Analgesic Effect of Coptis chinensis rhizomes (Coptidis Rhizoma) Extract on Rat Model of Irritable Bowel Syndrome.

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Abstract

Aim of study: *Coptis chinensis rhizomes* (Coptidis Rhizoma, CR), known as *Huang Lian*, is a common component of traditional Chinese herbal formulae used for the relief of abdominal pain and diarrhea. Yet, the action mechanism of CR extract in the treatment of irritable bowel syndrome is unknown. Thus, the aim of our present study is to investigate the effect of CR extract on neonatal maternal separation (NMS)-induced visceral hyperalgesia in rats and its underlying action mechanisms.

Materials and methods: Male Sprague-Dawley rats were subjected to 3-hr daily maternal separation from postnatal day 2 to day 21 to form the NMS group. The control group consists of unseparated normal (N) rats. From day 60, rats were administrated CR (0.3, 0.8 and 1.3g/Kg) or vehicle (veh; 0.5% carboxymethylcellulose solution) orally for 7 days for the test and control groups, respectively.

Results: Electromyogram (EMG) signals in response to colonic distension were measured with the NMS rats showing lower pain threshold and increased EMG activity than those of the unseparated (N) rats. CR dose-dependently increased pain threshold response and attenuated EMG activity in the NMS rats. An enzymatic immunoassay study showed that CR treatment significantly reduced the serotonin (5HT) concentration from the distal colon of NMS rats compared to the Veh (control)

group. Real-time quantitative PCR and western-blotting studies showed that CR treatment substantially reduced NMS induced cholecystokinin (CCK) expression compared with the veh group.

Conclusions: These results suggest that CR extract robustly reduces visceral pain that may be mediated via the mechanism of decreasing 5HT release and CCK expression in the distal colon of rats.

Keywords : cholecystokinin, colonic distension, coptidis rhizoma, irritable bowel syndrome, neonatal maternal separation, serotonin

1. Introduction

Irritable bowel syndrome (IBS) is a prevalent chronic functional gastrointestinal disorder affecting approximately 10%-15% of the world's population (Clarke et al., 2009; Cremonini and Talley, 2005). According to The Rome Committee for the classification of functional gastrointestinal disorders, IBS is characterized by recurrent abdominal pain or discomfort, change in the frequency of stool form, bloating, abnormal bowel habits (diarrhea, constipation or a combination of both) and related symptoms (Crowell et al., 2005; Sohrabi et al., 2010; Spiegel et al., 2010). These symptoms are thought to be associated with a distrubance of the brain-gut axis which contributes to visceral hypersentivity and dysmotility (Akbar et al., 2009; Manabe et al., 2009). The exact etiology of IBS is multi-factoral, including psychological stress (Barreau et al., 2007; Miranda, 2009), environmental factors, genetic predisposition, and diet **(**Morcos et al**.**, 2009; Saito et al., 2009; Talley, 2006).

Serotonin (5HT) is a neuropeptide that modulates GI tract smooth muscle contractions and the perception of pain (Akbar et al., 2009; Crowell and Wessinger, 2007; Sikander et al., 2009). Ca. 95% of 5HT are found in the GI tract, 90% of which is secreted from enterchromaffin (EC) cells and the remaining 10% are found in the serotonergic neurons of the myenteric pleus (Bertrand and Bertrand, 2010; Sikander et al., 2009). It has been reported that excess amount of 5HT level was found in IBS

patients as well as in rodent models (Bertrand and Bertrand, 2010; Kerckhoffs et al., 2008; Ren et al., 2007). In addition, 5HT recptor 3 (5HTR 3) antagonist or 5HTR 4 agonist can reduce IBS symptoms(Gershon and Liu, 2007; Harris and Chang, 2007). Thus, the 5HT signaling pathway is implicated in the development of IBS.

Cholecystokinin (CCK) is a brain-gut hormone that regulates various GI tract functions including satiety, digestion, motility and pain (Varga et al., 2004; Zhu et al., 2010).The action of CCK is mediated through the CCK receptors (CCKR), CCKR1 and -2. CCKR1 is primarily distributed in the GI tract (Cawston and Miller, 2010), while CCKR 2 is mainly found in the central nervous system (Fornai et al., 2007). Elevated CCK levels have been reported in patients with IBS in a clinical study (Zhang et al., 2008), suggesting that the CCK pathway is a protential target for IBS treatment.

The rhizomes of *Coptis chinensis* Franch. (Ranunculaceae) *officially recognized* as Coptidis Rhizoma, (CR), also known as *Huang Lian* (China, 2010) are used in traditional Chinese herbal formulae have been reported to exert various pharmacological actions including antihypertensive (Tsai et al., 2008), antibacterial (Kong et al., 2009), antioxidative (Jung et al., 2009), and antiinflammatory effects (Remppis et al., 2010), among others. CR is a key component of many traditinal Chinese medicine (TCM) prescriptions used to treat syndromes including abdominal

pain and diarrhea (Chen and Chen, 2004). However, their mechanisms of action have not been elucidated. Therefore, the aim of the present study is to examine the effect of CR on neonatal maternal separation (NMS)-induced visceral hyperalgesia rat model, a well established early-life stress model that mimic human IBS symptoms, and to elucidate possible mechanisms of action in the event of a positive pharmacological action. We hypothesized that CR might reduced the visceral hypersentivity by modulating 5HT and CCK pathways.

2. Materials and methods

2.1 Animal and neonatal maternal separation

The animal experimental procedures detailed below were approved by the Animal Ethics Committee of the Chinese University of Hong Kong and the Institutional Animal Care and Use Committee of the University of Maryland-Baltimore. All male Sprague-Dawley pups grouped to 6 pups per dam on postnatal day 2 (P2 ; date of birth is designated as P0). Pups were randomly assigned to neonatal maternal separation (NMS) or unseparated control (N) groups according to well established protocol (Ren et al., 2007; Tjong et al., 2010). In brief, pups in the NMS group were separated from their mothers and placed into individual cages in an adjacent room,

maintained at 22^{o}C, daily for 3 hours (09 :00-12 :00) on P2-P21. The pups were then returned to the maternal cages after the separation on each period day. While the N group of rats were allowed to remain in standard cages with their dams. All pups were weaned on day 22 and housed (5 animals per cage) on a 12 :12-hr light-dark cycle (Lights on at 06:00) with free access to food and water ad libitum.

2.2 Plant material

Coptis chinensis rhizomes (Coptidis Rhizoma, CR) were puchased from the Zhixin Chinese Pharmaceutical Co. Ltd. (Guangzhou, China) and authenticated macroscopically and mictroscopically according to the descriptions found in Chinese Pharmacopoeia (China, 2010) and the Hong Kong Chinese Materia Medica Standards (Department of Health, 2008). A voucher sample, IBS-09, was deposited at the herbarium of the School of Chinese Medicine, The Chinese University of Hong Kong. The rhizomes were stored in air-tight containers kept in air-conditioned environment until use.

2.3 Preparation of Coptidis Rhizoma (CR) extract

Sliced CR (600 g) was extracted with 600 mL of 70% ethanol for 1 hr by reflux. After cooling to room temperature, the mixture was filtered and the filtrate collected. The

extraction was then repeated two more times. The filtrates were combined, the ethanol removed by in *vacuo* evaporation to almost dryness, and the resulting aqueous concentrate was lyophilized to a powder.

2.4 Adminstration of Coptidis Rhizoma (CR) extract

At 2-month of age, the NMS rats ,weighing c.a.~250g, were randomly divided into 4 groups (7 rats per group), with three groups being dosed orally with CR (0.3g/Kg, 0.8g/Kg or 1.3g/kg in 10ml/Kg in 0.5% carboxymethylcellulose solution) in volume of 10ml/Kg per day for seven days. The 4th group, designated as "Veh", received the vehicle (0.5% carboxymethylcellulose solution at 10ml/Kg body weight). The N rats were orally dosed with carboxymethylcellulose solution at 10ml/Kg for 7 days.

2.5 Implantation of electromyogram electrode and colonic distension-induced visceral hyperalgesia

The visceral motor response to colonic distension was measured by recording the electromyogram (EMG). Rats were anesthetized by inhalation of 2% isoflurane (in oxygen, 0.5L/min). A pair of EMG electrodes were surgically implanted at the lower left abdominal area to expose the external oblique abdominal masculature and the electrodes were tunneled subcutaneously, exteriorized and secured at the back of the

neck for sebsequent EMG recording. The colonic distension (CRD) study commenced 7 days after EMG electrodes implantation. The response to visceral stimulus was quantitatively assessed by measuring the EMG signals, as described in previus and the other studies previously (Christianson and Gebhart, 2007; Tjong et al., 2010). The rats were anesthetized with 2% isoflurane (in oxygen, 0.5L/min) to facilitate placement of the inflatable balloon, constructed from a latex glove finger attached to a Rigiflex balloon dilator via a Y connector to a syringe pump and a sphygomanometer into the descending colon. The balloon catheter was inserted into the rectum. The rats were allowed to recover for 30 min prior to the CRD study. EMG signals in response to CRD were recorded with a PowerLab 16/30 instrument, and analysed using Chart software (AD instruments, Bella Vista, Australia). The pain threshold pressure was defined as minimum pressure that evokes an observatory signal. EMG signal responses to CRD were measured at 10, 20, 40, 60 and 80mmHg. (Tjong et al., 2010). The change of the EMG signal responses to CRD were determined by calculating the change of area-under-curve (AUC) of raw EMG amplitude responses to CRD, based on the formula ΔAUC % baseline (AUC during CRD/ AUC before CRD).

2.6 Tissure preparation

Rats were anesthetized immediately after the completion of the CRD study, the distal colon dissected, immediately frozen in liquid nitrogen, and stored at -70^{o}C.

2.7 Measurement of serotonin content

Frozen samples of the distal colon tissues were homogenized in 0.2N $HClO_4$ and centrifuged at 10,000g for 5 min at 4^{o}C. The supernatant was collected and filtered through a 0.2μm filter. One volume of the supernatant was neutralized to pH 7-8 with one volume of 1M borate buffer (pH 9.25), followed by centrifugation at 10,000g for 1 min at 4 ^{o}C. The serotonin (5HT) content of an aliquot (100μl) of the sample was analysed by ELISA kit according to the manufacturer's instructions (Beckman Coulter, Fullerton, CA, USA). The sample was measured spectrophotometrically by a microplate reader (BMG Labtech, Offenburg, USA) at 405nm. The absorbance of the sample was converted into concentration by standard calibration, and the 5HT content of the tissue was expressed as a function of wet weight (ng/g tissue).

2.8 Total RNA isolation

Frozen distal colon tissues were homogenized with 1ml of Trizol reagent (Invitrogen, USA) and the total RNA extracted following the manufacturer's instructions. In brief,

homogenates were mixed choroform and centrifuged at 12,000g for 15 min at 4 ^{o}C. The RNA was precipitated with isopropanol and centrifuged at 12,000g for 15 min at 4^{o}C and the precipitated RNA was treated with 75% ethanol, followed by centrifugation at 7,500g for 5 min at 4^{o}C. The total RNA pellet was air dried, resuspended in Rnase-free water and its concentration measured by use of a Fluostar Optima spectrometer (BMG Labtech, Offenbury, Germany). For cDNA synthesis, 1.5μg of total RNA was mixed with a reverse-transcription master mix using high-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA, USA). The reverse transcription reaction was performed in a thermal cycler (9800 Fast Thermal Cycler, Applied Biosystems, Foster City, CA, USA).

2.9 Real-time quantitative PCR

A cDNA sample (2μl) and the primer was added with the GeneAmp Fast PCR master mix (Applied Biosystems, Foster City, CA, USA) and primer to a final volume of 20μl. The primers and taqman fluorogenic probes were purchased from Applied Biosystems, and their sequences are shown in table 1. Real-time quantitative PCR amplification reactions were carried out in a Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). cDNA was empolyed to quantify mRNA encoding β-actin, which is used as a non-regulated reference gene. The

thermocycling reaction was carried out at 50 ^{o}C for 2min, 95 ^{o}C for 10min, followed by 40 cycles of 95 ^{o}C for 15s and 60 ^{o}C for 1 min. The data were analysed with the Step One software and relative gene expression was determined using the $2^{-\Delta\Delta}$ cycle threshold (C_T) Method(Schmittgen and Livak, 2008). In brief, the C_T values were normalized by substracting from the β-actin control (ΔC_T = C_T target gene mRNA- C_T β -actin control). The expression of mRNA of target gene in the NMS or CR groups was calculated by substracting the normalized C_T values in the N group from those in the NMS or CR groups ($\Delta\Delta C_T$=ΔC_T NMS or CR-ΔC_T N), and the relative expression ($2^{-\Delta\Delta CT}$) was thus determined.

2.10 Western-blotting study

Frozen distal colons were homogenized in the lysis buffer containing 10μl of protease inhibitor cocktail (Calbiochem, USA), and centrifuged at 15,000g for 20 minutes at 4^{o}C. The proteins were mixed with the Laemmi buffer, incubated at 70 ^{o}C for 3min. Protein samples were separated by using 10% SDS-polyacrylamide mini-gel. The membranes were transferred to polyvinylidene-difluoride membranes using a transblotting apparatus (Bio-Rad laboratories, USA) and blocked with 5% skimmed milk in TBS buffer at room temperature for 2 hours. The membranes were then incubated with primary antibody CCK, CCKR 1, CCKR 2, SERT, 5HTR 3a, 5HTR 3b

and 5HTR 4 or β-actin (Santa Cruz Biotechnology Inc, USA) overnight at 4^{o}C. Immunoreactivity was detected with the secondary antibody, anti-mouse, anti-rabbit or anti-goat IgG conjuated to HRP (Santa Cruz Biotechnology Inc, USA). 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).

2.11 Statistical Analysis

Data were expressed as mean$\pm$SE. Statistical analyses were performed using Prims 4.0 software (GraphPad sofware Inc, la Jolla, CA, USA). Statistical significances were determined by the unpaired *t*- test or one-way ANOVA and differences were considered significant at $^{*}P<0.05$.

3. Results

3.1 CR attenuates visceral hyperalgesia in NMS rats in response to CRD

NMS Veh rats were more sensitive to colonic distension than the N rats, as demonstrated by a reduction in threshold to pain (N=7 per group; $p<0.01$) (Figure 1). On average, the pain threshold pressures were 27.1$\pm$1.5mmHg and 14.6$\pm$1.3mmHg in N and NMS veh rats, respectively. Pre-treatment of CR at doses of 0.8 and 1.3g/Kg

substantially elevated threshold to pain by 65% (N=7; $p<0.01$) and 80% (N=7; $p<0.01$), respectively, compared with veh animals in the NMS rats (Figure 1). In the EMG recording, the veh group showed a significant response to CRD at 20mmHg (Figure 2A), and the EMG magnitude increased by 164.1±35.3%, 183.8±35.4% and 289.7±37.8% to CRD at 40, 60 and 80mmHg, respectively, as compared to the N group (Figure 2B-D). On the other hand, the CR dosed (0.8 and 1.3g/Kg) NMS groups significantly depressed the elevated EMG signals of the veh group at all pressure response to CRD (Figure 2A-D).

3.2 CR decreases 5HT content in the distal colon of NMS rats

The 5HT level from the distal colon was significantly increased in the veh rats in comparison to the N rats (100.9±9.1ng/g of tissue versus 176.4±12.0 ng/g of tissue, n=6, $p<0.01$) (Figure3) (Figure 3). The elevated 5HT content exhibited by the NMS veh group was substantially lowered in the NMS rats pretreated with CR at 1.3g/Kg (by 54.8+17.4%; $p<0.05$) (Figure 3).

3.3 CR does not change the expressions of SERT, 5HTR subtypes in NMS rats

The mRNA and protein levels of SERT, 5HTR 3a, 3b and 4 were determined by RT-PCR and western-blotting studies respectively. SERT, 5HTR 3a, 3b and 4 were all

present in the both the N and NMS groups (Figure 4 and 5). Yet, there were no apparent changes in the level of their expressions among the groups (Figure 4 and 5).

3.4 CR significantly elevates CCK expressions in NMS rats

Protein expressions of CCK and CCKR subtypes from the distal colon were determined by western-blotting study. Increased protein level of CCK was observed in NMS rats (Figure 6A). On average, the level of CCK expression from the distal colon of veh rats was increased by 32.0$\pm$3.2% compared to the N group (Figure 6A). The protein level of CCK in the NMS rats treated with CR at 1.3g/Kg was significantly less (by 25.0$\pm$5.8%) than that in the veh group (Figure 6B). CCKR1 and 2 were present in the distal colon of both of the N and NMS rats (Figure 6A). There were no apparent differences in CCKR 1 and 2 expressions among the groups (Figure 6C). The mRNA expression of CCK and CCKR subtypes were examined by RT-PCR. As shown as in figure 7A, CCK expression in the NMS veh rats was higher than that in the N rats (1.0$\pm$0.1 versus 2.3$\pm$0.3, n=6, $p<0.01$). Treatment with CR at 1.3g/Kg significantly decreased CCK expression by 39.7$\pm$2.0% from the distal colon of NMS rats as compared to the N rats (Figure 7A), while there were no significant changes in CCKR 1 and 2 expressions among the groups (Figure 7B).

4. Discussion

The present study is the first to show that the Chinese herb CR markedly reduced visceral pain in NMS rats in a dose-dependent manner. Recurrent visceral pain is one of the main clinical observation of IBS that affects the quality of patient's daily life (Wong and Drossman, 2010). Although the etiology of this symptom is not clearly understood, an increasing number of studies have reported that both pyschological and physical early-life stress contributes to the risk for devleoping IBS (Chitkara et al., 2008; Gareau et al., 2008; Miranda, 2009). In the present study, we observed decreased pain threshold and elevated EMG activity in NMS rats, which are consistent with the observations recorded in our previous studies, as well as those reported by others (Chung et al., 2007; Ren et al., 2007; Tjong et al., 2010) , and that NMS is a prominent stressor causing visceral hyperalgesia (Coutinho et al., 2002). Our study provides evidence that CR treatment reduced the pain threshold pressure and depressed EMG activity evoked by CRD. Thus, CR extract may be an effective herbal treatment for mitigating the visceral pain of IBS.

It is widely accepted that 5-HT is a key messenger in modulating visceral pecreption in the enteric nervous system via the paracrine, endocrine and neurocrine pathways (McLean et al., 2007). Our study found that 5HT content from the distal colon was elevated in NMS rats, which confirmed with published clinical findings of

presence of increased 5HT level in the distal colon of IBS patients (Bertrand and Bertrand, 2010; Kerckhoffs et al., 2008), suggesting that 5-HT plays an important role in the pathophysiology of IBS. It has been reported that 5HT exerts its action via 5HTR, in which 5HT3a, 3b and 4 play predominant role in the regulation of gut functions (Sikander et al., 2009). It is reported that 5HTR 3 mediates IBS by activation of extrinsic primary neurons (Gershon, 2005), while 5HTR 4 promotes cyclic AMP and increases the release of acetylcholine from the neurons to the gut (Tonini, 2005). The action of 5HT in the gut was terminated and reuptaked by serotonin transporter (SERT) (Martel, 2006). Excess 5HT or SERT could alter the signaling pathway for gut motility and pain transmission (Spiller, 2008), and hence 5HT can act as a biomarker for IBS. Our results also showed that treatment of CR of NMS rats substantially attenuated the colonic 5HT concentration. However we did not observe any significant effect of CR on SERT and 5HTR expressions in NMS rats, which suggest that the attenuated 5-HT release by CR may be mediated through the modulation of tryptophan hydroxylase activity in EC cells (Liu et al., 2008) rather than SERT and 5HTR.

CCK belongs to the gastrin family, which is synthesized and released from the endocrine cells in the GI tract. It has recently been found to be involved in the modulation of GI tract motility (Varga et al., 2004). Administration of CCK promotes

colonic motor response in healthy guinea pig, and in the ascending colon in human (Morton et al., 2002; Zhu et al., 2010). Our results show that CCK expression from the distal colon was elevated in NMS rats. Greisen at al also found serum CCK level was elevated in NMS rats (Greisen et al., 2005), suggesting that early-life stress causes excess CCK secretion that disrupt the brain-gut axis. Clinical studies also reported increased plasma CCK level in IBS patient (Van Der Veek et al., 2006; Zhang et al., 2008), and CCKR antagonists reduced visceral perception in IBS patients (Scarpignato and Pelosini, 1999). These findings indicate that CCK is also a critical factor involved in the pathophysiology of IBS. Our results showed a downregulation of CCK expression in NMS rats treated with CR. Yet, CR did not have any effect on neither CCKR 1 nor CCKR 2 from the distal colon of NMS rats, suggesting that CR not only reduced 5HT release but also CCK expression, in lowering the visceral sensitivity.

In conclusion, our present study provides evidence that CR substantially attenuates visceral hyperalgesia by lowering 5-HT release and CCK expression in the colon of NMS rats. These findings also suggest that CR may be an effective herbal preparation for the treatment of IBS. The present study on individual herb may also provide useful information for better formulating a Chinese formula for IBS and better design of future clinical trials.

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Figures and legends

Table 1. Primer sequences of CCK, CCK R1, CCK R2, SERT, 5HTR 3a, 3b and 4.

Figure 1. Effect of CR on pain threshold pressure among groups. Data are expressed as mean ± SEM. The pain threshold of NMS veh group was significantly decreased compared with N group and CR (dosage: 0.8 and 1.3g/Kg) significantly increased pain threshold pressure compared with NMS veh group. Statistical significance is indicated by $^{**}P<0.01$ unpaired *t*-test compared with N group and $^{\#\#}P<0.01$ versus NMS veh group, one way ANOVA.

Figure 2. Effect of CR on electromyographic activity response to CRD at pressure of 20, 40, 60 and 80mmHg (A-D). Data are presented as mean ± SEM. Statistical significance is indicated by $^{*}P<0.05$ or $^{**}P<0.01$ unpaired *t*-test compared with N group and $^{\#}P<0.05$ or $^{\#\#}P<0.01$ versus NMS veh group, one way ANOVA.

Figure 3. Effects of CR on the 5HT content in distal colon of rats. Data were expressed as mean ± SEM (ng/g tissue). The 5HT level in NMS veh group was significantly higher than that in N group. CR significantly reduced 5HT content in NMS rats. Statistical significance is indicated $^{**}P<0.01$ unpaired *t*-test compared with

N group and $^{\#}P<0.05$ versus NMS veh group, one way ANOVA.

Figure 4. (A) Protein levels of SERT, 5HTR 3a, 3b and 4 expressions in the distal colon of rats. Summary of levels of (B) SERT, (C) 5HTR 3a, 3b and (D) 5HTR 4 in N, NMS veh and CR groups.

Figure 5. mRNA expressions of SERT, 5HTR3a, 5HTR 3b and 4 in the distal colon of rats. Summary of the relative mRNA expression level of (A) SERT, (B) 5HTR 3a, 3b and (C) 5HTR 4 were all present among groups.

Figure 6. (A) Protein levels of CCK, CCKR 1 and 2 expressions in the distal colon of rats. (B) Summary of levels of CCK protein expression among groups ($^{**}P<0.01$ one way ANOVA compared with N group; $^{\#}P<0.05$ compared with NMS veh group unpaired *t*-test). (C) Summary of levels of CCKR1 and 2 protein expressions among groups.

Figure 7. (A) Summary of relative mRNA expression of CCK in distal colon among groups. ($^{**}P<0.01$ one way ANOVA compared with N group; $^{\#}P<0.05$ compared with NMS veh group unpaired *t*-test). (C) Summary of relative mRNA CCKR1 and 2 expressions among groups.

Table 1

Primer		Primer sequence 5’-3’
CCK	(forward)	5’- ACT GCT AGC CCG ATA CAT CC -3’
CCK	(reverse)	5’- ATC CAT CCA GCC CAT GTA GT -3’
	(sense)	5’- CAG GGC CTG GAC CCT AGC CA -3’
CCKR 1	(forward)	5’- ATG CTC ATT GTC ATC GTG GT -3’
CCKR 1	(reverse)	5’- GAG TCC CTG AGA GGT GCT TC -3’
	(sense)	5’- CCG TGT CAT ATG CCC GCC AG -3’
CCKR 2	(forward)	5’- TCC ATT GGA GAC TGT GGA AA -3’
CCKR 2	(reverse)	5’- GCT TCC CAT AAA CCA CAC CT -3’
	(sense)	5’- CCT GCC TCT CCT CCC TCA CCA -3’
SERT	(forward)	5’- AGG AGT TCT ACT TGC GCC AT -3’
SERT	(reverse)	5’- AAG ATG AGC ACG ATG CAG AG -3’
	(sense)	5’- CCA GGA CCT GGG CAC CAT CA -3’
5HTR 3a	(forward)	5’- CCT CAA CGT GGA TGA GAA GA -3’
5HTR 3a	(reverse)	5’- GGA TGG ACA ATT TGG TGA CA -3’
	(sense)	5’- TCG TCG GTC CAG AAC TGC CG -3’
5HTR 3b	(forward)	5’- TTC CGG AAC GAT TAG AAA CC -3’
5HTR 3b	(reverse)	5’- CAG GAA GCC CAG GTC TAT GT -3’
	(sense)	5’- CCA TCC AGG TGG TCT CCG CA -3’

Figure 1

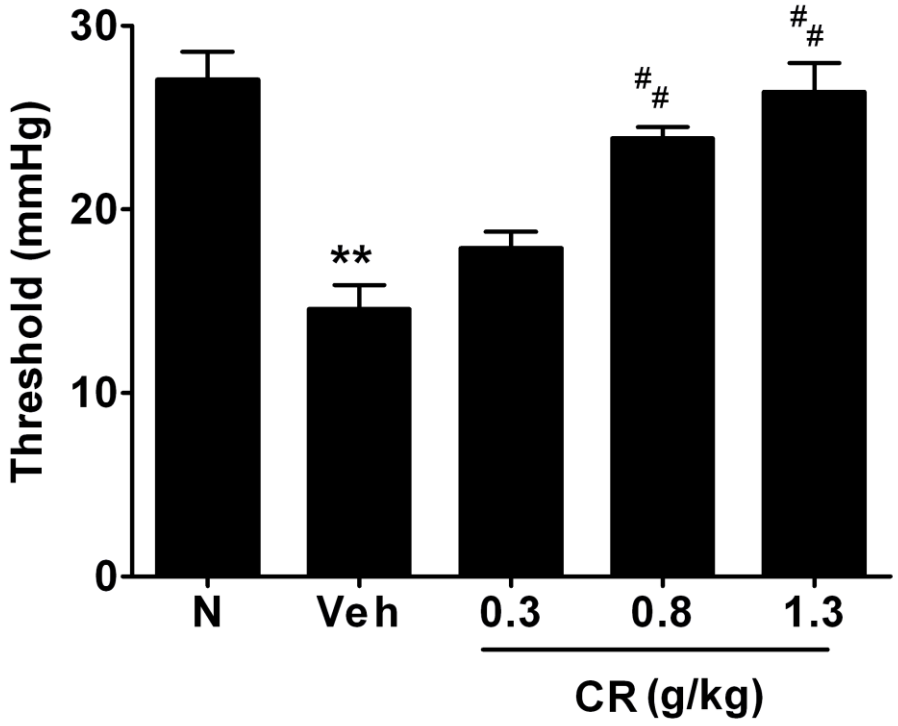


Figure 2

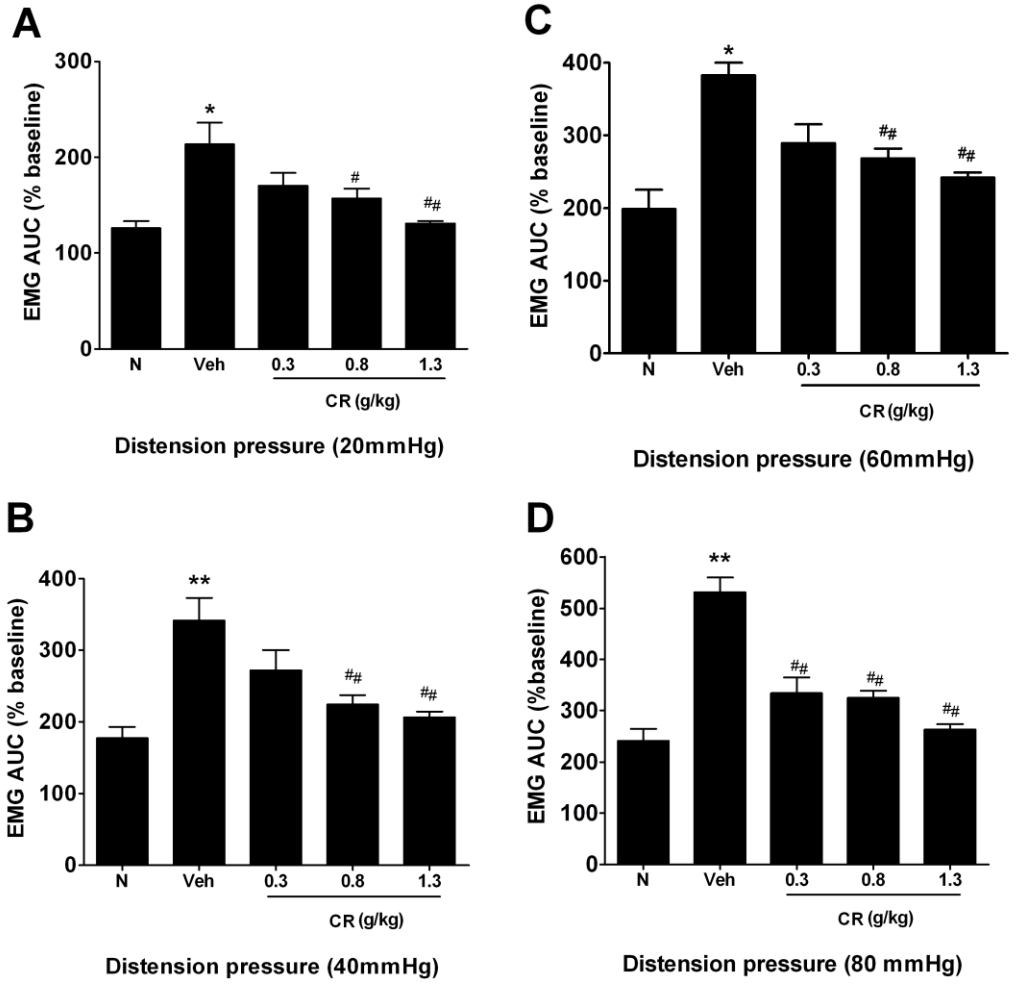
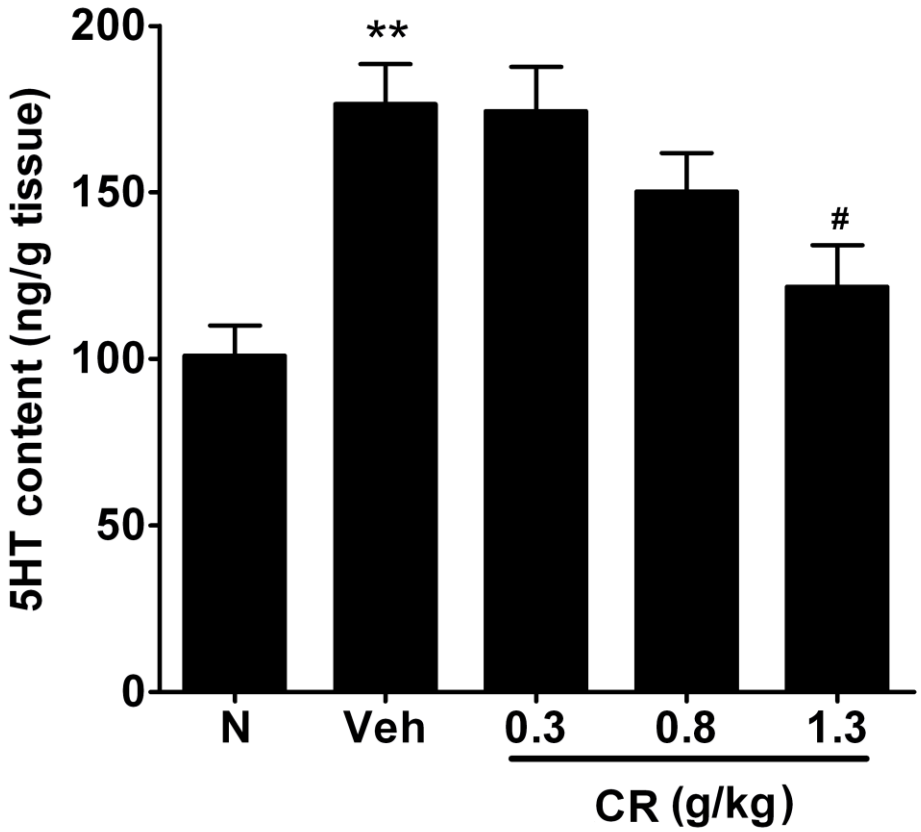

A
EMG AUC (% baseline)
300
200
100
0
*
#
#
N
Veh
0.3
0.8
1.3
CR (g/kg)
Distension pressure (20mmHg)
C
EMG AUC (% baseline)
400
300
200
100
0
*
#
#
N
Veh
0.3
0.8
1.3
CR (g/kg)
Distension pressure (60mmHg)
B
EMG AUC (% baseline)
400
300
200
100
0
**
#
#
N
Veh
0.3
0.8
1.3
CR (g/kg)
Distension pressure (40mmHg)
D
EMG AUC (%baseline)
600
500
400
300
200
100
0
**
#
#
#
N
Veh
0.3
0.8
1.3
CR (g/kg)
Distension pressure (80 mmHg)

Figure 3



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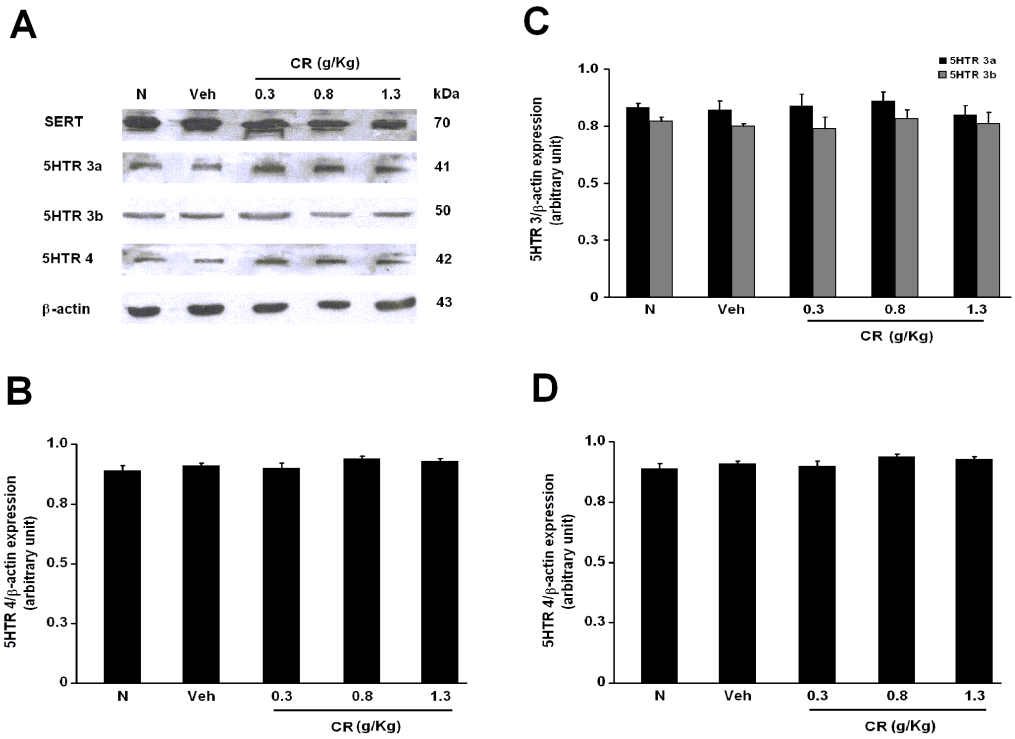

A
CR (g/Kg)
N
Veh
0.3
0.8
1.3
kDa
SERT
70
5HTR 3a
41
5HTR 3b
50
5HTR 4
42
β-actin
43
C
5HTR 3a
5HTR 3b
5HTR 3/β-actin expression (arbitrary unit)
1.0
0.8
0.5
0.3
0
N
Veh
0.3
0.8
1.3
CR (g/Kg)
B
5HTR 4/β-actin expression (arbitrary unit)
1.0
0.8
0.5
0.3
0
N
Veh
0.3
0.8
1.3
CR (g/Kg)
D
5HTR 4/β-actin expression (arbitrary unit)
1.0
0.8
0.5
0.3
0
N
Veh
0.3
0.8
1.3
CR (g/Kg)

Figure 5

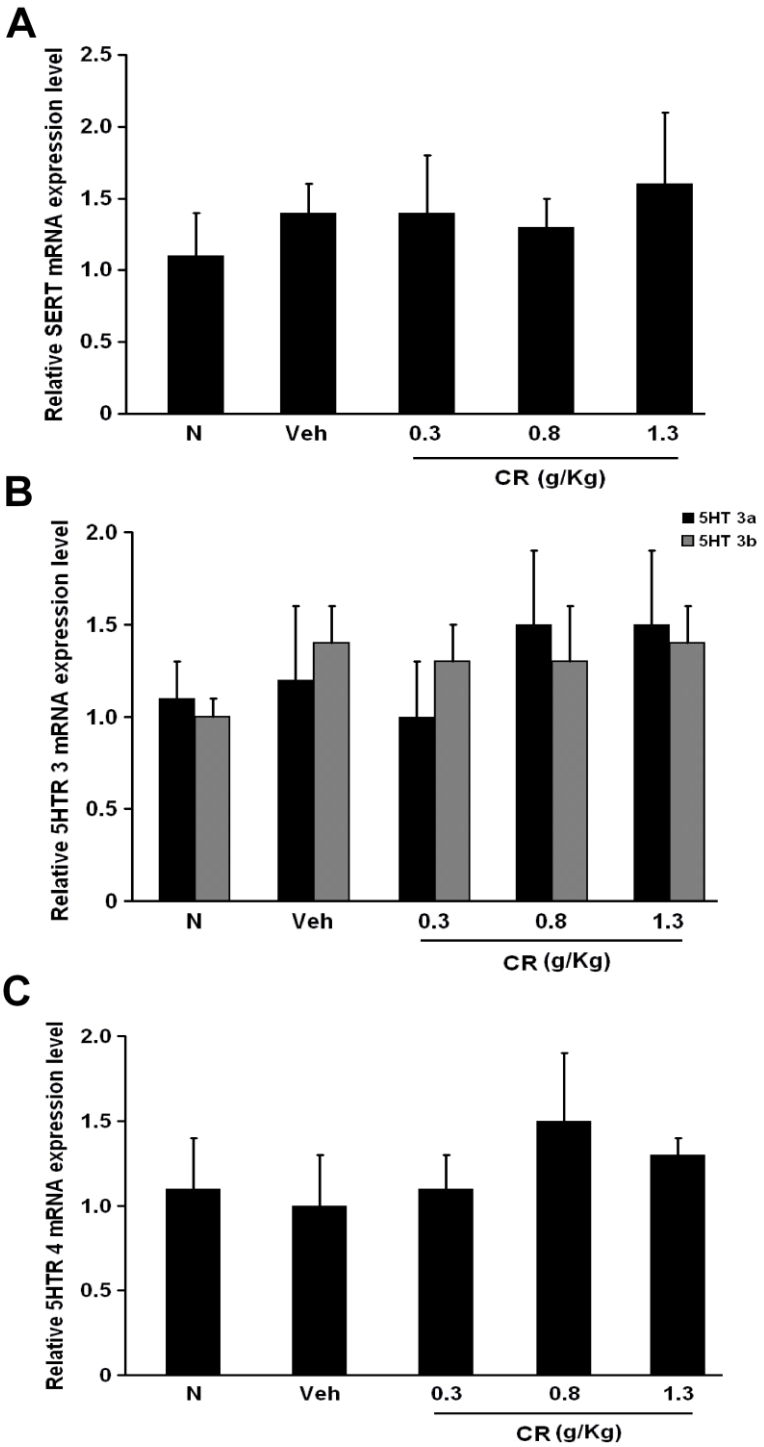

A
Relative SERT mRNA expression level
2.5
2.0
1.5
1.0
0.5
0
N
Veh
0.3
0.8
1.3
CR (g/Kg)
B
5HT 3a
5HT 3b
Relative 5HTR 3 mRNA expression level
2.0
1.5
1.0
0.5
0
N
Veh
0.3
0.8
1.3
CR (g/Kg)
C
Relative 5HTR 4 mRNA expression level
2.0
1.5
1.0
0.5
0
N
Veh
0.3
0.8
1.3
CR (g/Kg)

Figure 6

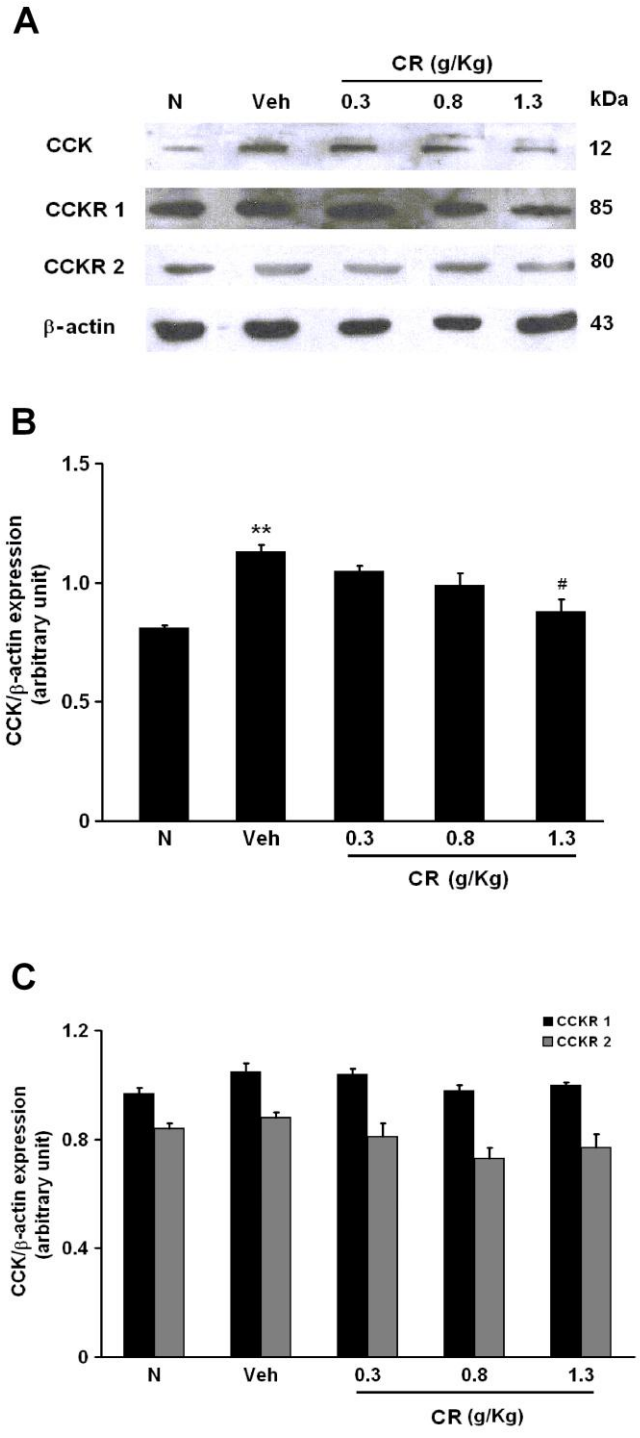
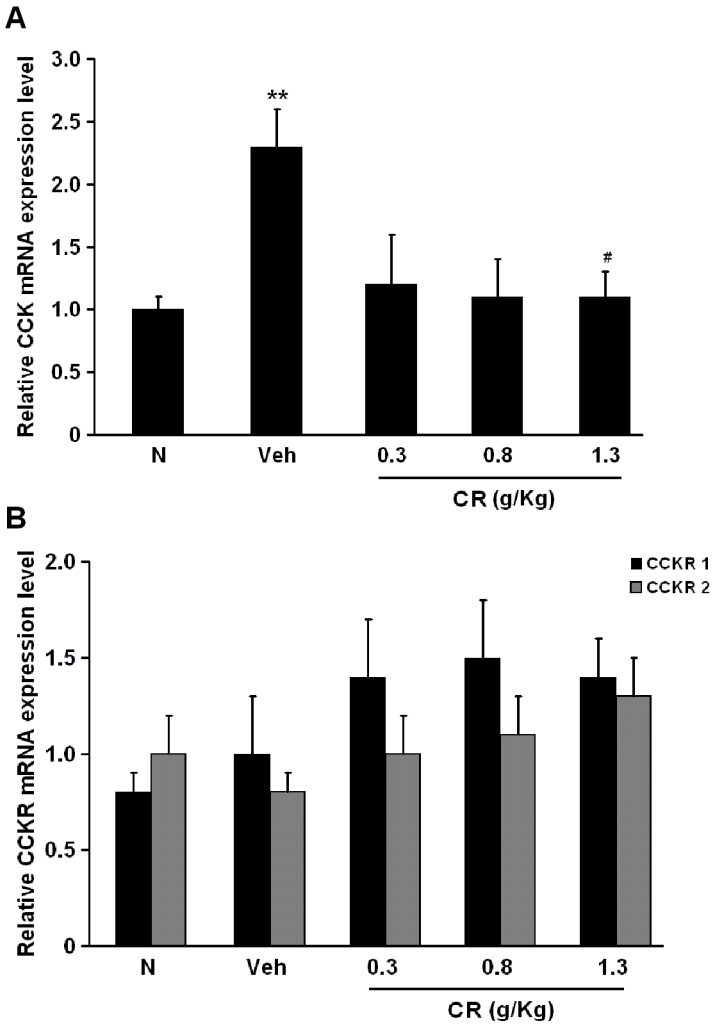

A
CR (g/Kg)
N
Veh
0.3
0.8
1.3
kDa
CCK
12
CCKR 1
85
CCKR 2
80
β-actin
43
B
CCK/β-actin expression (arbitrary unit)
1.5
1.0
0.5
0
**
#
N
Veh
0.3
0.8
1.3
CR (g/Kg)
C
CCKR 1
CCKR 2
CCK/β-actin expression (arbitrary unit)
1.2
0.8
0.4
0
N
Veh
0.3
0.8
1.3
CR (g/Kg)

Figure 7


A
Relative CCK mRNA expression level
3.0
2.5
2.0
1.5
1.0
0.5
0
**
#
N
Veh
0.3
0.8
1.3
CR (g/Kg)
B
Relative CCKR mRNA expression level
2.0
1.5
1.0
0.5
0
CCKR 1
CCKR 2
N
Veh
0.3
0.8
1.3
CR (g/Kg)