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ORIGINAL PAPER

The role of catecholaminergic neurons in the hypothalamus and medullary visceral zone in response to restraint water-immersion stress in rats

Dong-Qin Zhao · Chang-Liang Lu · Hong-Bin Ai

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Abstract The activity of catecholaminergic neurons in the hypothalamus and the medullary visceral zone (MVZ) in rats in response to restraint water-immersion stress (RWIS) was measured by use of dual Fos and tyrosine hydroxylase (TH) immunohistochemistry. In RWIS rats Fos immunoreactive (Fos-IR) nuclei dramatically increased in the paraventricular nucleus (PVN), the supraoptic nucleus (SON), the dorsal motor nucleus of the vagus (DMV), the nucleus of the solitary tract (NTS), the area postrema (AP), and the ventrolateral medulla (VLM). A small number of TH-immunoreactive (TH-IR) and Fos/TH double-labeling neurons in the PVN, and their absence from the SON, were observed in both RWIS and non-stressed rats. More TH-IR neurons were observed in the MVZ of RWIS rats than in nonstressed rats. In RWIS and nonstressed rats, the percentage of Fos-IR nuclei in TH-IR neurons was 38.0 and 14.3% in the DMV, 34.4 and 9.7% in the NTS, 18.6 and 4.5% in the AP, and 45.7 and 18.9% in the VLM, respectively. In conclusion, catecholaminergic neurons in the MVZ are involved in the response to RWIS; although the PVN and SON also participate in the response to RWIS, the mechanism is not via catecholaminergic neurons.

Keywords Fos · Hypothalamus · Medullary visceral zone · Restraint water-immersion stress · Tyrosine hydroxylase

D.-Q. Zhao · C.-L. Lu · H.-B. Ai (✉)
Key Laboratory of Animal Resistance of Shandong Province, College of Life Sciences, Shandong Normal University, Jinan 250014, Shandong, People's Republic of China
e-mail: aihongbin@sdnu.edu.cn

Introduction

Psychological stressors, for example water-avoidance stress, evoked strong Fos expression in the hypothalamus and the medullary visceral zone (MVZ), but no gastric lesions occurred under these experimental conditions [1]. Restraint water-immersion stress (RWIS), considered to be a complex of physical and psychological stressors, has been widely used to study the pathogenesis of stress-induced gastric lesions [2, 3]. RWIS induced remarkable Fos expression in the MVZ (including the dorsal motor nucleus of the vagus (DMV), the nucleus ambiguus (NA), the nucleus of the solitary tract (NTS), and the area postrema (AP)), and the hypothalamus (including the paraventricular hypothalamic nucleus (PVN) and the supraoptic nucleus (SON)) [4, 5]. Previous studies indicated that the abnormalities of gastric function induced by RWIS are not because of the hyperactivity of the hypothalamo–pituitary–adrenal (HPA) axis but because of the hyperactivity of vagal parasympathetic efferents, which largely originate in the DMV and partly in the NA [6–11]. It seems that hyperactivity of the DMV, NTS, and AP, which constitute the dorsal vagal complex (DVC), regarded as the primary nerve center regulating gastric function [12], leads to gastric lesions. Electrical and chemical stimulation of the DMV and NA inhibited gastric motility, however [13, 14]. The PVN and the NTS, DMV, and SON, and the NTS and DMV are reciprocally connected in rats [15–17]. So, does the hyperactivity of the higher center, mainly the PVN and SON, relieve the inhibition of gastric motility mediated by the primary nerve center? If so, what are the neurotransmitters during RWIS?

Catecholamine is an endogenous neurotransmitter and might be involved in processing and conducting visceral nociceptive information [18]. Tyrosine hydroxylase (TH) is

the rate-limiting enzyme in the biosynthesis of catecholamines (dopamine, noradrenaline, and adrenaline) and has been used as a marker of catecholaminergic (CA) neurons in the mammalian brain. Osmotic stimulus (i.p. hypertonic saline) or immobilization [19] and proximal colon distension [20] evoked Fos protein expression in CA neurons in specific brain nuclei. The PVN contains a dense network of CA terminals originating in the medullary visceral zone CA-cell groups, for example the NTS, the ventrolateral medulla (VLM), and locus coeruleus (LC) [15, 16, 21, 22]. The functional role of CA phenotypes of the hypothalamus and MVZ neurons, associated with the brain pathways activated during RWIS, has, however, not yet been explored. In this study, to evaluate the role of CA-neurons in the PVN, SON, DVC, and VLM during RWIS, the CA nature of activated neurons was determined by a double immunohistochemical method for colocations of TH and Fos, which is known as a marker of neural activation [23].

Materials and methods

Preparation of animals

Male Wistar rats weighing 180–220 g, were obtained from the Experimental Animal Center of Shandong University (Jinan, China) and housed two per cage in a room controlled for temperature (22 ± 2°C) under 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.

Stress protocols

Ten rats were randomly divided into two groups in accordance with the duration of RWIS. RWIS of rats was performed as previously described [3, 4]. Briefly, under light ether anesthesia, the four limbs of each rat in the stressed group were fixed gently but securely to a wooden board by use of medical adhesive tape. When the rats were conscious, they were immersed vertically in water (21 ± 1°C) to the level of the xiphoid for 1 h. Non-stressed rats, as a control group, were not stressed but were otherwise under identical conditions. To avoid the effect of diurnal variations on Fos expression, the experiment was performed between 9:00 and 11:00 a.m. All procedures were performed in accordance with the Japanese Physiological Society's Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences.

Tissue processing

At the end of the procedure, the rats were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg body weight) and perfused via the ascending aorta with 200 ml 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) followed by 500 ml 4% paraformaldehyde, 0.1% glutaraldehyde, and 14% saturated picric acid in 0.1 mol/L phosphate buffer (PB, pH 7.4). After perfusion, the brain was removed and post-fixed at 4°C for 4 h in the same fixative, and then infiltrated with 20% sucrose in 0.1 mol/L PB for 48 h at 4°C. Series of frozen coronal sections of the hypothalamus and MVZ were cut at 30 μm in a cryostat and collected in 0.01 mol/L PBS.

Immunohistochemistry

The immunoreaction of Fos plus TH was detected by a dual immunohistochemical technique. Briefly:

1. Free-floating sections were rinsed in 0.01 mol/L PBS followed by a preincubation in methanolic 3% H_2O_2 for 30 min at room temperature to eliminate endogenous peroxidase activity.
2. After rinsing in 0.01 mol/L PBS, the sections were incubated with blocking buffer containing 5% normal goat serum and 0.3% Triton X-100 in 0.01 mol/L PBS for 30 min, and then were incubated with rabbit anti-c-Fos antibody (sc-52; Santa Cruz Biotechnology, USA), diluted 1:2000 in 0.01 mol/L PBS containing 3% normal goat serum and 0.3% Triton X-100 for 24 h at 4°C.
3. At the end of this incubation period, the sections were rinsed in 0.1% Triton X-100 in 0.01 mol/L PBS (PBST) and then incubated with biotinylated goat anti-rabbit IgG (Zymed Laboratories, USA) for 1 h at room temperature.
4. Thereafter, the sections were rinsed in PBST followed by incubation with streptavidin–biotin–horseradish peroxidase complex (Zymed Laboratories) for 1 h at room temperature.
5. After several rinses in PBST, the sections were submitted to a diaminobenzidine hydrochloride (DAB; Sigma Chemical, St Louis, MO, USA), intensified with 0.05% cobalt chloride and 0.05% nickel ammonium sulfate for 4–5 min. This method produces a blue-black nuclear reaction product.
6. The Fos-immunoreactive (Fos-IR) sections were rinsed and then incubated for 24 h at 4°C with mouse anti-TH (Boehringer Mannheim, Germany) diluted 1:2000 in the same antibody diluent as the Fos antibody.

7. The sections were rinsed and then incubated with biotinylated goat anti-mouse IgG (Zymed Laboratories) for 1 h at room temperature.
8. Subsequent staining was performed using the above procedure except for visualization. Cytoplasmic TH-immunoreactive (TH-IR) neurons were detected with unintensified DAB that produces a brown reaction product.
9. Finally, the free-floating sections were mounted on gelatin-coated glass slides, air-dried, dehydrated in a series of alcohols, cleared in xylene, and placed under a coverslip with DPX (Sigma Chemical Co., St Louis, MO, USA).

Evaluation of immunostaining

Fos-immunoreactivity was identified as dark blue–black dots deposited in the nuclei, TH-IR neurons appearing in the cytoplasm as a brown color, and Fos and TH double-labeled neurons (Fos/TH-IR) as brown perikarya with a dark blue–black nucleus. Pictures of brain sections were taken under identical conditions with a Olympus (Japan) BX51 microscope 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. For quantitative assessment, the number of immunoreactive neurons, determined by use of Image-Pro Plus 6.0 (Media Cybernetics, USA), was counted at three levels for NTS and DMV: rostral (bregma −12.96 to −13.32 mm), intermediate (bregma −13.80 to −14.04 mm), and caudal (bregma −14.52 to −14.76 mm) [25]. The immunoreactive neurons in other nuclei were counted at one level, AP (bregma −13.80 to −14.04 mm), VLM (bregma −12.96 to −14.76 mm), PVN (bregma −1.80 to −1.92 mm), and SON (bregma −0.92 to −1.44 mm). In each nucleus, three kinds of neurons were counted—Fos-IR nuclei, TH-IR neurons, and Fos/TH-IR neurons. The number of immunoreactive neurons was counted in three near sections per animal and average numbers in 0.01 mm^2 are reported as the immunoreactivity.

Statistical analysis

Counting was performed on five rats under each condition and the results obtained from each animal were used to calculate group the mean ± SEM. Statistical procedures were performed with SPSS13.0 software (SPSS, Chicago, IL, USA). Statistical analysis of results from different parts of the NTS and DMV was performed by two-way analysis of variance (ANOVA) followed by S–N–K's post-hoc test, individually. Statistical analysis of results from the PVN, SON, AP, and VLM was performed by Student's *t* test. The statistical significance level was set at $P < 0.05$.

Results

Effect of RWIS on Fos, TH, and Fos/TH in the hypothalamus

PVN

Restraint water-immersion stress increased Fos-IR nuclei to 13.4 ± 1.1 (cells per 0.01 mm^2) in the PVN compared with 4.2 ± 0.7 in nonstressed rats, i.e. 2.2-fold ($P < 0.01$) (Fig. 1). The maximum number of Fos-IR nuclei occurred in the medial parvocellular part (PaMP) and the lateral magnocellular parts (PaLM) (Fig. 2a, b). Fos-IR nuclei in the PaMP were heterogeneous in size and had irregular profiles whereas those in the PaLM were large, round, and more homogeneous in size. Extensive Fos-labeling of diffuse neurons was also observed in the dorsal cap (PaDC), and in the ventral (PaV) and posterior (PaPo) subdivisions of the PVN. TH-IR neurons in the PVN were diffuse and had fusiform or round profiles with large numbers of TH-positive processes. TH-IR neurons within the PVN were mostly located in the periventricular part of the PVN (Pe) and a few were scattered in the PaV (Fig. 2a, b). In the PaMP, almost no TH-IR neurons were observed, although CA-nerve endings were relatively abundant. Overall, there were no differences between RWIS and nonstressed rats in TH-IR and Fos/TH neurons. Fos-IR nuclei in the PVN after RWIS were identified in 13.0 ± 5.5% TH-IR neurons with no difference compared with nonstressed rats (17.5 ± 1.9%) ($P > 0.05$) (Fig. 1).

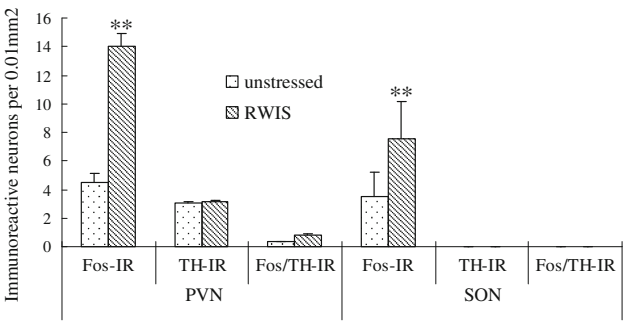

Fig. 1 Cell counts (cells per 0.01 mm^2) of Fos-IR and TH-IR neurons in the PVN and SON in rats either nonstressed or exposed to restraint water-immersion stress for 1 h. $n = 5$ rats per group. Values are mean ± SEM. $^{**}P < 0.01$ compared with the control group

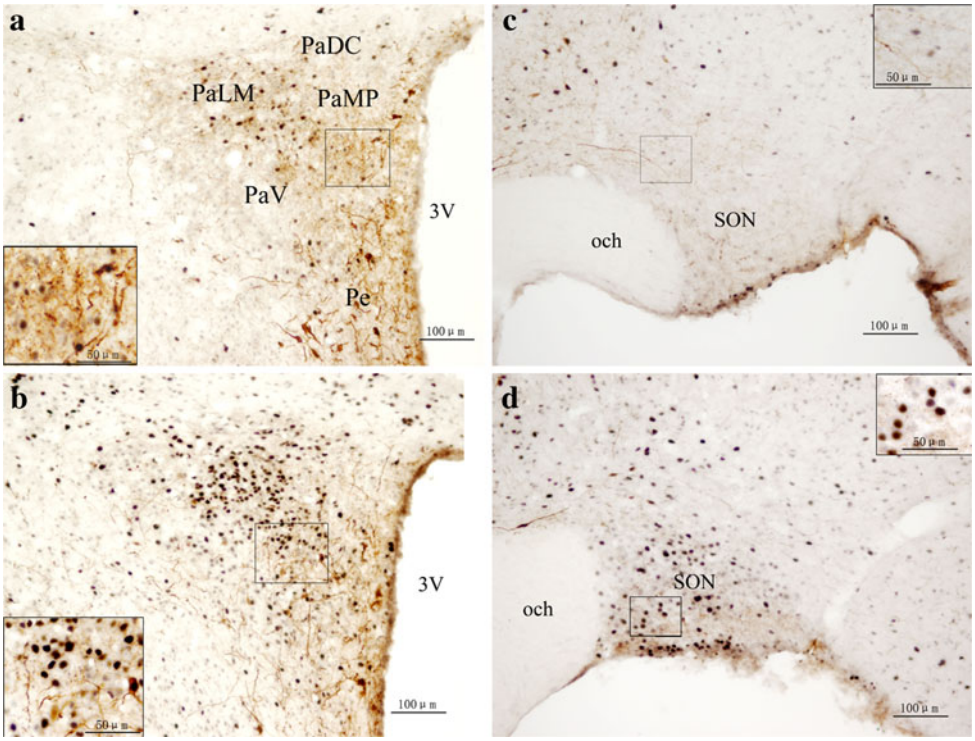

Fig. 2 Double immunohistochemical staining of Fos-IR and TH-IR neurons in the PVN (**a**, **b**) and SON (**c**, **d**) of the hypothalamus in unstressed rats (**a**, **c**) and rats exposed to restraint water-immersion stress for 1 h (**b**, **d**). The *insets* show higher magnification (×400) of cells in the *small boxes*. Fos-IR is localized in nuclei, and TH-IR is found in the cytoplasm. *PNV*, paraventricular hypothalamic nucleus; *PaLM*, lateral magnocellular part of the PVN; *PaV*, ventral part of the PVN; *PaMP*, medial parvocellular part of the PVN; *PaDC*, dorsal cap of the PVN; *Pe*, periventricular part of the PVN; *3V*, 3rd ventricle

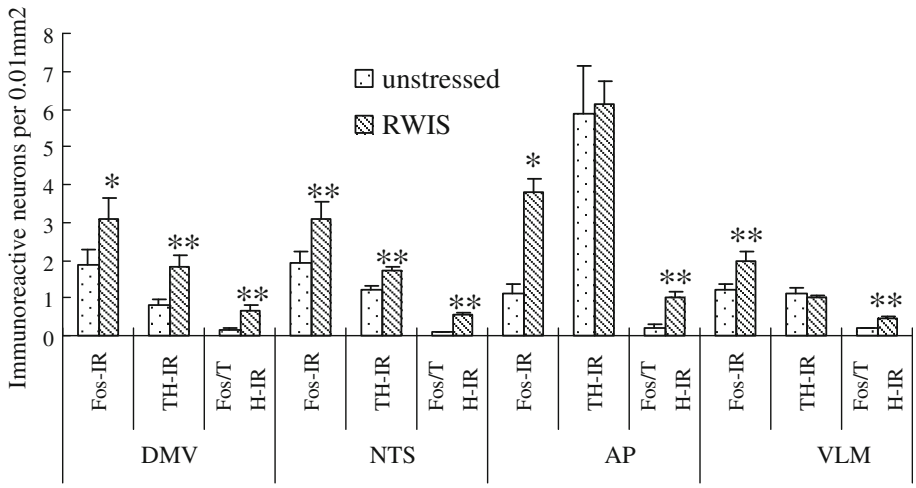

Fig. 3 Cell counts (cells per 0.01 mm^2) with double immunohistochemical staining of Fos-IR and TH-IR neurons in the DMV, NTS, AP, and VLM of rats either nonstressed or exposed to restraint water-immersion stress for 1 h; $n = 5$ rats per group. Values are mean ± SEM. $^{**}P < 0.01$ compared with the control group. $^{*}P < 0.05$ compared with the control group

SON

Restraint water-immersion stress for 1 h induced a robust 3.3-fold increase in Fos-IR nuclei in SON (15.9 ± 1.3 vs 3.7 ± 1.3 in the control group, cells per 0.01 mm^2) ($P < 0.01$) (Fig. 1). In contrast with the PVN, after RWIS, Fos-IR nuclei were evenly distributed in the SON (Fig. 2d). The labeled neuronal nuclei in the SON were large and round and similar in size. In the SON, the presence of TH-IR neurons was completely absent both in nonstressed rats and in rats stimulated with restraint water-immersion for 1 h. There were, however, large numbers of CA-nerve endings in the SON (Fig. 2c, d).

Effect of RWIS on Fos, TH and Fos/TH in the medullary visceral zone

DMV

In the DMV, there was induction of Fos-IR nuclei in RWIS rats compared with nonstressed rats ($P < 0.05$) (Fig. 3). The occurrence of Fos-IR nuclei was evident from the rostral to the caudal portions of the DMV (Fig. 4a–f). There was no significant difference in the number of Fos-IR nuclei in any portion of the DMV ($F_{2,24} = 2.499$, $P = 0.102$) (Table 1). TH-IR neurons in the DMV were rare and had fusiform or round profiles with a few neuronal

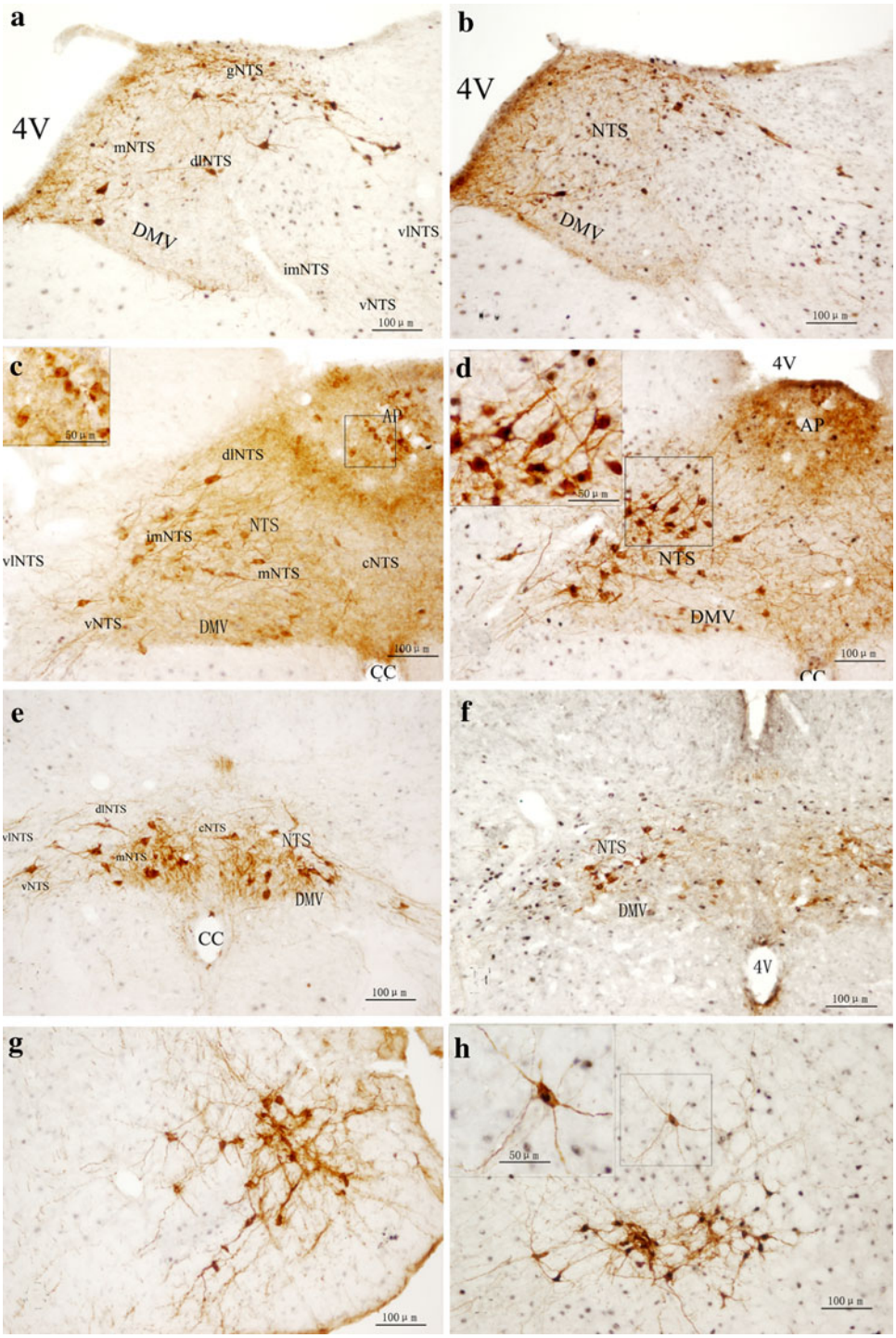

Fig. 4 Double immunohistochemical staining of Fos-IR and TH-IR neurons in the DVC and VLM of rats either nonstressed (**a**, **c**, **e**, **g**) or exposed to restraint water-immersion stress for 1 h (**b**, **d**, **f**, **h**). The *insets* show higher magnification (×400) of cells in the *small boxes*. Fos-IR is localized in nuclei, and TH-IR is found in the cytoplasm. *NTS*, nucleus of the solitary tract; *gNTS*, gelatinous nucleus of the solitary tract; *cNTS*, commissural nucleus of the solitary tract; *mNTS*, medial nucleus of the solitary tract; *imNTS*, intermediate nucleus of the solitary tract; *vNTS*, ventral nucleus of the solitary tract; *vlNTS*, ventrolateral nucleus of the solitary tract; *dlNTS*, dorsolateral nucleus of the solitary tract; *DMV*, dorsal motor nucleus of the vagus; *AP*, area postrema; *4V*, 4th ventricle; *CC*, central canal

processes (Fig. 4a–f). TH-IR neurons were greater in the intermediate and caudal portions of the DMV than in the rostral portion ($F_{2,24} = 4.903$, $P = 0.016$), whereas for Fos/TH-IR neurons there was no difference in different portions of the DMV ($F_{2,24} = 1.460$, $P = 0.251$) (Table 1). RWIS increased TH-IR and Fos/TH-IR neurons by 2.2 times and 4.1 times, respectively, compared with the control group ($P < 0.01$) (Fig. 3). Overall, Fos-IR nuclei in the DMV after RWIS for 1 h were identified in 38.0 ± 5.3% of TH-IR neurons, a 2.7-fold increase compared with the control group (14.3 ± 4.5%) ($P < 0.01$), and TH-IR neurons in the DMV after RWIS were identified in 17.3 ± 4.5% of Fos-IR nuclei, a 2.8-fold increase compared with the control group (6.2 ± 2.2%) ($P < 0.05$).

Table 1 Average numbers of Fos, TH, and Fos/TH immunoreactive neurons in the rostral, intermediate, and caudal parts of the DMV and NTS induced by restraint water-immersion stress (cells per 0.01 mm^2)

	DMV		NTS	
	Unstressed	RWIS 1h	Unstressed	RWIS 1h
Fos-IR				
Rostral	1.5 ± 0.4	3.4 ± 0.8*[a]	1.8 ± 0.3	3.4 ± 0.1*[ab]
Intermediate	2.9 ± 0.9	3.7 ± 0.7*[a]	1.8 ± 0.3	4.7 ± 0.6*[c]
Caudal	1.2 ± 0.2	2.2 ± 0.8*[a]	1.1 ± 0.9	2.1 ± 0.2*[bc]
TH-IR				
Rostral	0.5 ± 0.1	0.8 ± 0.1**[a]	0.6 ± 0.0	1.2 ± 0.1**[a]
Intermediate	0.8 ± 0.2	2.1 ± 0.6**[b]	1.7 ± 0.1	2.0 ± 0.1**[c]
Caudal	1.1 ± 0.3	2.5 ± 0.5**[b]	1.3 ± 0.1	1.8 ± 0.1**[b]
Fos/TH-IR				
Rostral	0.2 ± 0.1	0.3 ± 0.1**[a]	0.1 ± 0.0	0.4 ± 0.1**[a]
Intermediate	0.1 ± 0.0	0.6 ± 0.1**[a]	0.1 ± 0.0	0.6 ± 0.1**[b]
Caudal	0.2 ± 0.1	1.0 ± 0.3**[a]	0.1 ± 0.0	0.7 ± 0.1**[b]

Means in a column of the rostral, intermediate, and caudal portions of the DMV or the NTS of Fos-IR, TH-IR, and Fos/TH-IR neurons without a common letter are significantly different at $P < 0.05$, performed by two-way analysis of variance (ANOVA) followed by S–N–K's post-hoc test

** $P < 0.01$, * $P < 0.05$ compared with the control group

NTS

Restraint water-immersion stress for 1 h dramatically increased the number of Fos-IR nuclei in the NTS compared with that in nonstressed rats ($P < 0.05$) (Fig. 3). The occurrence of Fos-IR nuclei was evident in the rostral to caudal portions of the NTS. Fos expression was greater in the intermedial portion of the NTS than in the rostral and caudal parts ($F_{2,24} = 3.760$, $P = 0.037$) (Table 1). Fos-IR nuclei were mainly confined to the intermediate (iNTS), ventrolateral (vlNTS), and gelatinous subnuclei (gNTS) (Fig. 4a–f), with a few stained cells observed in the ventral (vNTS) and the medial subnuclei (mNTS) (Fig. 4a–f). TH-IR perikarya in the NTS were fusiform or round or triangular in profile with a number of long neuronal processes. Most TH-IR and Fos/TH-IR in the NTS were localized in the iNTS, vlNTS, and gNTS (Fig. 4a–f). TH-IR and Fos/TH-IR neurons were greater in RWIS rats than in nonstressed rats ($P < 0.01$) (Fig. 3). Numbers of TH-IR neurons were significantly different in the different portions of the NTS ($F_{2,24} = 43.637$, $P = 0.000$), and the number of Fos/TH-IR neurons was greater in the intermediate and caudal portions of the NTS than in the rostral portion ($F_{2,24} = 1.460$, $P = 0.251$) (Table 1). Overall, Fos-IR neurons in RWIS rats were identified in 34.4 ± 2.4% of TH-IR neurons, whereas in nonstressed rats they represented 9.7 ± 1.6% of OT-IR neurons, i.e. a 3.6-fold increase ($P < 0.01$). In addition, TH-IR neurons in RWIS rats were identified in 12.9 ± 0.8% of TH-IR neurons, whereas in nonstressed rats they represented 4.5 ± 0.6% of Fos-IR neurons, i.e. a 3.0-fold increase ($P < 0.01$).

AP

Fos immunoreactive nuclei were distributed evenly within the AP (Fig. 4c, d). Fos expression in the AP was significantly increased by RWIS for 1 h (3.8 ± 0.3 cells per 0.01 mm^2) compared with expression in nonstressed rats (1.1 ± 0.2 cells per 0.01 mm^2), i.e. 3.5-fold ($P < 0.01$) (Fig. 3). TH-IR perikarya in the AP were round with few evident neuronal processes (Fig. 4d). There was no difference in the number of the TH-IR neurons in RWIS (6.1 ± 0.6 cells per mm^2) and nonstressed rats (5.9 ± 1.3 cells per mm^2) ($P > 0.05$). The number of Fos/TH double-labeled neurons was significantly increased by RWIS for 1 h compared with that in nonstressed rats, i.e. 4.5-fold ($P < 0.01$) (Fig. 3). The percentage of Fos-IR neurons among the total number of TH-IR neurons in RWIS and nonstressed rats were 18.6 ± 6.1 and 4.5 ± 1.3% respectively, i.e. 4.1-fold ($P < 0.01$).

VLM

Restraint water-immersion stress for 1 h dramatically increased the number of Fos-IR nuclei in the VLM from 1.2 ± 0.1 to 2.0 ± 0.2 cells per 0.01 mm^2 compared with that in nonstressed rats, i.e. 1.7-fold ($P < 0.01$) (Fig. 3). The occurrence of Fos-IR nuclei was evident from the rostral to the caudal portions of the VLM, mainly the C1/A1 cell groups (Fig. 4g, h). TH-IR perikarya in the VLM were fusiform or polygonal in profile with a number of long neuronal protrusions, some of which projected into the ventrolateral region of the NTS. TH-IR neurons and Fos/TH double-labeled neurons were mainly located in the C1/A1 cell groups (Fig. 4g, h). Likewise in the case of the AP, there was no difference in the number of the TH-IR neurons in nonstressed rats and rats stimulated with RWIS ($P > 0.05$), and the number of Fos/TH double-labeled neurons was significantly increased in rats stimulated with RWIS compared with nonstressed rats ($P < 0.01$) (Fig. 3). RWIS activated 45.7 ± 2.3% of TH-IR neurons, compared with 18.9 ± 2.8% in nonstressed rats, i.e. 2.4-fold ($P < 0.01$).

Discussion

Restraint water-immersion stress evoked marked neuronal activation in specific populations of brain neurons assessed by Fos expression. The most intense Fos-IR neurons were observed in the PVN, SON, DMV, NTS, AP, and VLM, a

pattern consistent with that reported previously by us under similar conditions [3, 4]. These results indicate that these nuclei are important in mediation of signals induced by RWIS. Using double labeling, the characterization of these activated neurons in the PVN, SON, DVC, and VLM, on the basis of their morphological aspects and chemical coding, reveals that RWIS activates the MVZ CA-cell groups, which may project to the PVN and SON. In the following text, each of these nuclei will be discussed separately in more detail.

Psychological and physical stressors induce activation of different neuron populations in the hypothalamus

These results demonstrate that, in the hypothalamus, the marked activation of neurons induced by RWIS encompasses mainly the SON, the PaMP, and the PaLM, in agreement with the importance of the hypothalamus in response to a variety of stimuli. *c-fos* mRNA and Fos protein were strongly induced in the SON and the two subdivisions of the PVN, PaMP, and PaLM after osmotic stimulus (i.p. hypertonic saline) [26, 27]. Cold-restraint for 3 h induced gastric lesions and evoked substantial Fos protein expression in the PVN [28, 29]. The Fos protein expression induced by RWIS occupied mainly the magnocellular parts of the SON and PVN, and, to a lesser extent, the parvocellular part of the PVN [30, 31]. Water-avoidance stress for 1 h induced numerous Fos-IR cells in the dorsal and the medial parvocellular parts of the PVN, and only a few Fos-IR cells were observed in the Pe, the parvocellular portion of the PVN, and the SON, iNTS, and A1 cell groups in the MVZ [32, 33]. These results indicate that psychological stressors, for example water-avoidance stress, or only restraint stress or immobilization stress, evoke strong Fos-induction in the parvocellular portion of the PVN, except the SON. However, the physical stressors, including the osmotic stimulus, the cold stress, and the heat stress, induce extensive Fos activation in the SON and the magnocellular part of the PVN. RWIS, a mixture of physical and psychological stressors, activated the SON and the parvocellular and magnocellular parts of the PVN, which is consistent with the results reported above.

The role of CA-neurons in the hypothalamus during RWIS

In the hypothalamus, TH-labeled immunoreactive perikarya were the smallest population of cells in the PVN, and in the SON the presence of TH-IR perikarya was completely absent in both control rats or rats stimulated with RWIS. This indicates that hypothalamus CA-neurons are not important in mediation of signals induced by RWIS. The distribution and incidence of TH-IR perikarya in the hypothalamus in rats upon RWIS is consistent with previous results [19, 34]. However, the functional significance of these neurons in the PVN or SON is not yet fully understood. This different TH ratio in the neuroendocrine activation of the PVN expands previous studies showing different activation of TH-containing neurons in response to a variety of stimuli. For example, exposure to different states of arousal [35] and administration of glucose anti-metabolite 2-deoxy-D-glucose can induce increased activity of CA phenotype cells in the PVN [36]. These data suggest that TH-containing neurons in the PVN might be involved in more functional circuits in response to different stressors. However, the functionality of TH neurons in the hypothalamus and the phenotypic nature of activated neurons in RWIS rats can only be clarified by further studies.

The role of CA-neurons in the DVC and VLM during RWIS

Many recent studies have indicated that the DMV is important in integration of the gastric dysfunctions induced by a number of stressors. Cold-restraint for 3 h induced gastric lesions and evoked substantial Fos protein expression in the DMV [28, 29]. Our results showed that the Fos-IR nuclei occupied the rostral to the caudal portions of the DMV, mainly the intermedial subdivision. This result is in agreement with our previous report [5]. The expression patterns of Fos in different subdivision of the DMV suggest that neuronal activity in the different portions of the DMV are not the same during RWIS. Results from previous studies indicate that the vagal excitatory pathway originates in the rostral and intermediate subdivision of the DMV whereas the vagal inhibitory pathway originates in the caudal DMV [26, 37, 38]. RWIS for 1 h activated the DMV, especially the intermediate DMV, the vagal excitatory pathway, and then resulted in a series of gastric functional disorders.

The nucleus of the solitary tract is the major recipient of visceral afferent information arising from various regions of the gastrointestinal tract. Previous studies showed that when rats were exposed to RWIS, their body temperature decreased and gastric functions became abnormal [5, 39]. This information is relayed in the NTS and activates NTS neurons, which provide direct inhibitory and excitatory inputs to the DMV and then, in turn, control gastric functions via their efferent projections in the vagus nerve [40, 41]. Fos-IR nuclei in RWIS rats mainly occupied the intermediate portion of the NTS, then the caudal and rostral

NTS, probably because the intermediate and caudal potions receive the afferent information from gastrointestinal receptors whereas the rostral NTS is important in regulating the gustatory information [42].

The postrema area also receives inputs from the vagal sensory fibers of the stomach. The number of Fos-IR nuclei was significantly increased by RWIS compared with that in nonstressed rats. This result may imply that the AP is involved in responses to the incoming sensory information during RWIS.

The ventrolateral medulla is also a visceral motor nucleus and the vagal parasympathetic neurons innervating the stomach are partly located in the VLM [12]. Fos-IR nuclei in the VLM were found in the entire rostral-caudal column including the C1/A1 cell groups, and the number of the Fos-IR nuclei was significantly different in nonstressed rats and rats exposed to RWIS for 1 h, in agreement with results for proximal colon distension in rats [20].

In the MVZ, the marked activation of CA-neurons induced by RWIS encompasses mainly the NTS (A2/C2) and VLM (A1/C1), next the AP, and a few TH-IR were found in the intermediate and caudal portions of the DMV. In RWIS rats and nonstressed rats, the percentage of Fos-IR nuclei in TH-IR neurons was respectively 38.0 and 14.3% in the DMV (i.e. a 2.7-fold increase), 34.4 and 9.7% in the NTS (i.e. a 3.6-fold increase), 18.6 and 4.5% in the AP (i.e. a 4.1-fold increase), and 45.7 and 18.9% in the VLM (i.e. a 2.4-fold increase) (all the $P < 0.01$). Many previous studies have found that CA-neurons of the MVZ were involved in processing and conducting visceral nociceptive information. Proximal colon distension induced Fos in approximately 74 and 42% of CA-neurons in the VLM and the NTS, respectively [20]. RWIS induced substantial Fos protein expression in noradrenergic neurons of the MVZ (A1/A2 cell groups) [43]. Water-avoidance stress for 1 h induced a small amount of Fos protein expression in imNTS and A1 cell groups [33, 34]. These data strongly suggest that CA-neurons in the MVZ are related to stress-responsive signal transduction. There was no difference of Fos/TH-IR neurons in RWIS rats in the intermediate, caudal, and rostral portions of the NTS and DMV, and this may be due to that all portions of these nuclei take part in the stress-responsive signal transduction.

However, our observation that RWIS induces a quick increase of TH neurons in the both DMV and NTS than for the unstressed rats, while there was almost no change in the AP and VLM. The quick increase of the TH neurons may be due to that at least some portion of the RWIS effects on TH mRNA are directly related to NTS and DMV activation during the RWIS session. The differences in the number changes of TH neurons in the different nuclei may be due to a large, stable pool of TH mRNA at basal conditions [44].

CA-neurons in the DVC and VLM project to the PVN and SON

A1/C1 and A2/C2 cell groups in the MVZ are interconnected with the hypothalamus and major afferent innervation of the PVN originates in noradrenergic ascending fibers arising from the VLM and NTS [16, 17]. The VLM and NTS are reciprocally connected in rats [15]. In our study, a dense network of CA-terminals and CA neuronal processes were found in the hypothalamus and MVZ. Thus, the extensive connections of the CA-neurons in the hypothalamus and MVZ suggest that RWIS-induced CA neuronal activation might trigger the activation of complex circuitry involved in the modulation of gastric dysfunction.

In conclusion, the marked activation of neurons in the DVC and VLM induced by RWIS provided direct evidence that hyperactivity of the parasympathetic nervous system caused gastric dysfunction during RWIS [2]. The patterns of activated neurons in the different subdivisions of the NTS and DMV may also reflect their different roles in modulating gastric function. Activation of the CA-neurons in the MVZ may imply that the afferent and efferent limbs of the functional responses are associated with gastric dysfunction. A robust increase in Fos-IR nuclei in the PVN and SON induced by RWIS implies that the PVN and SON are important in the mediation of signals during RWIS. But a few of TH-IR neurons in the PVN and their absence in SON indicate that the CA-neurons in the PVN and SON may be not related to the stress-responsive signal transduction, and the phenotypic nature of the activated neurons in the hypothalamus must be further studied.

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