Neonatal maternal separation elevates thalamic corticotrophin releasing factor type 1 receptor expression response to colonic distension in rat

Yung Wui Tjong [1], **Siu Po Ip** [1], **Lixing Lao** [2], **Justin Wu** [3], **Harry H S Fong** [4], **Joseph J Y Sung** [3], **Brian Berman** [2], **Chun Tao Che** [1]

1 School of Chinese Medicine, The Chinese University of Hong Kong, Shatin, NT, Hong Kong
2 Center for Integrative Medicine, School of Medicine, University of Maryland, Baltimore, MA, USA
3 Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Shatin, NT, Hong Kong.
4 College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois, IL, USA.

Correspondence to: Yung Wui Tjong, PhD.
School of Medicine, The Chinese University of Hong Kong, Shatin,
New Territories, Hong Kong.
TEL: +852 31634458; FAX: +852 31634459; E-MAIL: jefftjong@cuhk.edu.hk

Abstract

OBJECTIVES: Early life psychological stress is an essential factor contributing to the development of irritable bowel syndrome (IBS), with corticotrophin releasing factor (CRF) having been implicated in this common gastrointestinal disorder. The aim of our study is to examine the effect of neonatal maternal separation (NMS), an early life stress model, on the brain CRF expression following visceral pain induced by colorectal distension (CRD) stimuli in male rats.
METHODS: Male neonatal Sprague-Dawley rats were subjected to 3-hr daily maternal separation on postnatal day 2–21, with unseparated normal (N) rats serving as controls. Electromyogram signals (EMG) in response to phasic CRD were measured. The results demonstrated an increased pain response and EMG magnitudes in NMS rats as compared to N rats in response to CRD stimulation. The mRNA and protein expressions of CRF in hippocampus, cortex and thalamus of NMS and N group following the CRD stress were determined by real-time quantitative PCR and western-blotting studies respectively.
RESULTS: There was an increased mRNA and protein level of CRF in thalamus of NMS rats but no apparent change in CRF expression in hippocampus and cortex of both groups. Furthermore, an increased expression of CRF type 1 receptor (CRF-R1) was observed in the thalamus of NMS rats.
CONCLUSION: These results suggested an up-regulation of thalamus CRF-R1 is associated with visceral hyperalgesia in the rat model of NMS.

Abbreviations :

AUC	- Area-under-curve
CI	- Coefficient intervals
CRD	- Colorectal distension
CRF	- Corticotropin-releasing factor
CRF R	- Corticotropin-releasing factor receptor
C_T	- Cycle threshold
EMG	- Electromyogram
HPA	- Hypothalamus-pituitary-adrenal axis
IBS	- Irritable bowel syndrome
NMS	- Neonatal maternal separation
PCR	- Polymerase chain reaction
SEM	- Standard error of mean

ORIGINAL ARTICLE

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional bowel disorder affecting 10–20% of population worldwide (Kearney & Brown-Chang 2008). It is characterized by recurrent abdominal discomfort and pain (Hammerle & Surawicz 2008), constipation, diarrhea or a combination of both (Spiller *et al.* 2007). The exact cause of IBS is unclear as the pathophysiological aspects of IBS are triggered by multiple factors including physical and psychological stress (Barreau *et al.* 2007), emotional state and environmental changes (Rey *et al.* 2008). Evidences have shown that stress and psychological disturbance are mediators capable of altering the brain-gut axis in the regulation of gut motility and visceral events, resulting in visceral hyperalgesia and motor dysfunction (Salmon *et al.* 2003; Chitkara *et al.* 2008). A neonatal maternal separation (NMS) rat model, in which early and periodic separation of infant rats from their mothers, has been developed to mimic the early life stress and hypersensitivity to mechanical distension of the colon (Barreau *et al.* 2007; Chitkara *et al.* 2008). Studies have shown enhancements of central sensitivity (Chung *et al.* 2007), neurochemical (Ren *et al.* 2007), and signal-related protein kinase response to colorectal distension (CRD) in NMS rats (Zhang *et al.* 2009), suggesting that elevated neuronal sensitivity contributes to the development of IBS.

Corticotropin-releasing factor (CRF) is an endogenous 41-amino acid neuropeptide, which is secreted from endocrine cells in the paraventricular nucleus of hypothalamus in response to stress (Aguilera *et al.* 2008). It initiates the signaling cascade via the hypothalamus-pituitary-adrenal (HPA) axis (Lightman 2008). The action of CRF is mediated by the CRF receptors, a member of the class II G protein-couple receptor family (Gallagher *et al.* 2008). Two major types of CRF receptors, CRF receptor type 1 (CRF-R1) and CRF receptor type 2, (CRF-R2) have been identified (Valverde *et al.* 2001). While CRF-R1 is distributed in various regions of the brain including the hippocampus, cerebral cortex, thalamus, pituitary, cerebellum and other areas etc (Bayatti *et al.* 2003; Auguilar-Valles *et al.* 2005). CRF-R2 is mainly in the hypothalamus and lateral septum (Wang & Kotz 2002). CRF have been linked to stress-related behavioral and physiological disorders (Tache & Brunnhuber 2008), and the central administration of CRF elevated visceral pain and irregular colonic motility in human (Nozu & Kudaira 2006). Yet there are brain regional variations in expression patterns and roles of CRF and its receptor subtypes in stress-induced visceral hyperalgesia (Tache & Bonaz 2007). Thus in the present study, we used well-established rat model of early life-stress to examine the brain regional expressions of CRF and receptors response to visceral stimulus. These findings provide the basis of neurological mechanisms of visceral hypersensitivity in IBS.

MATERIALS AND METHODS

Animal and neonatal maternal separation

The animal experimental procedures as detailed below were approved by the Animal Ethics Committee of the Chinese University of Hong Kong and the Institutional Animal Care and Use of Committee of the University of Maryland-Baltimore. Male Sprague-Dawley pups (postnatal day 1) were randomly assigned to neonatal maternal separation (NMS) or normal (N) groups. For NMS group, rats exposed to 3-hr of daily maternal separation (0900–1200) on postnatal day 2–21 inclusive. A separate group of N rats were placed in standard cage. On day 22, rats were weaned and the litter housed on a 12:12-h light-dark cycle (lights on at 06:00) with free access to food and water.

Implantation of electromyogram electrodes and colorectal distension-induced visceral hyperalgesia

Experiments aimed at assessing viscerosomatic sensitivity were performed beginning at 2-month of age (c.a. ~250 g). For the implantation of electromyogram (EMG) electrodes, rats were anesthetized with inhalation of 2% isoflurane (in oxygen, 0.5 L/min). A 1.5-cm incision was made at the lower left abdominal area to expose the external oblique abdominal musculature and a pair of electrodes with non-insulated tips was stitched in parallel into the external oblique abdominal musculature 5mm apart. The electrodes were tunneled subcutaneously and exteriorized and secured at the back of the neck for subsequent EMG measurements. The CRD study commenced 7 days after EMG electrodes implantation. The response to visceral stimulus was quantitatively assessed by measuring the EMG signals, as described before (Christianson & Gebhart 2007; Ren *et al.* 2007). The rats were anesthetized with 2% isoflurane (in oxygen, 0.5 L/min) to facilitate placement of the inflatable balloon into the descending colon. The balloon was constructed from a latex glove finger attached to a Rigiflex balloon dilator (Microvasive, Millford, Massachusetts, USA, 2 mm of diameter), connected via a Y connector to a syringe pump and a sphygmomanometer. The balloon catheter was inserted into the distal colon with the distal tip 1cm from the anal verge and secured to the base of the tail with duct tape. The rats were allowed to recover for 30 minutes. EMG signals in response to CRD were recorded with Powerlab 16/30 and analyzed using Chart software (AD Instruments, Bella Vista, Australia). EMG signals were calculated as the area-under-curve (AUC). The baseline of EMG signals in response to phasic CRD was defined as the AUC 10s prior to distension. The threshold pressures in response to CRD were determined using phasic ascending intensity of CRD 10, 20, 30, 40, 60 and 80 mmHg) was measured. The threshold was defined as the minimum pressure that evokes a signal at least 60% of the time. EMG signals response to CRD was measured at 10, 20, 40, 60 and 80 mmHg (pressure

was applied for 20 seconds every 4 minutes and was repeated twice for each intensity level).

Brain tissues preparation

After the experiment, rats were fully anesthetized, decapitated and the brains were quickly removed and chilled in an ice-cold saline solution. The cortex, hippocampus and thalamus were dissected and immediately frozen in liquid nitrogen and stored at –70 °C.

Total RNA isolation

Frozen samples of cortex, hippocampus and thalamus were homeogenized with 1ml of Trizol reagent (Invitrogen, USA), and total RNA were extracted following the manufacturer's instructions. Briefly, chloroform was added to homogenates and centrifuged at 12,000 g for 15 min at 4 °C. RNA was precipitated with isopropanol and centrifuged at 12,000 g for 15 min at 4 °C. The precipitated RNA was treated with 75% ethanol, followed by centrifugation at 7,500 g for 5 min at 4 °C. The resulting total RNA was air dried, resuspended in RNase-free water and its concentration was measured by a Fluostar Optima spectrometer (BMG Labtech, Offenbury, Germany). For cDNA synthesis, 1.5 µg of total RNA was mixed with reverse-transcription master mix using high-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA, USA). Reverse transcription reaction was perform in a thermal cycler (9800 Fast Thermal Cycler, Applied Biosystems, Foster City, CA, USA).

Real-time quantitative polymerase chain reaction

A 2 µl sample of cDNA sample was added to GeneAmp Fast PCR master mix (2×; Applied Biosystems, Foster City, CA, USA) and primer in a 20 µl final volume. The primers and taqman fluorogenic probes were purchased from Applied Biosystems, and their sequences were shown in table 1. Real-time quantitative PCR amplication reactions were carried out in a Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). cDNA was employed to quantify mRNA encoding β-actin, which is used as a non-regulated reference gene. The thermocycling reaction was 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The PCR results were analyzed with Step One Plus real-time PCR system. Relative gene expression was calculated by using the $2^{-\Delta\Delta}$ cycle threshold (C_T) Method (Livak & Schmittgen 2001). In brief, the C_T values were normalized by subtracting from β-actin control ($\Delta C_T = C_T$ CRH mRNA- C_T β-actin control). The expression of mRNA of CRF in NMS group relative to N group was calculated by subtracting the normalized C_T values in N group from those in NMS group ($\Delta\Delta C_T = \Delta C_T$ NMS – ΔC_T N) and relative expression ($2^{-\Delta\Delta C_T}$) was determined.

Western-blotting study

Frozen samples were homogenized in the lysis buffer containing 10 µl of protease inhibitor cocktail (Calbio-chem, USA), and centrifuged at 15,000 g for 20 minutes at 4 °C. The supernatant enriched proteins was transferred to the sample tube. The amount of protein was determined using protein assay (Bio-Rad laboratories, USA). The proteins were mixed with the Laemmi buffer contained lysis buffer, 10% 2-mercaptoehtanol and 2mg/ml bromophenol blue. 50 µl of each sample were loaded in each well of 12% SDS-polyacrylamide mini-gel. The gels were transferred to polyvinylidene-difluoride membranes using a transblotting apparatus (Bio-Rad laboratories, USA) for 30 minutes. Membranes were incubated for 2 hours at room temperature in TBS buffer with 5% non-fat dry milk (Bio-Rad laboratories, USA). The membranes were then incubated with primary polyclonal antibody CRF, CRF-R1, CRF-R2 (1:500; Santa Cruz Biotechnology Inc, USA) for overnight at 4 °C. Membranes were washed and incubated with secondary antibodies, anti-goat igG conjugated with HRP for CRF, CRF-R1 and -R2 (1:5,000; Santa Cruz Biotechnology Inc, USA) in TBS solution with 5% non-fat dry milk for 60 minutes and followed by washed with TBS solution. The blots were developed using a chemiluminescence's reagent, with the films being exposed and analyzed by using Image J (National Institutes of Health, USA). Results were expressed in relative optical density.

Statistical analysis

Data were analyzed with unpaired *t*-test. Values are expressed as mean ± standard error of mean (S.E.M) or 95% coefficient intervals (CI) where appropriate. Significant significances were set as at $p<0.05$.

RESULTS

Increased pain threshold and EMG activity response to CRD pressure in NMS rats

To determine whether NMS can cause visceral hypersensitivity in rats, increments of CRD pressure were applied to the colon of rats, and resulting EMG signals were recorded. The threshold pressure of the NMS rats was significantly lower than that of the N rats (Figure 1A). The average pain threshold of NMS and N groups were 28.1±1.2 mmHg (N=10) and 15.5±1.5 mmHg (N=10) respectively. With respect to viscerosomatic activity, the magnitude and elevation of EMG signals were pressure-dependent in both animal groups. However, the AUC of EMG signals in the NMS group (N=10) were significantly greater at each pressure point as compared to the N rats (N=10) (Figure 1B).

Elevated mRNA CRF in thalamus of NMS rats

To examine the changes of CRF expression in the brain of NMS and N rats, the mRNA encoding CRF in the hippocampus, cortex and thalamus were determined by real-time quantitative PCR. The relative expression of CRF was significantly increased in the thalamus of NMS group compared to that of the N group (Table 2).
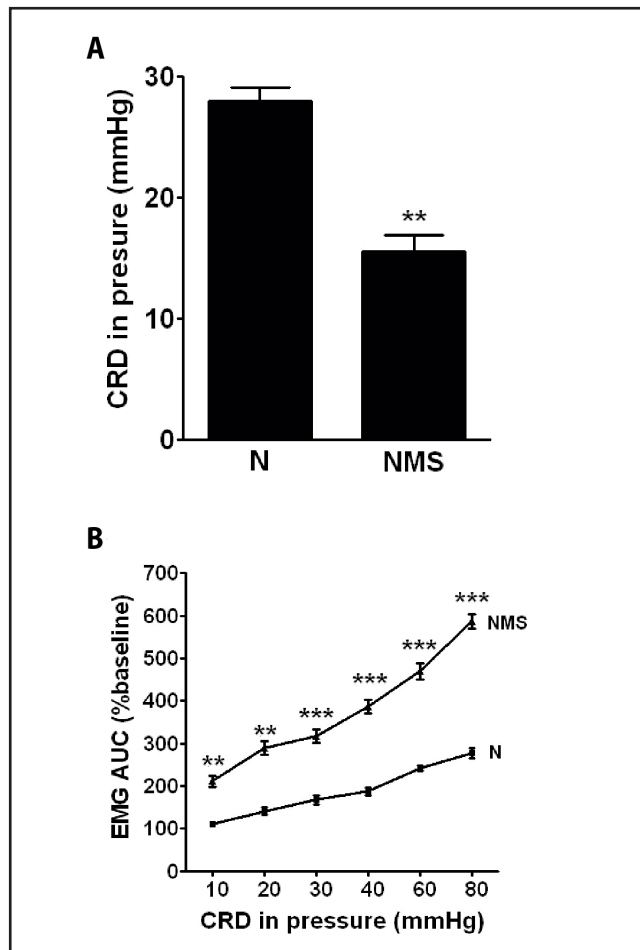
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Fig. 1. (**A**) Comparison of pain threshold pressure in NMS and N rats. Statistical significance (** *p*<0.01 compared with N group). (**B**) Visceromotor response to colorectal distension in NMS and N rats. Statistical significance (** *p*<0.01 or *** *p*<0.001 compared with N group at each CRD pressure).

Fig. 2. (**A**) Representative Western blot image of the semi-quantitative analysis for CRF receptor expression in thalamus, hippocampus and cortex of NMS and N groups. β-actin acts as internal control. (**B**) Summary of the level of CRF in NMS and N groups. Statistical significance (** *p*<0.01 versus N group, unpaired t-test).

No significant changes of CRF expression in hippocampus and cortex in both groups (Table 2).

Up-regulation of thalamic CRF expression in NMS rats

The protein expression of CRF in hippocampus, thalamus and cortex were confirmed by western-blotting analysis. Elevated protein level of CRF was observed in the thalamus of the NMS group (Figure 2A). On average, the expression of CRF in the thalamus of this group of rats was increased by 35.6% (N=3; 95%CI: –0.32 to –0.61; *p*<0.01) from that of the N group (Figure 2B). There were no apparent differences in CRF expression in the hippocampus and cortex of both groups (Figures 2A and B).

Elevated thalamic CRF-R1 expression in NMS rats

CRF-R1 and CRF-R2 expressions in thalamus, hippocampus and cortex were also determined. The level of thalamic CRF-R1 expression was elevated by about 25% in this NMS group compared to that of the N group (N=3; 95% CI: –0.28 to –0.07; *p*<0.01) (Figures 3A
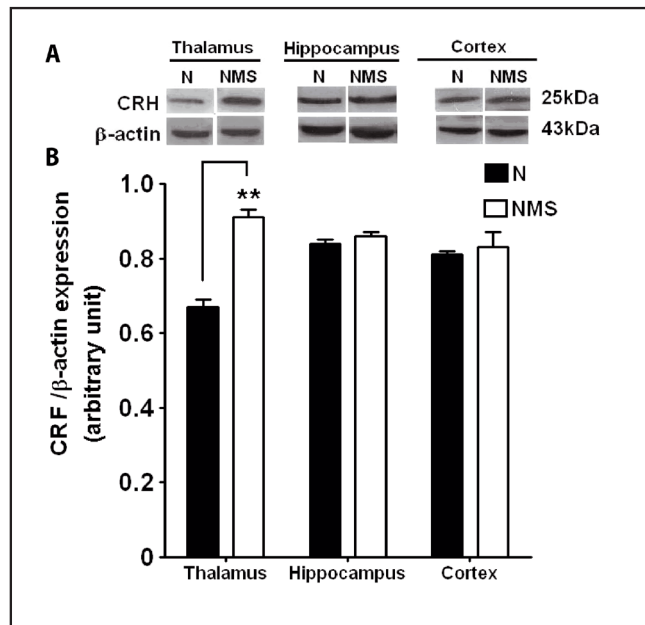
and B), whereas no significant change in the CRF-R1 in hippocampus and cortex (Figures 3A and B). There were no apparent differences in CRF-R2 expression in thalamus, hippocampus and cortex of both groups (Figures 4A and B).

DISCUSSION

The results of the present study provide evidence that NMS increases CRF-R1 expression in the thalamus of male rats in response to CRD. We demonstrated a reduced pain threshold and elevated EMG amplitude in the NMS rats induced by CRD stimuli, which is consistent with findings by others that NMS causes colonic hypersensitivity and irregular motor function reflecting the pathophysiologic features of IBS (Ren *et al.* 2007). Central CRF signaling pathways have been reported to be involved in stress-induced colonic motor response and pain (Nozu & Kudaira 2006; Yarushkina 2008), clinical studies showed that the administration of CRF antagonist alleviates abnormal EEG activity and gut motility of IBS patients in response to CRD stimuli (Sagami *et al.* 2004; Tayama *et al.* 2007)

Studies reported that early stress alters morphological and neuronal changes in several brain regions such as the hippocampus, cerebral cortex, and thalamus of IBS patients and rodent models (Hsu *et al.* 2001; Wilder-Smith *et al.* 2004; Chung *et al.* 2007; Ohashi *et al.* 2008). The thalamus is the site receiving visceral signal from colon via the splanchinic and pelvic pathways and mediates visceral nociception (Brierley *et al.* 2004). Study has
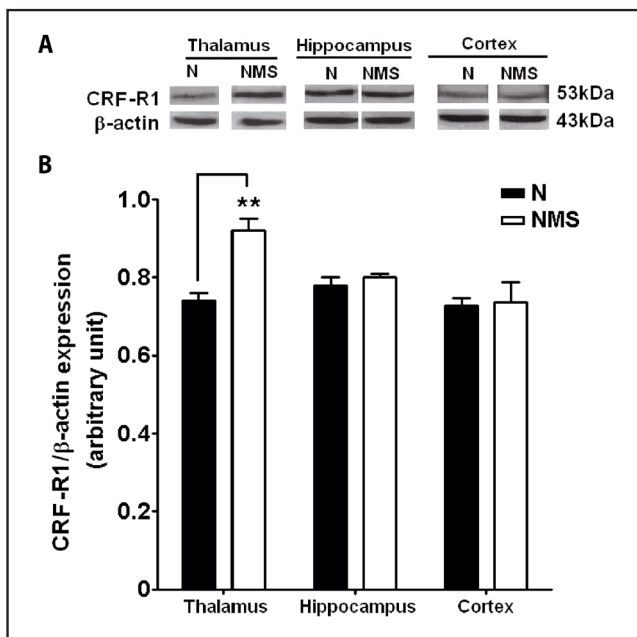
Fig. 3. (**A**) Western- blot band of CRF-R1 expressions in thalamus, hippocampus and cortex of N and NMS groups. β-actin acts as internal control. (**B**) Summary of the level of CRF-R1 expressions thalamus, hippocampus and cortex of NMS and N groups. Statistical significance (*$p<0.01$ versus N group, unpaired *t*-test).
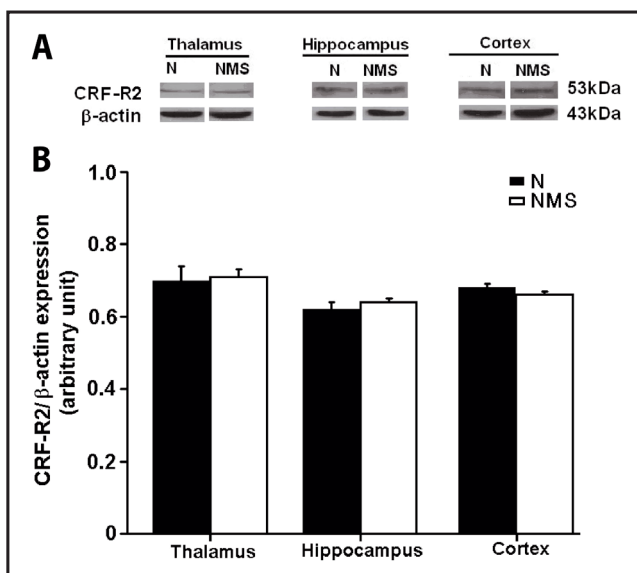

Fig. 4. (**A**) Western- blot band of CRF-R2 expressions in thalamus, hippocampus and cortex of N and NMS groups. β-actin acts as internal control. (**B**) Summary of the level of CRF-R2 expressions thalamus, hippocampus and cortex of NMS and N groups.

Tab. 1. Primer and probe sequences for quantitative real-time PCR.

Primer	Primer Sequence 5'–3'
CRF (forward)	5'-CAG CAA CAG GAA ACT GAT GG -3'
CRF (reverse)	5'-TGT GCT AAA TGC AGA ATC GTT -3'
Probe (sense)	5'-TGG CCA AGC GCA ACA TTT CA -3'
β-actin (forward)	5'- AGC AGA TGT GGA TCA GCA AG -3'
β -actin (reverse)	5'- AAC AGT CCG CCT AGA AGC AT-3'
Probe (sense)	5'- CCT CCA TCG TGC ACC GCA A-3'

Tab. 2. Real-time PCR analysis of CRF mRNA expression in thalamus, hippocampus and cortex of NMS and N rats. Values were expressed as mean ± SEM. Statistical significance (* $p<0.05$ versus N group, unpaired *t*-test).

Brain regions	N	NMS
Thalamus (n=3)	1.02±0.46	2.62±0.43*
Hippocampus (n=3)	1.09±0.38	1.60±0.60
Cortex (n=3)	1.23±0.56	1.82±0.68

mus nucleus in NMS rodents (Coutinho *et al.* 2002). It was also shown that elevated level of CRF concentration and adrenocorticotropic hormone, biomarkers of HPA axis, in NMS rats, suggesting that CRF mediates early-life induced IBS via HPA axis. CRF mediates its action via CRF receptors. CRF-R1 and CRF-2 play opponent roles in regulation of gastrointestinal activity response to stress. Activation of CRF-R1 cause anxiety, simulate gut motility visceral perception, whereas CRF-R2 reduces visceral sensitivity, induces anxiolysis (Fukudo 2007). Our results show increased CRF-R1 expression, but no difference in CRF-R2 in the thalamus of NMS rats. In addition, there were no apparent change in CRF-R1 and -R2 expressions in hippocampus and cortex. Experimental studies demonstrate central administration of CRF enhances colonic motility in rats. Brain injection of CRF-R1 antagonist reduced stress-induced visceral hyperalgesia in rodents (Ohashi *et al.* 2008), suggesting that the central CRF-receptor is involved in brain-gut axis dysregulation. These results are consistent with our hypothesis that up-regulation of CRF-R1 expression in thalamus of NMS rats play a role in altering the colonic sensitivity and motility.

It is well known that the thalamus plays an essential role in the regulation of the hypothalamo-pituitary-adrenocortical (HPA) axis (Maddux & Winstead 2005). Study has found that increased HPA axis activity in NMS rats (Yarushkina 2008), and that the thalamus plays an important role in the regulation of the HPA axis, suggesting that thalamic CRF-R1 is involved in HPA axis dysregulation. It has also been shown that central CRF promotes release of serotonin (5HT) via CRF-R1, which stimulates the gut motility through $5HT_3$ receptor (Nakade *et al.* 2007). However, to what

found elevated mRNA of CRF in thalamus in rats in response to stress. In our study, we found elevated CRF expression only in the thalamus of the NMS rat group, suggesting that NMS increases thalamic CRF which may play a crucial role in activation of somatic sensation in the thalamus in processing visceral pain and hypersensitivity in IBS. Another study has also reported increased mRNA of CRF in paraventricular hypothala-

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exact mechanistic pathway for driving hypersensitive and motility induced by early-life stress is needed to be further determined due to different experimental circumstances.

In conclusion, our present study shows up-regulation of thalamic CRF-R1, activating CRF signaling pathway that augments afferent reflex in thalamus and efferent signals in colonic motility in response to CRD, which is likely a pathway contributes to development of early life stress-induced IBS.

ACKNOWLEDGMENTS

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