Expression of genes encoding cytokines and corticotropin releasing factor are altered by citalopram in the hypothalamus of post-stroke depression rats

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Submitted: 2013-08-15 Accepted: 2013-11-03 Published online: 2014-01-15

Key words: **post-stroke depression; cytokines; corticotropin releasing factor; interleukin 1 beta; tumor necrosis factor alpha**

Neuroendocrinol Lett 2013; **34**(8):773–779 **PMID:** 24522018 NEL340813A05 © 2013 Neuroendocrinology Letters • **www.nel.edu**

Abstract

OBJECTIVES: To establish a rat model of post-stroke depression (PSD), and examine expression of genes encoding corticotropin releasing factor (CRF), interleukin 1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α) in the hypothalamus of PSD rats.

METHODS: Rats were subjected to middle cerebral artery occlusion (MCAO) and chronic mild unpredictable stress (CUMS). Open field test and sucrose preference were used to examine depressive-like behaviors. Observed changes in gene expression levels in the hypothalamus of PSD rats were evaluated.

RESULTS: MCAO with CUMS resulted in reduction of sucrose preference and locomotor activity. Genes encoding TNF-α, IL-1β and CRF were highly expressed in the hypothalamus of rats subjected to MCAO and CUMS. The antidepressant citalopram, a selective serotonin reuptake inhibitor, had inhibitory effects on the expression of the aforementioned genes. We observed a correlation between CRF and IL-1β mRNA levels in the citalopram-treated group of rats.

CONCLUSION: The etiology of PSD is associated with cytokine expression in the hypothalamus and with hypothalamic–pituitary–adrenal axis activity. Citalopram administration inhibited the expression of TNF-α and IL-1β transcripts in the hypothalamus, suggesting that selective serotonin reuptake inhibitors may be appropriate for PSD therapy.

Abbreviations:

CRF	- corticotropin releasing factor	PSD	- post stoke depression
TNF-α	- tumor necrosis factor alpha	HPA	- hypothalamus-pituitary-adrenal
IL-1β	- cytokines interleukin 1 beta	SSRIs	- selective serotonin reuptake inhibitors
CUMS	- chronic unpredictable mild stress	CNS	- central nervous system
MCAO	- middle cerebral artery occlusion	qPCR	- quantitative polymerase chain reaction

ORIGINAL ARTICLE

INTRODUCTION

Post-stroke depression (PSD) is one of the most common psychiatric disorders diagnosed, and is usually a consequence of stroke. Considering the negative impact on cognitive and functional performance of stroke patients, understanding the pathogenesis of PSD is important for clinical treatment and recovery. Clinical and pre-clinical studies have provided evidence that PSD is associated with pro-inflammatory cytokines such as interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and IL-6 (Hill *et al.* 1999; Su *et al.* 2012; Zaremba & Losy 2001). Following a stroke, TNF-α and IL-1β levels are increased in the blood of PSD patients (Su *et al.* 2012). Administration of cytokines, such as TNF-α and IL-1β, can induce depressive-like behavior in animals, and this behavior resembles symptoms of depression in humans (Dantzer 2001). The hypothalamus plays a key role in the etiology of depression, with the hypothalamus-pituitary-adrenal (HPA) axis thought to be involved in depressive symptomatology. Hyperactivity of the HPA axis is associated with corticotropin releasing factor (CRF) in the hypothalamus and could be influenced by cytokines (Bao *et al.* 2008), therefore, increased HPA axis activity could be related to PSD.

Selective serotonin reuptake inhibitors (SSRIs) are one type of drug currently recommended to treat depression (Ellis 2004). Citalopram is a commonly used SSRI, and is considered safe and tolerable for clinical use in patients. SSRIs can inhibit the inflammatory response of immune cells (Martensson & Nassberger 1993) and differentiation of human monocytes into macrophage-like cells *in vitro* (Ying *et al.* 2002). The inflammatory process in the brain originates from microglial cells (Xing *et al.* 2011). Antidepressants, however, have an anti-inflammatory effect on the central nervous system (CNS) by modulating production of TNF-α (Hwang *et al.* 2008). These previous findings suggest that the anti-inflammatory effects of SSRIs might play an important role in the therapeutic mechanism of PSD. Accordingly, in this study we investigated the behavioral improvement of PSD rats after treatment with citalopram, and the action of citralopram on production of TNF-α and IL-1β in the CNS of PSD rats.

MATERIALS AND METHODS

Animals

All animal experiments were conducted with the approval of the Animal Care Committee of the Chinese PLA General Hospital. Adult male Sprague-Dawley rats (240–260 g) were housed in cages (two rats per cage) and allowed to adapt to their environment (21–22°C, 12/12 h light/dark cycle, free access to food and water) for 1 week before surgery.

Surgical procedures

Rats were intraperitoneally (I.P.) anesthetized with sodium pentobarbital (40 mg/kg). Middle cerebral artery occlusion (MCAO) leading to cerebral ischemia in rats was conducted following the methods of Wang-Rischer (2009) and Wang et al (2009). The left common carotid artery (CCA) was exposed and a small incision made near the bifurcation of the external carotid artery and internal carotid artery (ICA). The prepared 3/0 nylon monofilament suture (Beijing Sunbio Biotech Co. Ltd., Beijing, China) was heat-blunted at the tip to a diameter of 0.34–0.36 mm and coated with poly-L-lysine, then gently inserted into the artery from the ICA incision. The suture length was 18.5±0.5 mm. The internal temperature of rats was monitored and maintained at 37°C throughout the procedure and during recovery. Rats in the sham group (n=8) were treated the same way except that the suture was inserted into the CCA. Rats were evaluated 24 h after surgery according to the 5-point scale proposed by Longa *et al.* (1989) : where 0 indicates no neurological deficit; 1 indicates a failure to fully extend the right forepaw; 2 indicates circling to the right; 3 signals falling to the right; and 4 indicates no spontaneous walking with reduced level of consciousness. The 24 surviving MCAO rats with a neurological score greater than or equal to 1 but less than 4 were randomly divided into three groups: MCAO (n=8); MCAO+CUMS (n=8); and MCAO+CUMS+CIT (n=8). For these treatment groups, CUMS represented chronic unpredictable mild stress, and CIT indicated treatment with citalopram. The MCAO+CUMS+CIT group was treated with citalopram (10 mg/kg I.P.) the day after surgery for 6 weeks (Papp *et al.* 2002). The morphology of ischemic lesions can be seen in Figure 4.

Sucrose preference test

For 6 weeks after surgery, anhedonia syndrome was evaluated every week using the sucrose preference test. All rats were trained to adapt to the taste of sucrose before the surgical procedure and as the baseline preference test. During the first training for the sucrose test, all rats were given a bottle of 1% sucrose and a bottle of water to adapt to the taste. Food and water were then removed, and after 23 h, rats were given a bottle of 1% sucrose and tap water. The consumption levels of 1% sucrose, and total liquids were measured for the next hour. Sucrose preference is presented as the ratio of consumed sucrose to total liquid consumed (Wang *et al.* 2010).

Open field test

We used an open field test to evaluate locomotor activity (Wang *et al.* 2010). The apparatus comprised an 80×80 cm area with four 30-cm high black walls, with the area divided into 16 squares. Rats were placed in the center of the field and observed for 4 min. The number of crossed squares and frequency of rearing were recorded.

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Chronic unpredictable mild stress (CUMS) procedures

Procedures for inducing CUMS were adapted from a previous study by Willner *et al.* (Willner *et al.* 1992; Willner 1997; Wang *et al.* 2010). Nine different stressors were randomly arranged for the three treatment groups over 18 consecutive days. These stressors were: 18 h of water deprivation; 17 h in a cage on a 45 ° tilt; overnight illumination; 21 h in a wet cage; 5 min of swimming in 8 °C water; 30 min of shocks to the feet at an intensity of 1.5 mA, and a duration of 2 s with 1-s intervals; 23 h of food and water deprivation; 1-min tail pinch; and 2 h of immobilization. No stressors were applied to control rats, which were housed under normal light and temperature conditions with free access to water and food except when the sucrose preference test was conducted. After CUMS procedures, rats were allowed 3 weeks to recover. The citalopram treatment was continued for 6 weeks. The open field and sucrose consumption tests were performed every week. The schedule for the animal experiments is shown in Figure 3.

Real time quantitative polymerase chain reaction (qPCR) assays

We used qPCR assays to analyze CRF, TNF-α and IL-1β mRNA expression levels. Total RNA from the ipsilateral hypothalamus of rat brains was isolated, and RNA samples subjected to reverse transcription using moloney murine leukemia virus and oligo(dT) primers (Fermentas, USA). The resulting cDNA templates were added to SYBR Green PCR MastMix (TianGen Biotech, Beijing, Co., Ltd). As an internal control we used β-actin. Gene expression levels were presented as relative expression values using the $2^{-\Delta\Delta Ct}$ method. Specific primers were used for the amplification of each gene (Table 1). Most primer pairs were designed to span the intron closest to the 3' end to avoid amplification of DNA templates. The thermal cycling profile involved an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of the amplification was verified using melting curve analysis and electrophoresis of the products on an 8% polyacrylamide gel. Sterile water, RNA samples lacking reverse transcriptase during cDNA synthesis, and DNA samples were used as controls.

Tab. 1. The PCR primer sequences.

Name	Primer sequences (5'–3')	GenBank Accession numbers	Amplicon (bp)
CRF	AGA ACA ACA GTG CGG GCT CAC CAA GGC AGA CAG GGC GAC A	NM_031019.1	117
β-actin	ACC GTG AAA AGA TGA CCC AGA T GTA ACC CTC ATA GAT GGG CAC A	NM_031144.3	164
TNF-α	CTC ATT CCT GCT CGT GGC TCC TCC GCT TGG TGG TTT	NM_012675.3	201
IL-1β	TGT GAT GTT CCC ATT AGA CAG CT TGG AGA ATA CCA CTT GTT GGC TTA	NM_031512.2	136

CRF, corticotropin releasing factor; β-actin, beta-actin; TNF-α, tumor necrosis factor alpha; IL-1β, interleukin-1 beta

Statistical analysis

Statistical analysis was conducted with SPSS (version 20, IBM Corporation). Gains in body weight, locomotor activity, sucrose preference and gene expression at the mRNA and protein level for CRF, TNF-α and IL-1β were analyzed using two-way analysis of variance (ANOVA). Association analysis was examined using Spearman's correlation coefficient and tests were two-tailed. Values were presented as means ± standard deviation (SD). A p-value less than 0.05 was considered statistically significant.

RESULTS

Body weight gain

Rats in the CUMS (49.2±7.3 g), MCAO (48.8±8.03 g), and MCAO+CUMS+CIT (43.5±11.5 g) groups experienced a reduction in body weight gain compared with the control (CON) group (109.5±9.5 g). Citalopram significantly increased body weight gain in the MCAO+CUMS+CIT group compared with the MCAO+CUMS group (p=0.011).

Sucrose preference test

There were significant reductions in sucrose preference for the CUMS and MCAO+CUMS groups (all p<0.01 at week 0; Figure 1a) after 3 weeks of testing, and these persisted until week 6. There were no significant differences between the MCAO and CON groups. Citalopram reversed the decrease in sucrose preference for the MCAO+CUMS+CIT group (p<0.01 *vs.* the MCAO+CUMS group at weeks 3 and 6; Figure 1a).

Open field test

The MCAO+CUMS group displayed a significant decrease in locomotor activity, indicated by a decrease in frequency of crossing squares and rearing after 1 week of stressor treatment (p<0.05 *vs.* baseline). Citalopram improved crossing activity compared for rats in the MCAO+CUMS group (p<0.0001 at weeks 3 and 6), and a significant improvement in rearing frequency (p=0.039 *vs.* MCAO+CUMS group) was seen at week 6. Rats in the CUMS group showed a decrease in crossing (p=0.000 vs. CON group at weeks 3 and 6) and rearing activities (p=0.002 *vs.* CON group at week 3, p=0.007 *vs.* CON group at week 6). The decrease in locomotor activity of CUMS rats was not significantly different between weeks 3 and 6 (crossing: p=0.199; rearing: p=0.402; Figure 1b and 1c).

CRF, TNF-α and IL-1β mRNA levels in the hypothalamus of PSD rats

The mRNA level of TNF-α in the MCAO+CUMS group was significantly increased (p=0.003 *vs.* CUMS; Figure 2a). Similarly, there was a significant

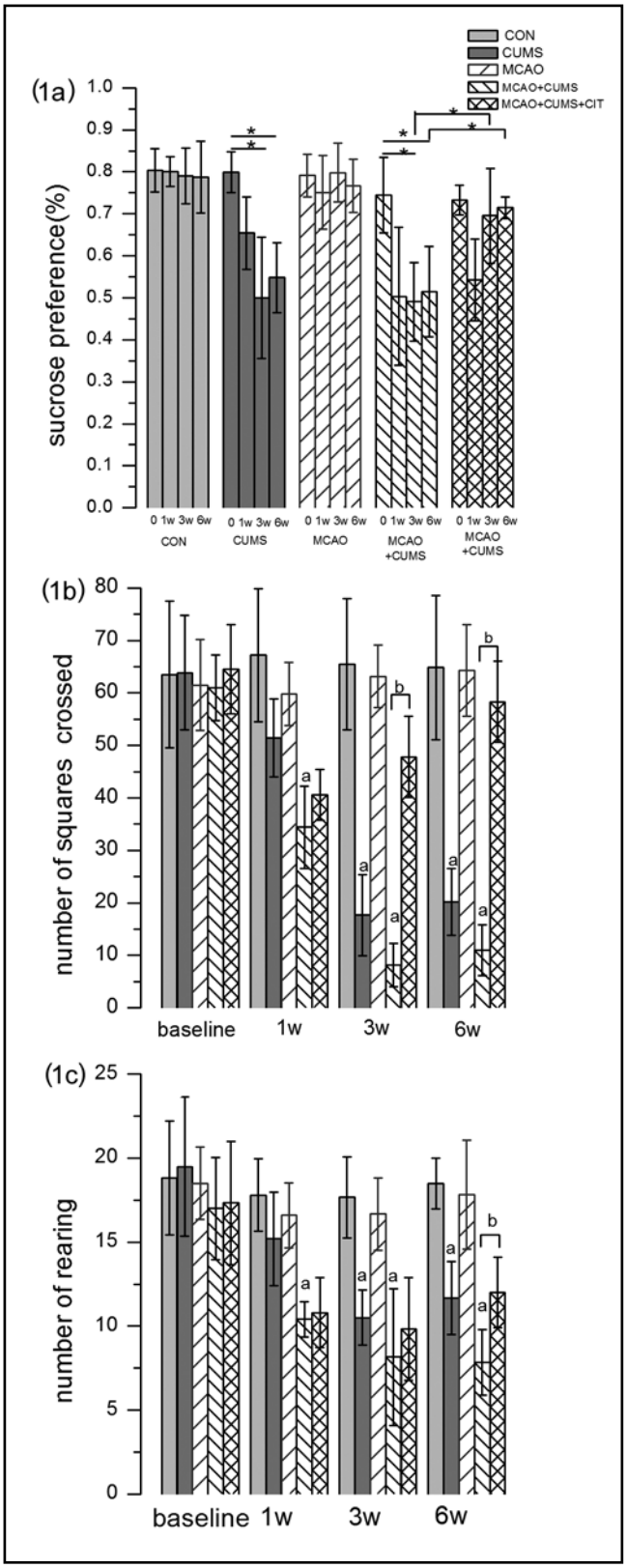

Fig. 1. Behavioral changes in MCAO+CUMS rats. (**a**) Significant reduction in sucrose preference compared with the CON group ($^{*}p<0.01$). Citalopram reversed decreases in sucrose preference for rats in the MCAO+CUMS+CIT group. (**b** and **c**) Significant decreases in crossing square and rearing frequency after 1 week of stress treatment. Citalopram improved crossing activity at weeks 3 and 6. There was significant improvement in rearing frequency at week 6. CUMS rats showed a decrease in crossing at weeks 3–6. $^{a}p<0.05$ *vs.* baseline; $^{b}p<0.05$ *vs.* MCAO+CUMS group.

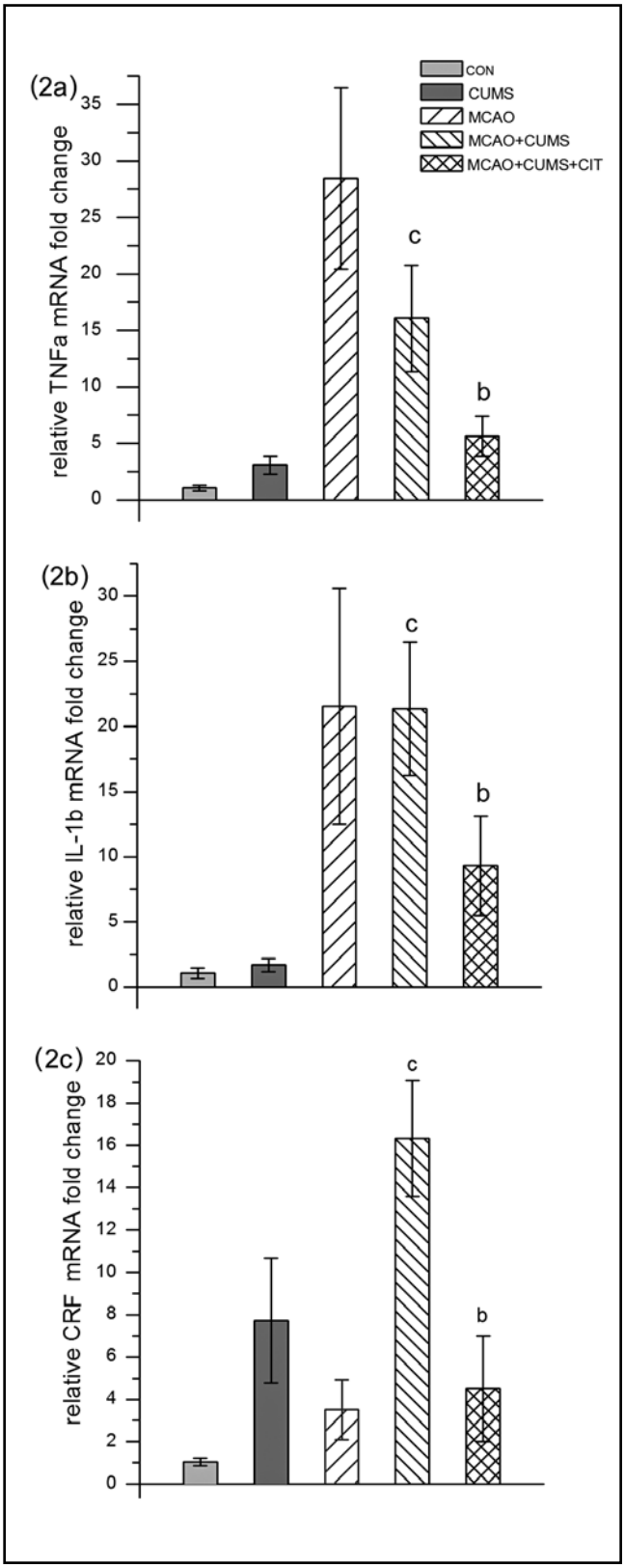

Fig. 2. Expression of cytokine and CRF genes in the hypothalamus of rats and the effects of citalopram. CRF, TNF-α and IL-1β mRNA transcript levels were increased in the hypothalamus of MCAO+CUMS rats, and reversed by citalopram. $^{c}p<0.05$ *vs.* CUMS group; $^{b}p<0.05$ *vs.* MCAO+CUMS group.

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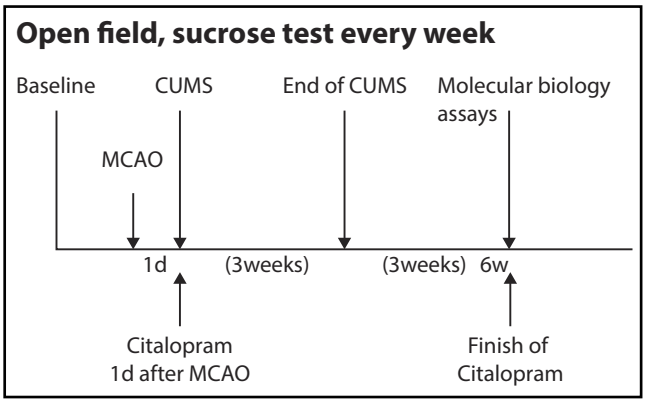

Fig. 3. Animal experiment procedures.

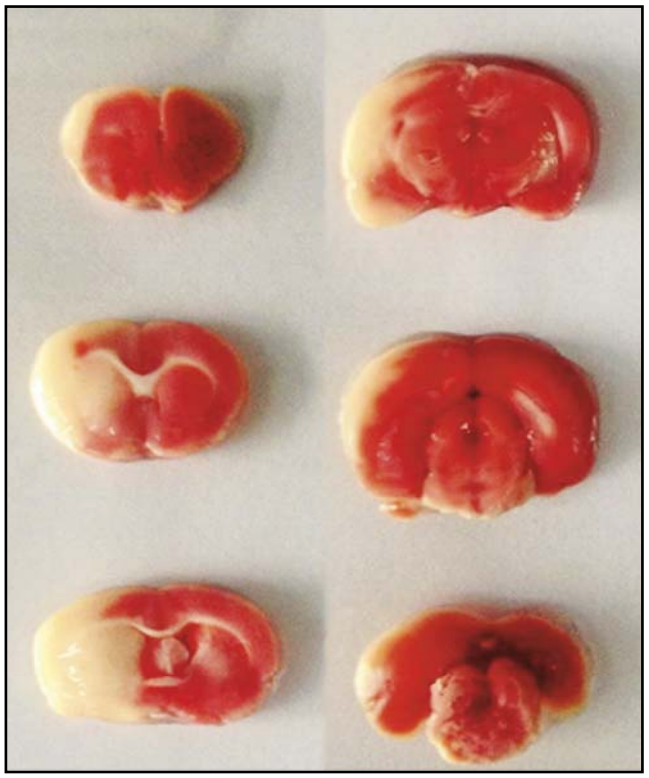

Fig. 4. Morphology of ischemic lesions.

increase in IL-1β mRNA levels in the hypothalamus of MCAO+CUMS rats (p=0.001 *vs.* CUMS; Figure 2b). Expression of CRF transcripts was significantly increased in the hypothalamus of MCAO+CUMS rats (p=0.000 *vs.* CON, p=0.000 *vs.* MCAO, p=0.005 *vs.* CUMS; Figure 2c). There was no significant increase in CRF mRNA levels in the MCAO group (p>0.05 *vs.* CON). We also analyzed correlations in gene expression between CRF, TNF-α, and IL-1β. We observed a correlation between CRF and IL-1β mRNAs in the MCAO+CUMS+CIT group (p=0.019, ρ=0.886). A similar correlation between CRF and IL-1β mRNAs was seen in the MCAO+CUMS and MCAO+CUMS+CIT groups (p=0.001, ρ=0.818). A correlation between TNF-α and CRF mRNA levels was seen for rats in the MCAO+CUMS and MCAO+CUMS+CIT groups (p=0.007, ρ=0.734).

Effects of citalopram on CRF, TNF-α and IL-1β mRNA expression in MCAO+CUMS rats

The levels of TNF-α mRNA transcripts (p=0.004) were significantly inhibited in the MCAO+CUMS+CIT group compared with the MCAO+CUMS group (Figure 2a). IL-1β mRNA levels was inhibited in the MCAO+CUMS+CIT group (p=0.02 *vs.* MCAO+CUMS; Figure 2b). CRF gene expression was decreased in citalopram-treated rats compared with MCAO+CUMS rats (p=0.000 for MCAO+CUMS+CIT *vs.* MCAO+CUMS; Figure 2c).

DISCUSSION

In this study we focused on changes in pro-inflammatory cytokine mRNA transcript expression, and on changes in the HPA axis in ischemic stroke rats with depression. MCAO combined with CUMS resulted in behavioral changes, including reduction of sucrose consumption and locomotor activity. We observed high expression levels for the cytokines and CRF in the hypothalamus of MCAO+CUMS rats. Citalopram had anti-inflammatory effects by inhibiting expression of TNF-α and IL-1β at transcripts.

The ischemic stroke animal model combined with mild stressors has been used in previous studies (Wang *et al.* 2012 ; Wang *et al.* 2008). Anhedonia and decreased motivation have been used as methods to evaluate depressive-like behaviors in animals. This is consistent with symptoms in post-stroke patients with depression. Anhedonia, which is a decrease in sucrose consumption, serves as a marker of reduced sensitivity (Willner *et al.* 1987; Willner 1997). Mild chronic stressors can mimic situations that reflect various states in post-stroke patients. A combination of biological and psychosocial factors can be considered the cause of depressive-like symptoms in post-stroke patients (Spalletta *et al.* 2006).

Pro-inflammatory cytokines are important mediators between the neuroendocrine and immune system in the CNS. Inflammatory challenge of ischemic stroke in the brain is one of the causes of immune stimulation. It has been shown that elevations in cytokine levels are involved in the etiology of PSD (Su *et al.* 2012). High levels of TNF-α and IL-1β mRNAs have been found in the hypothalamus of ischemic stroke rats, together with increased CRF mRNA expression levels. In particular, there was a strong association between IL-1β and CRF in this study. This would suggest enhancement of CRF production is influenced by TNF-α and IL-1β (Kakucska *et al.* 1993; Bernardini *et al.* 1990). These findings implicate that the immune system has been activated in the hypothalamus of PSD rats. Hyperactivity of CRF

neurons in the hypothalamus induces depressive-like symptoms (Bao *et al.* 2008; Wang *et al.* 2010). The CRF hypothesis of depression is supported in our study, *via* the increase in CRF mRNA levels in the hypothalamus of PSD rats. Cytokines could contribute to regulation of CRF gene expression in the hypothalamus (Kageyama *et al.* 2010), which in turn drives the HPA axis (Turnbull & Rivier 1999). This would indicate that there is a common etiology for PSD and depression. Therefore, cytokines could be stimulators of the HPA axis and play important roles in PSD pathophysiology.

Citalopram treatment improved depressive-like behaviors in MCAO+CUMS rats. It also has been shown that citalopram reverses depressive-like behaviors in the CUMS rat model (Papp *et al.* 2002). Multiple mechanisms possibly contributed to the beneficial effects of SSRI treatment for depression. These might include attenuation of the HPA axis, and neuroprotective effects (Kronenberg *et al.* 2012; Salazar-Colocho *et al.* 2008). The antidepressant citalopram has been investigated in terms of its immunomodulatory properties *in vivo* and *in vitro* (Tynan *et al.* 2012; Warner-Schmidt *et al.* 2011; Ying *et al.* 2002). We investigated citalopram treatment in rats with ischemic stroke and depressive-like behaviors; these rats showed improvement in sucrose preference and locomotor activity, but decreases in mRNA expression levels for TNF-α and CRF. However, we are unable to draw any conclusion that citalopram directly regulates CRF (Bah *et al.* 2011; Jensen *et al.* 1999; Tynan *et al.* 2012). The mechanism and interaction between pro-inflammatory cytokines and CRF should be determined by further studies, and the HPA axis should be further examined in PSD models.

There were some limitations to our study; CUMS rats were not treated with citalopram as a therapy control in our study. Citalopram can regulate HPA axis activity, however it appears to have a direct effect on other neural peptides in stressed rats according to previous studies (Jensen *et al.* 1999). We investigated the significant changes in transcript levels of cytokines and CRF in the hypothalamus of PSD rats. PSD is a complex disorder and CUMS procedures combined with ischemic stroke methods do not fully represent the situation in human PSD cases. Further molecular interaction studies between pro-inflammatory cytokines and the CRF gene should be conducted. It is likely that high expression levels of TNF-α, IL-1β, and CRF are essential for the etiology of PSD. We also believe that citalopram has partial therapeutic effects for PSD, but the relationship between citalopram and cytokine expression remains to be determined.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (Number: 81101011).

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