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Original Article

The effect of vagotomy on c-fos expression in the reticular formation areas following cystometry in cyclophosphamide-induced cystitis in rats

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Abstract: Background: The involvement of the vagus nerve in the supraspinal neural circuits that control the urinary bladder function, especially during pathological conditions, became increasingly evident. However, the role of brainstem areas in these circuits is not studied yet. Methods: In the present study, using c-fos immunohistochemistry, the roles of the vagus nerve to the responses of the reticular formation to cystometry in cyclophosphamide-treated rats were investigated. Results: Cyclophosphamide treatment significantly increased the c-fos expression in the lateral reticular nucleus (LRt), lateral paragigantocellular nucleus (LPGi), caudal part of the ventrolateral reticular nucleus (CVL), and gigantocellular reticular nucleus (Gi) following cystometry. However, cyclophosphamide treatment didn't have significant effect on c-fos expression in ventrolateral reticular nucleus (VL), rostral part of VL (RVL), raphe pallidus nucleus (RPa), and raphe obscurus nucleus (Rob). Vagotomy significantly demolished the effect of cyclophosphamide in the LRt and LPGi areas without having any significant effect on other reticular formation areas. Whereas, in comparison to normal animals, the vagotomised animals didn't show any significant changes in c-fos expression. Conclusion: The results of this study demonstrate the involvement of the reticular formation areas, particularly the ventral part, in processing urinary bladder function under cystitis condition. It also demonstrates the contribution of the vagus nerve in these processes.

Keywords: Cystitis, vagus nerve, urinary bladder, c-fos, reticular formation

Introduction

Many animal studies demonstrated that inputs through vagal afferents affect urinary bladder function. These effects were elicited through either electrical stimulation of the vagus nerve or mechanical stimulation of vagally innervated structures (e.g., esophagus) [1-3]. On the other hand, stimulation of the urinary bladder was able to modify the vagal efferent discharge [4, 5]. In addition, urinary bladder distention proved to modify different cardiovascular [6, 7] and respiratory [8, 9] functional parameters, mainly via the vagus nerve. These effects reflect the viscero-visceral interaction between the vagal and pelvic nerves territories, this interaction is part of functional coordination between viscera.

The interaction between the urinary bladder and other viscera is mainly controlled by supraspinal centers. Different areas of the reticular formation were proved to be involved in the supraspinal neural circuits that control bladder function. Electrophysiologic studies demonstrated that neurons in different medullary reticular formation nuclei receive convergent inputs from the urinary bladder and vagal afferents [10]. In addition, stimulation of other brainstem areas which are known to be involved in cardiovascular function, such as locus ceruleus, paraprachial area, ambiguus nucleus, solitary nucleus, and dorsal motor nucleus of vagus, are able to affect the urinary bladder function [11, 12].

Although most of the studies demonstrated indirect involvement of the vagus nerve in processing the urinary bladder function, some recent studies showed direct innervation of the vagus nerve to the urinary bladder. In an anatomical tracing study, cells in the nodose gan-

Table 1. Expression of c-fos in the reticular formation areas following cystometry

Brainstem area	Normal	Cyclophosphamide	Vagotomy
LRt	37.26 ± 3.39	66.50 ± 6.72*	43.18 ± 2.80
LPGi	23.48 ± 2.10	36.77 ± 1.93*	26.32 ± 1.75
VL	15.37 ± 1.65	19.54 ± 1.26	16.42 ± 1.16
CVL	13.25 ± 1.23	18.20 ± 1.68**	15.65 ± 0.93
RVL	17.43 ± 3.02	20.83 ± 1.86	17.35 ± 2.32
Gi	19.00 ± 1.84	26.84 ± 1.30***	23.93 ± 1.64
RPa	10.88 ± 0.65	12.54 ± 0.83	11.97 ± 0.75
ROb	3.93 ± 0.42	3.91 ± 0.49	3.20 ± 0.33

CVL; caudal part of the ventrolateral reticular nucleus, Gi; gigantocellular reticular nucleus, LPGi; lateral paragigantocellular nucleus, LRt; lateral reticular nucleus, ROb; raphe obscurus nucleus, RPa; raphe pallidus nucleus, RVL; rostral part of the ventrolateral reticular nucleus, VL; ventrolateral reticular nucleus. *Significantly Increased ($P < 0.001$) compared to normal and vagotomy groups. **Significantly Increased ($P < 0.03$) compared to normal group. ***Significantly Increased ($P < 0.002$) compared to normal group.

glion were labeled following injection of the urinary bladder wall with anterograde tracing material [13]. Other studies demonstrated direct vagal innervation to the urinary bladder under pathological conditions [14, 15]. As well, chemical irritation of the urinary bladder significantly changed the responses of the reticular formation areas to electrical stimulation of the vagus nerve [16, 17]. Collectively, these studies indicate that the vagus nerve could be part of the neural circuits that control the urinary bladder function during pathological conditions, such as cystitis. Also, the results of these studies indicate the possible role of the reticular formation in controlling bladder function under pathological conditions.

In the present study, the possible role of the vagus nerve in the responses of the reticular formation areas to urinary bladder function in a rat model of interstitial cystitis was investigated using c-fos gene expression.

Materials and methods

Twenty four Sprague Dawley male rats were used in the present study. Animals age was about eighth weeks at the day of terminal experiment. All animals were caged in optimal light/dark cycles with ad libitum access to food and water. All animal procedures were approved by the Deanship of Scientific Research, Al-Balqa Applied University. The guidelines of National Institute of Health (NIH, USA) for the use and care of laboratory animals were followed.

Animal grouping

Animals were divided equally into three groups (eight animals in each group): CY group, animals received cyclophosphamide treatment only; CY-Vx group, animals received cyclophosphamide treatment and had vagotomy procedure; and Control group, without any treatment or surgery.

Cyclophosphamide treatment

Each animal in the CY and CY-Vx groups received intraperitoneal cyclophosphamide 75 mg/Kg every third day for ten days before the terminal experiment [18]. The effectiveness of the cyclophosphamide treatment in developing the cystitis model was confirmed following the experiment by checking the urinary bladder grossly for acrolein accumulation, wall thickening (by noticing the rigidity of the bladder wall), increased weight (by weighing the urinary bladder following extraction at the end of the experiment), and development of hematuria (by checking the cage bedding for blood traces). The urinary bladder was also checked histologically of the epithelial erosion and edema accumulation in the wall layers [19].

Vagotomy procedure

Cervical vagotomy were performed for each animal in the CY-Vx group one hour before the cystometry procedure at the day of the final experiment. Vagotomy procedure was performed as described previously [2, 20]. Briefly, the cervical parts of the vagus nerves were accessed through a median incision of the cervical skin anteriorly. Each vagus nerve was identified just beside the carotid artery and dissected out. Using a micro-scissor, about 1 mm piece was cut off from each nerve. The separated nerve ends were visualized under the light microscope to confirm the cut.

Cystometry procedure

At the day of terminal experiment, each animal was anesthetized with 50% urethane at a dose

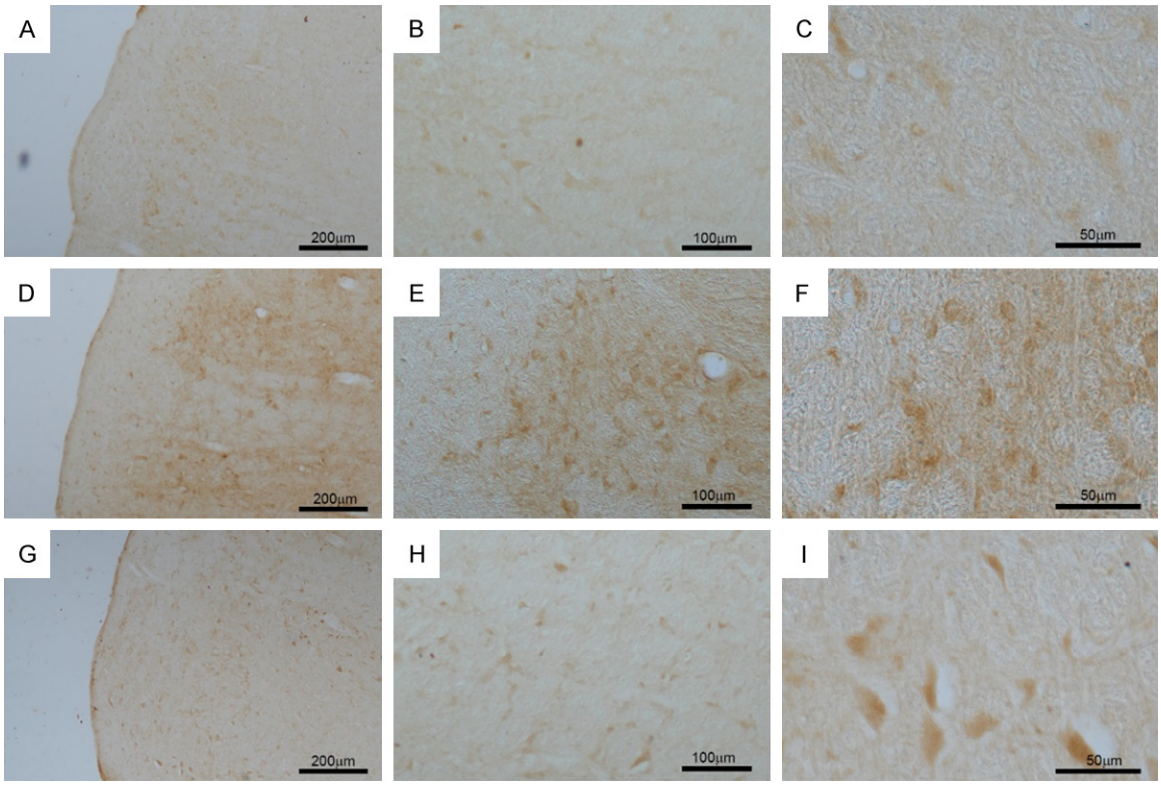

Figure 1. C-fos-stained sections of the lateral reticular nucleus (LRt). (A-C) are sections from the control animal. (D-F) are sections from the cyclophosphamide-treated animals. Note the increase of c-fos-stained cells. (G-I) are sections from the vagotomised animals. Note the decrease of c-fos-stained cells compared to the cyclophosphamide-treated animal.

of 1.2 g/kg. The anesthetic solution was injected subcutaneously (50%) and intraperitoneally (50%). The urinary bladder was accessed via an abdominal incision. After that, a catheter which is connected to a normal saline-filled syringe was installed to the bladder through its apex. The normal saline was pumped using a programmable pump (AL-1000; World Precision Instrument, Sarasota, FL) into the urinary bladder at a rate of 0.25 ml/minute. Cystometry was performed in each animal for 1 hour. At the end of cystometry procedure, each animal was perfused with heparinized saline followed by 4% paraformaldehyde.

C-fos Immunohistochemistry

Following animal perfusion, brainstem was dissected out and postfixed in 10% formalin overnight, then it was transferred into 30% sucrose for three days in preparation for sectioning. Brainstem was sectioned using a cryostat at a thickness of 34 μm. Every sixth section, starting 1 mm caudal to the obex and through the brainstem, was mounted at charged slides. Sections were incubated in the 1:200 diluted primary c-fos antibody (ab209794, Abcam, UK) for 48 hours at 4°C, then they were incubated with biotinylated secondary antibody for 30 minutes after which they were processed for DAP chromogen staining. Sections were blocked by hydrogen peroxide at the beginning and washed out throughout the immunohistochemistry procedure by buffer solution as necessary.

Stained cells were identified under light microscope (ECLIPSE Ni-U, Nikon, Japan) and viewed using NIS-Elements software program. Stained sections were counted and photographed by (DS-Fi3, Nikon, Japan). The left side of the mounted sections was considered for cell counting. The identification and counting of stained cells were done by a blinded person to avoid any bias. Data for each responsive reticular formation area were extracted for statistical analysis. The nomenclature and coordinates of the reticular formation areas in "The rat brain in

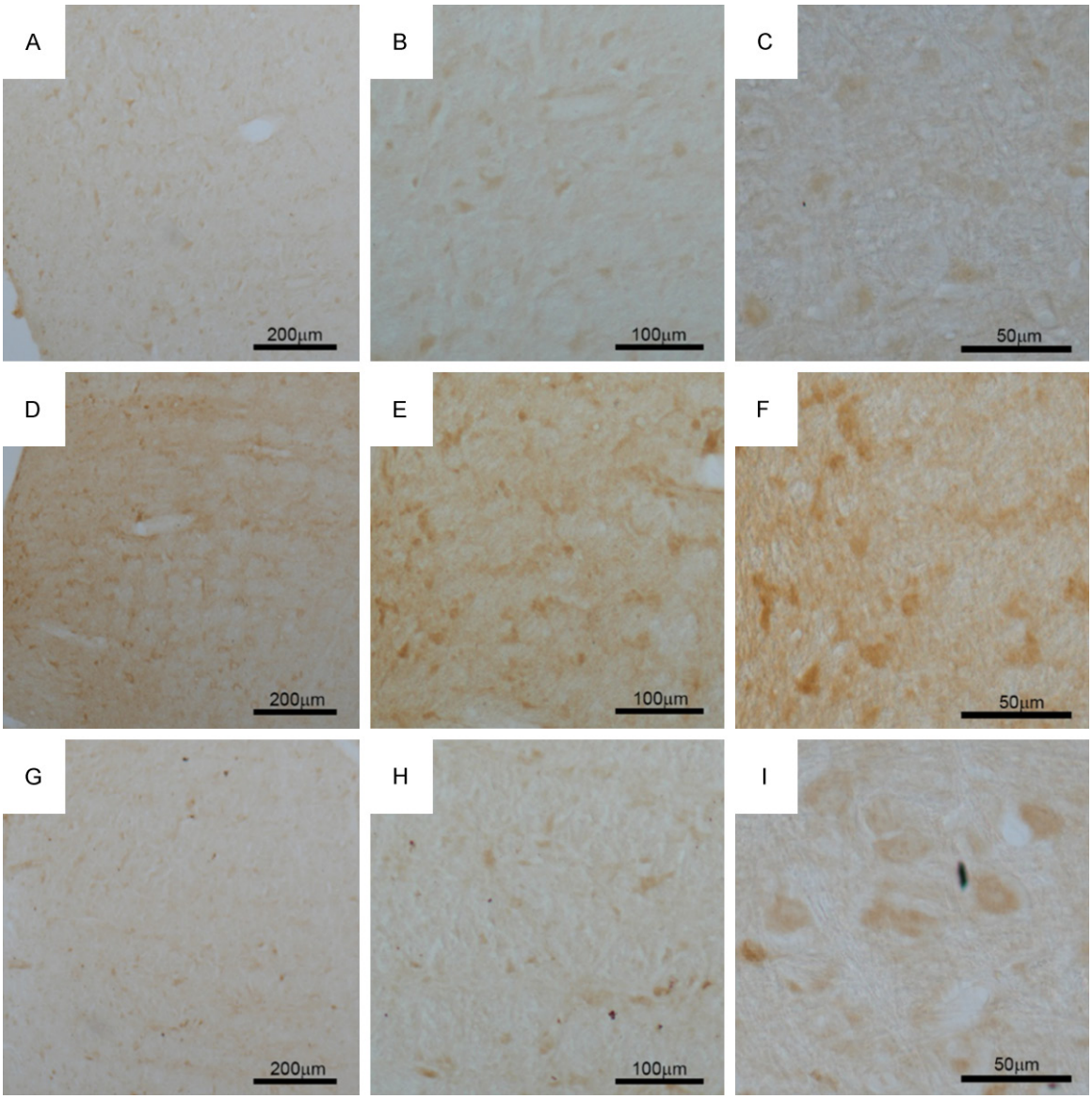

Figure 2. C-fos-stained sections of the lateral paragigantocellular nucleus (LPGi). (A-C) are sections from the control animals. (D-F) are sections from the cyclophosphamide-treated animals. Note the increase of c-fos-stained cells. (G-I) are sections from the vagotomised animals. Note the decrease of c-fos-stained cells compared to the cyclophosphamide-treated animal.

stereotaxic coordinates" book were adopted for this study [21]. Accordingly, the following areas and their rostral-caudal distances from bregma were considered for evaluation: LRt, lateral reticular nucleus (-13.2 to -13.6); LPGi, lateral paragigantocellular nucleus (-10.3 to -12.8); VL, ventrolateral reticular nucleus (-11.3 to -14.6); CVL, caudal part of the ventrolateral reticular nucleus (-12.8 to -14.6); RVL, rostral part of the ventrolateral reticular nucleus (-11.3 to -12.8); Gi, gigantocellular reticular nucleus (-10.2 to -13.3); Rob, raphe obscurus nucleus (-11.2 to -14.3); Rpa, raphe pallidus nucleus (-9.8 to -14.3). Stained cells in the destined areas were counted regardless to staining intensity. However, changes of the staining intensity between groups (if any) were noted in the results section.

Statistical analysis

All data for all regions were averaged and presented as mean ± standard error. One-way analysis of variance (ANOVA) was used for

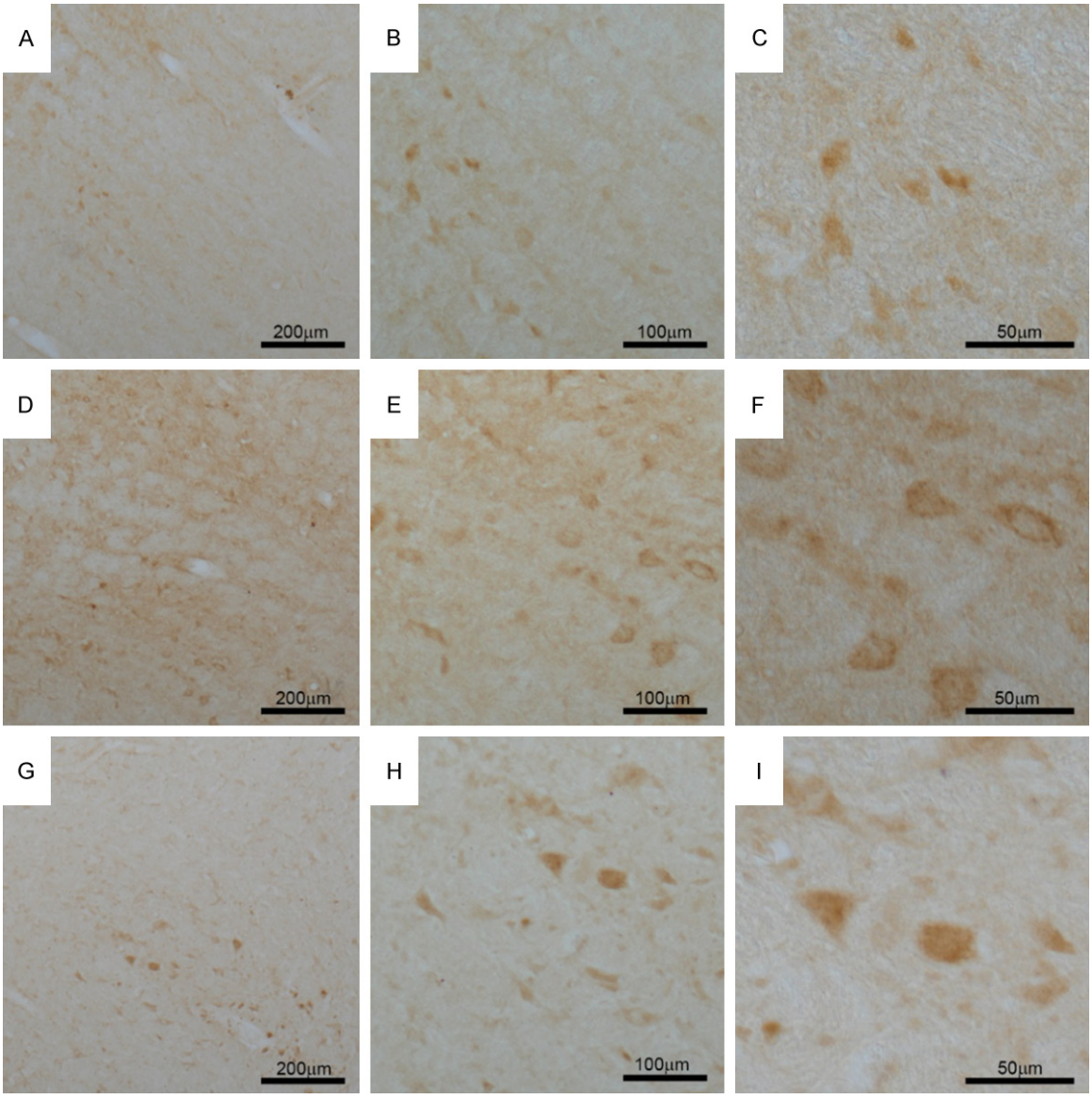

Figure 3. C-fos-stained sections of the caudal part of the ventrolateral reticular nucleus (CVL). (A-C) are sections from the control animals. (D-F) are sections from the cyclophosphamide-treated animals. Note the increase of c-fos-stained cells. (G-I) are sections from the vagotomised animals.

quantitative analysis. For the intra-group statistical analysis, Tukey's honestly significant difference (HSD) post-hoc test was used to reveal any significant changes. A *P* value less than 0.05 was adopted for consideration of significance.

Results

The expression results of c-fos in the areas of reticular formation in the different study groups were summarized in **Table 1**.

Following cystometry, neuronal cells in the lateral paragigantocellular nucleus (LPGi), the lateral reticular nucleus (LRt), the gigantocellular reticular nucleus (Gi), the subsets of the ventral reticular nucleus (VL); the caudal part of VL (CVL) and the rostral part of VL (RVL), and the subsets of the raphe nucleus; the raphe obscurus (RPa) and the raphe pallidus (RPa) nuclei showed c-fos expression.

Cyclophosphamide treatment significantly increased the expression of c-fos in the LRt,

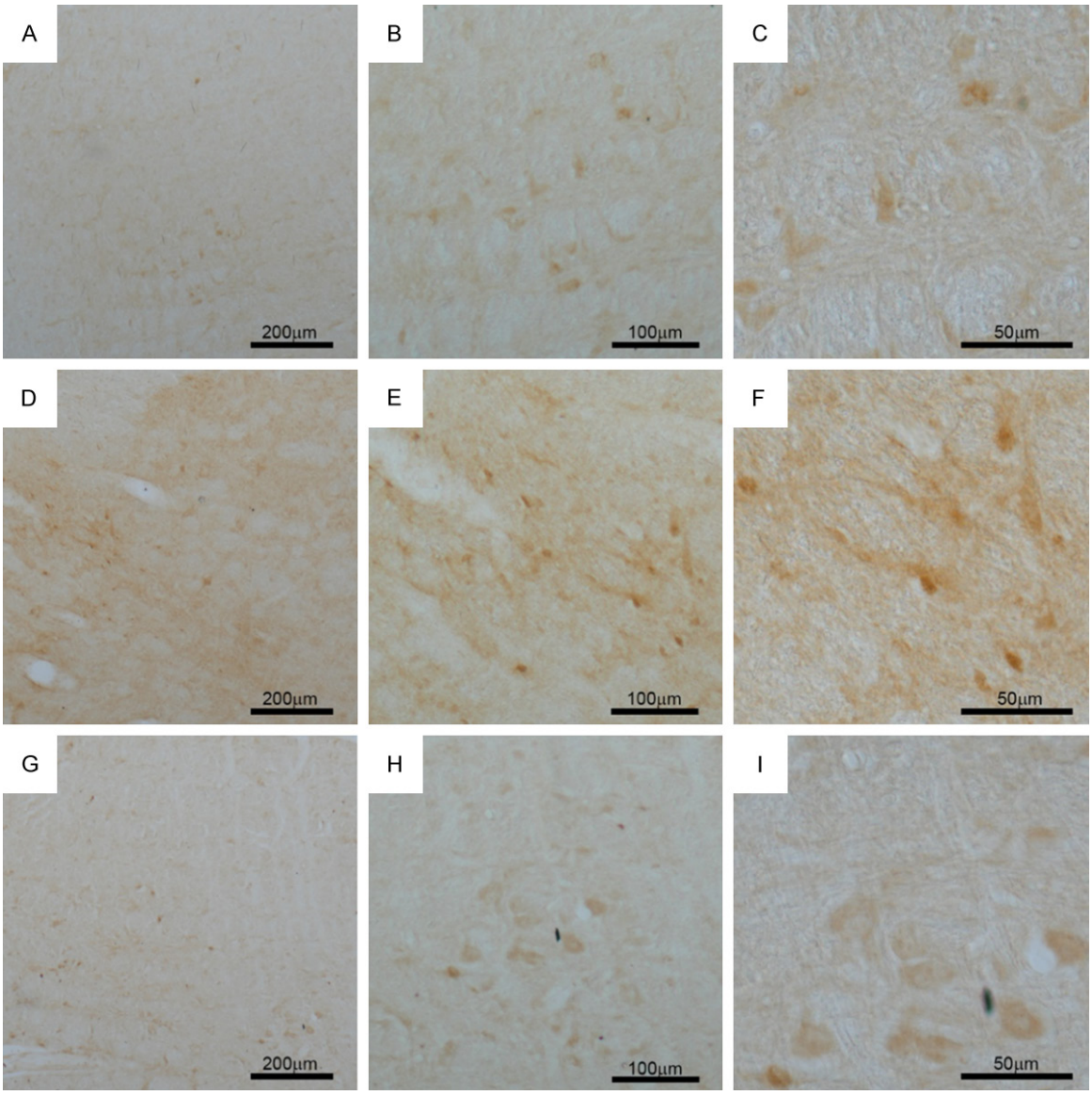

Figure 4. C-fos-stained sections of the rostral part of the ventrolateral reticular nucleus (RVL). (A-C) are sections from the control animals. (D-F) are sections from the cyclophosphamide-treated animals. (G-I) are sections from the vagotomised animals.

LPGi, Gi, and CVL areas of the reticular formation compared to the control group. However, the c-fos expression in RVL and raphe nuclei didn't have significant changes following cyclophosphamide treatment in comparison with the control group. It is generally noticed that cyclophosphamide treatment increased the staining intensity compared to normal and vagotomised groups.

Effect of vagotomy on c-fos expression

In the CY-VX group, vagotomy significantly decreased the expression of c-fos compared to CY group in the LRt and LPGi areas without any significant changes in the Gi and VL areas (CVL and RVL). On the other hand, there was no significant change in c-fos expression between CY-VX and control groups in all investigated reticular formation areas. Examples of histological sections showing the effect of cyclophosphamide treatment and vagotomy on c-fos expression in the reticular formation nuclei are presented in **Figures 1**-**6**.

Discussion

The present study demonstrates the role of the reticular formation areas in processing inputs from the urinary bladder during cystometry. The

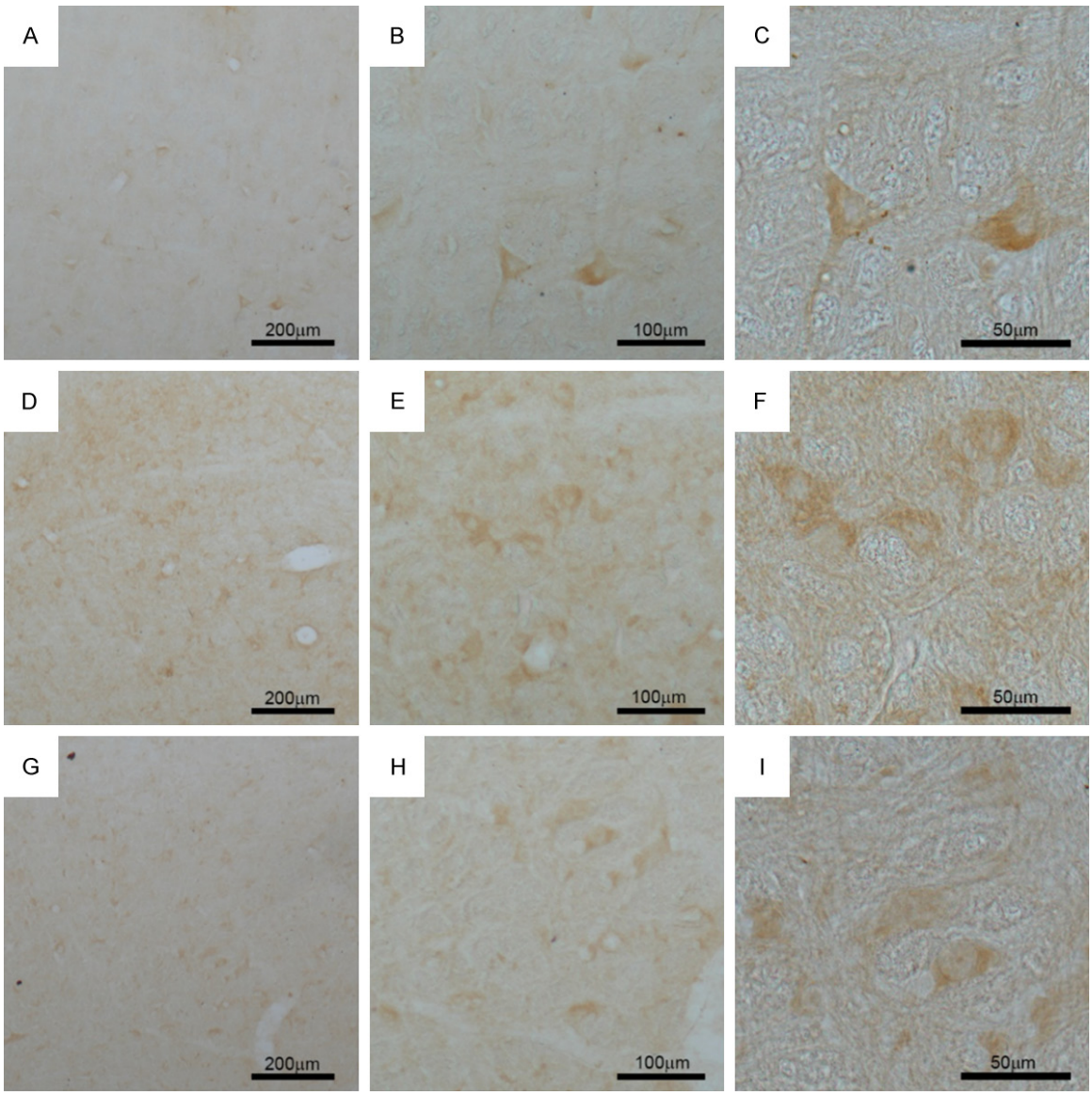

Figure 5. C-fos-stained sections of the gigantocellular reticular nucleus (Gi). (A-C) are sections from the control animals. (D-F) are sections from the cyclophosphamide-treated animals. Note the increase of c-fos-stained cells. (G-I) are sections from the vagotomised animals.

present study also demonstrates that the responses of these areas are significantly increased following cystitis. It also shows that part of these responses is conveyed via the vagus nerve.

The results of the present study showed significant increase of c-fos expression following cystometry in cyclophosphamide-treated animals compared to untreated animals. This increase was evident in the ventrolateral parts of the reticular formation and the gigantocellular reticular nucleus (Gi), but not in the raphe reticular nuclei. These results are consistent with other immunohistochemical studies [22, 23]. In addition, some of these areas were shown to receive inputs from normal and irritated urinary bladder upon distention using electrophysiological studies [10, 17]. The caudal part of the ventrolateral reticular formation showed significant increase compared to rostral part in the treated animals compared to normal.

The results of the present study demonstrate the impact of the vagus nerve in the responses of the reticular formation to cystometry follow-

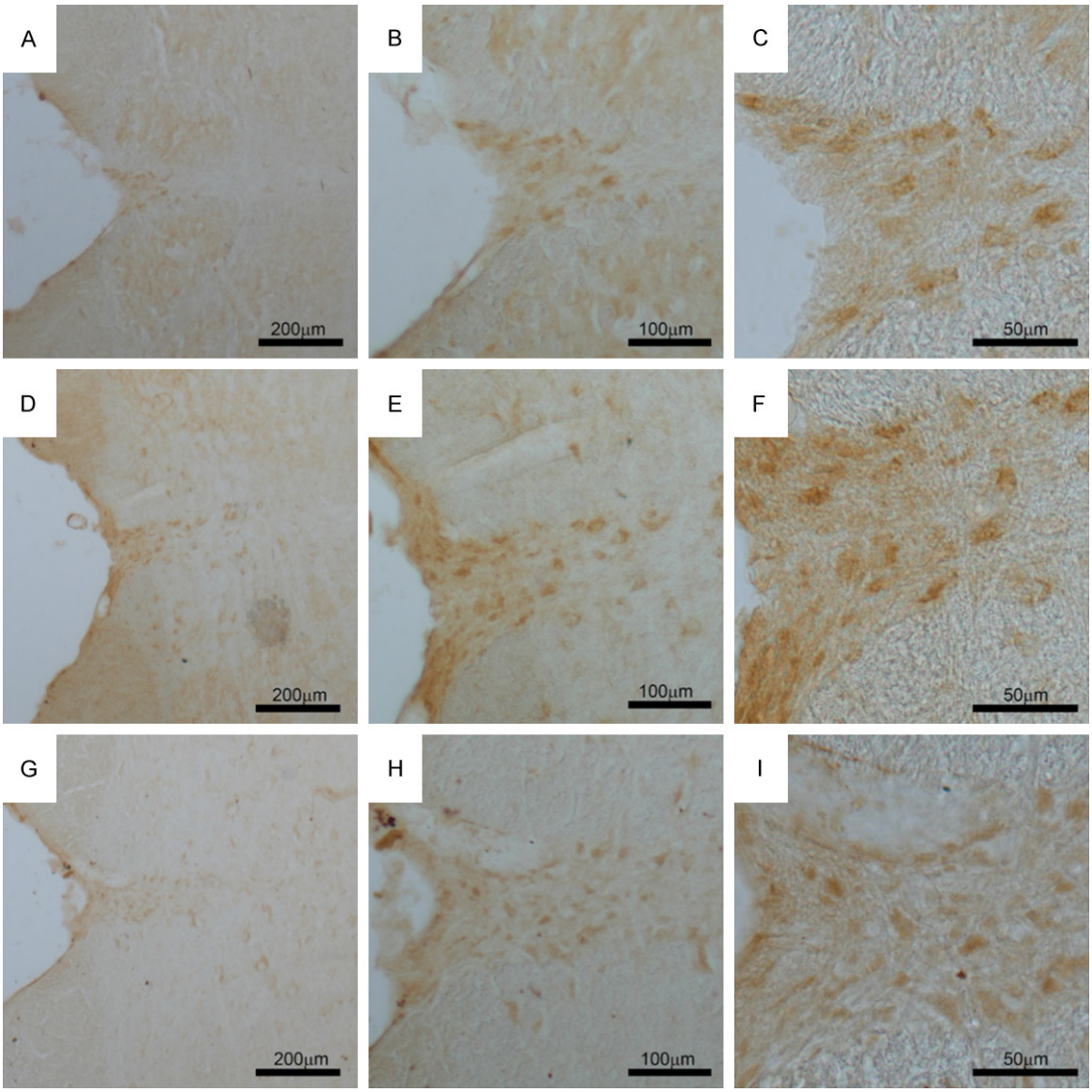

Figure 6. C-fos-stained sections of the raphe pallidus nucleus (RPa). (A-C) are sections from the control animals. (D-F) are sections from the cyclophosphamide-treated animals. (G-I) are sections from the vagotomised animals.

ing cyclophosphamide treatment. The results showed that vagotomy significantly reversed the effect of cyclophosphamide in the LRt and LPGi nuclei and decreased the effect of cyclophosphamide in the other reticular formation areas. However, vagotomised animals didn't show any significant change of c-fos expression compared to normal animals. The role of the vagus nerve in the neural circuits of the urinary bladder was demonstrated in many studies. Extracellular electrophysiological studies showed the convergence of bladder and vagal inputs into the reticular formation nuclei [10, 17]. This convergence was affected by urinary bladder irritation [16], which is concordant with the present results. The ventrolateral reticular formation areas were responsive to other vagally-innervated organs using c-fos immunoreactivity [24]. Others also demonstrated that some of these areas form part of the neural circuitry that controls cardiovascular functions [11]. The ventrolateral reticular formation areas may form a base for interaction between the urinary bladder and other viscera, such as the cardiovascular [11], respiratory [9], and gastrointestinal systems [3, 20] via the vagus nerve. On the other hand, many anatomical tracing studies demonstrated the direct innervation of

vagal afferents of the urinary bladder, although this innervation seems more prominent under pathological conditions [13, 14, 25].

The results of the present study are indicative of the role of the vagus nerve in innervating the urinary bladder during cystitis condition. The vagal afferents convey the nocuous inputs from the urinary bladder to the reticular formation, especially the ventral part. The convergence of inputs from the urinary bladder and other organs (e.g. heart, lungs, esophagus, etc) to the ventral part of the reticular formation may indicate the involvement of these areas in the viscero-visceral interaction between the urinary bladder and these organs during pathological conditions. This interaction is mediated through the vagus nerve. The role of the vagus nerve and reticular formation in the neural circuits thatcontrols the urinary bladder function during pathological conditions needs further investigations in awake animals. In addition, future studies are needed to investigate the characteristics of the reticular formation responses to the urinary bladder during cystitis condition.

In summary, the results of this study demonstrate the involvement of the ventral reticular formation in the supraspinal neural circuits that deal with the urinary bladder function under nocuous conditions. These results also demonstrate the important role of the vagus nerve in the processing of the reticular formation in response to the nocuous urinary bladder inputs. The understanding of the neural circuits that affect the urinary bladder function during pathological conditions can facilitate the management of these conditions.

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Disclosure of conflict of interest

None.

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