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Authors

Larauche, Muriel
Moussaoui, Nabila
Biraud, Mandy
et al.

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Brain CRF signaling: involvement in acute stress-induced visceral analgesia in male rats

M. Larauche[1,2], **N. Moussaoui**[1,2,3], **M. Biraud**[1,2,4], **W.K. Bae**[1,2,5], **H. Duboc**[1,2,6], **M. Million**[1,2], and **Y. Taché**[1,2]

[1]Department of Medicine, UCLA, G Oppenheimer Center for Neurobiology of Stress and Resilience and CURE: Digestive Diseases Research Center, Vatche and Tamar Manoukian Division of Digestive Diseases, David Geffen School of Medicine, Los Angeles, CA, United States

[2]VA Greater Los Angeles Healthcare System, Los Angeles, CA, United States

[3]Present address: Inserm U1048/I2MC Obesity Research Laboratory, 1 avenue Jean Poulhès BP 84225 31432 Toulouse Cedex 4, France

[4]Present address: 1060 William Moore drive CVM Main Building, RM C305, Raleigh, NC 27607, USA

[5]Present address: Department of Internal Medicine, Ilsan Paik Hospital, Inje University College of Medicine, Goyang, Korea

[6]Present address: CRI INSERM UMR 1149, University Paris Diderot, Sorbonne Paris Cité and DHU Unity, APHP, F-75890 Paris, France

Abstract

Background: Water avoidance stress (WAS) induces a naloxone-independent visceral analgesia in male rats under non-invasive conditions of monitoring. The objective of the study was to examine the role of brain CRF signaling in acute stress-induced visceral analgesia (SIVA).

Methods: Adult male Sprague Dawley rats were chronically implanted with an intracerebroventricular (ICV) cannula. The visceromotor response (VMR) to graded phasic colorectal distension (CRD: 10, 20, 40, 60 mmHg, 20 sec, 4 min intervals) was monitored using manometry. The VMR to a 1st CRD (baseline) was recorded 5 min after an ICV saline injection, followed 1h later by ICV injection of either CRF (30, 100, 300 ng and 1, 3 or 5 μg/rat) or saline and a 2nd CRD, 5 min later. Receptor antagonists against CRF_1/CRF_2 (astressin-B, 30 μg/rat), CRF_2 (astressin$_2$-B, 10 μg/rat), oxytocin (tocinoic acid, 20 μg/rat) or vehicle were injected ICV 5 min before CRF (300 ng/rat, ICV) or 15 min before WAS (1h).

Correspondence: Muriel Larauche, PhD, CURE: DDRC, VA GLA HS, 11301 Wilshire Blvd., Bldg. 115/Rm 111, Los Angeles, CA 90073, Tel. +1-310-478-3711 extension 41827, Fax: +1-310-268-4963, mlarauche@mednet.ucla.edu.

AUTHOR CONTRIBUTION:

ML designed the experiments, carried out research, analyzed data, and prepared the manuscript. NM, participated in the experimental research and writing of the manuscript; MB, WKB, HD performed components of the experiments; MM discussed and reviewed the manuscript; YT, designed the experiments, evaluated the data and prepared the manuscript. All authors read and approved the final version of manuscript.

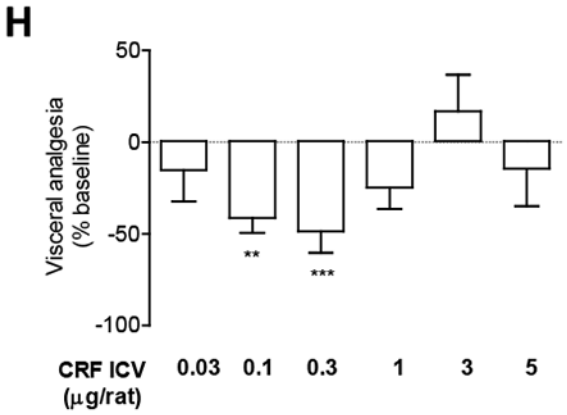
CONFLICT OF INTERESTS

The authors have nothing to disclose.

Key Results: ICV CRF (100 and 300 ng) reduced the VMR to CRD at 60 mmHg by −36.6±6.8% and −48.7±11.7% respectively vs baseline ($p<0.001$) while other doses had no effect and IP CRF (10 μg/kg) induced visceral hyperalgesia. Astressin-B and tocinoic acid injected ICV induced hyperalgesia and prevented the analgesic effect of ICV CRF (300 ng/rat) and WAS, while astressin$_2$-B only blocked WAS-induced SIVA.

Conclusions & Inferences: These data support a role for brain CRF signaling via CRF$_2$ in SIVA in a model of WAS and CRD likely mediated by the activation of brain oxytocin pathway.

Abbreviated abstract:



Recent reports indicate that WAS induces a naloxone-independent visceral analgesia in male rats under non-invasive conditions of monitoring, but the underlying brain neurochemical mechanisms are still unknown. When injected intracerebroventricularly (ICV) at low nanogram range doses, CRF induced a CRF$_1$ receptor mediated visceral analgesic response to colorectal distension that was prevented by ICV oxytocin antagonist. Similarly, acute WAS recruits endogenous CRF and oxytocin to induce SIVA.

Increasing the understanding of the neurochemical coding by which stress promotes visceral analgesia and how their dysfunction leads to visceral hyperalgesia may help developing new therapeutic modalities for patients with functional gastrointestinal disorders who exhibit abnormal endogenous pain modulation.

Keywords

corticotropin-releasing factor; stress-induced visceral analgesia; water avoidance stress; manometry; oxytocin

1 INTRODUCTION

Brain corticotropin-releasing factor (CRF) acts as a central mediator of stress by orchestrating several responses such as endocrine (hypothalamic-pituitary-adrenal axis), behavioral (anxiety and depression-like)[1], autonomic[2] and visceral.[3] CRF actions are initiated by binding to two subtypes of G-protein coupled receptors: CRF subtype 1 (CRF$_1$) and to a lesser extent subtype 2 (CRF$_2$).[4] The CRF receptors are expressed in several stress-

related brain regions.[5, 6] Stress is also known to modulate pain, inducing hyperalgesia or analgesia depending upon the nature, duration and magnitude of the stressor.[7, 8] Concerning stress-induced analgesia, in the somatic pain field, a number of studies demonstrated that exogenous CRF can induce analgesia and endogenous CRF signaling in the brain plays a role in acute physical and psychological stress-induced analgesia. [8–13] This response occurs through the recruitment of endogenous descending inhibitory pain pathways.[8–13] So far the role of CRF receptor subtype(s) involved has not yet been characterized except in one study indicative of a mediation through CRF_2 receptors.[14] By contrast, to this date, stress-induced visceral analgesia (SIVA) has been understudied. Existing literature in rodents indicates that exposure to a mild psychological stressor such as water avoidance stress (WAS)[15] or intracerebroventricular (ICV) injection of CRF promotes visceral hyperalgesia[16–17] in rodents. Of note, all those studies were performed using an invasive surgical technique to monitor the visceromotor response (VMR) to colorectal distension (CRD) by chronically implanting electromyographic (EMG) electrodes on the abdominal wall of rodents. Conversely, our recent studies, using a non-invasive method of VMR recording based on manometry,[18,19] highlighted the development of visceral analgesia in response to acute and/or repeated WAS in both mice and rats.[19, 20] Since then, other groups using WAS paired with CRD as a visceral painful stimulus have reproduced this visceral analgesic response in rats, using similar non-invasive VMR recording[21] or acute fixation of EMG electrodes on the external oblique muscles (30 min before CRD).[22] We and others also established that the visceral analgesia induced by acute and repeated WAS is opioid-independent in male rats. [20, 22] Outside of the reported role for brain-neurotensin signaling in rodents,[23–25] the underlying neurochemical substrata underpinning SIVA are still not well characterized. Of interest, the involvement of brain CRF signaling that contributes to a number of biological responses induced by stress,[1] remains largely unexplored in the SIVA response.

Therefore, in the present study, we first assessed the dose-response effect of CRF injected ICV on visceral pain by monitoring the VMR to CRD non-invasively in male rats. We tested several doses based on previous studies in the somatic pain field pointing to a narrow effective dose range.[12] Since previous studies established a brain to blood transport of CRF, [26] we injected CRF intraperitoneally to confirm that the ICV injection of CRF-induced SIVA was brain-mediated. Then, we characterized the receptor subtypes involved in the effect of centrally-injected CRF using the long acting CRF_1 and CRF_2 antagonist, astressin-B[27] and the selective peptide CRF_2 receptor antagonist, $astressin_2$-B.[28] The ICV injection of CRF causes oxytocin release,[29] and brain oxytocin (OT) is well established to be analgesic in different modalities of acute pain (neuropathic, somatic or inflammatory) in rodents and humans.[30–32] The role of OT in ICV CRF-induced visceral analgesia was examined using ICV injection of the OT receptor antagonist, tocinoic acid.[33, 34] Lastly, we performed ICV injections of non-selective CRF and selective CRF_2 and OT receptor antagonists to investigate the role endogenous brain CRF and OT signaling in the non-opiate form of SIVA. [20, 22] We used an acute exposure to an environmental aversive condition by applying WAS and the CRD test to assess visceral pain.

2 MATERIAL AND METHODS

2.1 Animals

Adult male Sprague-Dawley rats (Harlan Laboratory, San Diego, CA, USA) weighing 250-300 g were used for these experiments. 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 # 9906-020 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).

2.2 Treatments

Intracerebroventricular injection.—Rats were equipped with a chronic ICV cannula as previously described.[18] The guide cannula (22 gauge, Plastic One Products, Roanoke, VA) was implanted into the right lateral brain ventricle in animals anesthetized with an intraperitoneal (IP) injection of a mixture of ketamine hydrochloride (75 mg/kg; Ketaset, Fort Dodge Laboratories Inc., Fort Dodge, IA) and xylazine (5 mg/kg; Rompun, Mobay Corporation, Shawnee, KS) using the following coordinates (mm from bregma: antero-posterior, –0.8; lateral, –1.5; dorsoventral, –4.0). The guide cannula was maintained in place by dental cement anchored by four stainless steel jewelry screws fixed to the skull. Post-surgery, animals were housed individually on direct bedding and received subcutaneous injections of buprenorphine (0.03 mg/kg; Bedford Labs, Bedford, OH, USA) twice daily for 3 days. Thereafter, rats were handled for 5 min daily to habituate them to the manipulation of the cannula and the injection procedure. The ICV injection was performed in lightly hands restrained rat as in our previous studies.[18] A 28-gauge injection cannula, 1 mm longer than the guide cannula, was then connected to a 50-µl Hamilton syringe by a PE-50 catheter (Intramedic Polyethylene Tubing, Clay Adams, Sparks, MD) filled with distilled water. An air bubble (5 µl) was drawn at the distal end of the PE-50 catheter to separate the injected solution from the water and for visual inspection of the injections which were all performed over 30 s in 5 µl for each except otherwise stated. At the end of the experiments, the correct location of the cannula into the lateral ventricle was examined by injecting 10 µl of dye (0.1% toluidine blue). Visualization of dye on the wall of the lateral ventricle indicates correctness of the ICV injections. No rats were excluded due to the misplacement of the cannula.

Acute water avoidance stress.—The acute WAS was performed between 9 a.m. and 12 p.m. and lasted 1 h with all groups conducted in parallel to avoid experimental variations. The procedure for acute WAS was essentially as described previously in rats.[20] It consisted of placing rats individually on a rectangular platform (5.8-cm length × 5.8-cm width × 6.0-cm height, AMAC box M series #510C, AMAC Plastic, Petaluma, CA) affixed in the center to the bottom of a container (26.7-cm length × 48.3-cm width × 20.3-cm height, R20 rat

cage, Ancare, Bellmore, NY) filled with room temperature tap water (25°C) up to 1 cm below the top of the platform.

2.3 Measurement of visceral pain

Assessment of visceral pain response to CRD.—Visceral sensitivity to CRD was assessed using the non-invasive manometric method that we have previously developed and validated for use in mice and rats, which does not require chronic implantation of EMG electrodes.[18,19] 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),[18, 35] 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 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.[18, 20]

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 VMR was defined as the increase in 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,[36] ICP 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.[18, 20]

2.4 Compounds

The human/rat CRF, astressin-B and astressin$_2$-B (J. Rivier, Peptide Biology Laboratories, Salk Institute, La Jolla, CA) were stored in powder form at −80°C, and diluted in sterile saline (CRF) or water (astressin-B, astressin$_2$-B) immediately before use. Tocinoic acid (Sigma-Aldrich, St Louis, MO, USA) was dissolved in saline.

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 intracerebroventricular vs intraperitoneal injection of CRF on visceral sensitivity to CRD.—Groups of rats were equipped with ICV cannula at least one week before the experiments. Rats were injected ICV with saline (5 μl/rat) and the VMR to a 1st CRD was monitored 5 min later (baseline response). After 1h rest period, saline (5 μl/rat) or CRF (10, 30, 100, 300 ng/rat, 1, 3 and 5 μg/rat in 5 μl) was injected ICV and a 2nd CRD was performed 5 min later. In a separate study, naïve rats were used to assess the influence of peripheral administration of CRF. Rats were subjected to 1st CRD and 1h later were injected IP (0.2 ml/rat) with saline or CRF (10 μg/kg, i.e. ~3 μg/rat) and 15 min later, the 2nd CRD was performed in both groups. The doses of CRF were based on our previous studies showing dose-related (ICV) or maximal effect effects (IP) on gut function.[37, 38] CRF at 300 ng/rat inducing the maximal visceral analgesic effect was selected for all further ICV studies.

Effect of CRF receptor antagonists on ICV CRF-induced visceral analgesia.—In separate groups, ICV cannula-equipped rats had a baseline VMR to 1st CRD and after the 1h rest period, astressin-B (30 μg/rat), astressin$_2$-B (10 μg/rat) or sterile water (5 μl) was injected ICV 5 min before ICV CRF (300 ng/rat, 5 μl) and a 2nd CRD was performed 5 min later. The ICV doses of astressin-B and astressin$_2$-B were selected based on our previous studies showing complete reversal of ICV CRF on gut motor function and blockade of endogenous CRF receptors in rats.[39–41]

Influence of oxytocin antagonist on ICV CRF-induced visceral analgesia.—Rats equipped with ICV cannula were tested for baseline CRD. After the 1h rest period, tocinoic acid (20 μg/rat, 5 μl) or saline (5 μl) was injected ICV 5 min before the ICV injection of CRF (300 ng/rat, 5 μl) and a 2nd CRD was performed 5 min later. The dose of tocinoic acid was based on previous reports showing that such regimen of administration prevented WAS-induced colonic motility response in rats.[42]

Influence of astressin-B, astressin$_2$-B and tocinoic acid on WAS-induced visceral analgesia.—In other groups, after a baseline on day 0, 24 h later, astressin-B (30 μg/rat), astressin$_2$-B (10 μg/rat), tocinoic acid (20 μg/rat) or their respective vehicles (sterile water and saline), were injected ICV in 10 μl, 15 min before exposure to WAS for 1 hour. A 2nd CRD was performed within ~ 45 min of the stress ending as previously published.[20] The doses of receptor antagonists used were based on above experiment showing blockade of ICV CRF-induced visceral analgesia.

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). Data were analyzed using one-way ANOVA or 2-way ANOVA followed by Sidak *post hoc* test to assess the dose-dependent influence of ICV CRF on VMR and the interaction of different treatments (baseline vs ICV CRF, astressin-B, astressin-B+CRF, astressin$_2$-B+CRF, WAS, astressin-B +WAS or astressin$_2$-B+WAS) and CRD pressure on VMR, respectively. A p value < 0.05 was considered significant.

3 RESULTS

3.1 CRF injected ICV induces visceral analgesia to CRD, while IP CRF induces visceral hyperalgesia.

In male rats equipped with a chronic ICV cannula, the ICV injection of saline and 5 min later, a 1st CRD at 10, 20, 40 and 60 mmHg, induced a stimulus-intensity related increase in the VMR recorded by intraluminal colonic pressure. This baseline was not modified when the same protocol (ICV saline followed by 2nd CRD) was repeated 1 h later (Fig. 1A). By contrast, ICV CRF at 100 and 300 ng/rat resulted in a significant decrease of the VMR at 60 mmHg CRD compared to baseline (VMR in % control: 63.4±6.8% and 51.3±11.7% vs 100.0±0.0% for 100 and 300 ng/rat, $p<0.001$ each, respectively, Figs. 1C,D,H and 2). At 20 or 40 mmHg, VMR values were superimposed to those of baseline at the 100 ng/rat (Fig. 1C) or showed a trend to be lower at 40 mmHg after 300 ng of ICV CRF compared to baseline (VMR in % control: 100 ng/rat: 48.5±7.7% vs 47.4±7.0% for baseline, and 300 ng/rat: 27.6±4.2% vs 46.5±8.3% for baseline, respectively, Fig. 1 C,D and 2). However, ICV CRF at lower (30 ng/rat) and higher (1, 3, or 5 μg/rat) doses had no significant effect (Figs. 1B, E-G, H). The dose of CRF at 300 ng/rat ICV, being the most efficient to induce visceral analgesia, was selected for all subsequent ICV injection studies.

When tested peripherally, CRF (10 μg/kg, IP) increased the VMR to CRD at 20, 40 and 60 mmHg compared to baseline values by 59.9±30.7% ($p>0.05$), 111.7±22.0% ($p<0.01$) and 167.7±37.4% ($p<0.001$), respectively (Fig. 3A and2), while the vehicle (saline) injected IP had no effect (Fig. 3B).

3.2 Astressin-B induces visceral hyperalgesia and blocks ICV CRF-induced visceral analgesia while astressin$_2$-B has no effect

The ICV injection of CRF (300 ng) decreased significantly the VMR to CRD at 60 mmHg in ICV saline pretreated rats (−5 min) compared to baseline values (VMR in % control: 35.0±15.8% vs 100.0±0.0%; Fig. 4A) as we observed in previous experiment in non-ICV pretreated rats. By contrast, in rats pretreated ICV with the CRF antagonist, astressin-B, ICV CRF increased significantly the VMR to CRD at both 40 and 60 mmHg compared to baseline (VMR in % control: 116.2±26.0% and 148.1±32.0% vs 64.6±11.5% and 100.0±0.0%, respectively, $p<0.05$ each; Fig. 4B). Astressin-B injected ICV plus ICV saline increased the VMR to CRD only at 60 mmHg compared to baseline (VMR in % control: 155.1±11.0% vs 100.0±0.0%, $p<0.05$; Fig. 4C). The selective CRF$_2$ antagonist, astressin$_2$-B injected ICV as pretreatment (−5 min) did not influence the analgesic response to ICV CRF

as shown by the significant decrease of the VMR to CRD at 60 mmHg compared to baseline (VMR in % control: 37.1±16.5% vs 100.0±0.0%; Fig. 3D) and the peptide + ICV saline did not influence the VMR to CRD (Fig. 4E).

3.3 The ICV oxytocin receptor antagonist, tocinoic acid induces visceral hyperalgesia and blocks ICV CRF-induced visceral analgesia

The ICV injection of tocinoic acid (20 μg/rat) plus ICV saline increased the VMR to CRD at 40 mmHg (VMR in % control: 113.4±28.5% vs 45.1±16.6%, $p<0.01$) while at 60 mmHg the response did not reach statistical significance (VMR in % control: 138.7±25.7% vs 100.0±0.0%, $p>0.05$) (Fig. 5A). The ICV pretreatment with tocinoic acid, abolished the development of visceral analgesia induced by ICV CRF (Fig.5B) at 60 mmHg.

3.4 ICV CRF and oxytocin receptor antagonists block SIVA induced by WAS

We then assessed whether SIVA induced by WAS is modified by blockade of CRF and oxytocin receptors. In rats pretreated with saline, WAS for 1 h decreased the VMR to CRD at 60 mmHg by −47.4±14.3% compared with baseline when monitored at the end the stress exposure (VMR in % control: 44.1±13.5% vs 100.0±0.0%, $p<0.0001$; Fig. 6A). The ICV injection of astressin-B (Fig. 6B), astressin$_2$-B (Fig. 6C) and tocinoic acid (Fig. 5C) prevented WAS-induced SIVA and values of VMR were identical to those of baseline after injection of astressin-B and tocinoic acid, but showed a non-significant tendency for hyperalgesia after astressin$_2$-B.

4 DISCUSSION

The present study showed that ICV injection of CRF induces visceral analgesia in male Sprague Dawley rats. The ICV injection of CRF at 100 or 300 ng/rat significantly reduced the VMR at 60 mmHg by −36.6±6.8% and −48.7±11.7% respectively from ICV saline control while the 30 ng/rat dose had no effect. The analgesic response occurred at ICV doses of CRF known to have no effects on locomotor behavior.[43] These data indicate that at a nanogram dose range, there is a dose-related visceral anti-nociception effect of ICV CRF. However, CRF given at higher doses (1 to 5 μg/rat) no longer influenced visceral pain induced by CRD. These observations are consistent with several somatic pain studies reporting an anti-nociceptive effect of ICV CRF only at doses within the nanogram range in male rats.[10, 12, 44] However, our data contrasts with earlier studies in which ICV injections of CRF at 25, 62.5 and 125 ng/rat in male Wistar rats equipped chronically with EMG electrodes induced visceral hypersensitivity to CRD when monitored during the 30-50 min post injection.[16] Strain differences between these studies may have played a role as distinct visceral sensitivity to CRD has been reported previously between Fischer-344 and Wistar rats.[17] In addition, the distinct preconditions of the animals linked to the method used to monitor visceral pain (no abdominal surgery for intracolonic pressure recording of VMR in the present study vs surgical chronic implantation of EMG electrodes may have contributed to these differences as recently reviewed.[45] We previously reported that the surgical intervention and EMG electrodes implanted into the external oblique abdominal muscle then exteriorized in the back of neck modifies the visceral pain responses of rodents to WAS when assessed 5 days post-surgery.[19] Indeed, studies in the somatic pain field demonstrated

that the assessment of pain is not only the result of nociceptive input level but is also dependent upon the prior stress/nociceptive events that induce a latent pain sensitization switching stress-induced analgesia to hyperalgesia.[45–47] Under our conditions of testing, we were not able to detect a significant influence of the brain surgery for the chronic implantation of ICV cannula on the visceral pain response of rats. We hypothesize that the lack of sensitization of the visceral pain responses following brain surgery when compared with abdominal and paw surgeries[19, 48, 49] may be due to different projections and convergence of sensory pathways in the spinal cord.[50]

We then assessed whether ICV injection of CRF-induced reduction of VMR to CRD was mediated in the brain since previous studies established a brain-to-blood transport of CRF.[26] Two sets of evidence support that the peptide acts in the brain. First, CRF injected IP at ~3 μg/rat, results in a significant visceral hyperalgesia to CRD when monitored under similar conditions. These data expand our previous reports showing that that IP injection of the CRF_1 agonist, cortagine[51] induced a peripherally-mediated increase in the VMR to CRD in rats when monitored similarly, as in the present study.[18] Second, the decreased VMR to CRD is observed only at the lowest doses (100-300 ng/rats), and no longer at the highest (1, 3 or 5 μg/rat) which should result in a more prominent leakage of the peptide from the cerebrospinal fluid to the peripheral circulation.[26, 52] The latter resulting in hyperalgesia as demonstrated with the IP injection of CRF, and not analgesia. The lack of analgesic effect of ICV at high doses may be due to an increased leakage of the CRF peptide from the brain to the periphery [26] and the potential initiation of peripheral hyperalgesic mechanisms which counteract the central analgesic effect. Because some of the peptide may be bound by CRF binding protein present in the blood,[53] the hyperalgesic response may not be as strong as when CRF is injected directly intraperitoneally, but may be enough to mask the analgesic effect of central CRF. Numerous evidence support a role for increased intestinal permeability, activation of mast cells, increase of serotonin release by direct action on cells that express CRF_1 receptors in the hyperalgesic visceral response to peripheral injection of CRF or CRF_1 agonists (for review see [54]). The differential modulation of visceral pain by CRF injected into the brain or peripherally also implies that ICV CRF acts in the brain independently of pituitary hormones including β-endorphin that are released into the peripheral circulation by both routes of CRF administration.[55, 56]

Varying intensities of colon distension pressure (non-noxious to noxious) induce different brain activation patterns with selective recruitment of specific nuclei as monitored by Fos expression.[57] The fact that the analgesic response to ICV CRF is limited to the CRD pressure of 60 mmHg (with a trend at 40 mmHg), suggests that the analgesic effect of CRF is likely linked with higher intensity recruitment of noxious pathways. The neuroanatomical site(s) that subserve the visceral analgesic action of ICV CRF need to be identified. In the somatic pain field, two specific brain sites, the central amygdala and periaqueductal gray (PAG), well-established critical structures in stress-related analgesia through modulation of descending inhibitory pathways,[8–12, 58–62] are responsive sites to CRF.[11, 63, 64] CRF microinjected in the PAG induces an analgesic response to nociceptive thermal, mechanical or chemical stimuli applied to the rodent hindpaw.[64, 65] Likewise, CRF microinjected into the central amygdala was reported to evoke a CRF receptor-mediated analgesia as shown by the increased withdrawal latency to noxious thermal and mechanical stimulation in rats.[11, 58]

In CRF-Cre mice, selective activation of endogenous CRF in the central amygdala by adenovirus-associated approach also further increases the thermal pain threshold elevated by acute swim stress.[58]

Next, we examined the role of CRF receptors. The specific, long acting, non-selective peptide CRF_1 and CRF_2 receptor antagonist, astressin-B injected ICV abolished the ICV CRF-induced visceral analgesic response to CRD, leading to the hyperalgesia at the pressure of 60 mmHg. In addition, astressin-B injected ICV alone enhanced the visceral VMR to CRD at the highest pressures of CRD (40 and 60 mmHg). There is evidence that CRD at nociceptive range activates neurons in the limbic system (paraventricular nucleus of the hypothalamus or PVN and amygdala including CRF neurons).[66, 67] Other studies showed that CRD activates dorsal/pons/periaqueductal grey, a region involved in nociception.[68] Endogenous brain CRF signaling is recruited under exposure to visceral nociceptive stimuli such as CRD, thereby inducing SIVA either by activating descending inhibitory pathways and/or counteracting the pain pathways. Therefore, blocking the CRF receptors with astressin-B before CRD enhanced the visceral motor response by preventing the CRF-related analgesic response induced by this visceral stress. Our results support the existence of an endogenous inhibitory tone of central CRF via CRF signaling in visceral pain as shown in the somatic pain field.[10] By contrast, the selective peptide CRF_2 antagonist, $astressin_2$-B injected ICV had no effect against CRF-induced SIVA or under basal conditions. It is unlikely that the lack of antagonist effect of $astressin_2$-B is due to a submaximal regimen of administration (10 μg/rat). We previously reported that the injection of $astressin_2$-B into the rat cerebrospinal fluid at 3 or 10 μg/rat completely prevented intracisternally-injected CRF (300 ng)- or urocortin 2 (100 ng)-induced delayed gastric empting in rats.[39] Moreover, CRF is a preferential CRF_1 agonist with a 10- to 40-fold lower affinity to CRF_2 compared to CRF_1 receptors.[69] The visceral analgesia occurring mainly at low doses would be consistent with CRF interacting preferentially with CRF_1 receptors. Collectively, these findings demonstrate that the visceral analgesic response to ICV CRF is CRF receptor-mediated and support a primary involvement of CRF through an interaction with the CRF_1 receptor subtype. The role of CRF_1 receptors was also reported in the somatic analgesia induced by CRF microinjected into the PAG.[64]

The demonstrated analgesic action of exogenous CRF administered into the brain may have physiological relevance to SIVA. We show that the acute exposure to WAS mimicked the magnitude of VMR reduction to CRD at 60 mmHg observed at low doses of ICV CRF. This result is consistent with previous reports showing that a single or repeated daily 1-h session of WAS decreased the VMR to CRD monitored at the end of the stress period by manometry.[19–22] Importantly, the CRF antagonist, astressin-B pretreatment given ICV at a dose that blocked ICV CRF-induced visceral analgesia also abolished the visceral analgesic response induced by the acute WAS. However, contrary to rats injected with ICV CRF, blockade of central CRF receptors with astressin-B did not lead to hyperalgesia in WAS rats. Furthermore, $astressin_2$-B administered before exposure of the animals to acute WAS blocked the visceral analgesia. This supports the involvement of central CRF_2 receptors in WAS-induced SIVA, while we showed that CRF_2 had no effect in ICV CRF-induced SIVA. These findings indicate that the astressin-B sensitive SIVA induced by ICV CRF at low doses or WAS involved distinct CRF_1 and CRF_2 receptor pathways respectively. This may

be related to the preferential CRF_1 receptor action of exogenous CRF at low dose[70] while WAS combined with CRD recruit additional neuronal circuitries[67, 71, 72,] and/or higher level of CRF release able to activate CRF_2 receptors also located in PVN neurons.[5] Along with CRF, stress can indeed lead to the release of urocortins, which bind CRF_2 with higher affinity than CRF,[73] and may in turn lead to the recruitment of brain circuitries to induce SIVA which differ from the ones recruited by the stimulation of a proximal single pathway using ICV CRF as previously reported for other stressors.[74, 75]

We next assessed the underlying central mechanisms of SIVA. Stress and ICV injection of CRF induce the release of neuropeptides such as oxytocin (OT)[29, 76, 77] established to induce analgesic effect in different modalities of acute pain (neuropathic, somatic or inflammatory) in rodents and humans.[30, 31] Moreover, in rat brain, synaptic connections between OT and CRF containing neurons were identified in the PVN [78] and CRD stimulates the activity of both CRF and OT neurons as shown by the induction of Fos expression and double labeling.[66] Pharmacological and neuroanatomical evidence support the implication of hypothalamic OT in somatic analgesia[79–82] and a reciprocal regulation of OT and CRF hypothalamic systems in stress response.[83] No study has assessed their implication in SIVA, yet. Interestingly, using an OT receptor antagonist, tocinoic acid, we found that the antagonism of the oxytocinergic system could prevent ICV CRF and WAS-induced SIVA, suggesting that downstream oxytocinergic pathways play a role in the visceral analgesia induced by central injections of CRF or exposure to an environmental aversive stressor. The observation that tocinoic acid when injected alone induced a robust hyperalgesic response in rats suggests that a tonic endogenous oxytocinergic tone is modulating pain in animals exposed to nociceptive CRD. These results are further supported by our previous report showing a prominent activation of OT neurons in the hypothalamus of rats subjected to CRD.[66]

In conclusion, the present data show that ICV injection of CRF in nanogram doses range acts into the brain primarily through CRF_1 receptors to decrease visceral pain elicited by CRD at 60 mmHg when monitored by manometry in male rats. We found that the ICV CRF-induced visceral analgesic action is dependent of brain OT signaling. Moreover, the pharmacological blockade of CRF and OT receptors in the brain using antagonists, astressin-B, astressin$_2$-B and tocinoic acid, respectively, blocked SIVA induced by the 1-h acute exposure to WAS before CRD. These data support a role of endogenous brain CRF ligands and oxytocin signaling in SIVA, which may recruit central CRF_1 or CRF_2 receptors in a stress-differential manner. Increasing the knowledge of neurochemical mechanisms underpinning SIVA may have translational application in the context of irritable bowel syndrome (IBS) since clinical studies performed in IBS patients indicate a dysfunction of the analgesic response to both somatic and visceral noxious stimuli.[84–87]

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Abbreviations:

AUC:	area under the curve
CRD:	colorectal distension
CRF:	corticotropin-releasing factor
EMG:	electromyography
IBS:	irritable bowel syndrome
ICP:	intracolonic pressure
ICV:	intracerebroventricular
IP:	intraperitoneal
OT:	oxytocin
pICP:	phasic intracolonic pressure
PAG:	periaqueductal gray
SIVA:	stress-induced visceral analgesia
VMR:	visceromotor response
WAS:	water avoidance stress

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Key Points

- Recent reports indicate that WAS induces a naloxone-independent visceral analgesia in male rats under non-invasive conditions of monitoring, but the underlying brain mechanisms are still unknown.
- When injected intracerebroventricularly (ICV) at low nanogram range doses, CRF induces a CRF_1 receptor-mediated visceral analgesic response to colorectal distension that is prevented by ICV injection of the oxytocin antagonist. In contrast, acute WAS recruits endogenous CRF_2 and oxytocin receptor signaling to induce SIVA.
- Increasing the understanding of mechanisms by which stress promotes visceral analgesia and how dysfunction of this pathway lead to visceral hyperalgesia may help developing new therapeutic venues for patients with functional gastrointestinal disorders who exhibit abnormal endogenous pain modulation.

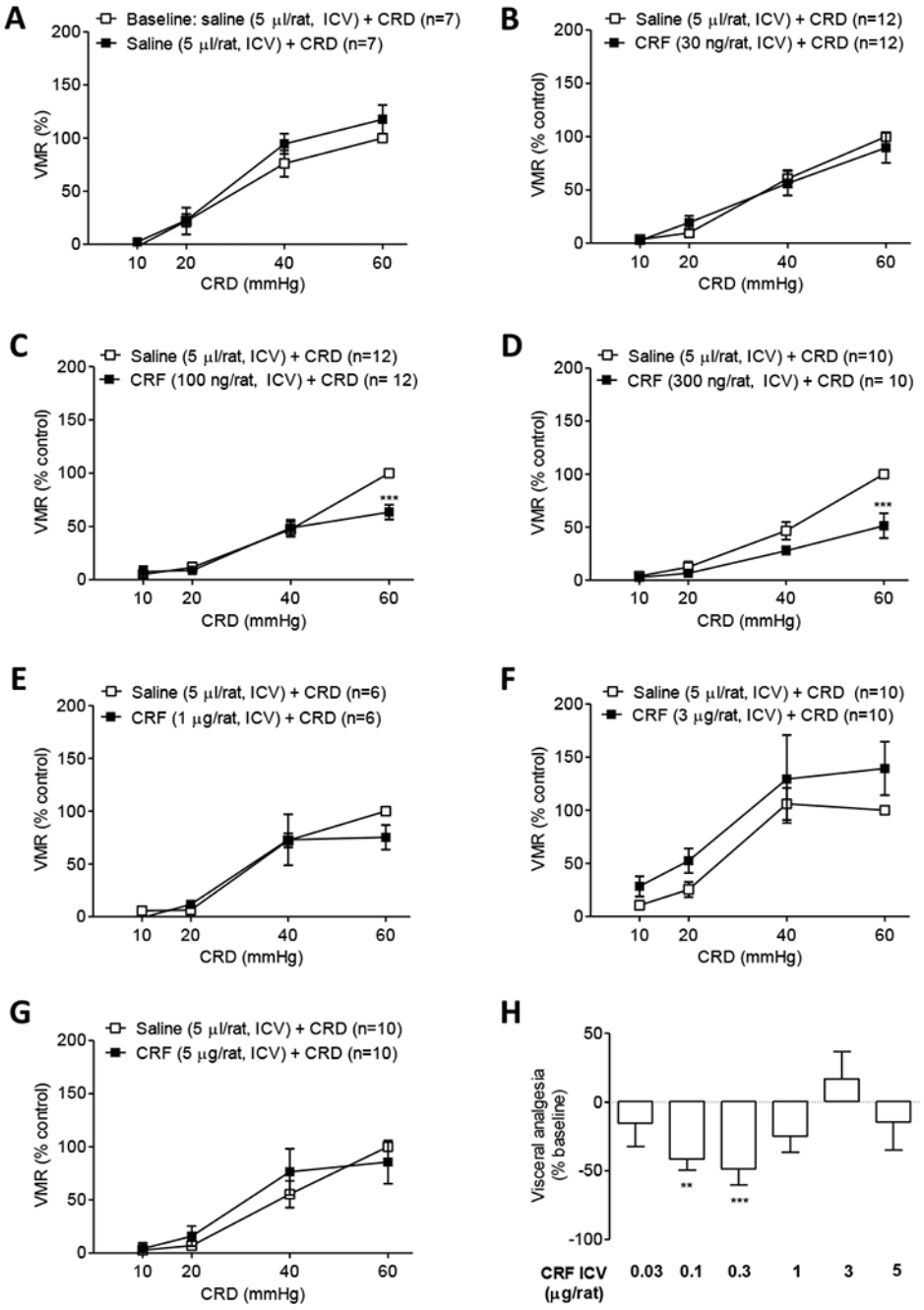

FIGURE 1.

Intracerebroventricular (ICV) CRF induced visceral analgesia at a narrow nanogram range in male rats chronically implanted with an ICV cannula. (A) One group received ICV saline and 5 min later, the visceromotor response (VMR) to a 1st colorectal distension (CRD) was monitored by manometry (white squares), then 1h later, a similar protocol was repeated with ICV saline and VMR to 2nd CRD (black squares). Other groups had the same experimental protocols, except after ICV saline (white squares, baseline), the second ICV injection was CRF (black squares) at 100 ng/rat (C), 300 ng/rat (D), 1 μg/rat, 3 μg/rat (F) and 5 μg/rat (G).

Data are means ± SEM, numbers of rats are indicated in parenthesis as n. ***p<0.001 vs respective baseline, 2-way repeated measures ANOVA and Sidak post hoc test. (H) Comparison of the visceral analgesic response (in % baseline) at 60 mmHg for the different doses of ICV CRF. Data are means ± SEM. **p<0.01, ***p<0.001 vs 30 ng/rat dose, one-way ANOVA and Dunnett *post hoc* test.

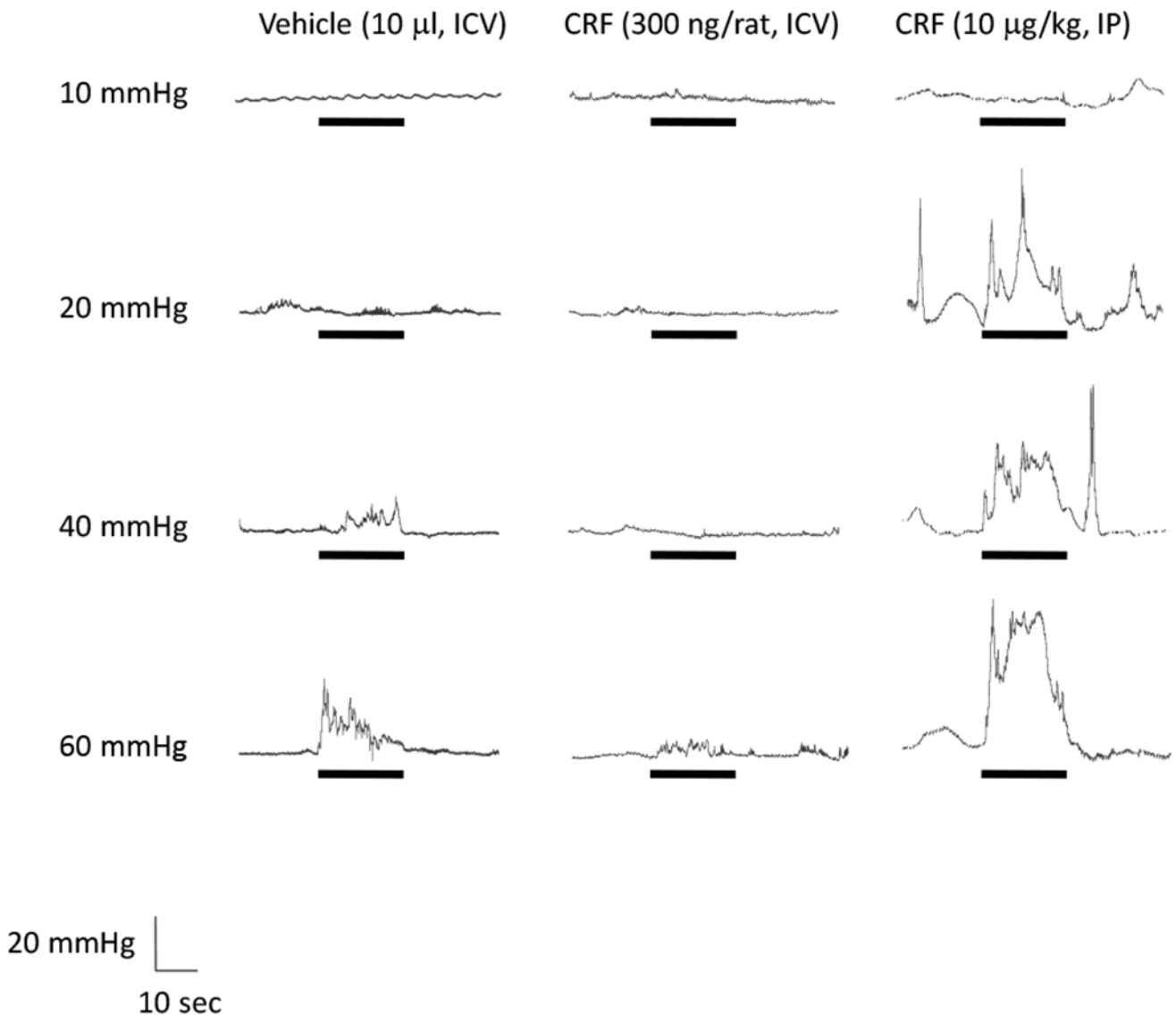

FIGURE 2.

Representative raw traces of intracolonic luminal pressure in response to colorectal distension (10, 20, 40 and 60 mmHg) in male Sprague-Dawley rats injected with vehicle (10 µl, ICV) or CRF centrally (300 ng/rat, ICV) and peripherally (10 µg/kg, IP).

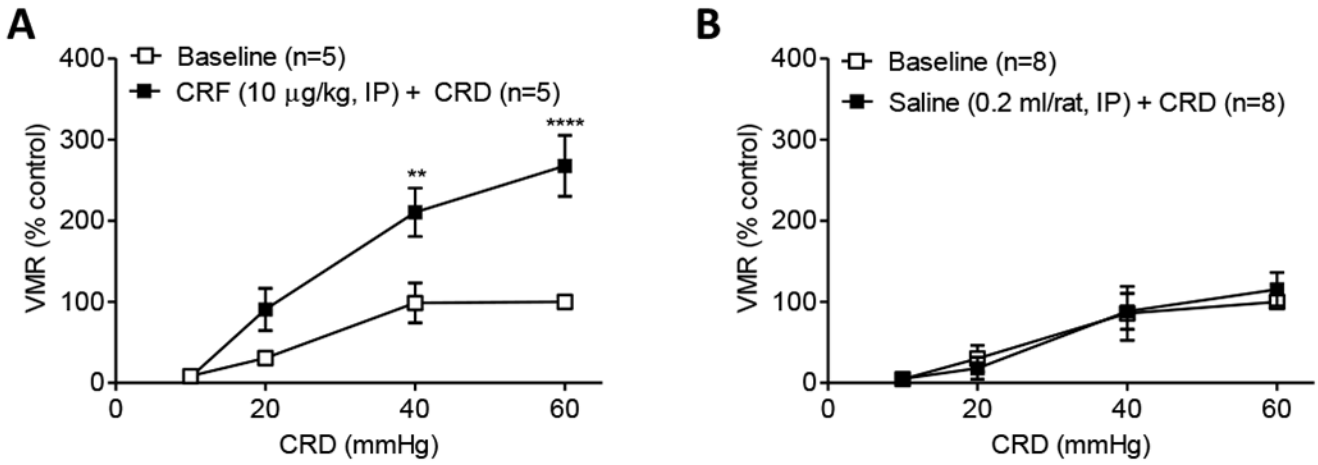

FIGURE 3.

Peripheral injection of CRF induced visceral hyperalgesia in male rats. A 1st CRD was performed (baseline, white square) followed 1h later with an intraperitoneal (IP) injection of CRF (10 µg/kg, IP) (A) or saline (B) and 15 min later a 2nd CRD (black square). Data are means ± SEM, numbers of rats are indicated in parenthesis as n. **p<0.01, ****p<0.0001 vs baseline, two-way repeated measures ANOVA and Sidak *post hoc* test.

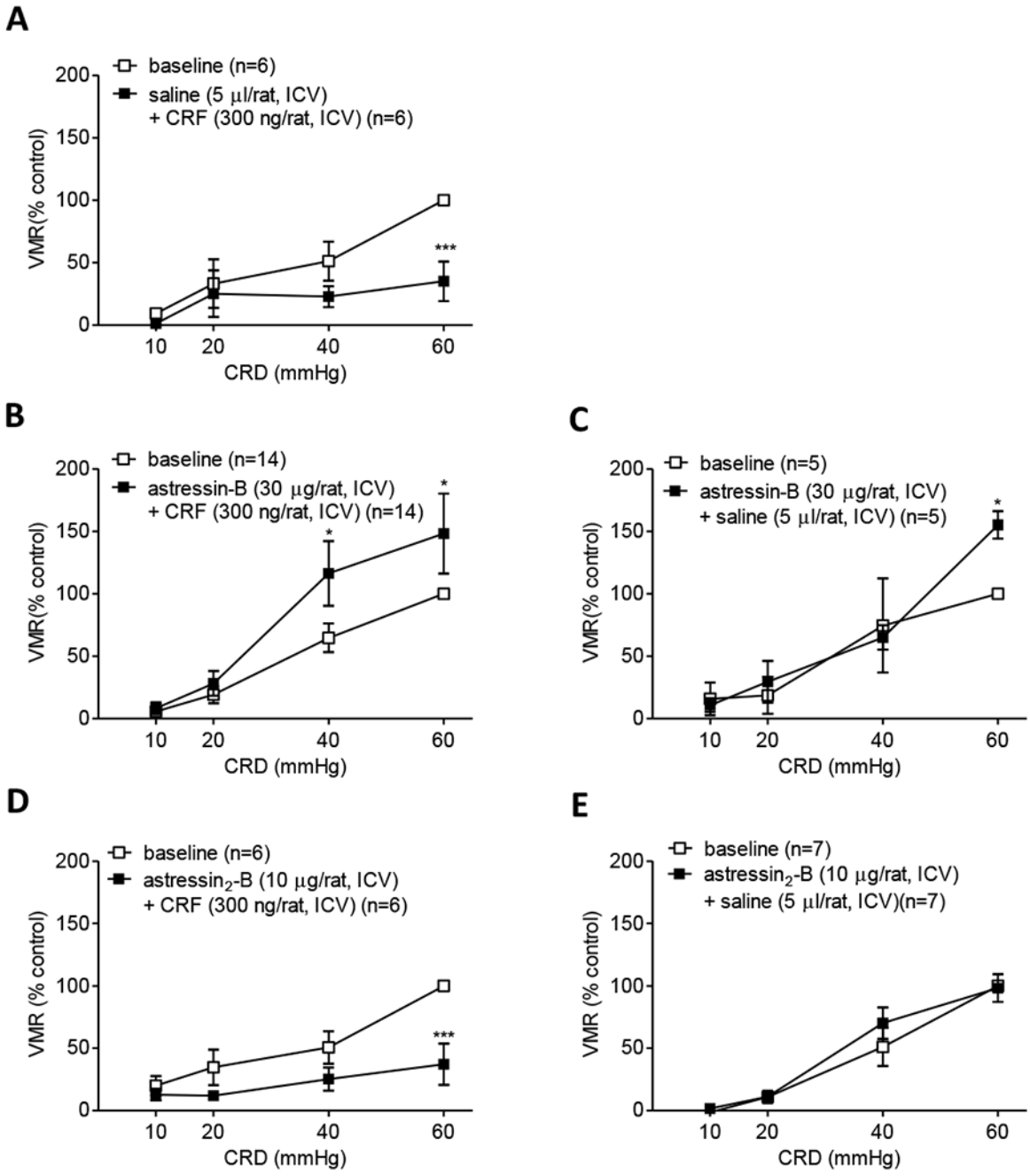

FIGURE 4.

Involvement of CRF receptors in ICV CRF-induced visceral analgesia. In male rats chronically implanted with an ICV cannula, a baseline CRD was performed. One hour later, rats received an ICV injection of saline (A), astressin-B (B) or astressin$_2$-B (D) followed 5 min after by ICV CRF (300 ng/rat) before the 2nd CRD. In other groups, the same protocol was applied except that astressin-B (C) or astressin$_2$-B (E) was given 5 min before ICV saline. Baseline: white squares, post-injection: black squares. Data are means ± SEM, of

number of rats as indicated in parenthesis. *p<0.05, ***p<0.001 vs respective baseline, 2-way repeated measures ANOVA and Sidak *post hoc* test.

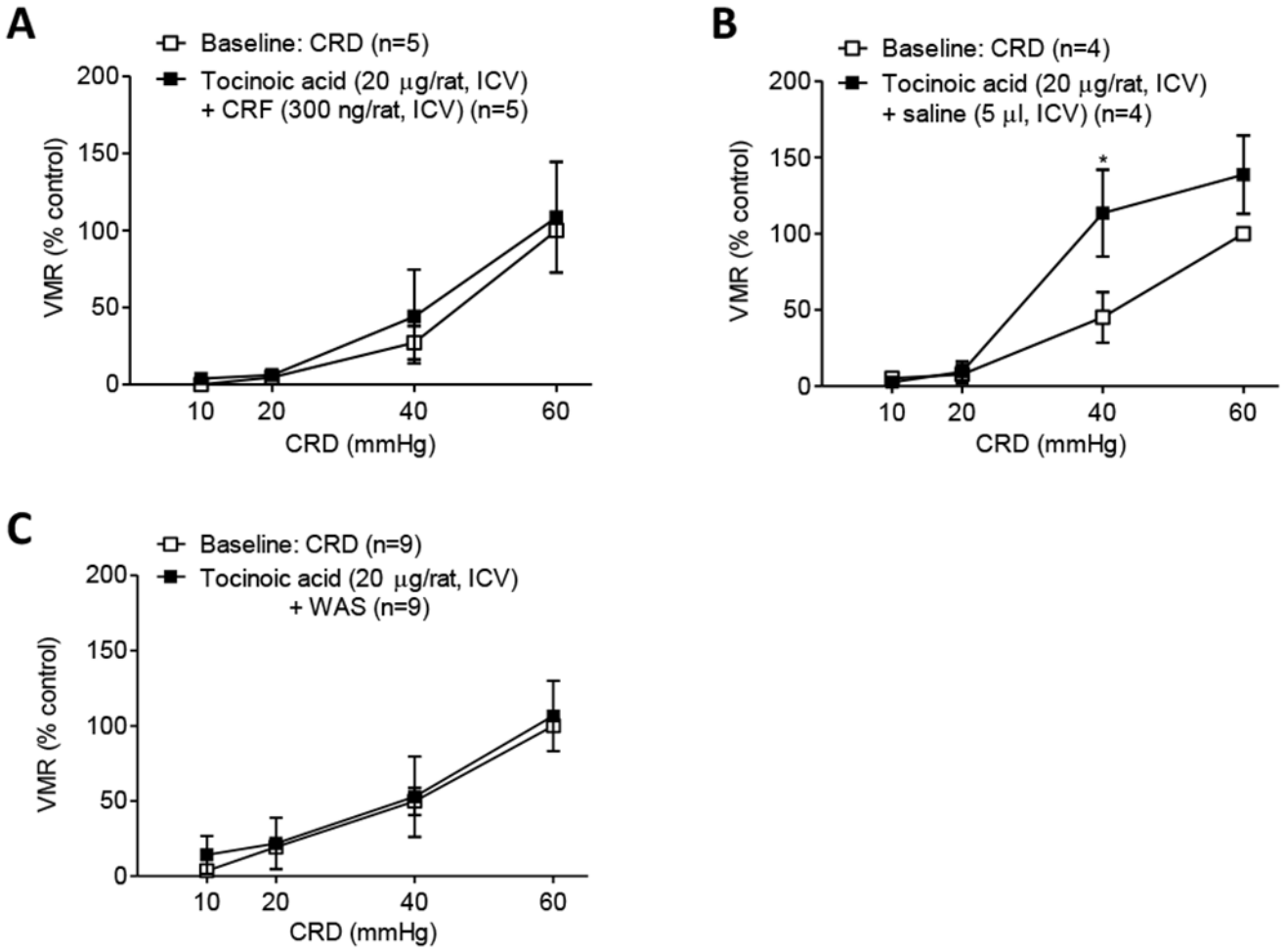

FIGURE 5.

ICV CRF recruits oxytocin to mediate ICV and WAS-induced visceral analgesia. In male rats chronically implanted with an ICV cannula, a 1st CRD was performed (baseline, white squares), and 1h later, groups of rats were injected ICV with tocinoic acid and 15 min later with ICV CRF (A) or saline (B) (black squares). In a separate group of animals, on day 0, a baseline CRD was performed. Twenty-four hours later, rats were injected ICV with tocinoic acid (C) and 15 min later, exposed to 1h of WAS followed by a 2nd CRD. Data are means ± SEM, n as indicated in parenthesis. *$p<0.05$ vs baseline, 2-way repeated measures ANOVA and Sidak post hoc test.

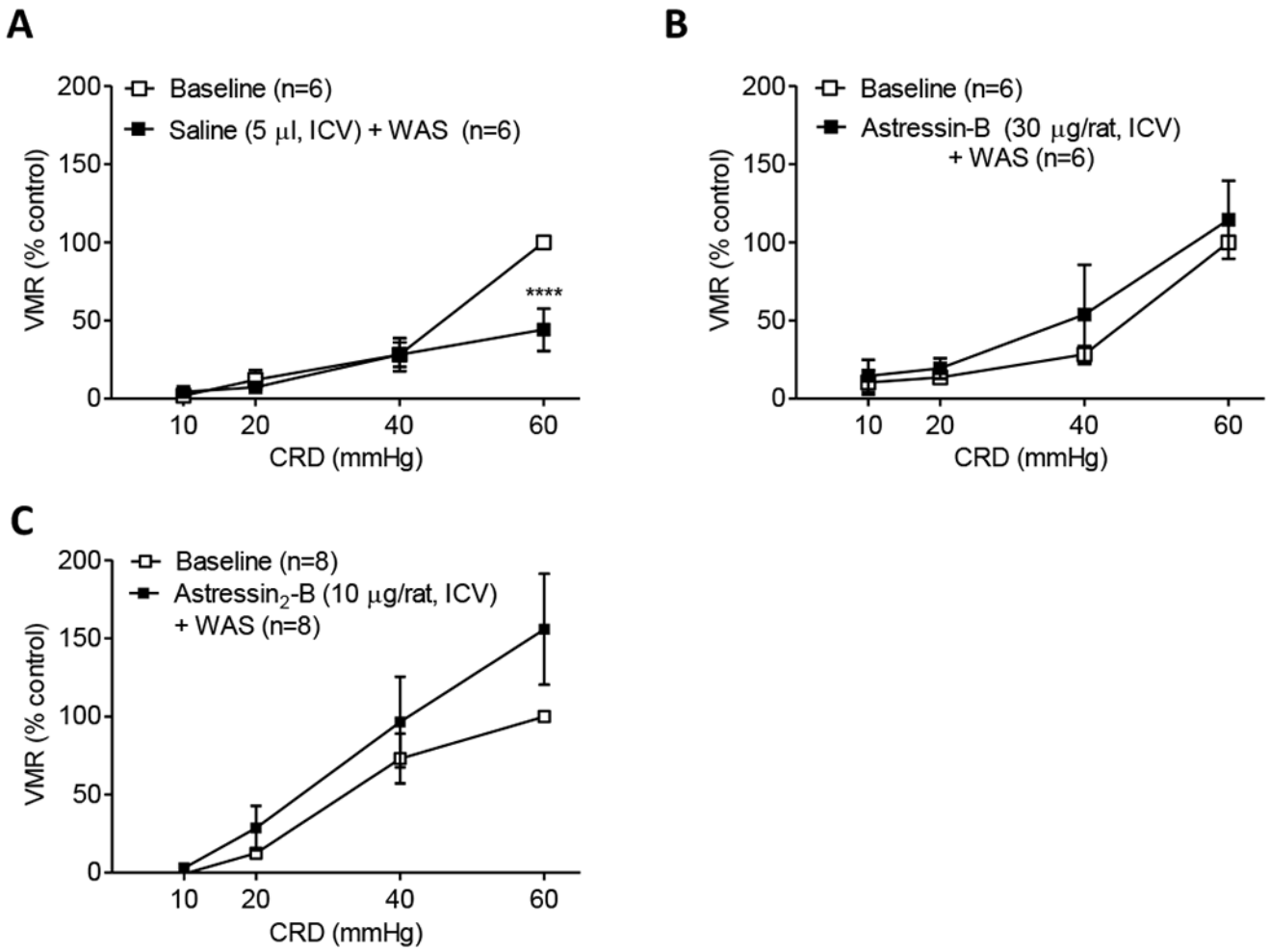

FIGURE 6.

Involvement of CRF receptors in acute WAS-induced visceral analgesia. On day 0, a baseline CRD was performed and 24 h later, rats were injected ICV with saline (A), astressin-B (B) or astressin$_2$-B (C) and 15 min later, exposed to 1h of WAS followed by a 2nd CRD. Baseline: white squares, post-injection: black squares. Data are means ± SEM, number of rats as indicated in parenthesis. ****p<0.0001 vs respective baseline, 2-way repeated measures ANOVA and Sidak post hoc test.