Neuronal expression of c-Fos protein in the brain after intraperitoneal injection of leptin in Wistar rats

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Abstract：Leptin has been recognized to be an important neuroendocrine signal in the regulation of food intake and energy balance. We aimed to survey central neurons that might be activated after peripheral administration of leptin, by examining the distribution of neurons expressing c-Fos protein. Leptin dissolved at a dose of 500 μg/kg in physiological saline was intraperitoneally injected in Wistar rats. One and a half hours after the injection, rats were transcardially perfused with saline and fixed with fixatives. The brain was removed and sectioned at 40 μm in thickness. Every fourth section was treated with anti-c-Fos antiserum, and c-Fos protein was immunohistochemically stained using the avidin-biotin complex method. Control rats were injected with saline solution, and brain sections were processed similarly as described above. It was found that leptin injected intraperitoneally induced the neuronal expression of c-Fos protein in several nuclei throughout the brain. In the central nucleus amygdala, ventromedial nucleus of hypothalamus, periaqueductal gray matter, lateral parabrachial nucleus, and the solitary tract nucleus, numbers of neurons expressing c-Fos protein were much more in the test experiments than those in the control experiments. Intraperitoneally injected leptin was found to stimulate central neurons that may play some roles in the regulation of such as a food intake.

Key words：Brain, Central nervous system, c-Fos, Leptin, Neurons,

INTRODUCTION

Leptin was an endogenous peptide secreted predominantly by adipocytes. This peptide was encoded by obese (ob) gene that was firstly identified from examination of naturally occurring mutant ob/ob mice[1)]. Leptin was known to act within the central nervous system (CSN) to decrease food intake and increase energy expenditure[2), 3)]. It was reported that leptin increased the sympathetic nervous activity not only to brown adipose fat, but also to other organs such as the kidneys, hindlimb and adrenal glands, suggesting broader autonomic functions of this peptide[4,5)]. Banks et al. suggested that leptin may act within the CSN by influencing the circumventricular organs that lack a blood brain barrier (BBB) and/or by acting on the specific sites behind the BBB by entering the cerebral spinal fluid via a saturable transport mechanism[6)]. Furthermore, leptin receptors

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were found to locate in the basal forebrain, hypothalamus, preoptic area and brainstem[7-9]. These experimental aspects on leptin urged us to investigate whether neurons in the CNS may be excited by peripherally administered leptin, or not.

It is well known that proto-oncogene c-fos induces c-Fos protein rapidly and transiently within neurons in response to kinds of activation[10,11]. We have studied expression of c-Fos protein in the central nervous system after a number of physiologically active peptides such as gastrin[12], galanin[13] and ghrelin[14]. In this series of experiment we chose leptin as a physiologically active peptide to study possible influence on the central neurons, and examined the expression of c-Fos protein in central neurons after intraperitoneal injection of leptin. So far, it was reported that intravenous injection of leptin induced expression of c-Fos protein in the hypothalamus and brainstem[15,16]. In the present study, we undertook to survey the neuronal expression of c-Fos protein throughout the CNS after intraperitoneal injection of leptin, not after intravenous injection of leptin. We found that after the intraperitoneal injection of leptin c-Fos protein was expressed in neurons in several nuclei in the CNS, some of which had not been reported in the experiment of intravenous injection of leptin.

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats weighing 240-260 g (9-10-weeks old, Center of Experimental Animals in Gunma University). To avoid any restlessness of a single rat, some rats were housed in a cage in quiet room under conditions of regulated illumination (LD12:12, light on at 08:00 h) and constant temperature (22 ± 2 ℃) with ad lib water and rat chow (MF, Oriental Yeast Co., Ltd., Tokyo, Japan). We undertook to examine which neurons in the CNS may express c-Fos protein after intraperitoreal injection of leptin (500 μg/kg, RD Systems. MN, USA) in 200 μl of physiological saline (0.9% NaCl solution) using Wistar rats (n=4). One and a half hour after the leptin injection rats were anesthetized by injection of intraperitoreal injection of pentobarbital sodium (80 mg/kg, Abbott, USA), and perfused via the left ventricle with about 20 ml of saline to flush out the blood. This was immediately followed by 100 ml of 0.5% glutaraldehyde and 4% paraformaldehyde (PFA, Merck, German) in 0.1 M phosphate buffer (PB, pH 7.4) under pressure of 100-120 mmHg, and 400 ml of 4% PFA in PB under hydrostatic pressure. The brains were removed and cut into two blocks at the level between superior and inferior colliculus. They were fixed in 4% PFA in PB for further 1.5 h at 4℃, then soaked stepwise in 10, 20, 25% sucrose in PB at 4℃. The brains were frozen and cut into serial transverse sections at 40 μm in thickness. The sections were collected in plates containing PB chilled by ice water. One group of every fourth section was rinsed in 0.1 M Tris-saline (TS, pH 7.4) three times, incubated with 0.5% bovine serum albumin (BSA) in TS for 20 min and incubated with sheep anti-c-Fos polyclonal antiserum (1:100; *sc*-52, Santa Cruz Biotech. Inc., USA) in 0.5% BSA at 4℃ for 16 h. Afterwards, the sections were rinsed three times in TS, and then incubated in avidin and biotin solution respectively for 15 min. Thereafter they were incubated for 20 min with 2% normal rabbit serum in TS to block nonspecific bindings, and incubated for 60 min with anti-sheep IgG in TS. After rinsed three times in TS, the sections were incubated for 60 min in Vectastatin ABC kit (Vector Lab., Burlingame, CA, USA), and then treated with diaminobenzidine-nickel solution containing 0.003% H_2O_2. After that, the sections were rinsed in TS twice and rinsed further in PB. They were mounted on glass slides and dried at room temperature. The sections on the slides were dehydrated in a graded ethanol series (50%, 70% 95%, 99%), infiltrated with xylene and coverslipped in Permount (Fisher Comp., USA). Control rats (n=4) were sham-operated and injected with 200 μl of saline. Brain sections were similarly processed as above. c-Fos protein localized in neuronal nuclei was visualized as black precipitates of nickel-intensified diaminobenzidine reaction products. c-Fos-immunoreactive (c-Fos-ir) neurons were surveyed under bright-field microscopy. Brain histology was checked against the rat brain atlas[17]. Mann-Whitney U-tests were performed to compare the data between the two groups.

RESULTS

Significant increases in the number of c-Fos-ir neuronal profiles compared to those in control experiment were observed in several nuclei in the brain from the medulla oblongata to the basal telencephalon after intraperitoneal injection of leptin (500 μlg/kg). Table I shows numbers of c-Fos-ir neurons counted in the central nuclei after the intraperitoneal injection of leptin in Wistar rats. Typical examples of c-Fos protein expressed in neurons in the ventromedial hypothalamus (VMH) were shown in Fig.1, and those in the periaqueductal gray matter (PAG) shown in Fig.2. Significantly much more numbers of c-Fos-ir neurons were found in the test experiments than in the control experiments in the following nuclei; central nucleus amygdala (CEA), anterior hypothalamic nucleus (AH), VMH, PAG, lateral parabrachial nucleus (LPB), and the solitary tract nucleus (NTS). Sites of these nuclei were shown in Fig.3. c-Fos-ir neurons were also found in dorsomedial hypothalamus (DMH), lateral hypothalamus (LH), paraventricular hypothalamus (PAH), the locus coeruleus (LC), and the ventrolateral medulla (VLM). However, no significant differences in the number of c-Fos-ir neurons in these nuclei were observed between the test experiments and the control experiments.

DISCUSSION

The present study showed that significant increases in the number of c-Fos-ir neuronal profiles compared to those in control experiment were observed in several nuclei in the brain from the medulla oblongata to the basal telencephalon after intraperitoneal injection of leptin. The nuclei where c-Fos protein was expressed were fundamentally in accordance with those reported previously when leptin was intravenously injected[14,15], except for a few nuclei.

It was to be noted to examine the pattern of expression of c-Fos-ir neurons in the VMH and LHA because leptin has been reported to be related with feeding behavior. The VMH is known to be as "satiety center", the lesion of which caused hyperphagia and satiety. The electrical stimulation of this nucleus induced the inhibition of feeding. On the other hand, the LHA is known to be as "feeding center", lesion of which is known to cause cibophobia. The electrical stimulation of the LHA is known to induce the feeding behavior[18]. In this study a significant increase in the number of c-Fos-ir neurons were observed in the VMH in the test experiment than the control

Table I : Numbers of c-Fos immunoreactive neurons in central nuclei after intraperitoneal injection of leptin (500 μg/kg) (test) and that of 0.9%NaCl solution (control). Results were expressed as mean ± S.E.. *P<0.05. Abbreviations: AH: anterior hypothalamic nucleus; CEA: central nucleus amygdala; DMH: dorsomedial nucleus hypothalamus; LC: locus coeruleus; LHA: lateral hypothalamic area; LPB: lateral parabrachial nucleus; NTS: solitary tract nucleus; PAG: periaqueductal gray matter; PAH: paraventricular nucleus hypothalamus; PVT: paraventricular nucleus thalamus; VLM: ventrolateral medulla; VMH: ventromedial nucleus hypothalamus.

nucleus	test	control
NTS	382=79*	155=24
VLM	47±15	11±4
LC	71±17	62±8
LPB	150=18*	91±8
PAG	980±120*	552=24
VMH	284=91*	92±6
AH	501=60*	242=31
PAH	217⊥19	248_21
CEA	260=58*	60±24
PVT	529 ⁞ 68	360 27
LHA	140 ⁞ 10	285 62
DMH	425 ⁞ 60	255 60

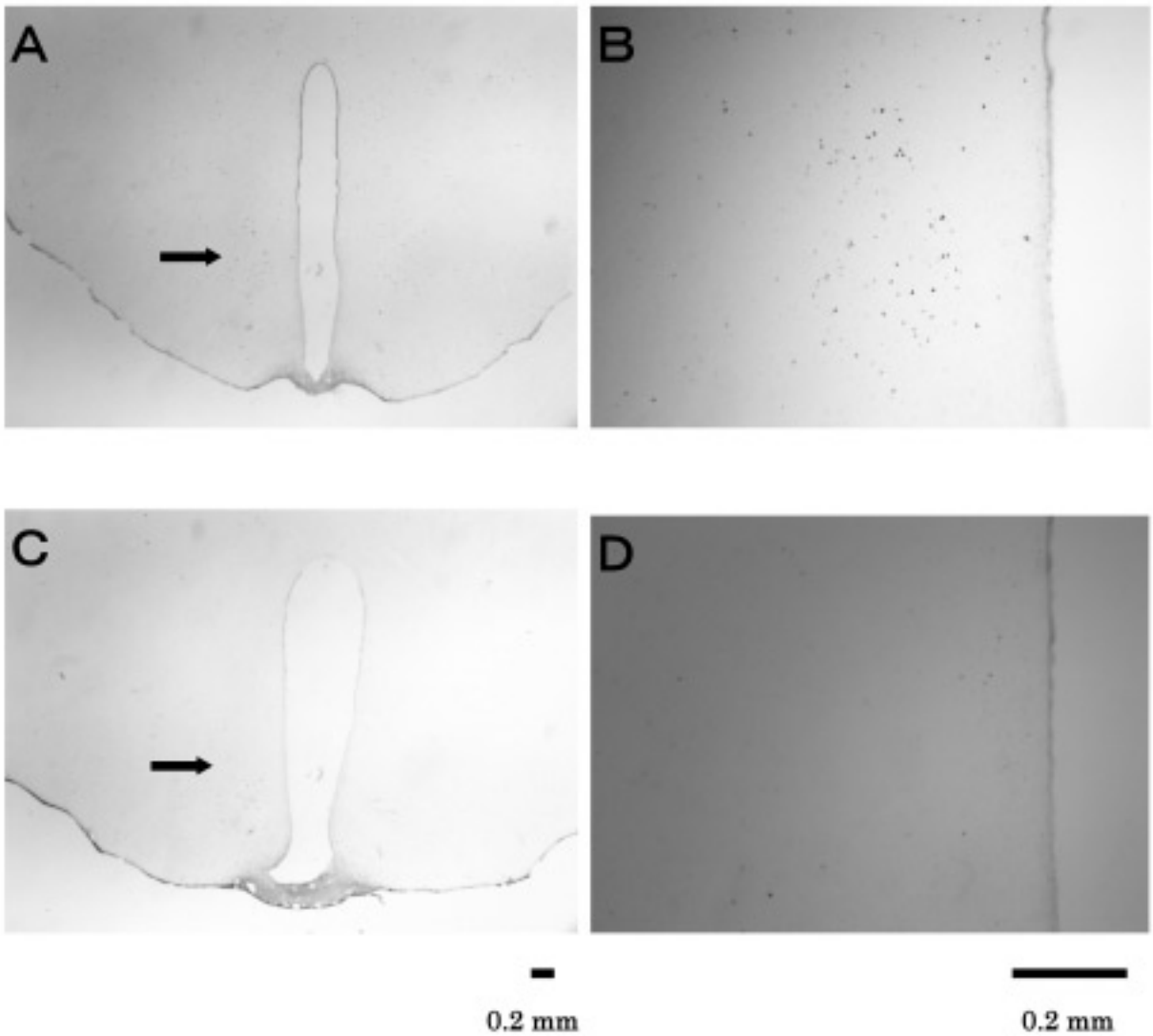

Fig. 1 Typical photomicrographs of c-Fos immunoreactive neurons in the VMH after intraperitoneal injection of leptin (A,B) and 0.9%NaCl solution (C,D). The sites indicated as arrows in A and C were enlarged in B and D, respectively.

experiments, but differences in the numbers of c-Fos-ir neurons in the LHA between the test and control experiments were not observed. This suggests the possibility of action of leptin to the VMH neurons under the suppression of feeding.

Significantly much more c-Fos-ir neurons were expressed in the PAG in the test experiment than in the control experiment. This has not been reported in other previous studies. The PAG was reported to be involved in the regulation of feeding or regulatory mechanism of metabolism. It was reported that the sympathetic descending pathway of the VMH - PAG - caudal reticular nucleus of medulla oblongata - intermediolateral nucleus in the spinal cord may be involved in the secretary function of the pancreas and adrenal medulla[19]. The present results suggest that intraperitoneally injected leptin may stimulate neurons in the VMH and PAG to play a role in the regulation of functions described above.

We found that leptin induced the neural expression of c-Fos protein in a large number of neurons in the CEA. Zawoiski et al. reported that the electrical stimulation of the same area elicited an increase in gastric acid secretion[20], suggesting that neurons in the CEA may relate to gastric acid secretion. It has been known that the CEA has dense connections of nerve fibers among various regions in the brain and many of these connections are reciprocal[21]. Anatomical studies showed that the CEA projects to the NTS-DMX[22] which projects to the stomach in turn[23,24]. It was also shown that CEA has reciprocal projections with the LPB and the NTS, and that the

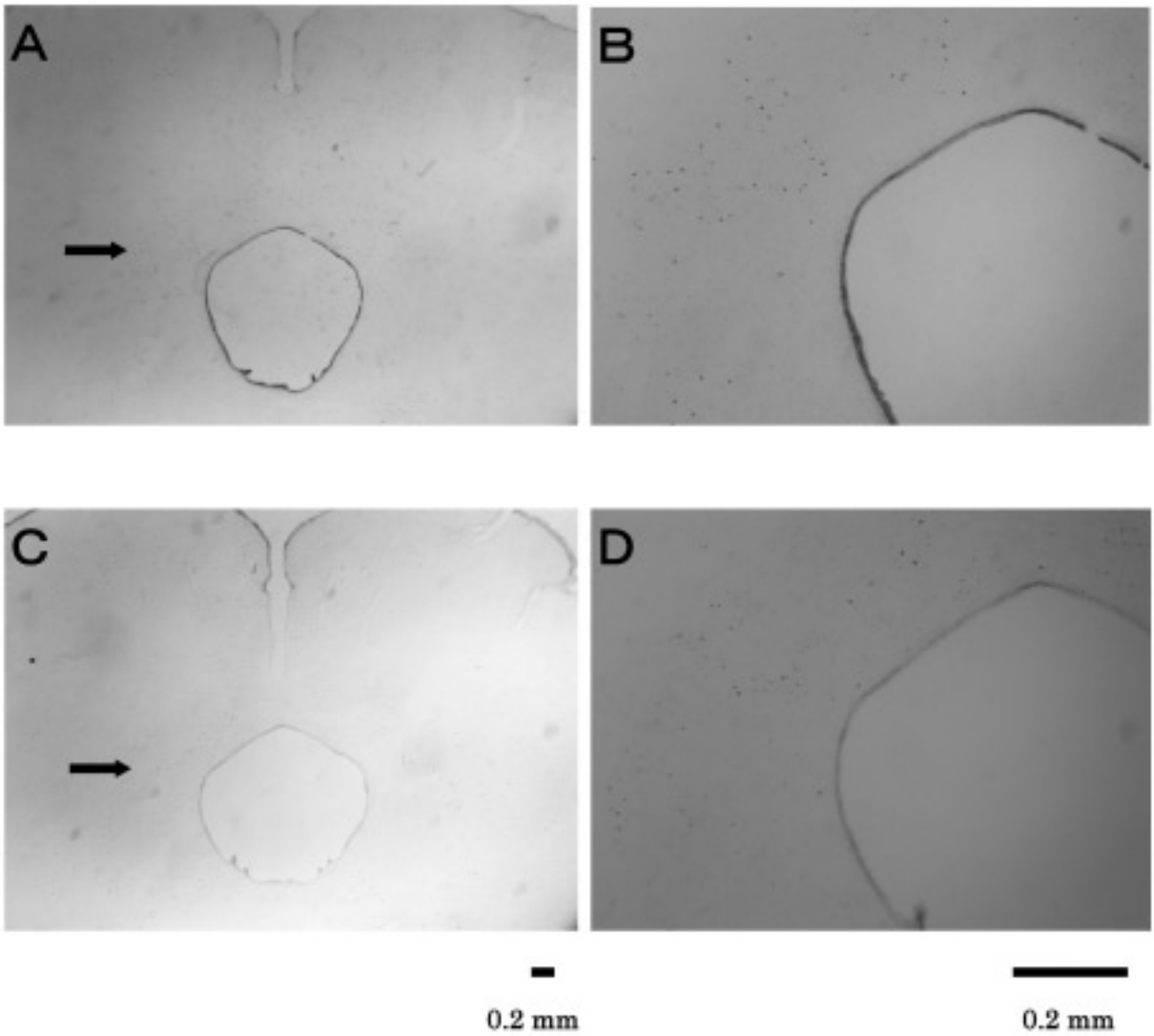

Fig. 2 Typical photomicrographs of c-Fos immunoreactive neurons in the PAG after intraperitoneal injection of leptin (A,B) and 0.9%NaCl solution (C,D). The sites indicated as arrows in A and C were enlarged in B and D, respectively.

projection from the LPB to the CEA was especially potent[21]. Taking these into consideration, it is suggested that leptin may stimulate CEA neurons to elicit some physiological functions.

Many c-Fos-ir neurons were found in the LPB. It has been known that the LPB has reciprocal connections with the CEA[25]. This suggests that c-Fos protein in the LPB neurons might be expressed by the excitatory inputs from the CEA neurons, which were stimulated by leptin. It addition, it was shown that the LPB neurons project to the NTS-DMX[26]. Michl et al. reported that the primary sensory neurons that signal an acute gastric acid insult to the brain are vagal afferents that terminate in the NTS and the AP, from which the information is carried to the LPB, the SFO and the CEA[27]. These suggest that the LPB neurons may be involved as an intermediate site between the CEA and the NTS-DMX.

We also found that c-Fos protein was expressed in many neurons in the NTS-DMX. The NTS-DMX is known as the key site for regulation of intestinal functions in the brain. The NTS receives major inputs of the vagus nerve as well as taste information from the oral cavity, and sends a direct projection to the DMX[28,29]. It is also known that the NTS has connections with the CEA, LPB. The DMX integrates visceral sensory input from the NTS and central sensory input arising from the hypothalamus. Vagal motor neurons originate in the DMX and innervate the stomach and intestine[22,23]. The vagus nerve is one of the final common pathways that transmit centrally integrated output from the brain to the stomach and

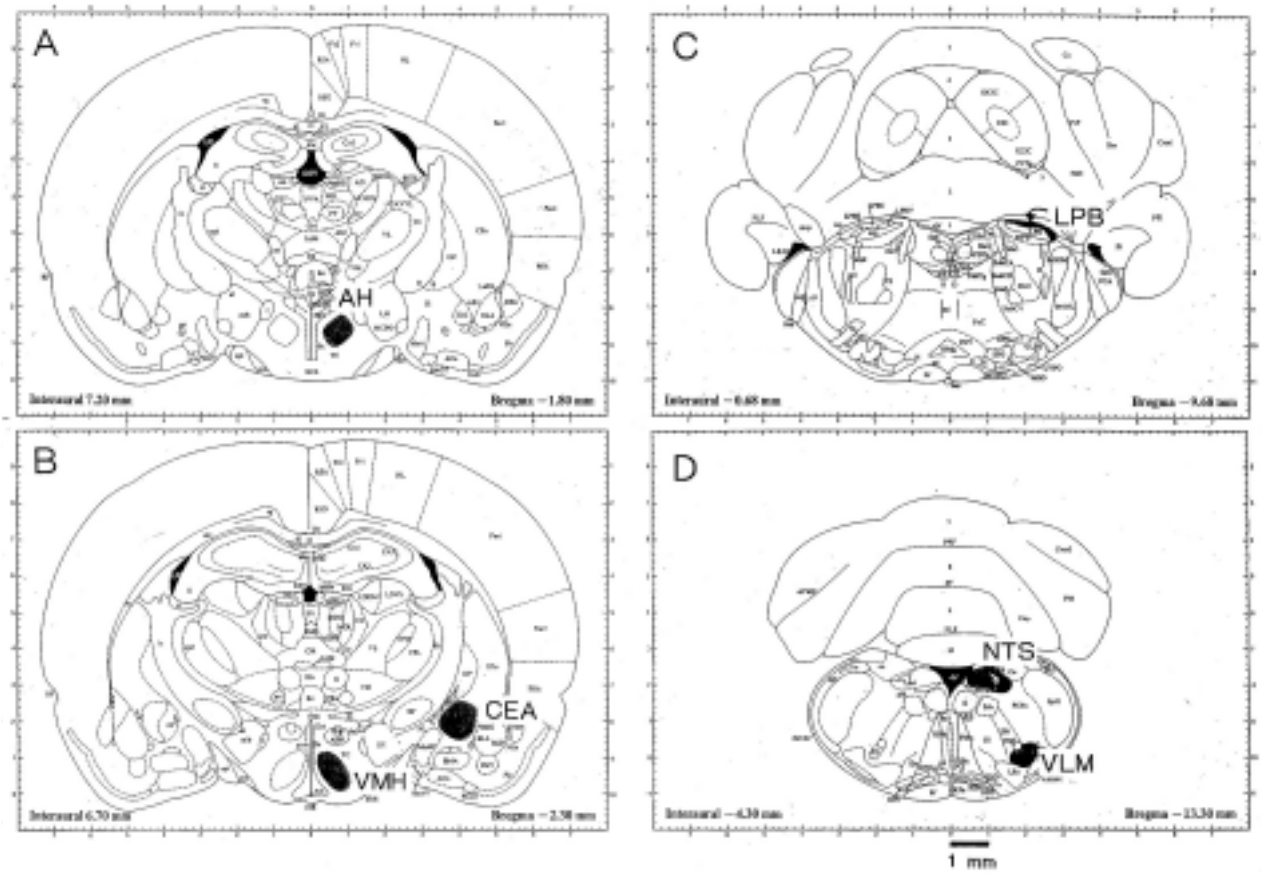

Fig. 3 Sites of nuclei where significantly much more numbers of c-Fos immunoreactive neurons were found in the test experiments than in the control experiments were shown as dark areas in A-D of the rat brain atlas[17]. Abbreviations: AH, anterior hypothalamic nucleus; CEA, central nucleus amygdala; LPB, lateral parabrachial nucleus; NTS, solitary tract nucleus; VLM, ventrolateral medulla oblongata; VMH, ventromedial nucleus hypothalamus.

intestine. Thus, the c-Fos-ir neurons found in the NTS-DMX complex after the intraperitoneal injection of leptin may be involved in some gut functions.

So far we have shown that the intravenous and intraperitoneal injection of gastrin, or ghrelin or apelin induced neural expression of c-Fos protein in the CEA, LPB and the NTS-DMX complex[12-14]. Gastrin[30], ghrelin[31] and apelin[32] have been known to be a gastrointestinal hormone which induced gastric acid secretion potently. Thus, the neural expression of c-Fos protein in the CEA, the LPB and the NTS-DMX after peripheral injection of these three peptides may be involved in gastric acid secretion. So far, it has not been determined whether leptin may induce gastric acid secretion. In the present study the intraperitoneal injection of leptin was found to induce c-Fos expression in the nuclei described above which might be involved in the secretion of gastric acid. Thus, the c-Fos-ir neurons in these nuclei after leptin injection may not be involved in the gastric acid secretion, but some other functions that remain to be clarified.

In conclusion leptin was found to induce expression of c-Fos protein in the VMH and the PAG, which might be involved in the regulation of feeding and metabolism.

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