DELAYED STRESS-INDUCED COLONIC HYPERSENSITIVITY IN MALE WISTAR RATS: ROLE OF NEUROKININ-1 AND CORTICOTROPIN-RELEASING FACTOR –1 RECEPTORS

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Short title: Delayed hypersensitivity – role of NK₁Rs and CRF₁Rs
ABSTRACT

The mechanism(s) underlying stress-induced colonic hypersensitivity (SICH) are incompletely understood. Our aims were to assess the acute and delayed (24 h) effect of water avoidance (WA) stress on visceral nociception in awake male Wistar rats and to evaluate the role of two stress-related modulation systems: the substance P/neurokinin-1 receptor (SP/NK$_1$R) and the corticotropin-releasing factor (CRF)/CRF$_1$ receptor (CRF/CRF$_1$R) systems, as well as the possible involvement of the sympathetic nervous system (SNS). Visceral pain responses were measured as the visceromotor response (VMR) to colorectal distension (CRD) at baseline, immediately after WA and again 24 h later. The NK$_1$R antagonists RP67580 and SR140333 and the CRF$_1$R antagonist CP154526 were injected 15 min before WA or 1 h before the CRD on day 2. Chemical sympathectomy was performed by repeated injection of 6-hydroxy-dopamine. WA stress resulted in a significant increase in the VMR on day 2, but no change immediately after WA. Injection of CP154526 abolished delayed SICH when applied either before WA stress or before the CRD on day 2. Both NK$_1$R antagonists only decreased SICH when injected before the CRD on day 2. Chemical sympathectomy did not affect delayed SICH. Our results indicate that in male Wistar rats, both NK$_1$R and CRF$_1$R activation, but not SNS activation, play a role in the development of SICH.

KEYWORDS: Visceral pain, stress
INTRODUCTION

Recurrent abdominal pain or discomfort in the absence of detectable organic abnormalities is one of the principal symptoms of irritable bowel syndrome (IBS) (10). Several studies have demonstrated that IBS patients have lowered colorectal perceptual thresholds, increased sensory ratings and larger viscerosomatic referral areas compared to healthy individuals, consistent with heightened perception of visceral stimuli (1; 33). As a consequence, visceral hypersensitivity has become widely accepted as a pivotal mechanism contributing to symptom generation in IBS (5).

Different types of stressors are known to play an important role in the development (8), maintenance and exacerbation of IBS symptoms (31). In addition, studies in IBS patients suggest that psychological (9), as well as physical (35), stressors can induce increases in the perceptual response to rectosigmoid distension. Taken together, these findings suggest that stress-induced modulation of visceral sensitivity, or stress-induced visceral hypersensitivity may contribute to the exacerbation of IBS symptoms. Experimental evidence suggests the possible involvement of several central and peripheral mechanisms in the development and expression of stress-induced colonic hypersensitivity (SICH), for instance the activation of the autonomic nervous system (18). Both the corticotropin-releasing factor (CRF)/CRF_1 receptor (CRF/CRF_1R) system (15) and the substance P/neurokinin-1 receptor (SP/NK_1R) system (2) are thought to be involved in the development of SICH, although the exact role of these systems and the site of involvement are not known.

CRF is a major mediator of the effects of stress on the GI tract involving both central and peripheral CRF receptors (44), and activation of the CRF/CRF_1R system may modulate pain
responses centrally (24), or peripherally (40). For example, central CRF release is associated with activation of the sympathetic nervous system (SNS), as well as with activation of the hypothalamic-pituitary-adrenal (HPA) axis (6; 29), both of which can have profound effects on peripheral targets. In rats, central administration of CRF mimics the effect of restraint stress in increasing the number of abdominal contractions to rectal distension (15), and preliminary results suggest that CRF$_1$Rs are involved in SICH in a rat model of neonatal stress (42). In humans, peripherally administered CRF decreased perception thresholds and increased intensity ratings in response to rectal distension in healthy volunteers (25). Bradesi et al. (2) recently demonstrated that an NK$_1$R antagonist (SR140333) significantly counteracted acute restraint stress-induced increase of abdominal contractions in response to rectal distension, suggesting that NK$_1$Rs are also involved in the modulation of visceral sensitivity in response to stress.

While CRF$_1$Rs and NK$_1$Rs located both centrally and peripherally may be directly involved in the mediation of SICH, activation of postganglionic sympathetic nerves is a plausible peripheral mechanism. In principal, SICH could result from stress-induced mediator release from postganglionic sympathetic nerves, such as prostaglandins (20), which could directly sensitize primary afferent terminals. Alternatively, postganglionic sympathetic nerves could trigger the release of mediators from mast cells, such as tryptase and histamine, or other immune cells which can produce primary afferent sensitization (4). Such SNS-mediated mast cell degranulation has been demonstrated in other organ systems (22).

In the current study, we examined the general hypothesis that activation of NK$_1$Rs and CRF$_1$Rs are involved in the stress-induced increase of colonic nociception in male Wistar rats, a rat strain
with increased anxiety-like behavior (14). Visceromotor response (VMR) to colorectal distension (CRD) was used to assess colonic nociception and water avoidance (WA), an established mild psychological stressor, in order to address the following questions: 1) Does an acute psychological stressor produce a delayed increase in colonic nociception, as assessed at 24 h after the stressor? 2) Is delayed SICH dependent on NK₁Rs and CRF₁Rs? 3) Do the delayed stress-induced changes in colonic nociception involve peripheral mechanisms such as activation of the SNS? Some of the data presented in this paper have previously been published in abstract form (41).
MATERIALS AND METHODS

Animals

Experiments were performed using male Wistar rats purchased from Harlan, San Diego, USA (weight: 240-260 g at time of purchase). The rats were housed in pairs with free access to food and water. A 12-h light dark cycle (lights on at 0600 h) was maintained. The animals were allowed to acclimate to the animal facility for 1 week prior to surgery. All protocols were approved by the Institutional Animal Care and Use Committee at the VA Greater Los Angeles Healthcare System, Los Angeles, CA.

Surgery

As previously described (7), electrodes (Teflon coated stainless steel, AstraZeneca, Sweden) for electromyographic (EMG) recording were stitched into the external oblique musculature immediately superior to the inguinal ligament. The cannula housing the electrode leads was externalized through a 4-mm incision on the left side of the abdominal wall for access during subsequent experimental measurements. Following surgery, rats were housed in pairs and allowed to recuperate for at least 7 days. Wounds were examined to ensure complete recovery prior to testing.

Assessment of visceral sensitivity - Colorectal distension

The visceral stimulus employed was distension of the descending colon and rectum using a method that has been previously described (36). Briefly, animals were lightly anesthetized with halothane and a lubricated flexible latex balloon (6 cm) was inserted intra-anally into the descending colon. The balloon was positioned such that its end was 1 cm proximal to the anus.
and was secured in place by taping the balloon catheter to the base of the tail. Animals were placed in a plexiglass cage and allowed to recover for approximately 30 min. The balloon pressure was continuously monitored online with the aid of a customized pressure control device (AstraZeneca R&D, Sweden). Colorectal distension in awake rats results in contraction of the abdominal and hind limb musculature (36), recorded as EMG activity in the external oblique musculature. Each distension lasted 20 s at 4-min inter-stimulus intervals. EMG activity was recorded 20 s before CRD, 20 s during CRD and 20 s after termination of CRD. The EMG activity was rectified and the increase in the area under the curve (AUC) of EMG amplitude (over baseline) was recorded.

**Water avoidance stress**

The rats were placed on a pedestal (8 x 8 x 10 cm) attached to the bottom of a plastic tank (25 x 25 x 45 cm). The tank was filled with fresh tap water at room temperature (21 °C) within 1 cm of the top of the block. This well-characterized test represents a psychological stressor associated with large elevations of ACTH and corticosterone within 30 min (34). Sham WA, the control condition, consisted of placing rats in the same, albeit dry, plastic tanks.

**Experimental protocol**

All animals were handled and habituated to the plexiglass cages for 30 min on three consecutive days including the day before baseline testing.

*Effect of stress on visceromotor response*
In the first series of experiments, two groups of male Wistar rats \((n = 12\) in each group\) were used. Baseline VMR to graded intensities of phasic CRD \((2 \times 60 \text{ mm Hg} \text{ and two ascending series of } 10, 20, 40, 60 \text{ mm Hg})\) were obtained. The same CRD protocol was used throughout the study. One group was submitted to WA stress. The second group was used as a control and subjected to sham WA. The VMR to CRD was recorded immediately after the stress or sham stress procedure and again 24 h later.

**Effect of the selective CRF\(_1\)R antagonist, CP154526**

The first set of experiments consisted of 4 groups of animals. The first group \((n = 12)\) was injected subcutaneously (s.c.) with the CRF\(_1\)R antagonist, CP154526, at 32 mg/kg (dissolved in 10% DMSO, 10% CremophorEL and saline) 45 min before WA. A second group \((n = 11)\), treated with vehicle, served as controls. The third group \((n = 12)\) was injected with the compound 1 h before the start of the CRD on day 2, with the fourth group \((n = 10)\) as vehicle controls. All experiments were repeated in sham stress condition to exclude a per se effect of the compound.

**Effect of the NK\(_1\)R antagonists, RP67580 and SR140333**

The first group \((n = 18)\) received the NK\(_1\)R antagonist RP67580 (dissolved in distilled water) at 3-mg/kg or vehicle intraperitoneally (i.p.) 15 min before the WA stress. Ten rats were injected with RP67580 (3 mg/kg) 1 h before the start of the CRD on the second day. Based on the results of the 3-mg/kg dose, another group of 10 animals was injected with 1 mg/kg before the CRD on the second day. Ten rats served as vehicle controls. To exclude a per se effect of the compound, sham stressed animals were injected with 3 mg/kg RP67580 or vehicle before the 24 h CRD.
To confirm our results with RP67580, and to rule out possible non-specific effects of this compound, we repeated the experiments outlined above with another specific NK\textsubscript{1}R antagonist, SR140333. Briefly, SR140333 (1 mg/kg i.p. dissolved in saline) or vehicle was injected 15 min before WA (\(n = 10\) in each group). Ten rats were treated with the compound (1 mg/kg) 1 h before the CRD on day 2. Based on the results of the 1-mg dose, one group of animals was injected with 0.1 mg/kg SR140333 before the CRD on day 2 (\(n = 10\)). A separate group (\(n = 10\)) was injected with vehicle. To exclude a per se effect of the compound, sham stressed animals were injected with 1 mg/kg SR140333 or vehicle before the CRD on day 2.

**Chemical sympathectomy**

Chemical sympathectomy was performed and validated as previously described (32). Ten rats were injected i.p. with four doses of 6-hydroxy-dopamine (6-OHDA) dissolved in 0.1% ascorbic acid in saline over 7 days (days 1 and 2, 50 mg/kg; days 6 and 7, 100 mg/kg; total dose 300 mg/kg; protocol modified from McCaffertey et al. (32)). A second group was treated with vehicle. The day after the last injection, a baseline CRD was performed. All animals were submitted to WA. CRD was repeated immediately after WA and again 24 h later. We assessed the effectiveness of chemical sympathectomy by examination of neuropeptide Y (NPY) immunoreactivity in perivascular nerves in the submucosa of the colon as previously described (12). Briefly, colonic tissue was pinned flat in a Sylgard Petri-dish and fixed overnight in 4% paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.4 (PB) at 4 °C. Fixed tissue was subsequently immersed in 20% sucrose at 4 °C, embedded with optimal cutting temperature compound embedding medium (Miles, Elkhart, IN), frozen on dry ice, and sectioned with a cryostat at 8 µm thickness.
Immunostaining for NPY was performed by incubating frozen tissue sections overnight at 4 °C with rabbit polyclonal NPY antibody (1:1000; CURE #8711) followed by incubation with Rhodamine (TRITC)-conjugated donkey anti-mouse IgG (1:100, Jackson ImmunoResearch, West Grove, PA) overnight at 4 °C. Nonspecific labeling was determined by using primary NPY antibody that had been preincubated with excess NPY peptide for 2 h at room temperature prior to applying to the tissue sections. All tissue sections were washed three times for 10 min in PB prior to the addition of subsequent antibodies. Stained sections were mounted with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA). Sections were examined with the examiner blinded to the treatment and analyzed for the degree and intensity of NPY immunostaining.

Drugs
6–Hydroxydopamine Hydrobromide was bought from Regis, USA. The CRF$_1$R antagonist, CP154526, was a gift from Pfizer. SR140333 was kindly supplied by Sanofi Synthelabo, France. The NK$_1$R antagonist, RP67580, was a gift from Aventis Pharma, France. In all experiments, intraperitoneal injections of drug or vehicle were given in a volume of 1 mg/ml. Doses used for each compound were selected according to relevant references in the literature.

Data analysis and statistics
The EMG amplitudes were normalized as percent of baseline to the averaged response at 60 mm Hg in the ascending series. The effect of stress on the VMR to CRD was analyzed by comparing the post stress measurements with the baseline within the same group of animals. The data were
analyzed by repeated measure ANOVA followed by Tukey post–comparison test. The overall effect of stress was determined by calculating the AUC of the raw EMG amplitude as a function of pressure before and after stress for each animal. From these values, the percent change in AUC was calculated by taking the difference between the AUC before and after stress, dividing by the AUC before stress and multiplying by 100. The effect of drug treatment on the post stress VMR was assessed comparing the mean changes from baseline between treatment groups using one-way ANOVA and post hoc t-tests. *P* < 0.05 was considered significant.
RESULTS

Effect of stress on visceromotor response

No significant change in the VMR was observed (figure 1A) immediately following the hour of WA stress; however, a significant increase in the VMR developed 24 h after the WA stress ($P < 0.01$ compared to baseline, repeated measure ANOVA, $n = 12$) reflected by a mean increase in AUC of $69.7 \pm 13.1\%$. To confirm that the observed increase in VMR to CRD is due to the earlier experience of stress rather than being related to other factors (such as repeated distensions or conditioned responses to the site of CRD), the influence of repeated CRD on two consecutive days without any psychological intervention, as well as 1 h sham WA (dry tanks) were evaluated. Repeated CRDs on two consecutive days without any stress intervention did not lead to a significant increase in VMR (data not shown). Sham WA had no effect on VMR immediately after the procedure or 24 h later (figure 1B). Comparing the mean change from baseline 24 h after stress, there was a significant increase in VMR in the stressed group at 40 mm Hg ($P < 0.05$) and 60 mm Hg ($P < 0.0001$) compared to sham-stressed animals (figure 1C).

Effect size and variability of stress-induced colonic hypersensitivity

Inter-animal variability in delayed SICH was assessed by calculating the percent change in AUC for the VMR. All rats that received vehicle treatment for the experiments described above were included. A total number of 102 rats were used in this analysis. The mean increase in the VMR was $47 \pm 5\%$. Eighty-six percent of animals exposed to WA (88 of 102) showed increases in AUC 24 h after the stress (figure 2), with 47% (48 of 102) exhibiting increases between 1 and 50% over baseline, and 39% (40 of 102) showing increases in AUC of more than 51% over baseline.
Effect of the CRF$_1$R antagonist, CP154526

The CRF$_1$R-selective antagonist CP154526 was used in a dose previously found to be effective in blocking SICH in a different rat model (42). The compound was administered s.c. either 45 min before WA, or 1 h before the start of CRD on day 2 to evaluate the dependence of delayed SICH on peripheral and/or central CRF$_1$Rs. Administration before WA ($n = 12$) resulted in a significant decrease in the VMR on day 2 at 40 mm Hg ($P < 0.05$) and 60 mm Hg ($P < 0.001$) compared to the vehicle group ($n = 11$, figure 3A). Similarly, injection before the start of the CRD on day 2 ($n = 12$) significantly decreased VMR on day 2 at 40 mm Hg ($P = 0.02$). At 60 mm Hg, a decrease in VMR was observed but did not achieve significance ($P = 0.07$, figure 3B). In both vehicle groups, stress significantly increased VMR on day 2 ($P < 0.05$ compared to baseline). The effect of the compound on sham stressed animals was assessed in a separate group of animals ($n = 10$ per group). Sham stress did not lead to an increase in colonic sensitivity on day 2 compared to baseline in any of the groups. Injection of CP154526 before the sham procedure or before the CRD on day 2 did not change VMR to CRD at 24 h (data not shown), suggesting that CP154526 does not have an effect on the VMR in non-stressed animals.

Effect of the NK$_1$R antagonists, RP67580 and SR140333

The doses of the NK$_1$R selective antagonist RP67580 were selected according to relevant references in the literature (16; 38). The compound was administered 15 min prior to the WA stress to evaluate the dependence of delayed SICH on NK$_1$Rs. RP67580 (3 mg/kg) given prior to WA stress on day 1 did not decrease the VMR assessed on day 2 (table 1). In both the vehicle and the treated group ($n = 9$ in each group), stress significantly increased VMR on day 2 ($P <$
0.05 compared to baseline). In contrast, injection of RP67580 at 3 mg/kg before the CRD on day 2 resulted in a significant decrease in VMR on day 2 compared to vehicle (at 40 mm Hg, $P < 0.01$; at 60 mm Hg, $P < 0.0001$, figure 3C). When the dose was reduced to 1 mg/kg, RP67580 still significantly decreased VMR on day 2 compared to vehicle (at 40 mm Hg, $P = 0.01$; at 60 mm Hg, $P < 0.001$, figure 3C). In the vehicle group ($n = 12$), stress significantly increased VMR on day 2 compared to baseline ($P < 0.05$). Sham stress did not lead to an increase in colonic sensitivity on day 2 compared to baseline. Administration of RP67580 (3 mg/kg) before the CRD on day 2 in sham stressed animals decreased the VMR on day 2 below baseline (data not shown), indicating that RP67580 may have an analgesic effect in this model.

To confirm our findings with RP67580 and rule out that the observed effects may be related to non-specific effects of this compound, we repeated the experiments using a different NK$1$R antagonist, SR140333. The compound was used in a dose previously shown to be effective in a model of SICH (2). Administration of SR140333 before WA (1 mg/kg) did not change VMR on day 2 compared to vehicle (table 2). Stress significantly increased the VMR on day 2 in both the vehicle and treated group ($P < 0.05$ compared to baseline). In contrast, injection of 1 mg/kg before the CRD on day 2 resulted in a significant decrease in VMR on day 2 compared to vehicle (at 40 mm Hg, $P < 0.05$; at 60 mm Hg, $P < 0.001$, $n = 10$ in both groups, figure 3D). Treatment with SR140333 at a dose of 0.1 mg/kg before the CRD on day 2 did not change VMR on day 2 significantly (figure 3D). In a separate group of animals ($n = 10$), the per se effect of the compound was assessed on sham stressed animals. Sham stress did not lead to an increase in colonic sensitivity on day 2 compared to baseline, and the administration of SR140333 (1 mg/kg)
before the 24 h measurement in sham stressed animals did not influence the VMR on day 2 (data not shown).

**Chemical sympathectomy**

We first determined if the repeated treatment of the animals with 6-OHDA successfully ablated the sympathetic innervation of the gut wall, by evaluating the immunohistochemical labeling of NPY, a neuropeptide co-localized in noradrenergic nerve terminals innervating the gut mucosa (12; 32). In control animals, NPY immunostaining was specifically localized to neural elements within the submucosa and muscularis layers of the colon (figure 4A). Rats treated with chemical sympathectomy demonstrated a markedly reduced amount and intensity of labeling compared to control treatment (figure 4B). Nonspecific labeling within the mucosa was present (figure 4C), but did not interfere with the interpretation of the specific NPY peptide immunolocalization and assessment of the changes during chemical sympathectomy. These data confirmed that chemical sympathectomy resulted in loss of NPY-containing neural components within the colonic wall.

Both the 6-OHDA and vehicle treated group exhibited increased VMR 24 h after stress ($P < 0.05$ compared to baseline, $n = 10$ in each group, table 3). There was no significant difference between the two groups comparing mean changes from baseline.
DISCUSSION

This study in male Wistar rats demonstrates that 1 hr WA, an acute psychological stressor, does not have an immediate effect on colonic nociception, but results in the delayed development of colonic hypersensitivity 24 h after the stress procedure. The development of this delayed SICH requires activation of CRF$_1$Rs, but not NK$_1$Rs, at the time of the initial stressor. In contrast, the expression of the delayed colonic hypersensitivity is dependent on activation of both CRF$_1$Rs and NK$_1$Rs. Further, the development and expression of the delayed hypersensitivity is not dependent on effects mediated by peripheral noradrenergic nerves.

Delayed stress-induced colonic hypersensitivity

In the current study, exposure to 1 hr WA stress resulted in delayed SICH in 84% of all animals tested, with an average increase in the nociceptive response over baseline of 47%. Thus, SICH in this model was both robust and inducible in a reliable fashion. The increase in colonic sensitivity 24 h after WA was only observed at noxious intensities of CRD (40 and 60 mm Hg) compared to the sham stressed controls, consistent with the definition of hyperalgesia (increased nociceptive response to stimuli of noxious intensity). While evidence for SICH has previously been reported (8; 15), to our knowledge this is the first study to demonstrate a delayed effect of an acute stressor on visceral sensitivity 24 h later. The observed increase in colonic sensitivity may not be due to the earlier experience of WA stress, but rather represent a conditioned fear response to the CRD testing environment. Alternatively, it may represent non-specific changes in colonic sensitivity due to repeated testing. To rule out these confounding factors, we performed two control experiments: a) measurement of the stability of the nociceptive response to repeated CRDs on two consecutive days without any intervention; b) the effect of a 1-h sham WA stress,
in which the rats were exposed to the WA tanks without water. Neither of these interventions showed any effect on the nociceptive response on day 2 indicating that delayed SICH is a specific stress-related phenomenon.

**Dependence of delayed stress-induced colonic hypersensitivity on receptors for CRF and SP**

The mechanism(s) underlying SICH are incompletely understood and may involve both central and peripheral components (30). Preliminary evidence suggests an involvement of central CRF release and activation of CRF$_1$Rs (42). In the current study, peripheral administration of the selective CRF$_1$R antagonist CP154526, which is capable of passing through the blood brain barrier (21), prior to the WA stress prevented delayed SICH. The dose of the antagonist was chosen based on the ability of the compound to prevent SICH in a different stress model (42). This observation suggests that activation of CRF$_1$Rs on the day of the stressor is a necessary condition for the development of subsequent colonic hypersensitivity. In contrast, inhibition of NK$_1$Rs had no effect on the development of delayed SICH. One interpretation of these findings is that stress-induced CRF$_1$R activation results in an up-regulation of the CRF/CRF$_1$R signaling system detectable at 24 h. Consistent with such a hypothesis are reports showing that immobilization stress can lead to an up-regulation of the CRF$_1$R expression in the paraventricular nucleus (PVN) of the hypothalamus and other regions of the rat brain (26; 27; 39). Furthermore, central administration of CRF increased CRF$_1$R mRNA in the PVN (17; 28) suggesting that CRF release directly, or indirectly may induce CRF$_1$R gene expression. For example, up-regulation of the CRF$_1$R in the amygdala by glucocorticoids has been reported (43). Finally, there is evidence that CRF may also result in CRF gene expression (17). Taken together,
it is plausible that the activation of the CRF/CRF\textsubscript{1}R system by the acute WA stress results in an up-regulation of this system by day 2, resulting in an enhanced central stress response to the visceral pain stimulus (37). Thus, inhibition of CRF\textsubscript{1}R activation induced by WA stress could prevent CRF and CRF\textsubscript{1}R up-regulation and thereby the development of delayed SICH. Consistent with this hypothesis is also the observation that the delayed colonic hypersensitivity was blocked by administration of CRF\textsubscript{1}R antagonist prior to CRD on day two of the study.

In contrast to the effect of CRF\textsubscript{1}R antagonism, inhibition of the NK\textsubscript{1}R was only effective in blocking the expression of the delayed SICH. A possible concern regarding the use of existing NK\textsubscript{1}R antagonists is their specificity. Several different compounds have been reported to have non-specific analgesic effects on neuronal responses, possibly via inhibitory effects on voltage-sensitive Ca\textsuperscript{2+} channels. Both doses of RP67580 decreased the VMR 24 h after stress as well as sham stress below baseline suggesting an analgesic rather than an antihyperalgesic effect of the compound in our model. Prior studies have demonstrated that RP67580 has potent analgesic activity similar to morphine in response to subcutaneous formalin injection and the phenylbenzoquinone-induced writhing test (13). In contrast, SR140333 abolished only stress-induced hypersensitivity but did not have an effect on baseline or in sham-stressed animals. The dose of the antagonist used in this study was based on previous reports of its effectiveness in a model of SICH (2). These observations suggest that SR140333 does not have analgesic properties and that the observed effects are mediated by inhibition of NK\textsubscript{1}Rs.

The ability of both CRF\textsubscript{1}R and NK\textsubscript{1}R antagonism to prevent delayed SICH suggests two intriguing possibilities: 1) Activation of the CRF/CRF\textsubscript{1}R system resulted both in its own up-
regulation, and in the expression of NK₁Rs involved in SICH. 2) Antagonism of both receptor systems blocked the expression of delayed SICH, which is consistent with the hypothesis that the CRF/CRF₁R and SP/NK₁R systems are operating “in series.” Up-regulation of the CRF and SP systems could result in colonic hypersensitivity regardless if these changes occurred peripherally, centrally or at both locations. Our experiments were not aimed at locating the site of action of the CRF/CRF₁R and SP/NK₁R systems in this model. Future studies will be needed to address this important question.

**Role of the sympathetic nervous system in delayed stress-induced colonic hypersensitivity**

Stress-induced activation of the SNS could play a role in the development of SICH by several mechanisms including direct or indirect sensitization of primary afferent nerve terminals. Sympathetically maintained pain is a well-described mechanism for some forms of chronic pain (19). The SNS is one arm of the stress response which has been shown to modulate peripheral immune function, for example, mast cell degranulation can be induced by neuronal stimulation of sympathetic ganglia (22). In order to evaluate a possible role of the peripheral sympathetic innervation of the colon in the development of delayed colonic hypersensitivity, we performed the same experiments with WA stress and CRD following a chemical sympathectomy with 6-OHDA. Completeness of the chemical sympathectomy was verified by performing immunohistochemistry for NPY in the colonic submucosa, a peptide co-localized with norepinephrine in a subset of noradrenergic nerves (12). Disappearance of NPY immunoreactivity in the gut mucosa has previously been used to document the effectiveness of chemical sympathectomy (32). Since chemical sympathectomy did not affect the development of SICH, it is unlikely that release of norepinephrine (or NPY) from postganglionic sympathetic
nerves plays a role in the development of SICH in this model. A possible role of other peripheral stress mediators, such as corticosterone or epinephrine, which have been shown to sensitize primary afferents in a model of mechanical hyperalgesia (23), was not evaluated in this study.

Summary and conclusions

In summary, we demonstrate that male Wistar rats develop delayed colonic hypersensitivity following a relatively mild psychological stressor. This stress sensitization appears to involve up-regulation of both the CRF/CRF$_1$R and SP/NK$_1$R systems. Increased cerebral spinal fluid levels of SP and CRF have been reported in various stress-sensitive disorders, including posttraumatic stress syndrome (3), and preliminary data from our group suggest increased cerebrospinal fluid levels of these neuropeptides in the majority of female IBS patients (11). Thus, up-regulation of these systems may play a role in mediating recurrent abdominal pain and discomfort in a subset of these patients.
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REFERENCES


FIGURE LEGENDS

Figure 1.

A. Effect of 1 h WA stress on VMR to graded, phasic CRD immediately after WA and on day 2
\((n = 12)\). Data was normalized as percentage of the baseline of the averaged response at 60
mm Hg in the ascending series. There was a significant increase in VMR 24 h after stress
compared to baseline \((P < 0.01)\).

B. Effect of 1 h sham stress on VMR immediately and 24 h after the procedure \((n = 12)\). There
was no change in VMR after sham stress.

C. Mean changes in EMG amplitude in stressed and sham-stressed animals. The mean baseline
EMG amplitude (in percent of baseline) was subtracted from the day 2 mean EMG amplitude
at each pressure step, in each group. These mean changes were compared between the two
groups using one-way ANOVA and post hoc t-tests. Data is presented as mean change ±
standard error of difference. * \(P < 0.05\) compared to sham stress. There is a significant
difference between stressed and sham-stressed animals at 40 and 60 mm Hg.

Figure 2. Effect size of stress-induced increases in VMR on day 2. The overall effect of stress
was determined by calculating the AUC of the raw EMG amplitude as a function of pressure
before and 24 h after stress for each animal. From these values, the percent change in AUC was
calculated by taking the difference between the AUC before and 24 h after stress, dividing by the
AUC before stress and multiplying by 100. All animals that received vehicle treatment were
included in this analysis. Data are frequency histograms of the % increase in AUC on day 2.
Figure 3. Mean changes in EMG amplitude between baseline and VMR on day 2 in drug-treated vs. vehicle-treated animals. The mean baseline EMG amplitude (in percent of baseline) was subtracted from the day 2 mean EMG amplitude at each pressure step, in each group. These mean changes were compared between the two groups using ANOVA and post hoc t-tests. Data is presented as mean change ± standard error of difference. * $P < 0.05$ compared to vehicle at the particular pressure.

A. CP154526 (32mg/kg) administered before WA ($n = 12$) significantly decreased the VMR on day 2 compared to vehicle ($n = 11$) at 40 and 60 mm Hg.

B. CP154526 (32mg/kg) administered before the CRD on day 2 ($n = 12$) significantly decreased VMR on day 2 compared to vehicle ($n = 11$) at 40 mm Hg. At 60 mm Hg, the decrease in VMR almost reached significance ($P = 0.07$). ** $P = 0.07$ compared to vehicle.

C. RP67580 (3 mg/kg or 1 mg/kg, $n = 10$ in each group) injected before CRD on day 2 significantly decreased VMR 24 h after stress compared to vehicle ($n = 10$) at 40 and 60 mm Hg.

D. SR140333 (1 mg/kg) injected before CRD on day 2 ($n = 10$) significantly decreased VMR on day 2 compared to vehicle ($n = 10$) at 40 and 60 mm Hg. At 0.1 mg/kg there was no significant change in VMR 24 h after stress compared to vehicle.

Figure 4: NPY immunostaining of colon mucosa.

A. Vehicle treated rats demonstrated substantial immunostaining of NPY peptide in the submucosa and myenteric layers of the colon wall.

B. Treatment with chemical sympathectomy resulted in a marked decrease in NPY immunostaining.
C. Pre-incubation of the primary antibody with NPY blocking peptide resulted in an absence of immunostaining in the submucosal and myenteric layers with some nonspecific staining within the mucosa.
Table 1. Effect of RP67580, injected before WA, on VMR on day 2.

<table>
<thead>
<tr>
<th>mm Hg</th>
<th>Baseline</th>
<th>Day 2 *</th>
<th>Mean Change</th>
<th>Baseline</th>
<th>Day 2 *</th>
<th>Mean Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5.1 ± 1.9</td>
<td>4.1 ± 1.2</td>
<td>-1.0 ± 2.3</td>
<td>2.5 ± 0.8</td>
<td>11.0 ± 6.7</td>
<td>8.4 ± 6.8</td>
</tr>
<tr>
<td>20</td>
<td>9.1 ± 2.5</td>
<td>44.9 ± 18.2</td>
<td>35.8 ± 18.3</td>
<td>13.0 ± 6.4</td>
<td>22.9 ± 6.8</td>
<td>9.9 ± 9.4</td>
</tr>
<tr>
<td>40</td>
<td>59.0 ± 9.4</td>
<td>83.4 ± 24.1</td>
<td>24.4 ± 25.9</td>
<td>37.9 ± 6.3</td>
<td>61.3 ± 7.4</td>
<td>23.4 ± 9.7</td>
</tr>
<tr>
<td>60</td>
<td>100 ± 0</td>
<td>132.2 ± 25.0</td>
<td>32.2 ± 25.0</td>
<td>100 ± 0</td>
<td>124.6 ± 5.0</td>
<td>24.6 ± 5.0</td>
</tr>
</tbody>
</table>

EMG amplitude percent of baseline at 10, 20, 40 and 60 mm Hg at baseline, on day 2 and mean change between baseline and day 2 for rats treated with RP67580 (3 mg/kg) before WA or vehicle (n = 10 in both groups). Data is presented as mean ± SEM, mean change ± SE of difference. * P < 0.05 compared to baseline.
Table 2. Effect of SR140333, injected before WA, on VMR on day 2.

<table>
<thead>
<tr>
<th>mm Hg</th>
<th>Baseline</th>
<th>Day 2 *</th>
<th>Mean Change</th>
<th>Baseline</th>
<th>Day 2 *</th>
<th>Mean Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.6 ± 0.8</td>
<td>1.7 ± 0.7</td>
<td>-1.9 ± 1.1</td>
<td>2.8 ± 0.9</td>
<td>11.3 ± 8.1</td>
<td>8.4 ± 8.2</td>
</tr>
<tr>
<td>20</td>
<td>8.4 ± 1.8</td>
<td>13.1 ± 3.4</td>
<td>4.7 ± 3.9</td>
<td>11.9 ± 7.3</td>
<td>22.6 ± 7.9</td>
<td>10.6 ± 10.7</td>
</tr>
<tr>
<td>40</td>
<td>31.9 ± 4.1</td>
<td>66.1 ± 8.1</td>
<td>34.1 ± 9.1</td>
<td>38.8 ± 7.6</td>
<td>61.0 ± 8.8</td>
<td>22.2 ± 11.6</td>
</tr>
<tr>
<td>60</td>
<td>100 ± 0</td>
<td>130.2 ± 8.4</td>
<td>30.1 ± 8.4</td>
<td>100 ± 0</td>
<td>126.6 ± 5.8</td>
<td>26.6 ± 5.8</td>
</tr>
</tbody>
</table>

EMG amplitude percent of baseline at 10, 20, 40 and 60 mm Hg at baseline, on day 2 and mean change between baseline and day 2 for rats treated with SR140333 (1 mg/kg) before WA or vehicle (n = 10 in both groups). Data is presented as mean ± SEM, mean change ± SE of difference. * P < 0.05 compared to baseline.
## Table 3. Effect of chemical sympathectomy on VMR on day 2.

<table>
<thead>
<tr>
<th>mm Hg</th>
<th>Chemical Sympathectomy</th>
<th>Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Day 2 *</td>
</tr>
<tr>
<td>10</td>
<td>4.2 ± 0.6</td>
<td>11.9 ± 2.6</td>
</tr>
<tr>
<td>20</td>
<td>19.7 ± 2.3</td>
<td>46.2 ± 7.4</td>
</tr>
<tr>
<td>40</td>
<td>62.4 ± 7.9</td>
<td>84.7 ± 4.9</td>
</tr>
<tr>
<td>60</td>
<td>100 ± 0</td>
<td>125.4 ± 7.6</td>
</tr>
</tbody>
</table>

EMG amplitude percent of baseline at 10, 20, 40 and 60 mm Hg at baseline, on day 2 and mean change between baseline and day 2 for rats treated with 6-OHDA or vehicle (n = 10 in both groups). Data is presented as mean ± SEM, mean change ± SE of difference. * P < 0.05 compared to baseline.
Figure 2

- % increase in AUC
  - Decrease
  - 1-50%
  - 51-100%
  - > 101%

- % of animals
  - Decrease
  - 1-50%
  - 51-100%
  - > 101%
Figure 3

A

![Graph A](graph.png)

B

![Graph B](graph.png)

C

![Graph C](graph.png)

D

![Graph D](graph.png)
Figure 4

A

B

C