Repeated exposure to water avoidance stress in rats: a new model for sustained visceral hyperalgesia

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Repeated exposure to water avoidance stress in rats: a new model for sustained visceral hyperalgesia. Am J Physiol Gastrointest Liver Physiol 289: G42–G53, 2005. First published March 3, 2005; doi:10.1152/ajpgi.00500.2004.—Chronic stress plays an important role in the development and exacerbation of symptoms in functional gastrointestinal disorders. To better understand the mechanisms underlying this relationship, we aimed to characterize changes in visceral and somatic nociception, colonic motility, anxiety-related behavior, and mucosal immune activation in rats exposed to 10 days of chronic psychological stress. Male Wistar rats were submitted daily to either 1-h water avoidance (WA) stress or sham WA for 10 consecutive days. The visceromotor response to colorectal distension, thermal somatic nociception, and behavioral responses to an open field test were measured at baseline and after chronic WA. Fecal pellets were counted after each WA stress or sham WA session as a measure of stress-induced colonic motility. Colonic samples were collected from both groups and evaluated for structural changes and neutrophil infiltration, mast cell number by immunohistochemistry, and cytokine expression by quantitative RT-PCR. Rats exposed to chronic WA (but not sham stress) developed persistent visceral hyperalgesia, whereas only transient changes in somatic nociception were observed. Chronically stressed rats also exhibited anxiety-like behaviors, enhanced fecal pellet excretion, and small but significant increases in the mast cell numbers and the expression of IL-1β and IFN-γ. Visceral hyperalgesia following chronic stress persisted for at least a month. Chronic psychological stress in rats results in a robust and long-lasting alteration of visceral, but not somatic nociception. Visceral hyperalgesia is associated with other behavioral manifestations of stress sensitization but was only associated with minor colonic immune activation arguing against a primary role of mucosal immune activation in the maintenance of this phenomenon.

chronic psychological stress; visceral nociception

STRESS-INDUCED VISCERAL HYPERALGESIA (SIVH) has been suggested as an important pathophysiological component underlying the cardinal symptoms of irritable bowel syndrome (IBS) (28). Whereas symptom exacerbation following chronic stress is a common finding in patients with IBS (4) as well as inflammatory bowel disease (22), the majority of studies in rodents have focused on transient changes in nociceptive responses to acute experimental stress (18, 19). Different forms of SIVH can be distinguished in animal models with a varying degree of face validity for the human disorder. The ability of an acute stressor to produce visceral hyperalgesia appears to be dependent on the type of stressor, as well as on several vulnerability factors, including genetic and perinatal environment (27). For example, in Wistar rats, a strain with high-stress responsiveness, acute partial restraint stress leads to an immediate, but transient increase in visceral sensitivity to colorectal distension (19). In the same rat strain, we recently reported the development of delayed visceral hyperalgesia 24 h following an acute psychological stressor (40). This effect was transient, and visceral nociception returned to baseline within a week (I. Schwetz, unpublished observations). Different peripheral and central pathophysiological mechanisms have been implicated in the development of SIVH (27). These include mucosal mast cell activation (19), upregulation of corticotrophin releasing factor (CRF)/CRF receptor type 1 (CRFR1) and substance P/neurokinin-1 receptor ligand/receptor systems (40), defective activation of descending opioidergic pathways (11), and altered expression of thalamic nuclei (38). In summary, the type and timing of the stressor (acute vs. chronic, psychological vs. physical, early life vs. adult) might be a key factor in the determination of the pathways engaged, which in turn, will produce transient or sustained modulation of nociceptive responses.

In the current study, we wanted to maximize face validity of the rodent model for the well-documented clinical observation of sustained visceral hyperalgesia following a chronic psychological stressor in IBS patients. Based on our earlier observations of delayed SIVH following a one-time water avoidance (WA) stress, we hypothesized that chronic WA stress might induce a more sustained form of visceral hyperalgesia. In addition, recently published data suggested that chronic psychological stress in some rat strains may be an initiating factor to intestinal inflammation by impairing epithelial defenses against luminal bacteria (42). Therefore, we hypothesized that changes in visceral nociception following chronic stress may be related to the sensitization of visceral afferent pathways through inflammatory mediators released by chronic stress exposure. By evaluating the effect of repeated WA stress over...
a period of 10 days on behavior, colonic motility, and visceral and somatic sensitivity, we aimed to answer the following questions. Does chronic exposure to WA stress result in a generalized and long-lasting modulation of visceral and somatic nociception? Does chronic WA produce more generalized stress sensitization in terms of colonic autonomic regulation and anxiety-like behavior? Is colonic mucosal immune activation associated with the expression of visceral hyperalgesia? We report the development of a sustained visceral hyperalgesia following repeated exposure to psychological stress. The early phase of increased visceral sensitivity was associated with a transient somatic analgesia, enhanced colonic motility, and increased anxiety-related behaviors. These findings were accompanied by evidence for low-grade mucosal immune activation in the colon. Parts of these results have previously been reported in abstract form (7).

**MATERIALS AND METHODS**

### Animals and Procedures

**Animals.** Adult male Wistar rats (200–250 g) were purchased from Harlan (Indianapolis, IN). Animals were maintained on a normal light-dark cycle, housed in pairs, and provided with food and water ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee at the Greater Los Angeles Veterans Affairs Healthcare System, Los Angeles.

**Surgery.** Adult male rats were deeply anesthetized with pentobarbital sodium (45 mg/kg, Nembutal; Abbott Labs, North Chicago, IL) administered intraperitoneally. Electrodes (Teflon-coated stainless steel wire; AstraZeneca, Molndal, Sweden) were stitched into the external oblique musculature, just superior to the inguinal ligament, for electromyographic (EMG) recordings as previously described (10). Electrode leads were then tunneled subcutaneously and externalized laterally for future access. Wounds were closed in layers with appropriate sutures. Following surgery, rats were housed in pairs and allowed to recover for at least 7 days. Wounds were tested for tenderness to ensure complete recovery from surgery before testing.

**Assessment and quantification of the visceromotor response to colorectal distension.** The visceral stimulus employed was distension of the descending colon and rectum (CRD) using a well-established and validated method for the evaluation and quantification of visceral nociceptive responses (34). Briefly, under light Halothane anesthesia, a flexible latex balloon (6 cm) was inserted intra-anally (after the distal part of the rectum was gently cleared by massage) such that its end was 1 cm proximal to the anus. Once recovered from anesthesia, animals equipped with the balloon were placed in a Plexiglas cylinder for 30 min before the CRD procedure was initiated. The CRD procedure consisted of two series of phasic CRD to constant pressures of 10, 20, 40, and 60 mmHg (20-s duration; 4-min interstimulus interval). The visceromotor response (VMR) to CRD was quantified by measuring EMG activity in the external oblique musculature. EMG activity was recorded 20 s before (baseline), 20 s during, and 20 s after termination of CRD. The EMG activity was rectified, and the increase in the area under the curve (AUC) of EMG activity during a function of pressure of distension. The AUC reflects the overall response during the course of the CRD test (from 10 to 60 mmHg pressure). The percent change of AUC from baseline indicates the degree of increase and/or decrease of the response at day 10 from baseline. In the following, we will use the term EMG referring to the VMR to CRD. Animals showing an EMG signal/noise ratio <0.05 were excluded from the study.

**Assessment of somatic pain sensitivity.** Somatic pain sensitivity was assessed using the thermal nociceptive tail-flick reflex. Animals were placed in Plexiglas cylinders in which they were awake and loosely restrained. Following a 45-min acclimation period, the tail-flick latency (TFL) was quantified by exposing the ventral surface of the tail to radiant heat and recording the time taken to withdraw the tail from the noxious thermal stimulus. Routinely, four TFL values were obtained at 5-min intervals for each animal, and the mean value was designated as TFL. A 10-s cutoff latency was used to avoid tissue damage.

**WA stress protocol.** The test apparatus consisted of a Plexiglas tank (45 cm length × 25 cm width × 25 cm height) with a block (10 × 8 × 8 cm) affixed to the center of the floor. The tank was filled with fresh room temperature water (25°C) to within 1 cm of the top of the block. The animals were placed on the block for a period of 1 h daily for 10 consecutive days corresponding to the chronic stress protocol (WA). Sham WA consisted of placing the rats similarly for 1 h daily for 10 days on the same platform in a waterless container. This well-characterized test represents a potent psychological stressor with large elevations of ACTH and corticosterone within 30 min (30).

**Measurement of fecal pellet output and rat weight.** We used a validated and previously described procedure to estimate autonomic regulation of distal colonic motility (26). Fecal pellets found in the tank were counted at the end of each 1-h WA stress or sham WA session. Rats were weighed every day before exposure to WA stress or sham WA to assess weight change from baseline.

**Open-field apparatus.** We used a validated and previously reported test to quantitate anxiety-like behavior of rats (14). The open-field arena was a white, translucent polyethylene box (model CB; Iris USA, Pleasant Prairies, WI), with internal dimensions of 69 cm long × 34 cm wide × 30 cm high. Black electrical tape attached to the underside of the floor marked eight squares (17 × 17 cm) visible from above. The arena was situated on a table in the center of a dimly lit room. Three lamps, each containing a single 100-W white light bulb, were positioned at one end of the table. One lamp was positioned 14 cm from the east or west walls, and 15 cm from the north wall. All three lamps were situated 18 cm above the base of the arena. When lit, the bulbs flooded one end of the open field with light, creating an illumination gradient across the arena. The experimenter could manipulate these lights from the adjacent room by using a switch. A 25-W red light bulb suspended from the ceiling illuminated the room. A fan provided background noise (65 dB, A scale). A camera was suspended from the ceiling directly above the center of the open field and connected to a monitor and VCR located in the adjacent room. Scoring was done after completion of the test.

**Quantitative RT-PCR for colonic cytokine message detection.** Full thickness segments of distal colonic tissue samples were preserved in RNA-later (Ambion) at −20°C until use. Total RNA was isolated from 100-mg samples of tissue using the TRizol reagent kit (GIBCO Ultra Pure) and reverse-transcribed with Superscript II RT (GIBCO-BRL, Gaithersburg, MD) using random hexamers and following the manufacturer’s instructions. Quantitative real-time PCR was performed using an Applied Biosystems GeneAmp 5700 sequence detection system and SYBR Green reagents (Applied Biosystems, Foster City, CA). The PCR program was: stage 1, 50°C, 2 min; stage 2, 95°C, 10 min; stage 3, 40 cycles, each consisting of 15 s at 95°C and 60 s at 60°C; ending at 25°C. Primer pairs were designed to cross intron-exon boundaries as predicted from the rat Genome Browser at University of California, Santa Cruz, CA (Jun G. assembly; http://genome.ucsc.edu/) with specific primers. The sequences ≤116 bp. The sequences were (forward/reverse): 5'-TGAAGCAGCTATGGGCAACTG-3'/5'-ACCTTTGGGTGTCGTTGT-CAGC-3' for rat IL-1β; 5'-AGGAAACACACGACACGCACT-3'/5'-GGAGCTTACATGGGGAGT TT-3' for IL-2; 5'-TCCAGAAATAA-CAAAAGAATGTGAT-3'/5'-GGTAGAAGGGGAACTCCAG- AAGAC-3' for IL-6; 5'-TTGGAATTCCTGTGAGAAG-3'/5'-TTT CACCTGTTCAAGGCT-3' for IL-10; 5'-GCATTTACAGCA CAAATCA-3'/5'-GGAGCTTCTGTCAGAGT-3' for IL-12 p35; 5'-GCCACACCAGCTTCTCTGTCGTGAGG-
GTCTGG-3’ for TNF-α; 5’-CGAATCGACCTGACTA-3’/5’-CTGGATCTGTGGGGTTCA-3’ for IFN-γ; and 5’-CCAGAGGTGCGTGGACATCA-5’/5’TGGGACCCTCTATGGA-3’ for the ribosomal phosphoprotein, 36B4. Primer pairs were optimized by using stock RNA prepared from inflamed rat colon, and the size of the predicted PCR product was verified by gel electrophoresis. Detection of PCR products was monitored by the increase in fluorescence caused by the binding of SYBR Green to double-stranded DNA. To verify the homogenous nature of the PCR product, melting-point determinations were evaluated at the end of each reaction. The change in cycle threshold (ΔCt) method was used to compare mRNA expression of each target gene relative to expression of a reference “house-keeping” gene (see ABI PRISM 7700 Sequence Detection System User Bulletin No. 2). We used the acidic ribosomal phosphoprotein 36B4 as the reference gene. ACt indicates the difference between the number of cycles necessary to just detect linear amplification of the PCR products for each cytokine and that of the 36B4. Data were expressed as 2^{−ΔCt} to give a linear estimate of the amount of target mRNA present in the tissue relative to the 36B4. The average expression levels for each cytokine in the chronic WA-stressed rats were normalized to the average of those in the sham WA-treated animals to give the fold change in expression expressed as 2^{−ΔΔCt}.

Histological examination of colonic samples. Samples of distal colon were fixed in 10% formalin, embedded in paraffin, sectioned at 5-μm thickness, and stained with hematoxylin and eosin. Sections were imaged with an Axioskop 2 Plus light microscope (Carl Zeiss, Göttingen, Germany) coupled with a Hamamatsu Digital Camera (Hamamatsu Photonics KK, Hamamatsu City, Japan). Polymorphonuclear cells were counted in the mucosa and submucosa area at three noncontiguous locations of each section with a micrometer grid (247 × 247 μm). The average thickness of the mucosa plus the submucosa was measured across two noncontiguous locations of each section with the Simple PCI imaging software (Comiplx, Cranberry Township, PA).

Immunohistochemistry for rat mast cell protease. Immunohistochemistry was performed on paraffin-embedded sections using an avidin-biotin-peroxidase technique. Endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in methanol. Tissue sections were incubated for 30 min at room temperature with 3% normal goat serum before incubation with primary mouse monoclonal antibodies to rat mast cell protease (RMCP II; Moredu Scientific, Scotland, UK) diluted 1:500 in PBS containing 0.3% Triton X-100 for 24 h at 4°C. Bound antibody was detected with biotin-substance P-conjugated goat anti-mouse IgG secondary antibody (1:500; Jackson ImmunoResearch, West Grove, PA). Sections were processed using a standard biotin-avidin-horseradish peroxidase methodology. Diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen, and immunoreactivity appeared as brown staining. Immunohistochemical controls were routinely performed following the same procedures, except that PBS was substituted for the primary antibody. All tissue sections were washed three times for 10 min in PBS before the addition of subsequent antibodies. Stained sections were counterstained with hematoxylin and eosin and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Sections were observed with an Axioskop 2 Plus light microscope (Carl Zeiss, Göttingen, Germany). Cells positive for RMCP II immunostaining (mast cells) were counted in the mucosa and the submucosa area at three noncontiguous locations in each section with a micrometer grid (247 × 247 μm).

Experimental Design

Effect of chronic stress on nociception and autonomic response. All rats tested for somatic and visceral nociception were habituated to the test environment for 3 days before the start of the experiments, including the day of baseline testing. Each day, animals were transported to the testing room, and placed for 30 min in the Plexiglas cylinders used in the TFL and CRD experiments.

Somatic nociception testing. Two groups of 16 rats were used for the TFL test. On day 0, animals were placed in Plexiglas cylinders in which they were awake and loosely restrained. Following acclimation to this condition, the TFL was measured to evaluate the baseline somatic pain sensitivity. From day 1 to day 10, rats were submitted daily to either 1-h WA for the stress group, or to 1-h sham WA for the control group. In both groups, we assessed TFL response immediately after the end of the last WA stress or sham WA session (day 10) and again, 24 h later (day 11).

Visceral nociception testing. Separate groups of Wistar rats were surgically equipped with electrodes in the abdominal muscles for EMG recording 7 days before the beginning of the experiment. On day 0, animals were placed in Plexiglas cylinders in which they were awake and loosely restrained, and baseline response to CRD was evaluated. From day 1 to day 10, rats were submitted daily to either 1-h WA for the stress group, or to 1-h sham WA for the control group. Immediately after the end of the last WA stress or sham WA session (day 10), we assessed EMG response to CRD in both groups.

The autonomic nervous system-mediated response of the distal colon to stress was evaluated in all rats by quantifying the fecal pellet output after each WA stress or sham WA session.

Time course of nociceptive response to chronic stress. To test if the change in visceral sensitivity following chronic stress is a long-lasting process, groups of rats were submitted either to 1-h WA stress or sham WA, daily for 10 consecutive days. EMG response to CRD was measured 1, 5, 10, 15, 20, 25, 30, 40 and 50 days after the last WA/sham WA stress session, corresponding to days 11, 15, 20, 25, 30, 35, 40, 50, and 60, respectively.

Effect of chronic WA stress on response to open-field exposure. Behavioral experiments were performed in naïve animals to avoid the influence of prior surgery or pain testing. Rats were habituated 30 min daily to the room adjacent to the testing room for 3 days preceding the test. An initial baseline response to the open-field exposure was recorded on day 0. Animals were exposed daily to 1-h WA stress or sham WA from day 1 to day 10 and tested again in the open field on day 11.

Testing began 20 min after the animals were transported to the room adjacent to the testing room. Rats were tested individually in a sequence counterbalanced for group treatments. The animal was placed in the center of the open field. The test began as the door of the test room was closed. For each experiment, the test consisted of an 8-min exposure to the open field. During the first 4 min the room was dark, and during the last 4 min the open field was illuminated by the light gradient stimulus. The open-field arena was cleaned with 5% sodium hydroxide and rotated 180° before each test. The different behaviors were assessed as follows.

CROSSOVER. To provide a measure of general activity in the open field, an observer counted the number of crossovers a rat performed during each minute of the test. A crossover was defined as a movement from one square to another that included all four of the rat’s paws. This measure provided an estimate of the distance traveled by each animal.

ACTIVITY RESPONSE. To estimate the rat’s change in locomotion in response to a change in illumination, the difference in crossovers between minutes 4 and 5 of the test were calculated. To provide more information on behaviors in open field, five behaviors (behaviors 1-5) were scored according to an instantaneous time-sampling procedure. Each animal was observed every 2 s during minute 5 of the test (the minute following stimulus change). These observations yielded an estimate of time spent engaging in each behavior: 1) grooming was defined as licking the paws or using the forepaws to wipe the face or body; 2) forward locomotion was defined as movement with all four limbs in a forward direction; 3) rearing was defined as standing on the rear limbs with the forelimbs off the ground and without movement in
any lateral direction; 4) immobility was defined as the absence of any visible movement except that required for sniffing, whisker movement, and breathing; and 5) stationary activity was defined as any movement of the body that was not grooming, forward locomotion, rearing, sniffing, breathing, or whisker movement.

Influence of chronic WA stress on immunohistology of the colon. In a different series of experiments, samples of distal colon were collected on day 11 from rats previously exposed to repeated WA stress or control sham WA stress. These rats were naïve of surgical manipulation or CRD procedure. Samples were processed for cytokine mRNA detection, hematoxylin and eosin staining, and RMCP II immunohistochemistry.

Data Analysis

Data were analyzed in two ways. First, to examine the pressure-response relationship, EMG amplitudes were normalized as percent of the baseline response for the highest pressure (60 mmHg) for each rat and averaged for each group of rats. This type of normalization has generally been used to account for interindividual variations of the EMG signal (34). The effect of stress on EMG response to CRD within one group of animals was analyzed by comparing the poststress measurements to the baseline values at each distension pressure using a repeated-measures two-way ANOVA followed by Bonferroni posttest comparisons. When comparing the response of stressed rats to control rats, data were expressed as the mean change from baseline for different pressure of distension and analyzed using a Student’s t-test.

Second, this method of analysis determined the overall effect of stress by calculating the AUC of the raw EMG amplitude response as a function of pressure for each individual animal at different times of testing. The AUC was calculated from the overall EMG response at 10 to 60 mmHg of distension. The change in response at different times after stress was determined by dividing the values by the baseline value for each rat. The resulting ratios were then expressed as the percent change from baseline and averaged for each group of rats on each day. Statistical analysis was performed by comparison of percent change at different time points to a theoretical value of 0 (corresponding to 0% change) using a Normality test followed by Wilcoxon signed-rank test, theoretical median = 0. Difference in percent change at different time points was determined using a repeated-measures analysis. This type of analysis for EMG measurements has been previously validated in a recently published article (40).

Responses to the tail-flick test were analyzed using a paired Student’s t-test. Similarly, a Student’s t-test was used to compare the fecal pellet output at days 1 and 10. Paired comparisons were performed when appropriate.

Cytokine expression and histological data were analyzed by comparing WA stress with sham WA data using an unpaired Student’s t-test. Behavioral data were analyzed using a paired Student’s t-test when comparing baseline and poststress response within the same group. When comparing the mean difference from baseline in acute vs. chronic WA models, a Student’s t-test was used. Paired comparisons were performed when appropriate.

RESULTS

Does Chronic WA Stress Result in Alteration of Visceral and Somatic Noceception?

Visceral nociceptive response following chronic WA stress. The VMR to graded intensities of CRD was recorded on day 0 before the start of the WA stress or sham WA (basal visceral sensitivity) and again on day 10 immediately after the last WA stress session (stress-induced visceral modulation). We found a significant increase of the VMR at day 10 compared with baseline in the stressed animals (P < 0.05 at 40 mmHg and P < 0.01 at 60 mmHg) (Fig. 1A). In contrast, repeated exposure to sham WA for 10 consecutive days had no effect on VMR at day 10 (Fig. 1B). The VMR mean change from baseline at day 10 in the WA stress group (27.0 ± 14.6 and 30.2 ± 13.7% for the pressure 40 and 60 mmHg, respectively) was significantly higher (P < 0.05, Normality test followed by Student’s t-test) compared with that of the sham WA group (5.8 ± 8.6 and 0.0 ± 8.6% for the pressure 40 and 60 mmHg, respectively) consistent with visceral hyperalgesia. When expressed as percent change of AUC from baseline in sham WA and WA groups, stressed animals exhibit a significant increase of overall response (46.0 ± 14%, P = 0.0244), whereas no significant change was observed in the sham WA group (−5.4 ± 9%). Of the 19 rats initially included in this study, 3 were excluded on the basis of an EMG signal/noise ratio of <0.05.

Time course of repeated WA SIVH. The VMR to CRD was measured in the WA group, 1 to 50 days after the last WA stress session. A similar protocol was applied in rats previously exposed to the sham WA procedure to confirm the effect of
WA stress on VMR and to verify that repeated CRD does not change the VMR over time. As previously described in MATERIALS AND METHODS, the distension protocol consisted of series of phasic CRD to constant pressures of 10, 20, 40, and 60 mmHg (20-s duration; 4-min interstimulus interval). No additional stress or sham stress session was given during the post-10-day period of time and the rats were left undisturbed between each CRD test. Repeated exposure to WA stress for 10 consecutive days resulted in sustained enhancement of VMR as shown by the increased AUC in poststress measurements compared with baseline. As shown in Fig. 2A, an increased VMR ($P < 0.05$) was observed from day 11 to day 40, compared with a theoretical value of 0. The mean overall increase was $63.0 \pm 16.7\%$ at day 11, $79.9 \pm 31\%$ at day 15, $95.0 \pm 22.0\%$ at day 20, $66.7 \pm 25.8\%$ at day 25, $108.0 \pm 38.6\%$ at day 30, $81.4 \pm 36.7\%$ at day 35, and $54.7 \pm 21.8\%$ at day 40. Analysis of the EMG amplitude as a function of pressure of distension revealed that significant increase was consistently observed for the pressures 40 and 60 mmHg (data not shown). The VMR normalized thereafter and values were not different from baseline on day 50 ($6.2 \pm 11.1\%$ relative to baseline) and day 60 ($4.7 \pm 25.4\%$; Fig. 2A). Repeated exposure to sham WA had no effect on the VMR observed at the different time points compared with baseline. In addition, responses to CRD at the different times were not different from each other, indicating that repeated CRD every 5 days did not induce any sensitization (Fig. 2B). A total of 10 rats in each group (sham WA and WA) were included in the analysis (from baseline to day 60). Of the 16 rats in each group that were initially assessed at baseline, 6 were excluded during the course of the study on the basis of EMG signal/noise ratio <0.05.

Somatic nociceptive responses following chronic WA stress. To determine whether the 10-day WA stress resulted in an analogous, sustained modulation of somatic nociception, we measured TFL in both sham WA and WA-stressed rats at baseline on day 0, on day 10 immediately after the last session (stress-induced somatic pain modulation), and 24 h later on day 11 without any preceding additional stressor (sustained somatic pain modulation). This measure was performed in a separate group of animals to eliminate possible interactions between somatic pain testing and visceral nociceptive response. The 10-day course of repeated WA stress sessions was associated with a significantly increased TFL immediately following the 10th WA stress session (5.65 $\pm 0.19$ s, from a baseline value of $3.4 \pm 0.17$ s at day 0, $P < 0.0001$; Fig. 3A), indicating the development of stress-mediated somatic analgesia. As shown in Fig. 3B, the TFL in the sham WA group at day 0 was $3.2 \pm 0.18$ s and was not affected by the repeated exposure to sham WA ($3.00 \pm 0.15$ s at day 10; $3.17 \pm 0.13$ s at day 11). In contrast to the sustained increase in visceral sensitivity, there was no evidence for a sustained alteration of somatic nocicep-
tion 24 h following the last stress session, when the TFL had returned to 3.60 ± 0.44 s, a value not different from baseline.

**Is Chronic WA Stress Associated with Increased Colonic Motor Activity?**

Stress-induced changes in fecal pellet output, a validated measure of autonomic nervous system modulation of distal colonic motility (31), were quantified at the end of each sham WA or WA stress session from day 1 to day 10. Higher fecal pellet output was observed in the WA stress group compared with the sham WA group at both day 1 and day 10. A significant decrease of fecal pellet output was observed in both groups when comparing day 10 to day 1. Data are expressed as number of fecal pellets per hour, means ± SE, n = 16 (*P < 0.05 compared with sham WA, #P < 0.05 compared with baseline (day 1), Student’s t-test).

**Is Repeated Exposure to WA Stress Associated with Increased Anxiety-Like Behavior?**

To determine whether the model of repeated WA stress is associated with anxiety-like behavior, we assessed the response to open-field exposure at baseline and again after the end of the stress procedure. During both baseline and poststress testing (day 11), the rats explored the dark open field in the first 4 min and displayed a transient increase in general activity in response to the onset of the light gradient (Fig. 5). When anxiety-like behaviors were recorded during minute 5, chronically stressed rats showed a robust and highly significant decrease in forward locomotion (21.26 ± 2.8 vs. 36.25 ± 3.0% of baseline).

![Fig. 3. Influence of chronic WA stress or sham WA on somatic nociception. A: repeated exposure to WA induces increased tail-flick latency (TFL) on day 10 compared with baseline (day 0). TFL returned to baseline when measured on day 11. B: TFL is not affected by repeated exposure to sham WA when measured on days 10 and 11, compared with baseline (day 0). Data are the latency before tail withdrawal in seconds expressed as means ± SE, n = 16 (*P < 0.05, repeated-measures Student’s t-test).](#)

![Fig. 4. Influence of chronic WA stress or sham WA on fecal pellet output. Fecal pellets were quantified at the end of each sham WA or WA stress session from day 1 to day 10. Higher fecal pellet output was observed in the WA stress group compared with the sham WA group at both day 1 and day 10. A significant decrease of fecal pellet output was observed in both groups when comparing day 10 to day 1. Data are expressed as number of fecal pellets per hour, means ± SE, n = 16. *P < 0.05 compared with sham WA, #P < 0.05 compared with baseline (day 1), Student’s t-test.)](#)

![Fig. 5. Response to exposure to open-field and light gradient at baseline and day 11 in the chronic WA group. General activity estimated by the number of crossovers counted per minute. Data are expressed as number of crossovers, means ± SE, n = 8.](#)
time spent in behavior, \( P = 0.0003 \) accompanied by increased stationary activity \((42.09 \pm 1.7 \text{ vs. } 31.25 \pm 1.9\% \text{ time spent in behavior, } P = 0.0012)\) compared with baseline (Table 1). These changes in behavior are indicative of increased anxiety. In addition, there was a trend for lower number of crossovers in response to stimulus onset \(\text{(minute 5)}\) after chronic stress compared with baseline \((26.9 \pm 1.5 \text{ at baseline vs. } 22.9 \pm 2.4 \text{ on day 11, } P = 0.06)\) also consistent with decreased general activity. There was a higher number of crossovers during \(\text{minute 6}\) in the poststress condition compared with baseline \((20 \pm 1.6 \text{ vs. } 13.43 \pm 2.2, \ P = 0.049)\), suggestive of a lower ability to recover from the stimulus change. After repeated sham WA stress, no differences were observed at \text{day 11} compared with baseline (data not shown).

To determine whether “behavioral habituation” occurred to repeated WA stress exposure, the response to the open-field test was compared following acute WA and following chronic WA stress. The chronic WA group showed evidence for greater general activity compared with the acute WA group. At \text{minute 5}, immediately after the onset of light, rats previously exposed to chronic WA displayed \(22.9 \pm 2.4\) crossovers compared with \(12.0 \pm 1.6\) in acutely stressed rats \(P = 0.007)\). In addition, change in time spent for certain categories of behavior (rearing and stationary activity) were significantly attenuated after chronic stress compared with acute stress (Table 2).

**Is Chronic WA Stress Associated with Colonic Immune Activation?**

**Histology and immunohistochemistry.** There was no significant difference in the thickness of the mucosa plus submucosa layer between the WA group \((252.1 \pm 18.7 \mu m)\) and the control group \((290.5 \pm 16.2 \mu m)\). In general, the structural histology of the colon was not altered in samples from WA-stressed rats compared with control animals. There was no significant difference in the number of polymorphonuclear cells in hematoxylin and eosin-stained sections of the submucosa plus mucosa layer of colonic samples between WA-stressed \((2.7 \pm 0.4)\) and sham WA rats \((2.1 \pm 0.4)\). Mast cells, identified by positive immunostaining for RMCP II, were found primarily in the colonic mucosa and were of significantly higher number in samples from the WA stress group \((7.2 \pm 0.7)\) compared with the control group \((4.3 \pm 0.6, P = 0.0102, \text{Student’s } t\)-test).

**Expression of colonic cytokine mRNA.** Gene expression levels of seven cytokines were measured in extracts of the distal colon from WA-stressed and sham WA groups using quantitative real-time RT-PCR. To avoid potential changes in inflammatory cytokatoes due to surgical placement of EMG electrodes with an externalized catheter, as well as repeated distension of the distal colon, behavioral changes in visceral pain sensitivity were not measured in these rats. We chose to investigate the expression levels of proinflammatory cytokines (IL-1β, TNF-α), cellular immunity-promoting (T helper [Th] type 1) cytokines (IL-2, IL-12, IFN-γ), and humoral immunity-promoting (Th type 2) cytokines (IL-6, IL-10). We found relatively low levels of expression for all cytokines in both groups. Increased levels of mRNA for IL-1β \((2.0 \pm 0.4\text{-fold, } P = 0.03)\) and IFN-γ \((3.4 \pm 0.9\text{-fold, } P = 0.02)\) were observed in the WA group compared with the control group. In contrast, there was no significant group difference in the expression of IL-2, IL-6, IL-10, IL-12a, and TNF-α. Expressed as \(2^{-\Delta \text{CT}}\), which gives a ratio of the expression of each cytokine relative to the ribosomal housekeeping gene 36B4, IL-1β was \(2.10 \pm 0.30 \times 10^{-3}\) in the sham WA group vs. \(4.22 \pm 0.82 \times 10^{-3}\) in the WA group, and IFN-γ was \(9.29 \pm 1.15 \times 10^{-5}\) in the sham WA group vs. \(31.5 \pm 8.6 \times 10^{-5}\) in the WA group (Fig. 6).

**Effect Size and Variability of SIVH and Related Measures**

Interanimal variability of response to stress was assessed by calculating the percentage of animals developing increased response over baseline or compared with control sham WA group, for the following measures: visceral sensitivity to colonic distension, number of RMCP II positive cells in the colon, IL-1β and IFN-γ expression in the colon, and fecal pellet output. For each measure (except fecal pellet output), the percentage of rats showing increases in the range of \(<0, 1–50, 51–100, \text{ and } \geq 101%\) was calculated for \text{day 11}, the time point at which the molecular and histological studies were performed.

### Table 1. Effect of chronic water avoidance on the response of rats to open-field exposure

<table>
<thead>
<tr>
<th></th>
<th>Grooming</th>
<th>Forward Locomotion</th>
<th>Rearing</th>
<th>Stationary Activity</th>
<th>Immobility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.41±0.4</td>
<td>36.25±3.0</td>
<td>28.34±2.2</td>
<td>31.25±1.9</td>
<td>3.7±1.1</td>
</tr>
<tr>
<td>WA day 11</td>
<td>0.82±0.5</td>
<td>21.26±2.8*</td>
<td>28.33±2.1</td>
<td>42.09±1.7*</td>
<td>7.5±2.0</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>0.41±0.7</td>
<td>−15.0±4.15</td>
<td>−0.01±3.02</td>
<td>10.84±2.6</td>
<td>3.76±2.38</td>
</tr>
</tbody>
</table>

Values are means ± SE in percent, \( n = 8 \) rats. Percent time for each category of behavior during \text{minute 5} of the open field test was calculated at baseline and poststress conditions, where behaviors were scored according to an instantaneous time sampling procedure. Each animal was observed every 2 s during \text{minute 5} of the test. Following chronic water avoidance (WA) stress, rats exhibited significantly fewer forward behaviors and more stationary behaviors \((P < 0.01, \text{repeated measures Student’s } t\)-test) compared with baseline.
Visceral hyperalgesia. Eighty-two percent of the rats tested developed increased VMR over baseline. Twenty-four percent exhibited an increase in AUC between 1 and 50% over baseline, 35% showed increased AUC in the range of 51–100%, and 23% had an increase in AUC ≥101%. Of the 32 rats initially equipped with electrodes, 7 animals showing a EMG signal/noise ratio <0.5 were excluded. In the sham WA group, 39% of the rats tested showed unchanged or decreased VMR over baseline. Fifty-six percent exhibited increased response between 1 and 50% at day 11 compared with baseline (average increase of 26 ± 5%) and 5% showed increased response in the range of 51 to 100%. The average change of VMR at day 11 over baseline for the whole group (n = 18) was 0.4 ± 10%.

Mucosal cytokine expression. Fifty percent of the stressed animals showed increased expression of IL-1β mRNA in the colon; a 1–50% increase was observed in 37.5% of tested rats and a 51–100% increase was seen in 12.5% of animals. Increased expression of IFN-γ mRNA was observed in 87.5% of stressed animals; 25% showed increase in the range of 51–100% and 62.5% of rats showed increase ≥101%.

RMCP II positive cell numbers in colonic mucosa. Eighty-six percent of the stressed animals exhibited an increased number of RMCP II positive cells in the colon. Fourteen percent showed a 1–50% increase, 57% showed a 51–100% increase, and 14% showed a increase ≥101%. (Both the analysis for cytokine expression and mast cell number indicate percent change of response relative to control sham WA animals). Eight rats in each group were used.

Stress-induced increase of fecal pellet output (assessed on day 10). In the WA group, 50% of animals had 0–3 fecal pellets at day 10, 36% had 4–6 pellets, and 14% had 7–9 pellets. Sixteen rats were included in this study. None of the animals exposed to sham WA at day 10 had more than 3 fecal pellets.

DISCUSSION

Repeated WA stress in male Wistar rats resulted in a sustained increase of the nociceptive response to CRD, consistent with chronic SIVH. This long-lasting upregulation of the nociceptive response was selective for visceral sensitivity, because only a transient effect of the stressor on somatic nociception was observed. Visceral hyperalgesia following the repeated stressor was associated with increased anxiety-like behavior and evidence for low-level immune activation of the colon. The present study is, to our knowledge, the first demonstration of a sustained and selective enhancement of visceral nociception following exposure to a repeated psychological stressor in adult rats.

Long-Lasting Sensitization of Visceral Nociceptive Responses to CRD Following Repeated Exposure to WA Stress

Visceral hyperalgesia in response to 10 days of repeated WA stress was observed immediately after the last stress session (day 10) and was found to be long-lasting, taking up to 40 days to return to baseline values. This change in visceral sensitivity appeared to be related to the WA stressor and not to other potentially stressful aspects of the paradigm, because repeated sham WA stress was not associated with the development of visceral hyperalgesia. The negative results with sham WA stress also demonstrate that there was no conditioning effect of the repeated exposure to the test environment. The effect of the chronic stressor on visceral sensitivity was robust and reproducible. Determination of the effect size of the stressor on visceral sensitivity revealed that 82% of the rats studied exhibited an increased VMR to CRD at day 11. Fifty-eight percent of the stressed animals exhibited a response increase of at least 51%, corresponding to a group of “high responder” animals. This analysis was performed at day 11, a time at which behavioral, histological, and molecular studies were performed. These latter parameters were assessed in rats, naive for CRD, to avoid interactions between painful stimulation and behavioral responses, as well as the possibility of surgical or distension-mediated mucosal inflammatory responses. Unfortunately, this study design precludes a direct correlation of each parameter with visceral hyperalgesia on an individual rat basis. However, with the exception of IL-1β expression, the assessment of the response to stress in terms of increased IFN-γ expression, increased mast cell population, and increased fecal pellet output revealed a percentage of “high responder” animals in a range consistent with the one observed for visceral hyperalgesia. Future studies will need to address whether the observed biochemical changes are directly related to the observed enhancement in visceral nociception.

Whereas acute, transient sensitization of the nociceptive response to visceral stimulation by a single psychological stressor has previously been demonstrated in different animal models of visceral nociception (19, 40), we believe that this is the first report of a long-lasting effect of repeated psychological stress on visceral sensitivity in the adult rat. For example, acute partial restraint stress was found to result in enhanced EMG response to CRD in Wistar rats (19) or guinea pigs (18). The time course of visceral hyperalgesia in these studies was not reported. We have recently shown that a one-time WA stress in male Wistar rats can result in the development of visceral hyperalgesia 24 h later (40), which persisted for less than 1 wk following the stressor (I. Schwetz, unpublished observations).
Effect of Chronic Stress Is Specific for Visceral Nociception and Does Not Involve Long-Lasting Modulation of Somatic Nociception

Using a cutaneous thermal pain stimulus, we demonstrated that rats developed stress-induced somatic (cutaneous) analgesia following the repeated course of WA stress, but not the sham WA stress. However, this effect was transient and only observed immediately after the last stress session, arguing against a generalized sensitization of nociception. We have previously shown a transient somatic analgesia following acute WA exposure with nociceptive responses returning to baseline within 20 min (41). In the present study, we found that TFL had returned to baseline 24 h following the 10-day WA stress, suggesting that in contrast to the observed sustained visceral hyperalgesia, the repeated stress-induced changes in somatic nociception were transient. Depending on the nature, intensity, duration, and location of the stimuli, different types of stress-induced somatic pain modulation have been reported (47). For example, chronic stress has been associated with somatic hyperalgesia in models of repeated forced swimming or restraint stress, both of which include physical and psychological stress components (29, 15). In contrast, other studies have shown no changes or somatic analgesia following repeated homotypic stress exposure along with a reduction of the behavioral and physiological responses (5, 12). Whereas differences in rat strains, as well as in the type and severity of the stressor, may explain these different findings, our findings clearly demonstrate a qualitative difference of chronic WA stress on visceral and somatic nociceptive responses. This difference suggests the activation of a sensitization mechanism that specifically modulates a sustained visceral response, rather than a generalized hyperalgesic state.

Chronic Stress is Associated with Altered Autonomic and Behavioral Responses

We found that the initial phase of visceral hyperalgesia observed following chronic stress was associated with two well-established measures of anxiety-like behavior: 1) increased fecal pellet output (26) and 2) altered general activity and behavioral response to open-field exposure (14). The findings are in agreement with similar reports of anxiety-like behavior in different animal models of visceral hyperalgesia (1, 11, 21).

Fecal pellet output is a well-characterized parameter of stress-induced stimulation of the rat colon by sacral parasympathetic outflow and physiological readout of anxiety-like behavior (7, 31). The measure was elevated following each session of the 10 days of WA exposure compared with sham WA. We found an ~30% decrease in the magnitude of the stress response over the 10-day stress period, suggesting partial habituation of the autonomic nervous system response to the chronic stressor. Anxiety-like behavior was also observed in the form of an altered locomotion activity (in the form of a higher number of stationary activity behaviors) and behavioral response to open-field exposure (14). Similar to the habituation observed in fecal pellet output, we found attenuated behavioral response to stimulus after chronic stress (less rearing and stationary activity) compared with the one observed following a one-time WA stress. It is not clear why these changes are not reflected by the measure of forward locomotion, which was expected to be higher after chronic stress. We do not have a straightforward explanation for this inconsistency except the potential bias associated with the subjective component of visual observation. In general, these findings are consistent with behavioral habituation, showing that the anxiety-like response in chronically stressed rats is less than one observes in acutely stressed rats immediately after the stressor.

The habituation observed in both test paradigms is consistent with a large literature showing that animals repeatedly exposed to the same (homotypic) stressor exhibit decreased responses, also called adaptation, to the stressor (43). Habituation has been described for autonomic nervous system, as well as the hypothalamic-pituitary-adrenal axis, responses to stress, and for anxiety-like behavioral responses. It seems to depend on the nature of the challenge, and different outputs of the stress response can be modulated independently of each other (44). Consistent with such differential habituation, no significant habituation for the altered visceral pain response was observed; the EMG response to CRD after chronic stress was increased by an average of 63 ± 16% at day 11 compared with baseline, whereas a 47 ± 5% increase was previously described after acute WA stress exposure (41).

Possible Mechanisms Underlying the Development of SIVH

The current report summarizes the general properties of a novel chronic visceral hyperalgesia model, and future studies will have to identify the specific mechanisms underlying the sustained component of this phenomenon. Based on reports in the literature and some preliminary findings in the current study, we propose two possibly interrelated mechanisms that could be involved in the development of visceral hyperalgesia following chronic stress: 1) colonic immune activation and 2) central stress sensitization.

Colonic immune activation. One plausible explanation for the development of selective visceral hyperalgesia following chronic WA stress could be stress-induced immune activation of the colon, resulting in secondary sensitization of visceral afferent pathways by immune mediators, including proinflammatory cytokines (16, 24). Evidence for chronic stress-induced intestinal immune activation following 10 days of WA stress had previously been demonstrated by Soderholm et al. (42) in a different rat strain. Similar to our findings, these authors reported hyperplasia and activation of mast cells, in addition to infiltration of neutrophils and mononuclear cells, increased myeloperoxidase activity in the mucosa, and ultrastructural changes in epithelial cells. The increased mast cell numbers found in the current study are in accordance with numerous reports showing stress-induced mast cell activation at the gut level (13, 48, 19) and with increased mast cell numbers in colonic biopsies from some IBS patients (3). Mast cell products have been shown to play a role in altered gut permeability and resultant mucosal immune activation (51), and mast cell protease-induced-activation of protease-activated type 2 receptors in colonic mucosa has been found to result in hypersensitivity to CRD (9). Thus increased mast cell number can be considered a potential candidate involved in the sustained SIVH observed in our model, which deserves further investigation.
Although we did not perform ultrastructural evaluations of the colon tissues following repeated stress, the number of polymorphonuclear cells was unchanged and histological analysis revealed similar structural characteristics in both groups, with no significant alteration of mucosa or submucosa thickness. However, we identified two cytokines (IL-1β and IFN-γ) whose mRNA expression was significantly increased after stress, suggesting low-grade immune activation. Severalfold higher levels of IL-1β than the ones observed in the current study have been found in the distal colonic mucosa of rats with experimentally-induced colitis and in the intestine of patients with Crohn’s disease (32, 33, 53). We also found a trend for an increase in TNF-α, another potent proinflammatory cytokine released by mast cells and macrophages, and IL-6, a Th2 cytokine which is produced by a variety of cell types including macrophages. However, the increased levels of expression for these cytokines (and even IL-1β and IFN-γ) are small and may or may not reflect a change in protein expression.

Thus we cannot exclude the possibility of an initial mucosal immune activation during the early phase of the stress procedure. Stress cannot only reactivate inflammation in colitis models (37), but can also cause primary inflammation in a naïve host (39). Although a relatively mild psychological stress might not be expected to induce an inflammatory reaction comparable to that observed after chemically-induced colonic inflammation, a mucosal immune reaction during the early phase of stress exposure might be an initiating factor for sensitization of primary and secondary visceral afferent pathways (24) as well as sustained activation of central pathways involved in descending pain facilitation (36). Our data do not permit us to conclude whether the mucosal immune activation observed at day 11 persists in correlation with the sustained visceral hyperalgesia. We hypothesize that a transient change of mucosal immune mediators at the colonic level may trigger a sensitization process involved in the sustained modulation of visceral nociceptive response. A time course study of the level of immune activation from day 1 up to day 40 as well as pharmacological interventions aimed to counteract proinflammatory mediators would be required to verify this hypothesis.

Stress sensitization. Another plausible mechanism which might underlie the observed constellation of visceral hyperalgesia, autonomic nervous system hyperreactivity, and anxiety-like behavior is stress sensitization, a phenomenon which has been observed in response to various types of acute and chronic stressors (43, 45), and in which modulation of the CRF/CRF1 system might be involved (40). Central structures involved in stress sensitization include the amygdala complex and interconnected brain stem nuclei (such as the locus coeruleus complex), which are involved in descending pain modulation (50). For example, stereotaxic delivery of corticosterone into the amygdala was found to produce colorectal hypersensitivity in rats (17), supporting the role of stress-sensitive supraspinal structures in modulation of visceral sensitivity. More recently, a stress-induced increase in neurotensin signaling in several supraspinal regions of pain modulation circuits has been implicated in SIVH (20). Future studies should address the role of stress mediators such as CRF and substance P in the development and maintenance of the chronic visceral hyperalgesic state.

In summary, the present study describes the differential modulatory role of repeated psychological stress on visceral and somatic nociception in rat; chronic WA stress leads to a transient somatic antinociceptive response associated with sustained visceral hyperalgesia. The early phase of enhanced visceral nociception consecutive to 10 days WA stress was observed in parallel with changes of mucosal immune status of the colon, increased colonic motor function and increased anxiety-like behavior. Based on the available literature, colonic immune activation or central sensitization are both potential mechanisms in the development of increased visceral nociception following chronic psychological stress. Whether these pathways are involved in the developmental phase or of the sustained component of visceral hyperalgesia remains to be characterized.

Validity of Findings as an Animal Model for IBS

The findings in this animal model of repeated stress have excellent face and construct validity for IBS. First, the long-lasting enhancement of visceral sensitivity following chronic psychological stress observed in the present study is consistent with clinical studies showing that chronic, sustained, stressful life events play a major role in the first onset or exacerbation of symptoms in IBS patients (4, 28). Second, it is well documented that patients with IBS frequently have anxiety and depressive and/or psychiatric disorders as comorbid conditions (23, 46). The findings of increased anxiety-like behavior in rats following chronic WA stress in the current study are in agreement with several other animal models of chronic hyperalgesia (2, 25). Third, several recent studies have reported evidence for mild immune activation (8, 49) as well as increased mast cell numbers in colonic biopsies from IBS patients (35, 52). Although the cause and effect relationship between colonic immune activation and the development of visceral hypersensitivity and IBS symptoms remains to be determined, the findings in the current study are consistent with those reported in human patients.

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