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Peripheral CRF-R1/CRF-R2 antagonist, astressin C, induces a long-lasting blockade of acute stress-related visceral pain in male and female rats

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Abstract

Peptide CRF antagonists injected peripherally alleviate stress-induced visceral hypersensitivity (SIVH) to colorectal distension (CRD) in rodents. Here we further evaluated the dose and time-dependent inhibitory activity of several long-acting peptide CRF receptor antagonists related to astressin on SIVH, focusing on astressin C (AstC), which previously showed high efficacy on stress-related alterations of HPA axis and gut secretomotor functions. Male and female Sprague-Dawley rats pretreated subcutaneously (SC) with AstC were injected intraperitoneally (IP) with CRF 15 min later. The visceromotor responses (VMR) to graded phasic CRD (10, 20, 40 and 60 mmHg) were monitored at basal, 15 min and up to 1–8 days after pretreatment. Two other astressin analogs, hexanoyl-astressin D (Hex-AstD) and [CαMeVal^{19,32}]-AstC, were also tested. The response to IP CRF was sex-dependent with female rats requiring a higher dose to exhibit visceral hyperalgesia. Pretreatment with AstC (30–1,000 μg/kg) resulted in a dose-related inhibition of IP CRF-induced SIVH and diarrhea in both sexes. The highest dose prevented SIVH and diarrhea up to 5–7 days after a single SC injection and was lost on day 7 (females) and day 8 (males) but reinstated after a second injection of AstC on day 8 or 9 respectively. [CαMeVal^{19,32}]-AstC and Hex-AstD (1,000 μg/kg in males) also prevented SIVH. These data show the potent long-lasting anti-hyperalgesic effect of AstC in an acute model of SIVH in both

male and female rats. This highlights the potential of long-acting peripheral CRF antagonists to treat stress-sensitive irritable bowel syndrome.

Keywords

astressin; CRF antagonists; diarrhea; peripheral CRF; rats; sex difference; visceral pain

1. Introduction

Disorders of the brain-gut interaction (DGBIs) previously known as functional gastrointestinal (GI) disorders, [1] represent an important yet unmet medical problem. They exert a significant impact on patients' quality of life while putting excessive financial pressure on healthcare systems [2, 3]. Irritable bowel syndrome (IBS), one of the most common bowel DGBIs, affects between 4–10% of the world population depending on the Rome criteria used [2, 3]. It is characterized by recurrent abdominal pain and disturbances of bowel function with a higher prevalence in women than men [4]. Visceral pain is a cardinal symptom in patients with IBS and one of the main drivers of health care seeking [5], but current treatments are scarce and often lack efficacy [6].

IBS pathogenesis is multifactorial and complex, involving GI barrier and immune dysfunction, altered gut microbiota and brain-gut axis signaling, peripherally-initiated visceral hypersensitivity, and psychosocial factors [4]. Stress is a well-known contributor and exacerbating factor in the onset, development and/or maintenance of symptoms in patients with IBS [7]. The orchestration of a stress response on the body is primarily mediated by the activation of the corticotropin-releasing factor (CRF) signaling system [8]. CRF acts on two distinct membrane bound G-protein receptors, CRF-R1 and CRF-R2 that are distributed both in the brain and periphery [9]. We previously showed that the pathophysiology of visceral hypersensitivity involves the activation of peripheral CRF signaling within the gut [10]. Using noninvasive intraluminal colonic pressure monitoring, a hyperalgesic response can be induced by a peripheral injection of CRF or CRF-R1 receptor agonist, cortagine, in rats [10–13]. The role of peripheral CRF signaling in the modulation of stress-induced visceral sensitivity is also associated with an increase in paracellular and transcellular permeability in the colon and mast cell activation in rodents as well as in humans [14, 15]. The effect of both acute (restraint, WAS) and chronic stress (WAS 4–10 days, maternal separation) on visceral sensitivity related to an increase in colonic permeability can be abolished by pretreatment of male rodents with the peripheral administration of the nonselective CRF-R1/CRF-R2 antagonist, astressin [16, 17]. These previous studies provide experimental evidence that the activation of peripheral CRF signaling via CRF-R1 is involved in the underlying mechanisms leading to visceral hyperalgesia. Neuroanatomical support is provided by the receptor expression of CRF-R1/ CRF-R2 on colonic mast cells, enterochromaffin cells and enteric neurons of the colon in rodents and human and their upregulation during stress [18, 19].

Over the past two decades, numerous non-peptide CRF-R1 antagonists, orally active and crossing the blood brain barrier have been developed and tested to interfere with stress-

related behavioral, endocrine and visceral responses to stress [20, 21]. In rodent preclinical models of IBS, these non-peptide CRF-R1 antagonists have been shown to be potent and efficacious to prevent visceral pain [22]. So far, however, in a few clinical trials, these CRF-R1 antagonists did not curtail IBS symptoms in IBS patients [23, 24]. In this regard, several early CRF-R1 small molecule antagonists generally had high lipophilicity, fast dissociation rates and failed Lipinski's 'rule of five' criteria for drug candidates suggesting that if these parameters were addressed different outcomes may be possible [23, 25–27].

Recently, Dr. Rivier's group developed long lasting peptide CRF-R1/CRF-R2 antagonists, astressins [28]. *In vitro* and *in vivo* pharmacological data support the higher efficacy of these peptides, in particular astressin C (AstC), in modulating the hypothalamic-pituitary axis response as well as stress-induced alterations of colonic secretomotor function and gastric emptying compared to previously developed peptide CRF receptor antagonists in male rats [28]. So far, however, although we have previously demonstrated that peripherally-administered astressin reduced stress-induced visceral hyperalgesia in rats [17], the influence of AstC or other astressin-related compounds on visceral pain has not yet been assessed. Therefore, the goal of the present study was to test the influence of AstC and related astressins, [CαMeVal^{19,32}]-astressin C and hexanoyl-astressin D (compounds 2, 17 and 35, Table 1) administered subcutaneously (SC) in a rodent model of acute stress-induced visceral pain in both male and female rats [10] to address their potential translational application to alleviate stress-sensitive functional bowel disorders.

2. Materials and methods

2.1. Animals

Experiments were performed in adult male (250–300 g) and cycling female (225–275 g) Sprague-Dawley (SD) rats (Envigo, San Diego, CA, USA). Animals were maintained group-housed (2/cage), unless otherwise indicated, under controlled conditions of illumination (12:12h light-dark cycle starting at 6 a.m.), temperature (21–23°C) and humidity (3–35%) and had *ad libitum* access to a standard rodent diet (Prolab RMH 2500 LabDiet, PMI Nutritional, Brentwood, MO) and tap water. Animals were acclimated to the animal facility for 1 week after their arrival. Experiments followed NIH guidelines according to the protocol # 03004–18 approved by the Institutional Animal Care and Use Committee (IACUC) of the VA Greater Los Angeles Healthcare System under the auspice of the Office of Laboratory Animal Welfare - Assurance of Compliance (A3002–01). Rats were not tested for estrous cycle stage to reduce differential stressors to the animals.

2.2. Compounds

All peptides were synthesized as previously described [28]. The rat/human CRF (r/hCRF), cortagine, astressin C (AstC), hexanoyl-astressin D (Hex-AstD), and [CαMeVal^{19,32}]-astressin C ([CαMeVal^{19,32}]-AstC) (J. Rivier, Peptide Biology Laboratories, Salk Institute, La Jolla, CA; Sentia Medical Sciences Inc., La Jolla, CA) [28] were stored in powder form at −80°C, and diluted in sterile saline (r/h CRF), sterile water (cortagine) or vehicle (DMSO 20%/mannitol 5% in sterile water) (AstC, Hex-AstD, [CαMeVal^{19,32}]-AstC) immediately

before use. The volume of injection was 0.2 ml and 0.3 ml/rat for intraperitoneally (IP) and subcutaneously (SC) respectively.

2.3. Visceral hypersensitivity

Model of acute visceral hypersensitivity: CRF or cortagine intraperitoneal injections.—Rats were injected IP with saline, CRF (10 or 50 μg/kg) or the selective CRF-R1 agonist, cortagine (10 μg/kg) [10, 29]. The doses of CRF and cortagine were based on our previous studies showing maximal effects on gut function in male rats [10, 30].

Assessment of visceral pain to CRD.—This was assessed using the non-invasive manometric method that we have previously developed and validated for use in mice and rats that does not require the chronic implantation of electromyographic electrodes [31, 32]. Briefly, a PE50 catheter was taped below (3.5 cm) the pressure sensor of a miniaturized pressure transducer catheter (SPR-524 Mikro-Tip catheter; Millar Instruments, Houston, TX). A custom-made balloon (2 cm wide x 5 cm long), [32] prepared from an infinitely compliant polyethylene plastic bag was tied over the catheter at 1 cm below the pressure sensor with silk 4.0 (Henry Schein Inc., Melville, NY). At the beginning of each experiment, the "balloon-pressure sensor" was calibrated at known pressures of 0, 20, 40 and 60 mmHg using a barostat (Distender Series II, G&J Electronics Inc, Toronto, Canada), and the voltage output was converted to pressure using CED digital analog convertor (Micro1401, Cambridge Electronic Design, Cambridge, UK) and Spike 2 software (CED, Ltd., Cambridge). On the day of the experiment, rats were briefly anesthetized with isoflurane (3% in O_2) and the lubricated "balloon-pressure sensor" catheter was introduced into the colorectum such that the distal end of the balloon was at 1 cm from the anus, and the catheter was secured to the tail with tape. Rats were placed in an individual Bollman cage to which they had been habituated for the past 3 consecutive days (1h/day). Animals were covered with a light tissue blanket and left to rest for 30 min before the CRD procedure. Each balloon was connected to the barostat and the miniaturized pressure transducer to a preamplifier (model 600; Millar Instruments, Houston, TX). The intracolonic pressure (ICP) signal was acquired using CED Micro1401/SPIKE2 program. The CRD protocol for rat consisted of two CRDs at 60 mmHg to unfold the balloon immediately followed by two series of graded phasic distensions to constant pressures of 10, 20, 40 and 60 mmHg (20 s duration, 4 min inter-stimulus interval). Similar CRD paradigms have been used previously to assess visceral pain-related responses in rats [10, 32].

Data analysis.—The phasic component of the intracolonic pressure (pICP) was extracted from the ICP signal recorded by applying the "DC Remove" Process in Spike 2 with a time constant of 1 s, to exclude the slower, tonic changes in ICP resulting from colonic smooth muscle activity, and by applying the "RMS amplitude" process with a time constant of 1 s to the resulting trace. The visceromotor response (VMR) was defined as the increased area under the curve (AUC) of pICP during CRD over the mean value of pre- and post-distension 20 s periods and was quantified using the "modulus" process in Spike 2. As each CRD pressure was repeated 2 times, the pre-post CRD and during CRD values were averaged for each pressure. To examine the pressure-response relationship and adjust for inter-individual variations of the signal, [33] pICP amplitudes were normalized for each animal to the

highest pressure (60 mmHg) in the 1st set of CRD. This value served as 100% response (control) in the baseline period of data collection and represented the baseline VMR [10, 32].

2.4. Assessment of diarrhea

Diarrhea was assessed based on a modified score used in our previous study [30] due to the fact that it was monitored at the end of the CRD procedure. The diarrhea scores were established based on the presence of watery feces (score = 2/3) or regular feces (score = 0) (absence of diarrhea).

2.5. Experimental protocols

All experiments were performed in the morning, between 8 a.m. and 12 p.m. each day to avoid variations due to the circadian rhythm.

Influence of acute CRF or cortagine IP on visceral sensitivity in male and female rats.—After 3 days of training, male and female Sprague-Dawley rats were assessed for their VMR to graded phasic CRD at 10, 20, 40, 60 mmHg (20 sec duration, 4 min intervals) monitored using manometry. On day 0, a first CRD was performed (baseline), followed by an hour of rest. Rats were then injected SC with vehicle followed 15 min after by IP CRF (10 μg/kg or 50 μg/kg), cortagine (10 μg/kg) or saline, and a second CRD was done 15 min later.

Dose response and time course of astressins influence on acute CRF IP-induced visceral hypersensitivity.—After 3 days of training, male and female Sprague-Dawley rats were assessed for their VMR to graded phasic CRD at 10, 20, 40, 60 mmHg (20 sec duration, 4 min intervals) monitored using manometry. On day 0, a first CRD was performed, followed by one hour rest period. Rats were then injected (SC) with astressin compounds namely, AstC (30–1,000 μg/kg), Hex-AstD (300 and 1,000 μg/kg), [CαMeVal^{19,32}]-AstC (1,000 μg/kg) or vehicle (DMSO 20%/mannitol 5% in sterile water). The SC doses of astressin compounds were based on previous studies showing the inhibition the endocrine and gut motor response to peripheral CRF [28]. Fifteen min later, male and female rats were injected IP with CRF (10 μg/kg for males, 50 μg/kg for females) and second CRD was done 15 min later. On day 1, 24h after astressin compounds or vehicle SC injection, rats were tested again for their baseline CRD response, and after 1 h of rest were injected IP with CRF and another CRD was performed 15 min later. A similar protocol to day 1 was repeated on days 3, 5, 7 and 8 post astressin(s) or vehicle SC injection. In some experiments, after the extinction of the inhibitory effect of CRF antagonist pre-treatment (day 7 in females or 8 in males), AstC 1,000 μg/kg was re-administered SC and 15 min, 24 h and 3 days later IP CRF (10 μg/kg in males and 50 μg/kg in females) was injected and 15 min later another CRD performed. Diarrhea was monitored at the end of the distension procedure.

2.6. Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com) and SAS 9.4 (Cary, NC).

Data were analyzed using one-way ANOVA or 2-way ANOVA followed by Sidak *post hoc* test to assess the dose-dependent influence of SC astressin(s) on IP CRF on VMR and the interaction of different treatments (baseline vs SC vehicle or astressins plus IP CRF) and CRD pressure on VMR, respectively. Generalized linear mixed effect model was used to analyze diarrhea (y vs n) incidence over time. Using this model, 4 treatment groups were compared to vehicle control group and sex was included as a covariate. Scheffe and Tukey-Kramer adjustment were applied to group (treatment groups vs control) and time (pairwise comparisons) respectively in *post hoc* analysis. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Intraperitoneal CRF induces visceral hyperalgesia in a sex-dependent manner

In male SD rats pretreated SC with vehicle 15 min before, the peripheral injection of CRF (10 μg/kg, IP) increased the VMR to CRD at 20, 40 and 60 mmHg compared to baseline values by 314% (p<0.05), 102% (p<0.0001) and 79% (p<0.0001), respectively (Fig. 1A, Table 2, Suppl. Fig. 1A), while the SC vehicle + IP vehicle (saline) had no effect (data not shown). In contrast, under the same conditions, female rats injected with CRF at 10 μg/kg, IP did not show significant changes in their VMR to CRD (Fig. 1D). Similarly, while the selective CRF-R1 agonist, cortagine, injected at 10 μg/kg increased the VMR to CRD at 40 mmHg by 158% in male rats (p<0.05) and showed a trend at 60 mmHg (Fig. 1B), the peptide did not influence the visceral pain responses in females (Fig. 1E). At 50 μg/kg, IP, CRF induced a strong visceral hyperalgesic response to CRD in both males (Fig. 1C) and females (Fig. 1F and Table 3) with an increase of the VMR to CRD at 20, 40 and 60 mmHg compared to baseline values by 744% (p=0.07), 185% (p<0.05) and 132% (p<0.01) in males and 330% (p<0.01), 233% (p<0.0001) and 76% (p<0.001) in females. Male rats exhibited diarrhea in response to CRF IP injection to a greater extent than females (OR = 15.24, 95% CI [5.81, 39.99], p<0.0001 (Figs. 2E and 4E). Therefore, the subsequent experiments were performed using IP injections of CRF at 10 μg/kg in males and 50 μg/kg in females.

3.2. Dose- and time-related preventive effect of AstC injected SC on IP CRF-induced visceral hyperalgesia to colorectal distension and diarrhea in male and female rats

In male rats, AstC duration of action was assessed after a SC pretreatment with the CRF antagonist at different doses 15 min, 1, 3, or 5 days before that of IP CRF (10 μg/kg). There was no effect at 30 and 100 μg/kg at any time point (Fig. 2A–D). At 300 μg/kg, the CRF-induced hyperalgesic response to CRD at both 40 and 60 mmHg was prevented 1 day (Fig. 2B) and no longer observed at day 5 after pretreatment (Fig. 2D). AstC at the dose of 1,000 μg/kg was the most efficacious as shown by the complete blockade of the VMR to CRD at 20, 40 60 mmHg (Suppl. Fig.1B) that lasted up to 7 days after pretreatment (Fig. 3 A–E) and was lost at day 8 (Fig. 3F). After the extinction of AstC pretreatment efficacy, a new SC injection of AstC 1,000 μg/kg at day 9 reduced the VMR response to IP CRF given 15 min (Fig. 3G) and prevented it 3 days later (Fig. 3H).

In females, AstC pretreatment at lower doses (30, 100, 300 μg/kg) showed a trend to reduce CRD induced VMR that reached significance at the lowest dose given 15 min prior to

CRF injection (50 µg/kg; Fig. 4-A–D). AstC at 1,000 µg/kg completely suppressed the CRD-induced VMR when given from 15 min to 5 days before the IP injection of CRF (Fig. 5A–D) and after 7 days, there was a significant increase of VMR to IP CRF at 40 mmHg (Fig. 5E). Another SC administration of AstC at 1,000 µg/kg at day 9 after the first injection blocked the visceral response to CRD to IP CRF monitored 24 h later (Fig. 5F)

In both sexes, AstC dose-dependently inhibited diarrhea (Fig. 2E, 4E). At 30 µg/kg, AstC had no effect while with doses of 100, 300 and 1,000 µg/kg, the number of rats responding to IP CRF with diarrhea was reduced by 86% (OR = 0.14, 95% CI [0.04, 0.51], p=0.012), 89% (OR = 0.11, 95% CI [0.03, 0.46], p=0.011) and 99% (OR = 0.01, 95% CI [0.00, 0.14], p=0.002), respectively compared to the vehicle group, adjusting for time and sex. AstC inhibitory influence on diarrhea was time-dependent with rats having the lower odds of exhibiting diarrhea at day 1 (OR = 0.12, 95% CI [0.03, 0.47], p=0.001), day 3 (OR = 0.06, 95% CI [0.01, 0.29], p<0.0001) and day 5 (OR = 0.18, 95% CI [0.05, 0.69], p=0.006) post injection compared to 15 min post injection. AstC inhibitory influence on diarrhea was maximal at day 3, the odds of rats to develop diarrhea were 52% compared to day 1 (OR = 0.52, 95% CI [0.11, 2.53], p=0.710) and 34% compared to day 5 (OR = 0.34, 95% CI [0.07, 1.64], p=0.287).

3.3. Dose- and time-related preventive effect of Hex-AstD and [CαMeVal^{19,32}]-AstC injected SC on IP CRF-induced visceral hyperalgesic and diarrheic response to colorectal distension in male rats

Based on the dose response of AstC, we selected the effective doses (300 and 1,000 µg/kg) to test two other astressin related peptides. In male rats, [CαMeVal^{19,32}]-AstC at 1,000 µg/kg partially inhibited the visceral hyperalgesic response to IP CRF 15 min post administration at 20 mmHg and 40 mmHg, but not 60 mmHg. The antihyperalgesic effect was maximal on days 1 and 3, but was lost on day 5 (Table 2). Hex-AstD demonstrated intermittent activity up to day 3 at 1,000 µg/kg in males (Table 2). Interestingly, the lower dose of 300 µg/kg showed better efficacy from 24h to day 5 in males (Table 2) and up to 24h in females (Table 3). Reinjection of hex-AstD at 300 µg/kg at day 7 in males only showed intermittent activity when tested up to 3 days after (Table 2).

4. Discussion

In this study the influence of long-acting peptide CRF-R1/CRF-R2 antagonists, including AstC [28] was tested on acute stress peptide-induced visceral hyperalgesia in male and female rats. We tested 3 astressin-derived compounds (see Table 1) and demonstrated that AstC at 1,000 µg/kg SC was the most effective compound/dose found to prevent the development of visceral hyperalgesia induced by IP CRF in both male and female rats up to 5–7 days post a single SC injection and to suppress the diarrheic response.

Over the past two decades, preclinical studies led by our group and others have provided convergent evidence that the activation of peripheral CRF receptors mimics colonic responses induced by stress and IBS-related symptoms including visceral pain, increased motility, intestinal permeability and induction of diarrhea [9]. However, most of these studies, if not all, were performed on male rodents using doses of CRF or CRF-R1 agonist(s)

ranging between 3 and 50 μg/kg [10–12, 34]. Interestingly, we found that low doses such as 3–10 μg/kg were unable to produce visceral hyperalgesia in naïve SD female rats. However, by increasing the dose of CRF IP to 50 μg/kg, s, there was a significant visceral hyperalgesic response in females, also accompanied by signs of sympathetic nervous system activation (spiky hair, swollen eyes and redness)(data not shown). It is possible that these data may in part be due to sex differences in CRF receptor signaling. Previous studies showed that CRF-R1 activation is implicated in visceral sensitivity and gut secretomotor responses whereas peripheral CRF-R2 activation exerts inhibitory effects on these responses in rodents. Thus, a balance theory has been proposed that would determine the overall response based on the degree of CRF-R1 and CRF-R2 activation [11, 35–37]. In this regard, sex differences in CRF-R1 and CRF-R2 expression levels have been described in various brain regions [38] suggesting that if similar changes in CRF-R2 over CRF-R1 expression occur in the gut, more CRF may be required in females to overcome the presumed inhibitory effects of CRF-R2 activation on visceral hypersensitivity. However, the observation that cortagine, which is considered to be a CRF-R1 selective agonist, did not result in a visceral hypersensitivity response at 10 μg/kg in females (Fig. 1), may argue somewhat against this hypothesis, although the selectivity may not have been sufficient to fully avoid CRF-R2 activation. Of potential relevance, in females, CRF-R1 preferentially signals through Gs-related pathways and has decreased ability to associate with β-arrestin 2. In contrast, in males CRF-R1 can associate with β-arrestin 2 and is biased towards β-arrestin 2–related signaling pathways [38–41]. In this regard, β-arrestin 2 signaling has been implicated in morphine-induced pain perception in that β-arrestin 2 deficient mice display enhanced and prolonged morphine-induced antinociception in spinal (tail flick) and supra-spinal (hot plate) responses to a noxious thermal stimulus [42–46]. In addition, antinociceptive potentiation and attenuation of tolerance has been reported by intrathecal β-arrestin 2 small interfering RNA in rats [47]. Thus, although Gs-mediated pathways are implicated in pain responses [43], the extent of β-arrestin 2 signaling may tip the balance resulting in enhanced CRF-induced visceral hyperalgesia in male compared to female rats. In addition, receptor desensitization differences between CRF-R1 relative to CRF-R2 may also play a role and the lack of β-arrestin 2 coupling reported in female rats may contribute to this [48]. Thus, it is plausible that these previously reported sex differences in receptor signaling, and expression may contribute to the differences we observed in visceral sensitivity between male and female rats. In contrast to males, diarrhea was not as frequent in females at the high IP CRF dose and absent at the low dose. This is in line with recent studies showing that male mice had higher baseline colonic ion secretion and greater secretory responses to similar doses of stress-related peptides (CRF and urocortins) than female mice [49]. Taken together these data may partly explain the higher prevalence of diarrhea in men with irritable bowel syndrome [50–52].

The CRF-R1 gene expresses one known functional variant, both in humans and rodents (CRFR1α, generated by deletion of exon 6) and several nonfunctional variants, which are produced by differential splicing of various exons [53–55]. The CRF-R2 has three functional splice variants in human (α, β, and γ) and two in rat (α and β) that are produced by the use of alternate 5′ exons [56–60]. Moreover, the expression pattern in the gut of CRF-R1 and CRF-R2 as well as the CRF and urocortin ligands themselves has been

reviewed for comparison across species and the evidence suggests that there are multiple common overlapping areas of expression in the rodent and human gastrointestinal systems [61]. Also, AstC has previously been reported to be a very potent antagonist acting on both CRF-R1α and CRF-R2β [28]. Thus, from both a receptor expression and potency perspective our results in rodents are encouraging for potential translation in humans and suggests that future clinical studies are warranted. In the preclinical setting, consistent evidence demonstrated that non-peptide (np) CRF-R1 antagonists alleviate stress-related visceral hyperalgesia and colonic alterations (bowel movements) [9, 10, 22, 62]. These findings stimulated mounting interest in the therapeutic potential of non-peptide (np) CRF-R1 antagonists to treat IBS [63]. However, placebo-controlled, double-blind clinical trials in IBS-diarrhea (IBS-D)-predominant patients performed using the small molecule CRF-R1 antagonists, pexacerfont (BMS-562,086) and emicerfont (GW 87,008, GSK Clinical Study Register - Study CRI105626) showed disappointing outcomes with lack of efficacy to improve IBS symptoms including only a trend to reduce visceral pain [24]. In this regard, the possible inadequacies of the early selective CRF-R1 antagonists have been reviewed and include poor chemical properties, poor bioavailability and fast dissociation rates, although other newer generation short-acting CRF-R1 antagonist(s) have now shown significant promise for other indications [23].

Astressins are newly developed peptide CRF-R1/CRF-R2 antagonists [28] which offer distinct relevant features that were not achieved by the selective np CRF-R1 small molecule antagonists tested so far. First, the characteristics of the binding interaction between selective np CRF-R1 small molecules and peptide antagonists display different attributes. The peptide antagonists interact directly with the extracellular N-terminus of CRF receptor subtypes and therefore block directly the initial event of CRF ligand binding [64] as well as interacting with the J-domain of the receptor [65]. By contrast, the np CRF-R1 antagonists are allosteric modulators and bind exclusively to sites located centrally within the transmembrane J domain and separated from the binding sites of CRF [66, 67]. Thus, their inhibitory action depends on the conformational state of the CRF-R1 induced by different agonists [68]. It is therefore possible that in some circumstances direct competitive binding at the orthosteric binding site may provide a more potent and effective means to block CRF-R1/CRF-R2 receptors. Second, some astressins have demonstrated long-acting (up to 1 week) dose-dependent effects assessed in an *in vivo* model of endogenous CRF-induced chronically elevated ACTH release in adrenalectomized rats and peripheral CRF-induced GI motor alterations [28], and visceral hyperalgesia (present study). Third, unlike the CRF-R1-selective small molecule antagonists, the astressins block both CRF-R1 and CRF-R2. This is of importance as mucosal mast cells in the human colon express both CRF-R1/CRF-R2 and both receptors contribute to the regulation of mucosal barrier function, colonic contractility, increased passage of colonic bacteria and the development of visceral pain [69–74]. In addition, CRF-R2 polymorphisms have been associated with IBS [75]. Thus, both CRF-R1 and CRF-R2 modulation may be beneficial to prevent gut motility and pain responses in humans. This is supported by the original report of Fukudo *et. al.* [76] in IBS patients using the peripheral administration of the first peptide CRF-R1/CRF-R2 antagonist, α-helical CRF$_{9-41}$ developed by Dr. Rivier [77] showing that the visceral pain response to colonic distention was blunted [78, 79] contrasting with the report that

the selective np CRF-R1 antagonist, pexacerfont failed to "decrease subjective symptoms of bloating, gas, or abdominal pain in IBS patients" [24]. Similarly to these discrepant effects, *in vitro* studies using human colonic biopsies demonstrated that α-helical CRF$_{9-41}$ prevented the horseradish peroxidase flux induced by CRF through interaction with mast cells while the np CRF-R1 antagonist antalarmin was much less effective [69].

In a rodent model of stress-induced visceral hyperalgesia following IP CRF injection, we showed that three astressin peptides significantly inhibited the visceral hyperalgesic response in a time-related manner. In both males and females, AstC was found to be the most efficient to produce long-lasting effects, protecting against the development of visceral hyperalgesia up to 7 days and diarrhea after one single subcutaneous injection. These effects were recapitulated by a second injection, indicating that the preventive effects of astressins can be maintained and reproduced with repeated administrations.

Our study has some limitations, the first being that we only addressed the preventive influence of astressins against an acutely induced visceral hyperalgesia. Further studies assessing the therapeutic effects of astressins in models of chronic visceral hyperalgesia are warranted. Secondly, the antihyperalgesic influence of astressins was assessed against a model of visceral hyperalgesia induced by IP CRF, one in which we are expecting CRF antagonists to block the pathways recruited by CRF. The use of different models of visceral hyperalgesia induced by peripheral stressors such as inflammation or infection are currently being considered.

Together, our data demonstrate a potent long-lasting antihyperalgesic effect of some astressin peptides administered SC, in particular AstC, in an acute model of stress-induced visceral hyperalgesia induced by peripheral injection of CRF in both male and female rats. These findings highlight the potential of astressin compounds to provide long-acting peripheral treatment for stress-sensitive IBS patients as an alternative to the current approved orally short-acting drugs and other compounds in development that have limited efficacy with significant side effects [20].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

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Highlights

- Peripheral CRF plays a key role in stress-induced gastrointestinal alterations.

- Male rats are more responsive to CRF-induced hyperalgesia than female rats.

- Peripheral astressin antagonists prevent acute stress-related diarrhea in rats.

- Single astressin C injection abolishes CRF-related visceral pain for 5–7 days in both sexes.

- Dose-dependent effect of astressin C can be reinstated with subsequent injections.
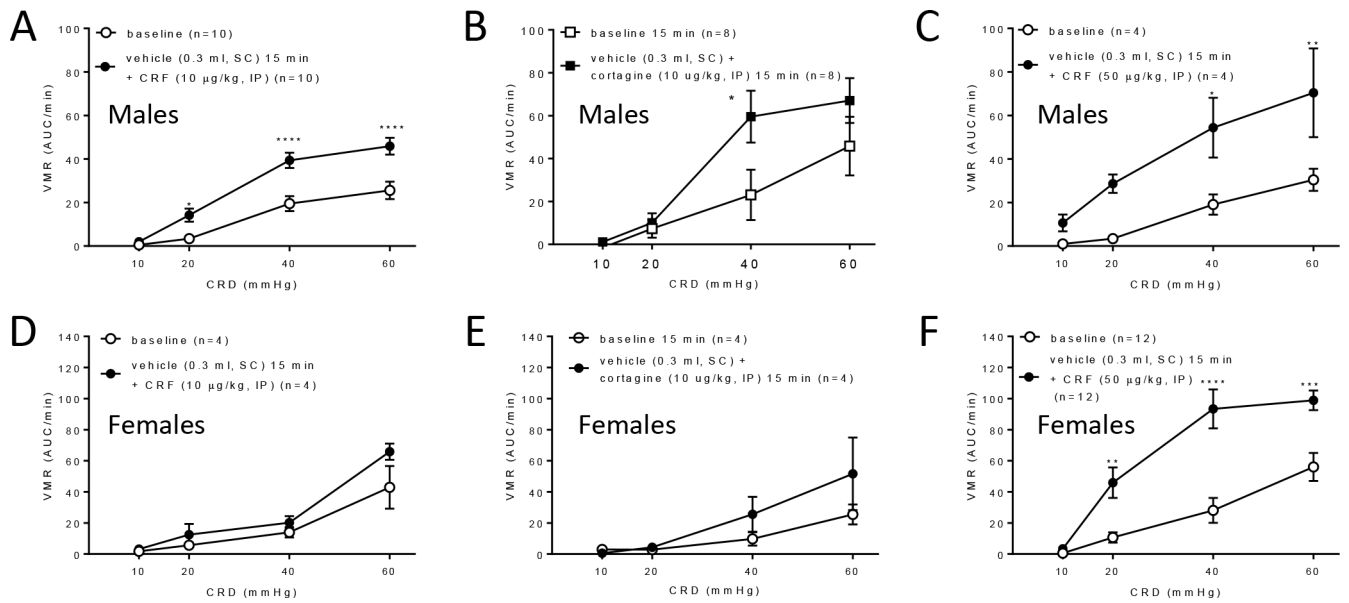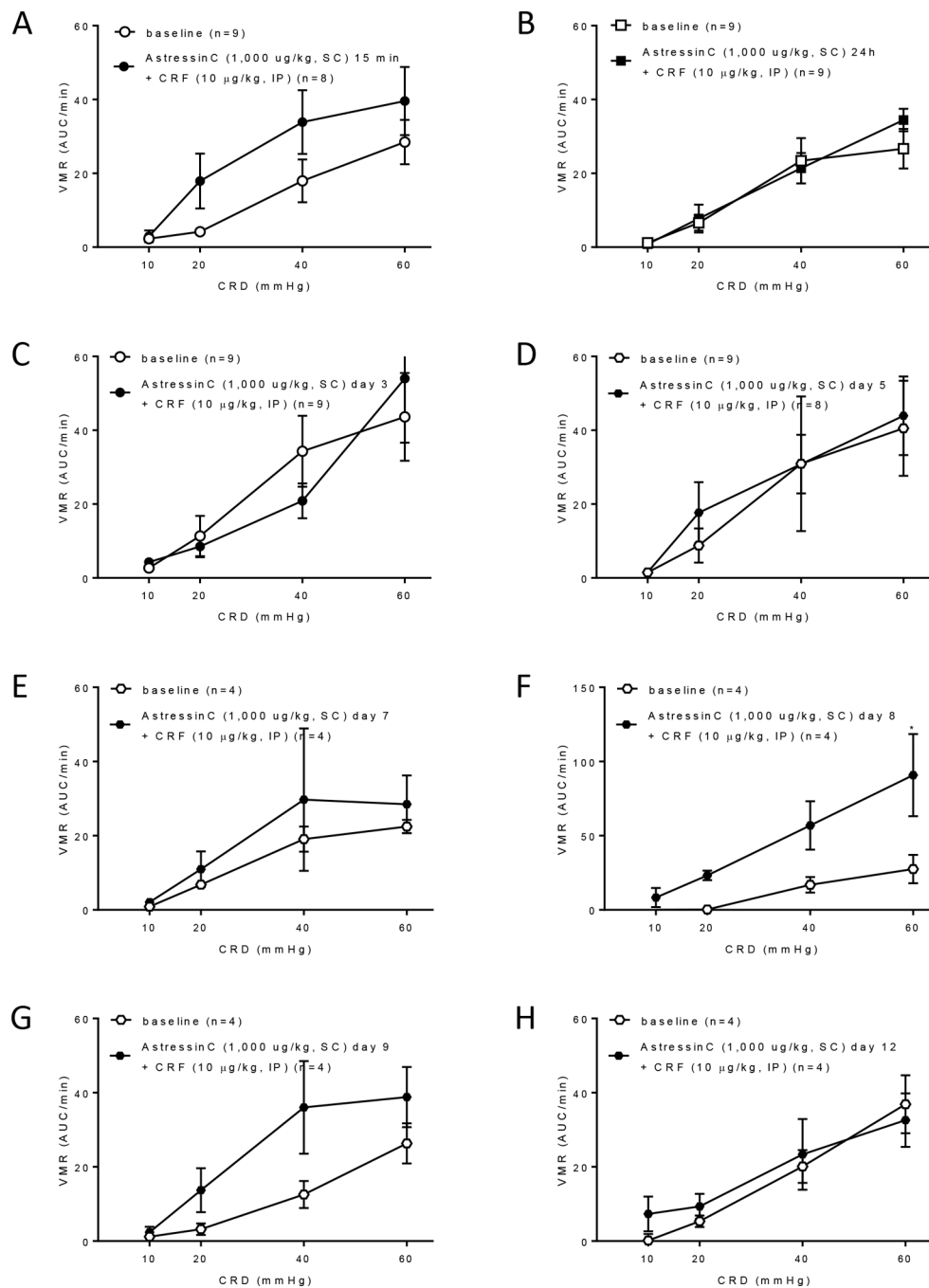
Figure 1.

Influence of intraperitoneal (IP) CRF or cortagine on visceral motor response (VMR) to colorectal distention (CRD) in male (A, B, C) and female (D, E, F) rats. On day 0, a first CRD was performed (baseline), followed by an hour of rest. Rats were then injected SC with vehicle followed 15 min after by IP CRF at 10 μg/kg, (A, D) or 50 μg/kg (C, F) or cortagine (10 μg/kg, B, E) or saline, and a second CRD was done 15 min later. Data are represented as means ± SEM, n=4–12 as indicated in parenthesis for each group. * p<0.05, ** p<0.01, *** p< 0.001 vs respective baseline, 2-way repeated measures ANOVA and Sidak *post hoc* test.
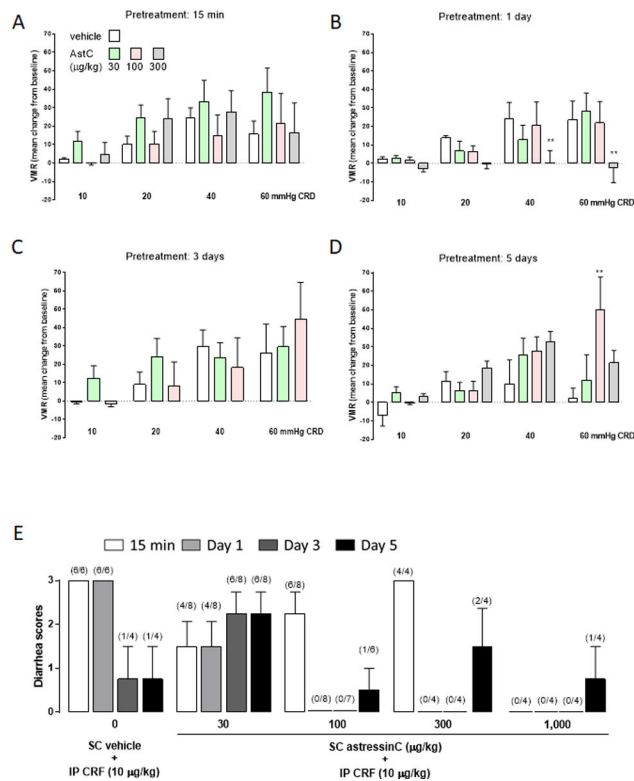
Figure 2.
Influence of dose and time of astressin C (AstC) pretreatment on acute CRF-induced
visceral hypersensitivity and diarrhea in male SD rats. After a baseline CRD and a rest
period of one hour, VMR to CRD was monitored 15 min after CRF (10 μg/kg, IP) in
rats pretreated with AstC (30, 100 or 300 μg/kg, SC) 15 min (A), 1 (B), 3 (C), and 5
(D) days before. Diarrhea scores in response to CRF IP were assessed at the end of the
CRD procedure in rats pretreated with AstC (30, 100, 300 or 1,000 μg/kg, SC) or vehicle.
Numbers in parenthesis represent the number of rats that developed diarrhea over the total
number of rats tested (E). Data are represented as means ± SEM, vehicle (n=3–6), AstC 30
(n=8), AstC 100 (n=6–8), AstC 300 (n=4). ** p<0.01 vs vehicle, 2-way repeated measures
ANOVA and Bonferroni *post hoc* test.

Figure 3.
Time-dependent influence of astressin C (AstC) pretreatment at 1,000 μg/kg, SC on acute
CRF -induced visceral hypersensitivity and effect of a repeated pretreatment in male SD
rats. After a baseline CRD and a rest period of one hour, VMR to CRD was monitored
15 min after CRF (10 μg/kg, IP) in rats that were pretreated with AstC (1,000 μg/kg) 15
min (A), 1 (B), 3 (C), 5 (D), 7 (E) and 8 (F) days before. On day 9, rats were reinjected
with AstC (1,000 μg/kg, SC) and the visceral pain to IP CRF was again assessed 15 min
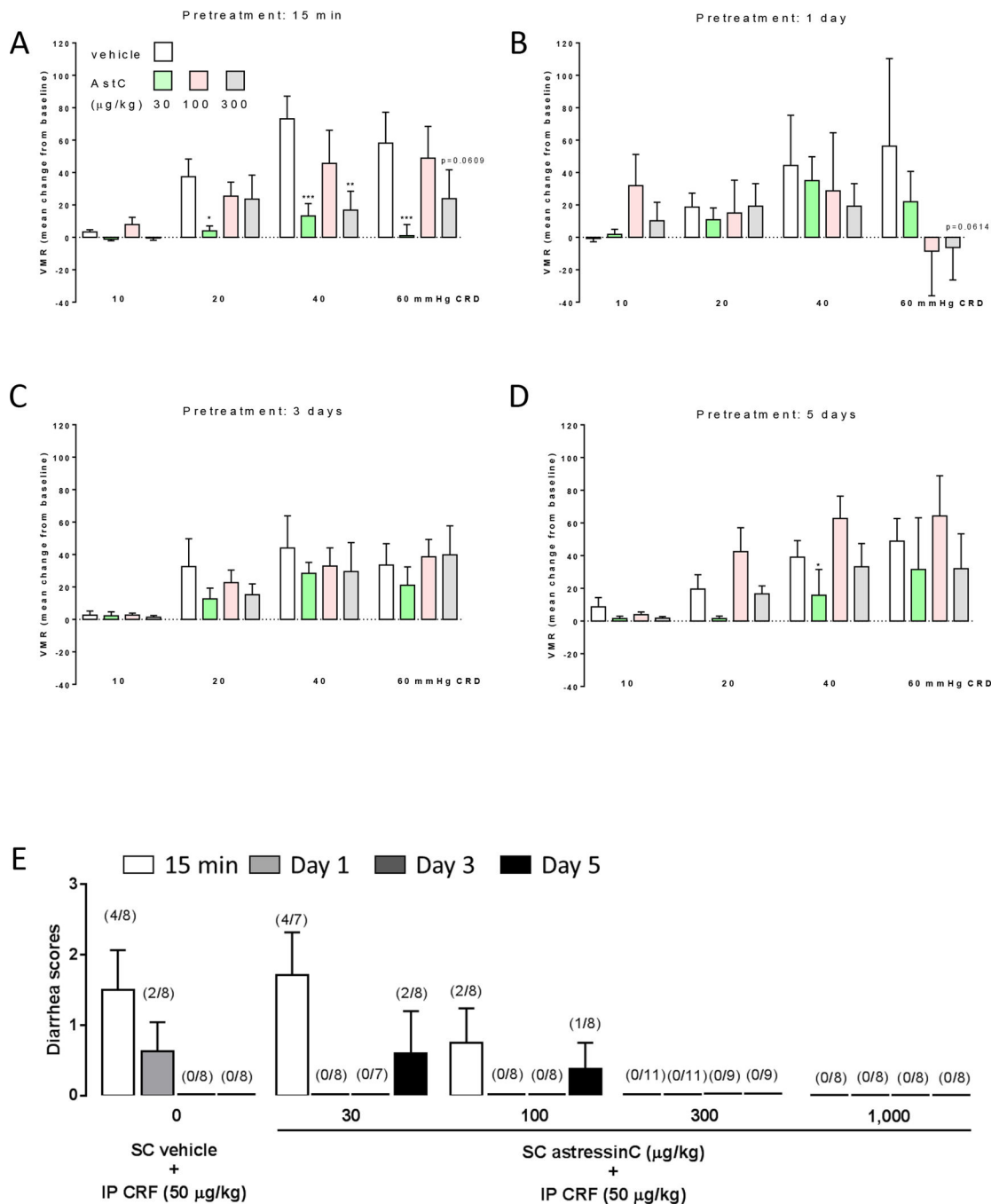(day 9)(G) and 3 days (day 12)(H) later. Data are represented as means ± SEM, n=4–9

as indicated in parenthesis for each group. *p<0.05 vs baseline, 2-way repeated measures ANOVA and Sidak *post hoc* test.

Figure 4.
Influence of dose and time of astressin C (AstC) pretreatment on acute CRF-induced visceral hypersensitivity and diarrhea in female SD rats. After a baseline CRD and a rest period of one hour, VMR to CRD was monitored 15 min after CRF (50 μg/kg, IP) in rats pretreated with AstC (30, 100 or 300 μg/kg, SC) 15 min (A), 1 (B), 3 (C), and 5 (D) days before. Diarrhea scores in response to CRF IP were assessed at the end of the CRD procedure in rats pretreated with AstC (30, 100, 300 or 1,000 μg/kg, SC) or vehicle. Numbers in parenthesis represent the number of rats that developed diarrhea over the total

number of rats tested (E). Data are represented as means ± SEM, vehicle (n=7–11), AstC 30 (n=6–8), AstC 100 (n=8), AstC 300 (n=8), *p<0.05, ** p<0.01, ****p<0.0001 vs vehicle, 2-way repeated measures ANOVA and Bonferroni *post hoc* test.
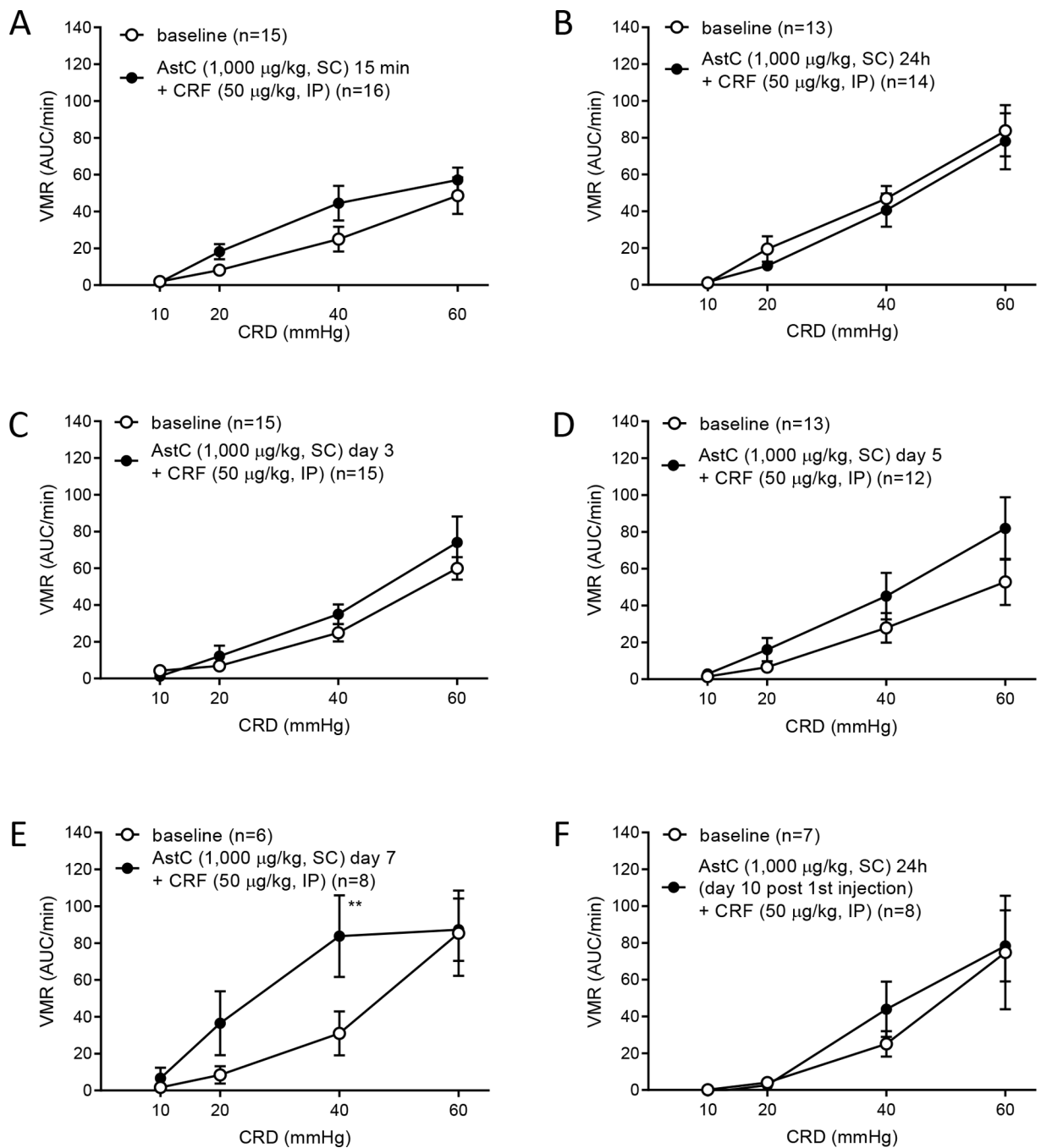
Figure 5.
Time-dependent influence of astressin C (AstC) at 1,000 μg/kg, SC on acute CRF-induced visceral hypersensitivity and effect of a repeated pretreatment in female SD rats. After a baseline CRD and one rest period, rats received an acute injection of AstC followed 15 min later by CRF. The VMR to CRD was assessed again after 15 min (A), 1 (B), 3 (C) and 5 (D) days. A second injection done at day 9 reversed the hyperalgesic influence of CRF IP when tested 24h later (F). Data are represented as means ± SEM, n=6–16 as indicated

in parenthesis for each group. ** p<0.01 vs respective baseline, 2-way repeated measures ANOVA and Sidak *post hoc* test.

Table 1.

Amino acid sequence and chemical modifications of the main three peptides tested.

AstC	Ac-DLTfHLLREVLEXARAEQZAQEABKNRKLXEZI-NH$_2$	[Aib^{24}]-AstB
[CαMeVal^{19,32}]-AstC	Ac-DLTfHLLREVLEXARAEQOAQEABKNRKLXEOI-NH$_2$	
Hex-AstD	Hex-DLTfHLLREVLEXARAEQBAQEABKNRKLXEBI-NH$_2$	[Aib^{19,32}]-Hex-AstC

f=DPhe; X=Nle; Z=CαMeLeu; O=CαMeVal; B=Aib; Hex=hexanoyl; EABK lactam bridge between E and K

Table 2:

Influence of astressin compounds on the visceromotor response to CRD in male Sprague-Dawley rats injected with CRF (10 µg/kg, IP). Data are mean ± SEM of the visceromotor response expressed in AUC/min of the number of animals as indicated in parenthesis in each column for post-injection/baseline respectively.

Males (CRF 10 µg/kg IP)	(µg/kg SC)	Days	CRD pressure (in AUC/min, n in parenthesis for post-injection/baseline)			
			10 mmHg	20 mmHg	40 mmHg	60 mmHg
Vehicle	0	15 min	1.9 ± 0.5 vs 0.6 ± 0.4 (10)	**14.2 ± 3.1 vs 3.4 ± 0.8** *(10)	**39.4 ± 3.5 vs 19.5 ± 3.5** ****(10)	**45.9 ± 3.9 vs 25.6 ± 4.0** ****(10)
		24h	1.7 ± 0.7 vs 0.9 ± 0.6 (7)	24.9 ± 5.7 vs 8.6 ± 2.8 (7)	**41.8 ± 9.9 vs 23.9 ± 7.5** *(7)	**61.9 ± 11.0 vs 33.3 ± 6.7** *(7)
		Day 3	0.8 ± 1.0 vs 1.2 ± 0.7 (4)	10.8 ± 6.3 vs 1.7 ± 2.2 (4)	**42.2 ± 9.0 vs 12.6 ± 1.7** *(4)	**52.8 ± 15.7 vs 26.8 ± 2.9** *(4)
		Day 5	0.2 ± 1.2 vs 7.4 ± 5.5 (4)	15.7 ± 4.5 vs 4.5 ± 3.0 (4)	29.9 ± 8.8 vs 20.0 ± 9.7 (4)	24.7 ± 3.9 vs 22.7 ± 4.0 (4)
HexAstD	300	15 min	4.1 ± 1.9 vs 0.3 ± 0.3 (17)	23.6 ± 6.6 vs 5.9 ± 1.5 (17)	**57.3 ± 10.3 vs 30.2 ± 5.5** **(17)	**60.4 ± 9.3 vs 36.3 ± 4.5** *(17)
		24h	1.1 ± 0.7 vs 1.6 ± 0.7 (17/15)	7.8 ± 1.7 vs 6.5 ± 1.7 (17/15)	30.8 ± 4.3 vs 27.4 ± 3.5 (17/15)	51.9 ± 7.5 vs 43.6 ± 6.1 (17/15)
		Day 3	1.6 ± 0.8 vs 0.3 ± 0.6 (17/16)	12.3 ± 5.3 vs 6.9 ± 2.9 (17/16)	39.7 ± 10.5 vs 22.5 ± 6.7 (17/16)	58.3 ± 10.3 vs 38.4 ± 9.2 (17/16)
		Day 5	3.2 ± 1.3 vs 2.1 ± 1.1 (17/13)	15.3 ± 4.2 vs 6.3 ± 1.4 (17/13)	36.9 ± 6.7 vs 27.7 ± 7.5 (17/13)	51.7 ± 9.5 vs 34.7 ± 5.9 (17/13)
		Day 7	0.5 ± 0.7 vs 1.7 ± 0.9 (8)	19.4 ± 8.7 vs 6.3 ± 2.5 (8)	**47.6 ± 7.3 vs 22.4 ± 7.7** *(8)	53.1 ± 14.7 vs 39.0 ± 8.5 (8)
		Day 8 (#15 min)	2.4 ± 0.9 vs 0.4 ± 0.3 (7)	12.2 ± 3.1 vs 6.7 ± 5.2 (7)	47.0 ± 10.0 vs 30.2 ± 9.9 (7)	**83.5 ± 19.0 vs 45.8 ± 11.1** ***(7)
		Day 9 (#24h)	1.1 ± 1.5 vs −0.5 ± 0.7 (7)	2.1 ± 1.3 vs 2.2 ± 0.4 (7)	**39.4 ± 12.6 vs 23.3 ± 6.9** *(7)	48.0 ± 11.8 vs 40.9 ± 7.0 (7)
		Day 11 (#day 3)	−1.1 ± 0.9 vs 1.5 ± 0.6 (4)	2.5 ± 1.9 vs 2.8 ± 1.5 (4)	30.6 ± 13.9 vs 30.4 ± 7.7 (4)	82.1 ± 12.7 vs 54.4 ± 13.7 (4) (p=0.0624)
	1,000	15 min	7.1 ± 2.8 vs −0.1 ± 0.4 (8)	11.7 ± 2.5 vs 7.9 ± 2.0 (8)	**43.2 ± 7.2 vs 30.2 ± 10.1** *(8)	36.2 ± 3.0 vs 35.7 ± 1.7 (8)
		24h	48.8 ± 7.9 vs 1.3 ± 0.5 (8)	39.1 ± 7.9 vs 6.4 ± 1.3 (8)	**6.4 ± 2.0 vs 17.7 ± 3.0** *(8)	0.3 ± 0.5 vs 35.8 ± 5.4 (8)
		Day 3	28.6 ± 2.5 vs 2.6 ± 1.0 (8)	21.6 ± 3.9 vs 3.4 ± 1.4 (8)	6.5 ± 1.9 vs 16.9 ± 4.5 (8)	1.9 ± 1.0 vs 28.8 ± 3.6 (8)
		Day 5	10.4 ± 5.3 vs 1.2 ± 0.7 (8/5)	20.0 ± 8.9 vs 11.3 ± 2.2 (8/5)	**54.8 ± 9.6 vs 26.7 ± 7.8** *(8/5)	36.6 ± 9.1 vs 45.3 ± 11.1 (8/5)
[CαMeVal[19,32]]-AstC	1,000	15 min	0.9 ± 0.8 vs 0.8 ± 0.6 (8)	4.6 ± 1.9 vs 3.8 ± 1.4 (8)	16.2 ± 4.0 vs 16.5 ± 3.7 (8)	**40.6 ± 9.6 vs 21.2 ± 4.5** *(8)
		24h	1.8 ± 0.4 vs 2.8 ± 2.2 (7/8)	7.8 ± 2.8 vs 5.9 ± 2.5 (7/8)	20.5 ± 1.9 vs 15.5 ± 5.2 (7/8)	29.0 ± 3.9 vs 37.2 ± 7.3 (7/8)
		Day 3	3.2 ± 1.9 vs 3.5 ± 1.8 (7/8)	11.7 ± 4.0 vs 6.0 ± 2.8 (7/8)	22.9 ± 5.2 vs 14.4 ± 5.4 (7/8)	35.3 ± 5.0 vs 26.1 ± 3.8 (7/8)
		Day 5	1.1 ± 1.4 vs 0.5 ± 0.6 (7/6)	17.4 ± 6.8 vs 4.3 ± 1.8 (7/6)	**49.2 ± 11.2 vs 8.1 ± 1.4** ***(7/6)	50.7 ± 8.2 vs 22.6 ± 4.6 (7/6)

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p<0.05

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p<0.01

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p<0.001

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p<0.0001 vs baseline, 2-way ANOVA and Sidak's *post hoc* test.

Table 3:

Influence of astressin compounds on the visceromotor response to CRD in female Sprague-Dawley rats injected with CRF (50 µg/kg, IP). Data are mean ± SEM of the visceromotor response expressed in AUC/min of the number of animals as indicated in parenthesis in each column.

Females(CRF 50 µg/kg IP)		Days	CRD pressure (in AUC/min, n in parenthesis for post-injection/baseline)			
			10 mmHg	20 mmHg	40 mmHg	60 mmHg
vehicle	0	15 min	4.5 ± 1.3 vs 1.1 ± 0.4 (22)	44.5 ± 10.6 vs 7.0 ± 2.7 ** (22)	94.5 ± 13.2 vs 21.3 ± 4.5 **** (22)	111 ± 16.2 vs 52.8 ± 10.1 **** (22)
		24h	1.5 ± 1.0 vs 2.1 ± 1.8 (19)	23.0 ± 8.6 vs 4.3 ± 0.9 * (19)	75.6 ± 28.8 vs 31.2 ± 11.3 ** (19)	129.2 ± 51.1 vs 73.0 ± 18.0 * (19)
		Day 3	3.8 ± 2.5 vs 1.2 ± 0.5 (19/11)	34.8 ± 17.2 vs 2.3 ± 0.9 * (19/11)	61.9 ± 19.2 vs 17.8 ± 4.7 ** (19/11)	80.4 ± 8.9 vs 46.9 ± 9.8 **** (19/11)
		Day 5	8.8 ± 5.6 vs 0.1 ± 0.7 (9/11)	26.3 ± 8.4 vs 6.8 ± 2.6 (9/11)	57.5 ± 9.0 vs 18.4 ± 4.6 ** (9/11)	89.0 ± 9.6 vs 40.1 ± 9.9 *** (9/11)
HexAstD	300	15 min	−0.2 ± 1.4 vs 0.8 ± 0.3 (10/12)	15.6 ± 6.8 vs 4.3 ± 1.5 (10/12)	49.0 ± 19.4 vs 25.0 ± 5.3 (10/12)	66.6 ± 14.4 vs 39.7 ± 8.7 (10/12)
		24h	0.7 ± 0.5 vs 0.7 ± 0.7 (8/5)	2.4 ± 1.9 vs 6.0 ± 2.8 (8/5)	21.0 ± 5.4 vs 25.1 ± 5.8 (8/5)	50.1 ± 10.7 vs 60.3 ± 7.7 (8/5)
		Day 3	−0.4 ± 0.7 vs 0.4 ± 0.7 (8/7)	10.6 ± 2.4 vs 0.7 ± 0.9 (8/7)	54.0 ± 11.9 vs 18.5 ± 7.4 * (8/7)	87.2 ± 14.9 vs 50.1 ± 17.2 * (8/7)
		Day 5	7.4 ± 5.1 vs 0.4 ± 0.9 (8)	32.5 ± 16.3 vs 4.5 ± 2.8 (8)	58.8 ± 22.1 vs 26.2 ± 6.0 (8)	82.9 ± 27.2 vs 62.9 ± 13.4 (8)

*
$p < 0.05$

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$p < 0.01$

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$p < 0.001$

****
$p < 0.0001$ vs baseline, 2-way ANOVA and Sidak's *post hoc* test.