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Comparative Study of c-Fos Expression in Rat Dorsal Vagal Complex and Nucleus Ambiguus Induced by Different Durations of Restraint Water-Immersion Stress

Yu-Yu Zhang*, Guo-Hong Cao*, Wen-Xing Zhu, Xi-Yun Cui, and Hong-Bin Ai

Key Laboratory of Animal Resistance of Shandong Province
College of Life Sciences, Shandong Normal University
Jinan 250014, Shandong, The People's Republic of China

Abstract

Restraint water-immersion stress (RWIS) of rats induces vagally-mediated gastric dysfunction. The present work explored the effects of different durations of RWIS on neuronal activities of the dorsal vagal complex (DVC) and the nucleus ambiguous (NA) in rats. Male Wistar rats were exposed to RWIS for 0, 30, 60, 120, or 180 min. Then, a c-Fos immunoperoxidase technique was utilized to assess neuronal activation. Resumptively, c-Fos expression in DVC and NA peaked at 60 min of stress, subsequently decreased gradually with increasing durations of RWIS. Interestingly, the most intense c-Fos expression was observed in the dorsal motor nucleus of the vagus (DMV) during the stress, followed by NA, nucleus of solitary tract (NTS) and area postrema (AP). The peak of c-Fos expression in caudal DMV appeared at 120 min of the stress, slower than that in rostral and intermediate DMV. The c-Fos expression in intermediate and caudal NTS was significantly more intense than that in rostral NTS. These results indicate that the neuronal hyperactivity of DMV, NA, NTS and AP, the primary center that control gastric functions, especially DMV and NA, may play an important role in the disorders of gastric motility and secretion induced by RWIS.

Key Words: restraint water-immersion stress, dorsal vagal complex, nucleus ambiguus, c-Fos expression, gastric dysfunction

Introduction

Restraint water-immersion stress (RWIS) of rats, considered to be a complex and psychological stressor, induces vagally-mediated gastric hypercontractility, hypersecretion of gastric acid and acute gastric erosions within a few hours (1, 2, 9-11, 18). However, pretreatment with bilateral subdiaphragmatic vagotomy or atropine inhibited gastric hypercontractility and hypersecretion of gastric acid and prominently alleviated gastric erosions under RWIS in rats (1, 18), while pretreatment with hypophysectomy, adrenalectomy or phenoxybenzamine failed to affect gastric hypercontractility, hypersecretion of gastric acid and gastric erosions under RWIS (1, 8). These studies indicated that the abnormalities of gastric functions induced by RWIS were not due to the hyperactivity of the hypothalamo-pituitary-adrenal (HPA) axis, but due to the hyperactivity of vagal parasympathetic efferents (5, 23).

The vagal parasympathetic neurons which innervate the stomach are largely located in the dorsal motor nucleus of the vagus (DMV) and partly in nucleus ambiguus (NA) (14, 16, 22). Moreover, the nucleus of the solitary tract (NTS) receives a part of the afferent information from the stomach. The area

Corresponding author: Prof. Hong-Bin Ai, College of Life Sciences, Shandong Normal University, Jinan 250014, Shandong, The People's Republic of China. E-mail: physiology@sdnu.edu.cn
*Y.-Y. Zhang and G.-H. Cao contributed equally to this work.
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postrema (AP) is the chemoreceptor trigger zone of the vomitive reflex and also receives inputs from the vagal sensory fibres of the stomach. The adjacent DMV, NTS and AP have complicated neuronal contact and close correlation in function, so that they constitute the dorsal vagal complex (DVC) (4). Thus, DVC and NA are the primary nerve centres that regulate gastric functions. Nevertheless, whether the neurons of DMV, NTS, AP and NA are excited, and characterization of the temporal-spatial pattern of neuronal activities in these four nuclei under RWIS, have not been reported to date.

The mapping of changes in c-Fos expression has become an established method used to visualize neuronal activation induced by diverse stimuli including stress and neurotransmitters (21). Bonaz and Wang and their colleagues found cold (4°C) restraint stress for 3 h induced intense c-Fos expression in DMV, raphe pallidus (RPa), locus coeruleus (LC) and hypothalamic paraventricular nucleus (PVN) in rats (3, 30). However, the patterns of c-Fos expression in DMV, NTS, AP and NA of the rat during RWIS are still unknown. In the present study, we investigated the effects of different RWIS durations on the neuronal activities in the four nuclei using c-Fos expression as a marker of the temporal-spatial activation pattern of the neurons.

Materials and Methods

Subjects

The subjects consisted of male Wistar rats, weighing 170-200 g. All rats were purchased from the Experimental Animal Center of Shandong University, Jinan, Shandong, People's Republic of China. They were individually housed in cages at an ambient temperature of 22 ± 2°C with a normal day/night cycle for at least 7 days before the experiments. The animals had *ad libitum* access to pelleted food and tap water. Before stress, the rats were fasted for 24 h, but allowed free access to water. All stresses were finished between 0800 and 1200 to minimize circadian rhythm-related variations in the stress response. All procedures were performed in accordance with the Chinese Psychological Association's ethical standards for the use of animals in research[a].

Grouping and Stress Protocols

Twenty-six rats were randomly divided into 5 groups designated according to the duration of RWIS, 0, 30, 60, 120 and 180, with five rats in each of the first four groups and six rats in group 180. Under light ether anesthesia, the four limbs of each rat in Groups 30, 60, 120 and 180 were bound on a wooden board gently but securely with medical adhesive tape. After the rats were conscious, they were vertically immersed in water (21 ± 1°C) to the level of the xiphoid for 30, 60, 120 or 180 min, respectively. Group 0 rats, as a control group, were also fasted and anesthetized, but not stressed. At the end of the stress, the rats were deeply anesthetized by overdose of pentobarbital sodium (100 mg/kg body weight, intraperitoneally).

Additionally, another group (Group 30-30), in which 5 rats were given RWIS for 30 min and then replaced in their home cages for 30 min before sacrifice, was established for comparison to Group 60, to determine the intrinsic kinetics of c-Fos expression.

c-Fos Immunohistochemistry. Rats were perfused transcardially with 0.01 M phosphate-buffered saline (PBS, pH 7.4) followed by 500 ml freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (4°C). Afterwards, the brainstems were removed immediately, post-fixed in the same fixative for 4 h and cryoprotected overnight in 20% sucrose in 0.1 M PB at 4°C until sectioning. Series of 25 μm coronal sections of the brainstems at the level of the DVC were then cut with a freezing microtome and collected into 0.01 M PBS. The free-floating sections (a 1-in-4 series of the brainstem sections taken from each animal) were pretreated for 30 min in methanolic 3% H_2O_2 to eliminate endogenous peroxidase activity. After being rinsed in 0.01 M PBS, they were incubated with a blocking buffer (5% normal goat serum and 0.3% Triton X-100 in PBS) for 30 min, then with rabbit anti-c-Fos antibody (sc-52, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) at a dilution of 1:2000 for 24 h at 4°C. Subsequently, the sections were incubated with the biotinylated goat anti-rabbit IgG (Zymed Laboratories Inc, San Fransisco, CA, USA) for 1 hour at room temperature and then with streptavidin-biotin-horseradish peroxidase complex (Zymed) for 1 h at room temperature. The sections were submitted to a diaminobenzidine reaction, yielding a brown nuclear deposit. Between steps, the sections were rinsed completely in PBS containing 1% Triton X-100 (PBST). Sections were mounted on gelatin-coated glass slides, restained with hematoxylin, dehydrated in a series of alcohols, cleared in xylene, and coverslipped. The specificity of the immunostaining was verified by incubating of the brain sections with normal goat serum or PBS, which produced no staining.

[a]Chinese Psychological Association. Ethical standards for psychological professionals. Taipei: Chinese Psychological Association, 1996

Table 1. The number of Fos-IR neurons in the DVC and the NA of rats in five groups (number/0.01 mm^2) and the multiples of controls

	Group 0 (n = 5)	Group 30 (n = 5)	Group 60 (n = 5)	Group 120 (n = 5)	Group 180 (n = 6)	F	*P*
DMV	0.59 ± 0.05^{a}	2.14 ± 0.44^{bc}	3.11 ± 0.51^{c}	1.55 ± 0.30^{ab}	1.12 ± 0.19^{ab}	8.415	0.000
	1.00 ± 0.09	3.64 ± 0.75	5.28 ± 0.86	2.63 ± 0.51	1.91 ± 0.32		
NTS	1.04 ± 0.09^{a}	1.69 ± 0.23^{ab}	2.76 ± 0.37^{c}	1.72 ± 0.19^{ab}	2.18 ± 0.18^{bc}	7.577	0.001
	1.00 ± 0.09	1.64 ± 0.22	2.67 ± 0.36	1.66 ± 0.19	2.11 ± 0.17		
AP	2.32 ± 0.54^{a}	3.23 ± 0.73^{ab}	4.57 ± 0.80^{b}	2.20 ± 0.31^{a}	4.92 ± 0.51^{b}	4.546	0.008
	1.00 ± 0.23	1.39 ± 0.31	1.97 ± 0.34	0.95 ± 0.13	2.12 ± 0.22		
NA	0.38 ± 0.11^{a}	0.89 ± 0.12^{b}	1.35 ± 0.18^{c}	0.90 ± 0.11^{b}	0.71 ± 0.09^{ab}	7.828	0.001
	1.01 ± 0.29	2.33 ± 0.31	3.53 ± 0.47	2.36 ± 0.29	1.85 ± 0.23		

For each nucleus, the data in the top row represent the number of Fos-IR neurons, the data in the bottom row represent the multiples of control. Means in a row without a common letter represent significant difference at $P < 0.05$.

Counting Fos-immunoreactive (Fos-IR) Neurons

Pictures of the brainstem sections were taken under identical conditions with a BX51 Olympus microscope (Olympus Corporation, Tokyo, Japan) coupled to an Olympus DP70 camera. The nomenclature and nuclear boundaries defined in the rat brain stereotaxic atlas of Paxinos and Watson (24) were used in this study. Fos-IR nuclear profiles in the DVC and the NA were counted using Image-Pro Plus 6.0 (Media Cybernetics Inc, Siler Spring, MD, USA). The number and integrated optical density (IOD) of Fos-IR neurons within each nucleus were counted bilaterally (where possible) in all immunostained sections per animal and the average values of them in 0.01 mm^2 are reported as number and IOD of Fos-IR neurons which denote the intensity of c-Fos expression.

The DMV and the NTS are generally divided into three different zones (7, 17), a rostral zone, an intermediate zone and a caudal zone, so we also counted the number of Fos-IR neurons in each zone of DMV or NTS to compare neuronal activities in different zones during RWIS.

Evaluation of Gastric Mucosal Damage

After the rats were killed, the stomachs were removed and filled with 10 ml of 1% Formalin. Thirty minutes later, each stomach was opened along the greater curvature, rinsed lightly with physiological saline and spread. The gastric mucosal damage was examined with a hand magnifying lens and the erosion index (EI) was evaluated by the score system reported by Guth *et al*. (12). Scores were made according to the length of the lesion: the length ≤ 1 mm as 1 score, 1 mm < the length ≤ 2 mm as 2 score, and the others were deduced in turn. The score was multiplied by 2 when the width of the lesion was larger than 1 mm. The cumulative scores of all lesions in a rat served as the erosion index of the rat.

Statistical Analysis

All data are presented as means ± SEM. The statistical procedures were performed with SPSS13.0 software (SPSS Inc. Chicago, IL, USA). Time course data (Groups 0, 30, 60, 120, 180) were analyzed with one-way or two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test individually. Group 60 and Group 30-30 were compared by two-tailed independent sample *t*-tests. The level of significance was set at $P < 0.05$.

Additionally, in order to compare the reactive extent of the above four nuclei to the stress, the multiples of controls were calculated, that is, the quantum of c-Fos expression of each rat was divided by the mean values of the control group.

Results

Effects of Different RWIS Durations on c-Fos Expression in DVC and NA

Neuronal activation was assessed on the basis of Fos-immunoreactivity, seen as a brown deposit in the cell nucleus. The number of Fos-IR neurons in the DVC and NA of rats and the multiples of controls are shown in Table 1. One-way ANOVAs indicated a significant effect of RWIS duration on c-Fos expression for each observed nucleus ($P < 0.05$). Post hoc tests showed that c-Fos expression in DMV, NTS, AP and NA all peaked at 60 min of the stress, subsequently decreased gradually following the

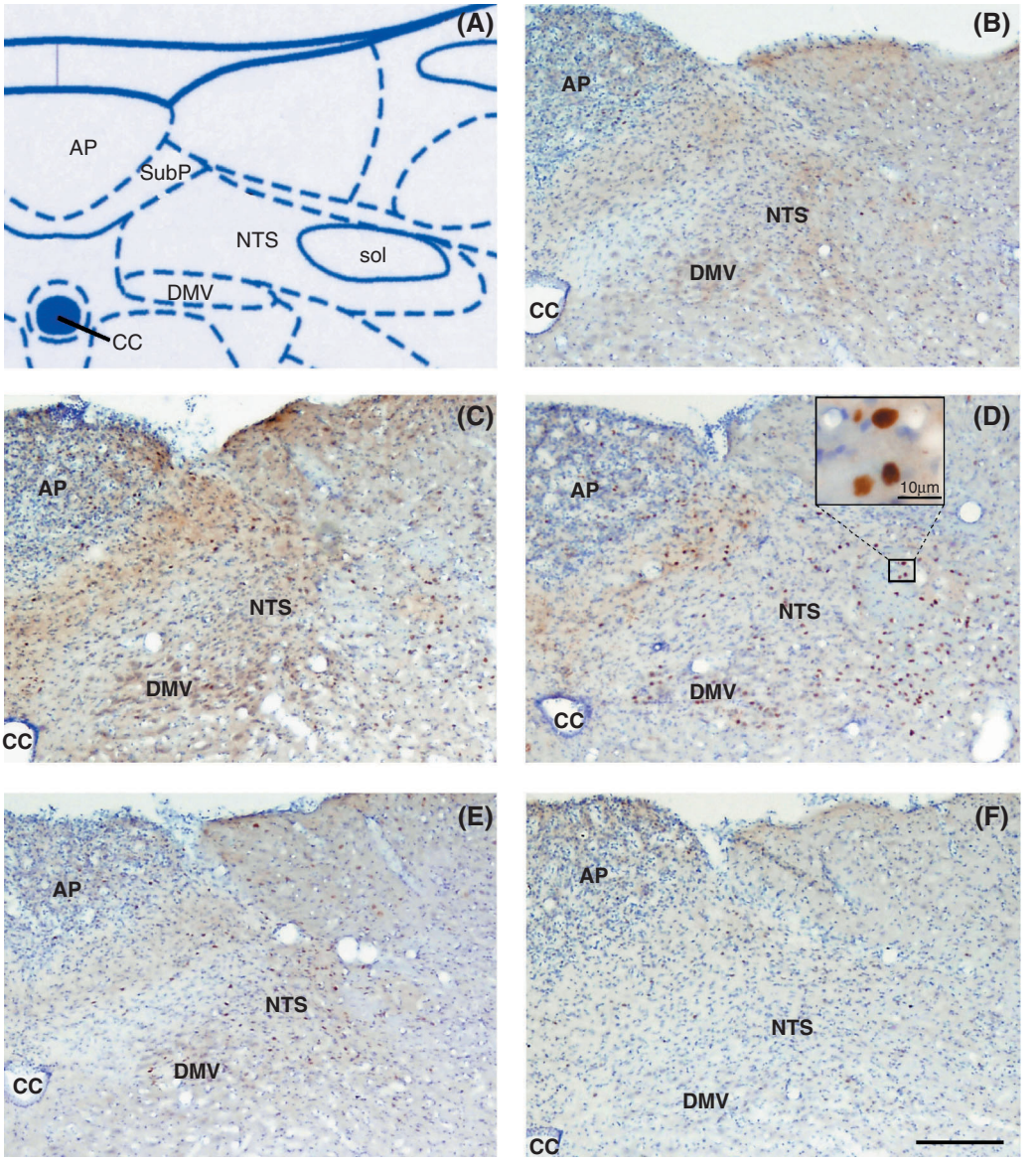

Fig. 1. Representative sections of Fos immunoreactive distribution in the dorsal vagal complex induced by RWIS for 0 min (B), 30 min (C), 60 min (D), 120 min (E) and 180 min (F). Panel (A) showed the exact positions of the dorsal motor nucleus of the vagus (DMV), nucleus of solitary tract (NTS) and area postrema (AP) in the rat brain atlas. In panel (D), Fos-IR neurons in a rectangle were magnified in higher magnification. The c-Fos expression in DVC peaked at 60 min of the stress, subsequently decreased gradually following the prolonging of RWIS duration. Scale bars = 200 μm. CC: central canal, sol: solitary tract, SubP: subpostrema area.

prolonging of RWIS (except AP) (Table 1, Fig. 1). According to the values of F and the multiples of controls, it was very obvious that c-Fos expression in DMV induced by RWIS was the most intense ($F_{4, 21}$ = 8.415), next in NA ($F_{4, 21}$ = 7.828), NTS ($F_{4, 21}$ = 7.577), and the weakest is in AP ($F_{4, 21}$ = 4.546) (Table 1).

The pattern presented by the IOD of Fos-IR neurons in DVC and NA induced by RWIS was identical with that presented by the number of Fos-IR neurons (Fig. 2).

In addition, rats stressed for 30 min and given a 30 min recovery period (Group 30-30) had significantly less c-Fos expression in DVC and NA than rats stressed continuously for 60 min (Group 60) (Table 2).

Differences of c-Fos Expression in Different Zones of DMV

The number of Fos-IR neurons in the rostral, intermediate and caudal zones of DMV induced by

Table 2. The number and the IOD of Fos-IR neurons in the DVC and the NA of rats given shorter or longer stress durations but equal total time from stress onset to death (number or IOD/0.01 mm^2) (n = 5)

	Group 30-30 Number	Group 60 Number	Group 30-30 IOD	Group 60 IOD
DMV	0.60 ± 0.07	3.11 ± 0.51*	1.74 ± 0.25	18.32 ± 3.95*
NTS	1.19 ± 0.10	2.76 ± 0.37*	4.05 ± 0.46	18.36 ± 3.91*
AP	1.77 ± 0.25	4.57 ± 0.80*	6.17 ± 0.96	28.92 ± 6.94*
NA	0.73 ± 0.14	1.35 ± 0.18*	2.00 ± 0.40	10.25 ± 2.41*

*$P < 0.05$ compared to Group 30-30 for each nucleus.

Table 3. The number of Fos-IR neurons in the rostral, intermediate and caudal zones of DMV induced by RWIS (number/0.01 mm^2)

	Group 0 (n = 5)	Group 30 (n = 5)	Group 60 (n = 5)	Group 120 (n = 5)	Group 180 (n = 6)
The rostral DMV	0.63 ± 0.06	1.40 ± 0.25	2.59 ± 0.40	1.11 ± 0.19[a]	1.02 ± 0.13
The intermediate DMV	0.63 ± 0.07	2.67 ± 0.60	3.47 ± 0.62	1.18 ± 0.20[a]	1.09 ± 0.26
The caudal DMV	0.56 ± 0.15	1.65 ± 0.38	2.93 ± 0.47	4.17 ± 0.78[b]	1.36 ± 0.22

In group 120, means without a common letter represent a significant difference at $P < 0.05$.

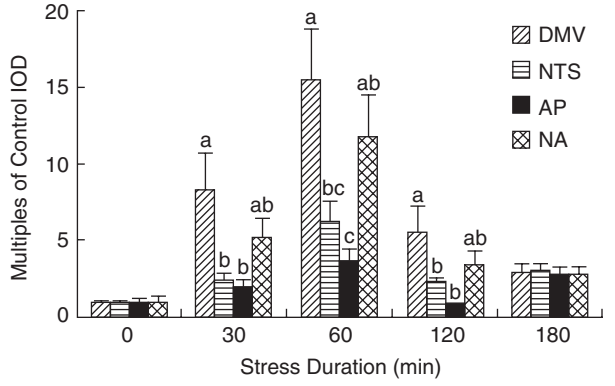

Fig. 2. The relative intensity of c-Fos expression in the DVC and the NA of rats in the five five groups (multiples of the corresponding control IOD). The IOD of Fos-IR neurons in the DMV, NTS, AP and NA all peaked at 60 min of the stress. At 30 min to 120 min of the stress, c-Fos expression in DMV was the most intense, next in NA, NTS, the weakest is in AP. Statistical analy were done by ANOVA and Tukey's *post hoc*, and the data are presented as means ± SEM. For each stress duration, means without a common letter represent significant difference at $P < 0.05$.

RWIS is shown in Table 3. RWIS induced a time-dependent Fos expression in different zones of DMV with peak expression apparently slower in caudal DMV (120 min) than in rostral and intermediate DMV (60 min). In addition, in Group 120, Fos-IR neurons in caudal DMV were significantly more than those in rostral and intermediate DMV ($F_{2, 12}$ = 13.491, P = 0.001), while in each of the other groups, no significant difference of c-Fos expression was observed among the three zones of DMV.

Differences of c-Fos Expression in Different Zones of NTS

The number of Fos-IR neurons in the rostral, intermediate and caudal zones of the NTS induced by RWIS is shown in Table 4. Different from DMV, the c-Fos expression in the three zones of NTS all peaked at 60 min of the stress. Interestingly, the c-Fos expression in intermediate and caudal NTS was significantly more intense than that in rostral NTS during RWIS ($F_{2, 18}$ = 27.726, P = 0.000).

Changes of Gastric Erosions

There was no macroscopic gastric mucosal lesion in the control group. Scattered spot or lineal hemorrhage and lesions were observed in the corpus mucosa along the folds in the stress groups. One-way ANOVA showed a significant effect of time of RWIS on the erosion indices ($F_{4, 21}$ = 24.691, P = 0.000). Erosion indices tended to increase with increasing durations of RWIS (Fig. 3).

Discussion

In the present study, RWIS induced remarkable expression of c-Fos in DVC (including DMV, NTS and AP) and NA, which suggested that many neurons in those regions were excited during RWIS. DVC and NA are the primary nerve centres that regulate

Table 4. The number of Fos-IR neurons in the rostral, intermediate and caudal zones of NTS induced by RWIS (number/0.01 mm^2)

	Group 0 (n = 5)	Group 30 (n = 5)	Group 60 (n = 5)	Group 120 (n = 5)	Group 180 (n = 6)
The rostral DMV	0.87 ± 0.16	0.95 ± 0.25^{b}	1.83 ± 0.31^{a}	0.71 ± 0.09^{a}	1.41 ± 0.20^{a}
The intermediate NTS	1.06 ± 0.07	1.90 ± 0.27^{b}	2.94 ± 0.39^{ab}	1.88 ± 0.18^{b}	2.41 ± 0.21^{b}
The caudal NTS	1.18 ± 0.10	2.30 ± 0.24^{b}	4.09 ± 0.58^{b}	2.24 ± 0.32^{b}	2.25 ± 0.31^{b}

In each of the stress groups, means without a common letter represent a significant difference at $P < 0.05$.

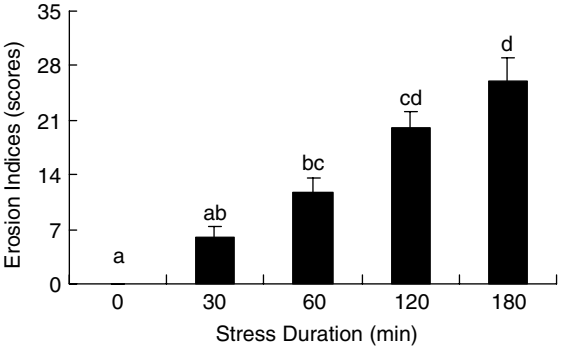

Fig. 3. Gastric erosions induced by RWIS. Data are presented as means ± SEM (n = 5 or 6). Erosion indices (EI) were increased time-dependently during RWIS. Tukey's post hoc test, columns without a common letter represent a significant difference at $P < 0.05$.

gastrointestinal functions and the final efferent pathway of the central parasympathetic nervous system which modulates visceral activity. Then it is possible that those excited neurons in DVC and NA are involved in gastric dysfunction induced by RWIS.

The c-Fos expression in DVC and NA peaked at 60 min of the stress, subsequently decreased gradually with increasing durations of RWIS. This result is in agreement with previous reports that the c-Fos expression in medullary visceral zone induced by only restraint stress peaked in rats restrained for 1 h, then began to decrease, at 6 h of only restraint, significantly decreased (29). Additionally, rats stressed continuously for 60 min had greater c-Fos expression in DVC and NA than rats stressed for 30 min followed by 30 min of recovery, which must be able to somewhat reflect more neuronal activation in these regions of rats given 60 min of RWIS than those of rats given 30 min of RWIS. Crane *et al.* (6) observed the pattern of c-Fos expression in the brains of the rats which were restrained for 30 or 60 min and killed 2 h after the onset of restraint, found a greater number of Fos-positive corticotrophin-releasing factor (CRF) cells in the PVN and medial amygdala (MeA) of rats restrained for 60 min compared with rats restrained for 30 min, but no significant difference in the numbers of Fos-positive A2 noradrenergic neurons between rats of the two groups. All these suggest the expression of c-Fos is sensitive to stressor duration, but this sensitivity varies with brain region (6, 19). The presence of Fos protein is widely accepted to be indicative of recent activation, therefore the duration of stressor is a critical factor that influences the pattern of activation in the central nervous system (6).

Consistent with other stimuli such as restraint (6), metrazol-induced seizures (20) and injection of hyperosmotic solution (28), time course of c-Fos expression induced by RWIS is also transient even in the continuation of stress. Therefore, the first one hour of RWIS may be more important in inducing the c-Fos expression in DVC and NA. Transient expression of c-Fos activate the transcriptions of AP-1-containing genes in the neurons of stressed animals to cause a long term alteration in cell function. There is some evidence for c-fos expression to be inhibited through an autoregulatory negative feedback effect (27). Thus, the temporal pattern of stress-induced c-Fos expression may be limited in the extent to which it reflects sustained neuronal activity (31).

Interestingly, the most intense c-Fos expression was observed in DMV, next in NA, NTS and AP. These results show that some of the neurons in DMV, NTS, AP and NA were excited and the intensity of neuronal activity in individual nuclei was different. Consistent with this inference was the finding of Bonaz and Tache (3) that after cold (4°C) restraint stress for 3 h, many Fos-IR nuclei were observed in DMV, whereas only a few Fos-IR nuclei were found scattered in NTS. Our previous study found RWIS could also induce activation of many neurons in restricted regions of the limbic system, especially the anterior part of the hypothalamus which was the center of the parasympathetic nervous system (unpublished observations). The activation of many neurons in the anterior part of the hypothalamus and DMV and NA provided a neuroanatomical basis for the information of vagally-mediated gastric erosions induced by RWIS. All the results indicate that RWIS mainly induces the hyperactivity of the parasympathetic nervous system which was contrary to the

activation of the sympathetic nervous system in other stresses (1, 5, 8, 18).

DMV is a visceral motor nucleus. The expression patterns of c-Fos in different zones of DMV suggested that neuronal activities in the different zones of DMV were not the same during RWIS. The peak of neuronal activities in caudal DMV appeared at 120 min of the stress, slower than that in rostral and intermediate DMV, and the neuronal activities in caudal DMV were significantly more intense than those in rostral and intermediate DMV. Previous studies found microinjection of L-glutamate into different areas of DMV rostral to *calamus scriptorius* (CS) or obex (that is, rostral and intermediate DMV) resulted in vagally mediated excitatory effects on gastric motility, while microinjection of L-glutamate into DMV caudal to CS or obex (that is, the caudal DMV) led to vagally mediated inhibition of gastric motility (7, 15, 32). These studies demonstrated that the vagal excitatory pathway originated in rostral and intermediate DMV and the vagal inhibitory pathway originated in caudal DMV. The difference in neuronal activities in different zones of DMV may be due to their different roles in modulating the gastric functions during RWIS. At 30 and 60 min of RWIS, a lot of DMV neurons were activated, which may result in a series of gastric functional disorders including gastric hypermotility. Then, those signals were fed back to DVC and the higher brain centre modulate visceral function (such as the hypothalamus). Our previous study showed that the neuronal activities on higher brain levels, especially the hypothalamus, tended to a peak at 60 min and 120 min of RWIS as indicated by the expression of c-Fos (unpublished observations). Therefore, late modulation by higher brain centers may dampen neuronal activity in rostral and intermediate DMV, and strengthen neuronal activity in caudal DMV, with the effect of limiting the gastric hypercontractility induced by the stress. However, the other causative factors (for example, the hypersecretion of gastric acid) induced by the stress may continue to aggravate gastric erosions.

NTS is the major recipient of visceral afferent information arising from various regions of the gastrointestinal tract. When a rat is exposed to RWIS, its body temperature decreases and gastric functions become abnormal. The sensory information is relayed in NTS. The NTS neurons provide direct inhibitory and excitatory inputs to preganglionic parasympathetic neurons in DMV that in turn control gastric functions *via* their efferent projections in the vagus nerve (25, 26). The c-Fos expression in intermediate and caudal NTS was significantly more intense than that in rostral NTS during RWIS, likely because the medial and commissural parts (mainly located in the intermediate and caudal NTS) receive the afferent information from gastrointestinal receptors. Some studies showed that the neuronal excitation of the medial and commissural parts of NTS produce vagally mediated inhibition of gastric motility (7), but rostral NTS plays an important role in relaying or regulating the gustatory information (13). AP is also a sensory nucleus and the c-Fos expression in AP during the stress may reflect response to incoming sensory information.

In conclusion, the remarkable expression of c-Fos in DVC (especially in DMV) and NA induced by RWIS provided a direct evidence for the hyperactivity of the parasympathetic nervous system produce gastric dysfunction during RWIS. The patterns of c-Fos expression in different zones of DMV and NTS may reflect their different roles in modulating gastric functions.

Acknowledgments

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