses. Mice were tested before and 1, 2, 4, 6, 8, 12, and 24 hr following 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 (figure 4B). Mustard oil, capsaicin (figure 4B), dextrose in water, and saline (not shown) all failed to significantly affect responses to CRD over the time course studied ($p>0.05$ vs baseline, two-way ANOVA for factor time within treatment).

Accordingly, we further characterized the hyperalgesia produced by 30% ethanol. Stimulus-response functions were recorded before and 3 and 24 hr and 3, 5, 7, and 14 days after intracolonic instillation of saline or 30% ethanol in saline (figure 5A 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 factors time and treatment). EMG
recordings from the same mouse before and 24 h after instillation of 30% ethanol show typical enhancement of the response to 60 mmHg CRD (figure 5B inset). In untreated mice, EMG activity typically increases immediately (<0.5 sec) following the onset of CRD and lasts 2-5 sec, followed by a period of enhanced activity that persists until termination of the stimulus (also see figure 1A inset). As illustrated, EMG recordings from ethanol-treated mice show significantly enhanced background activity (recorded in the 10 sec prior to distension) and CRD-evoked visceromotor responses. Activity ceases abruptly upon termination of the distending stimulus, and is less than the pre-distension background activity. This is consistent with response characteristics of pelvic nerve afferent fibers upon termination of CRD in the rat (Sengupta and Gebhart 1994).

Colon inflammation. Myeloperoxidase (MPO) activity, an index of inflammation, was assayed in the colons of mice following no treatment (naive), 1 hr 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 units/g, p=0.82 and 0.69, respectively, one-way ANOVA) nor did repetitive CRD (anesthesia only, 1 hr; 1.4 ± 0.5 units/g, p=0.102).

The colons of separate groups of mice were tested for MPO activity following intracolonic saline or 30% ethanol treatment at the same time points tested for behavioral hyperalgesia (3 and 24 hr, and 3, 5, 7, and 14 days). Colon MPO activity was significantly
enhanced (vs saline) at all time points tested (except 14 days) following ethanol treatment (figure 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 hrs) 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 was irregular crypt depth, mucosal edema (especially in the lamina propria) with neutrophil infiltration, and atrophy of the muscularis externa (figure 6).

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

The present study demonstrates that visceromotor responses 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 as the 129S6 and C57BL6 strains of mice are among the least sensitive strains to visceral/somatic noxious chemical stimuli (writhing tests; Mogil et al. 1999). In addition, the visceromotor response to CRD was reduced dose-dependently by opioid receptor agonists (morphine and U69,593), 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 (e.g., Mobil and Wilson 1997, Elmer et al. 1998). Accordingly, CRD in the mouse represents a reliable model of acute visceral nociception. Further, 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 (e.g., Sengupta et al. 1999, Friedrich and Gebhart 2000), 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 irritant (e.g., a dilute solution of acetic acid; Koster et al. 1959, Taber et al. 1969). Similar to CRD, chemical irritants produce contractions of the abdominal musculature, but affect unknown somatic and visceral structures. Additionally, hollow organ distension does, whereas chemically induced writhing does not, reproduce a natural visceral stimulus. Second, CRD is an easily controlled stimulus of predetermined, short duration. I.p. injection of chemical irritants produces writhes that are typically counted for 30 min after
injection (e.g., Martinez et al. 1999) but can persist for 6 hours (Koster et al. 1959). Given available alternatives, institutional review committees are increasingly hesitant to approve the use of an uncontrolled noxious stimulus.

Third, because constant pressure CRD is easily controlled by the experimenter, it can be reliably repeated. This has a clear advantage to pharmacologic studies as it allows the testing of both the magnitude and duration of drug effect. Moreover, a range of stimulus 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 (Laird et al. 2001a). Like i.p. injection of chemical irritants, mice are tested only once and humanely 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 mice 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 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 (Woodrow et al. 1972, Robin et al. 1987, Walker and Carmody 1998), although this is not a universal finding (Neri and Agazzani 1984, Lautenbacher and Strian 1991) and may depend on stimulus modality.
(Lautenbacher and Rollman 1993, Rollman and Lautenbacher 2001). 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 kappa opioid receptor agonists are more efficacious and/or potent in females than males in both rodents (e.g., Binder et al. 2000) and humans (e.g., Gear et al. 1996, 1999). Thus, we anticipated that antinociception produced by the kappa opioid receptor agonist U69,593 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 pre-drug responses were normalized (to remove this possible confound), U69,593 produced a more robust antinociception in female than male 129S6 mice.

The kappa opioid receptor has been considered especially relevant to visceral pain primarily because kappa opioid receptor knockout mice writh more than their wild type littersmates after i.p. injection of acetic acid (Simonin et al. 1998, Black and Trevethick 1998) and kappa opioid receptor agonists uniformly reduce visceral nociception (writhing or CRD; e.g., Tyers 1980, Schmauss et al. 1983, Ward and Takemori 1983, Barber et al. 1994, Broadbear et al. 1994, Su et al. 1997, Burton and Gebhart 1998, Sengupta et al. 1999, Wells et al. 2001). In contrast, studies of kappa opioid receptor agonist effects in cutaneous models of nociception report an antinociceptive effect (Tyers 1980, Kavaliers and Choleris 1997, Simonin et al. 1998, Binder et al. 2000), no effect (Schmauss et al. 1983, Ward and Takemori 1983, Barber et al. 1994) or even a hyperalgesic effect (Ramabadran 1984). In studies that have directly compared the antinociceptive effect of kappa agonists in models of visceral and cutaneous nociception, ED$_{50}$ values were not different (e.g., Tyers 1980, Ward and Takemori 1983, Barber et al. 1994). While we did not test the effect of U69,593 in a cutaneous pain model, the effective dose range
Colon inflammation. Exposure of the GI tract to ethanol has been used in a variety of animal species to produce inflammation and ulceration of the stomach, duodenum, ileum, or colon (e.g., Davies et al. 1997, Lu et al. 1997, Franco and Doria 1998, Myers et al. 1998, Boku et al. 2001). When neutrophil infiltration into the colon was examined at various times after intracolonic instillation of 30% ethanol, MPO activity was maximal at 24 hr and returned to baseline at two weeks. Histologic examination of colons 24 hr 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; Ritche 1973) and has since been reported for all functional bowel disorders (see Mayer and Gebhart 1994 for review). Similarly, inflammatory bowel disorders and colitis are often associated with discomfort and pain (Spriggs et al. 1951, Snape et al. 1980). 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 (Coutinho et al. 1996), sensitization of pelvic nerve colon afferents (Coutinho et al. 2000), and activation of silent fibers (Gebhart 1999). Centrally, spinal NMDA (Kolhekar and Gebhart 1996, Coutinho et al. 1996, Zhai and Traub 1999, Ji and Traub 2001), neurokinin (Julia et al. 1999, Kamp et al. 2001a, Laird et al. 2001b), opioid (Ness and Gebhart 1988) and CCK\textsubscript{B} receptors (Friedrich and Gebhart 2000) and
nitric oxide (Coutinho and Gebhart 1999) have been shown to modulate behavioral or electrophysiologic responses to noxious colon distension.

In summary, the visceromotor response to CRD is a reproducible, supraspinally-mediated contraction of the abdominal musculature that is graded to stimulus intensity and attenuated by opioids (morphine and U69,593). Responses to CRD were significantly increased for at least two weeks 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” as 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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References


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Figure 1: The visceromotor response (VMR) to colorectal distension (CRD) is reproducible, reliable, graded to stimulus intensity, and reveals strain and gender differences. A: The VMR to graded CRD (15-60 mmHg, 20 sec) was recorded 3 and 10 days following electromyographic (EMG) electrode implantation (n=6). Responses were not different on days 3 and 10 (two-way repeated measures ANOVA, F1,15=0.0274, p=0.875). Background (resting) activity was not significantly different between groups (p=0.795) and data were pooled for presentation (shaded bar). The inset shows a representative EMG record before, during and after 60 mmHg CRD. B: The VMR to graded CRD was recorded from adult male 129S6 (n=10), C57BL6 (n=5), B6.129 (n=8), and female 129S6 mice (n=8). Again, background activity was not significantly different between groups (p=0.789), so data were pooled for presentation (shaded bar). The inset is an area under the curve (AUC) presentation of the stimulus-response functions in B (see Methods). *p<0.05 vs male 129S6 and B6.129 (one-way ANOVA with the Bonferroni correction for multiple comparisons).

Figure 2: Effects of morphine on the visceromotor response (VMR) to colorectal distension (CRD). A: The VMR to CRD (45 mmHg, 20 sec) was recorded before and for one hour after morphine (1, 3 or 10 mg/kg s.c.) or vehicle (saline; n=7-10/treatment group) in male 129S6 mice. The AUC for each dose was determined to construct dose-response functions. B: Summary of dose-dependent effects of morphine (AUC analysis of the time courses of effect; see Methods). There were no differences in the effect of morphine between mouse strains (two-way ANOVA with the Bonferroni correction for multiple comparisons).
Figure 3: Effects of U69,593 on the visceromotor response (VMR) to colorectal distension (CRD).  A: The VMR to CRD (45 mmHg, 20 sec) was recorded before and for one hour after U69,593 (0.02, 0.2, 2 or 20 mg/kg s.c.) or vehicle (saline; n=7-8/treatment group) in male 129S6 mice.  The AUC for each dose was determined to construct dose-response functions.  B: Summary of dose-dependent effects of U69,593 (AUC analysis of the time courses of effect; see Methods).  The rank order of potency of U69,593 in the strains tested was: B6.129=female 129S6>male 129S6>C57BL6.

Figure 4: Effects of intracolonic treatment on the visceromotor response (VMR) to colorectal distension (CRD).  A: The VMR to graded CRD (15-60 mmHg, 20 sec each) was recorded before (not shown) and 1 hour 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 (two-way ANOVA, F3,60=1.227, p=0.308; n=3-6/treatment group).  B: The VMR to CRD (60 mmHg, 20 sec) was recorded before and 1-24 hr after intracolonic instillation of 30% ethanol, 0.25% mustard oil, or 0.003% capsaicin.  Only 30% ethanol enhanced responses to CRD.

Figure 5: Effect of intracolonic instillation of 30% ethanol on the visceromotor response (VMR) to graded colorectal distension (CRD).  A&B: The VMR to graded CRD (15-60 mmHg, 20 sec each) was recorded before (baseline) and 3 hr 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, two-way repeated measures ANOVA).  In ethanol-treated mice, responses were significantly enhanced (vs. baseline) at all times from 3 hr to 14 d following ethanol treatment (two-way repeated measures ANOVA).  The inset in B shows representative EMG records of the VMR to 60 mmHg CRD recorded from the same mouse before and 24 h following intracolonic instillation of 30% ethanol.  C: Quantification of colon inflammation by
myeloperoxidase (MPO) activity 3 h to 14 d following saline- or ethanol-treatment. MPO activity was significantly increased from 3 h to 7 d following ethanol-treatment (vs saline-treatment; n=6-12). The mean MPO activity of naive (untreated) mice is illustrated for comparison (dotted line). The ANOVA for factors treatment and time was significant (p<0.001); * p<0.05 for saline-vs ethanol-treatment at that time point.

Figure 6: Photomicrographs of colons from naive, saline-, and ethanol-treated mice, A, B, and C, respectively. Examples were taken 24 h following saline- or ethanol-treatment.

Figure 7: The potency of morphine is increased in male 129S6 mice with visceral hyperalgesia. A: The VMR to CRD (45 mmHg, 20 sec) was recorded before and 24 hr after intracolonic instillation of 30% ethanol (EtOH), after which morphine (1, 3 or 10 mg/kg s.c.) or vehicle (saline; n=7-8/treatment group) was given and responses to CRD were recorded over the next 60 min. The AUC for each dose (relative to vehicle-treated mice) was determined to construct a dose-response function. B: Comparison of dose-dependent effects of morphine in EtOH-treated mice and male 129S6 mice with no intracolonic pre-treatment (naïve, taken from figure 2B). Morphine was significantly more potent in EtOH-treated mice (p<0.001, two-way ANOVA for factor treatment).