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—Original—

Glucoprivation-Induced Fos Expression in the Hypothalamus and Medulla Oblongata in Female Rats

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Abstract. Glucoprivation induced by 2-deoxy-*D*-glucose (2DG) suppresses pulsatile luteinizing hormone (LH) secretion in female rats. The suppression is enhanced in the presence of estrogen. In the present study, 2DG-induced Fos expression was examined in the solitary tract nucleus (NTS), hypothalamic paraventricular nucleus (PVN), raphe obscurus nucleus (ROb) and raphe pallidus nucleus (RPa), which have been previously suggested to be involved in glucoprivation-induced suppression of LH secretion in female rats. Ovariectomized (OVX) or estrogen-primed ovariectomized (OVX+E_2) rats were injected intravenously with 2DG (400 mg/kg BW). The brain was removed 1 h after the injection. The number of Fos-like-immunoreactive (Fos-li) cells in the PVN and NTS was significantly increased in OVX+E_2 rats compared with control groups, but did not show a significant increase in the OVX group. Few Fos-li cells were observed in the ROb and RPa in all groups. All of the Fos-li cells in the PVN and NTS were neurons because they had immunoreactivities to microtubule-associated protein 2. Some Fos-li cells (8.3%) had tyrosine hydroxylase-like immunoreactivities in the NTS in 2DG-treated OVX+E_2 rats. These results suggest that neurons in the PVN and NTS are involved in the estrogen-dependent neural cascade mediating glucoprivic suppression of LH secretion in female rats.

Key words: c-Fos, Glucoprivation, Estrogen, Solitary tract nucleus, Hypothalamic paraventricular nucleus

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A competitive inhibitor of glycolysis, 2-deoxy-*D*-glucose (2DG), suppresses pulsatile luteinizing hormone (LH) secretion via the noradrenergic input to the hypothalamic paraventricular nucleus (PVN) [1]. The 2DG-induced suppression of LH secretion is potentiated by the circulating level of estrogen [2]. Noradrenergic cell bodies in the A2 region of the solitary tract nucleus (NTS) have been reported to project to the PVN [3]. Glucoprivation increases estrogen receptor α (ERα) immunoreactivities in the A2 region of the NTS and PVN in female rats [4]. These results strongly suggest that the A2 region of the NTS and PVN is involved in the neural cascade mediating estrogen-dependent suppression of LH secretion during glucoprivation.

The cascade would also involve a glucose-sensing mechanism in the brain. According to the previous results, a glucose-sensing mechanism could be located in the medulla oblongata to regulate LH secretion, food intake and counterregulatory response to blood glucose level [5, 6]. In addition, immunoreactivities to pancreatic glucokinase (GKP), which is a key protein for glucose sensing in pancreatic β-cells, have been found in ependymocytes and serotonergic neurons in the raphe nuclei of the medulla oblongata in rats, including the raphe obscurus nuclei (ROb) and

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raphe pallidus nuclei (RPa) [7].

The present study aimed to determine the neural cascade mediating physiological responses to glucoprivation using c-Fos protein expression as a marker of neuronal activation [8]. We chose the NTS, PVN, ROb and RPa as candidates for the nuclei involved in glucoprivic suppression of LH secretion in female rats.

Materials and Methods

Animals and treatments

Adult female Wistar-Imamichi strain rats weighing 225–275 g were individually housed under a controlled environment (14 h light and 10 h darkness; lights on at 0500 h; temperature, 24 ± 2 C). Animals had free access to food (Labo-MR stock, Nihon Nosan Kogyo Co., Yokohama) and water until the day of brain sampling. On the day of brain sampling, food was deprived for 3 h before 2DG or xylose injection.

Two weeks before the brain sampling, rats having shown at least two consecutive 4-day estrous cycles were bilaterally ovariectomized (OVX). Some of the OVX rats were given subcutaneous Silastic (i.d., 1.5 mm; o.d., 3.0 mm; length, 25 mm; Dow Corning, MI, USA) implants containing estradiol-17β (E_2; Sigma, MO, USA) dissolved in peanut oil at 20 μg/ml to mimic a physiological estrogen level at diestrus [9].

Experimental protocols

2DG (400 mg/kg BW, Sigma) was dissolved in physiological saline and injected at 1300 h through an indwelling atrial cannula which was inserted on the day prior to brain sampling. The dose of 2DG was previously confirmed to suppress pulsatile LH release in OVX and OVX+E_2 rats [2]. Control animals were injected with an isoosmotic concentration of xylose (400 mg/kg BW, i.v., Sigma), a non-utilizable sugar in monogastric animals.

One hour after 2DG or xylose injection, rats were deeply anesthetized with sodium pentobarbital (40–50 mg/kg) and perfused transcardially with 0.05 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.05 M phosphate buffer (PB). Brains were immediately collected, post-fixed with the same fixative overnight at 4 C and cryoprotected in 30% sucrose in 0.05 M PB. Serial coronal sections (50 μm in thickness) of the medulla oblongata and hypothalamus were made on a cryostat and stored in a cryoprotectant at – 20 C.

Fos immunohistochemistry

For Fos immunohistochemistry, free-floating sections were treated with 0.3% hydrogen peroxide (H_2O_2) in 0.05 M PBS for 30 min at room temperature to remove endogenous peroxidase activity. Sections were incubated with 5% normal-rabbit serum (NRS) in 0.05 M PBS for 1 h at room temperature to block non-specific binding, then incubated with sheep anti-Fos serum (1:4,000; Genosys Biotechnology Ltd., Cambridge, UK) in 0.05 M PBS containing 5% NRS and 0.1% Triton X-100 for 72 h at 4 C. They were incubated in biotinylated rabbit anti-sheep IgG (1:500; Vector Laboratories Inc., CA, USA) in 0.05 M PBS for 1 h at room temperature. Subsequently, the sections were incubated with avidin-biotinylated horseradish peroxidase complex solution (Vectastain elite ABC kit, Vector Laboratories) for 1 h at room temperature. They were then rinsed in 0.1 M Tris buffer solution (TB). The Fos-like immunoreactivity was visualized by 0.05% 3,3-diaminobenzidine (Sigma) and 2.5% nickel chloride in 0.1 M TB with 0.01% H_2O_2. The sections were rinsed three times in 0.1 M TB, mounted onto gelatin-coated glass slides, air-dried, dehydrated through graded ethanol solutions, cleared in xylene and coverslipped with Entellan new (Merck KGaA., Darmstadt, Germany). The sections were rinsed four times (15 min each) in 0.05 M PBS between each step unless otherwise stated.

Immunofluorescence dual immunohistochemistry

For fluorescence immunohistochemistry, free-floating tissue sections were incubated with 5% normal donkey serum (NDS) to block non-specific binding for 1 h at room temperature. The sections were incubated with a mixture of sheep anti-Fos serum (1:4,000; Genosys Biotechnology) and one of the following: mouse anti-microtubule-associated protein 2 serum (MAP2; 1:500; Sigma), a neuronal marker; rabbit anti-glia-fibrillary acidic protein serum (GFAP; 1:5; DAKO Co., CA, USA), a marker for glial cells; or rabbit anti-tyrosine hydroxylase serum (TH; 1:3,000; Charles River Laboratories, MA, USA) for 72 h at 4 C. They were incubated for 2 h at room temperature with a mixture of

indocarbocyanine (Cy3)- conjugated donkey anti-sheep IgG (1:800; Jackson ImmunoResearch Laboratories, Inc., PA, USA) and either fluorescein isothiocyanate (FITC)- conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories) or FITC-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories). The sections were then mounted onto glass slides with FluoroGuard (Bio-Rad, CA, USA) and observed under a confocal laser microscope (MRC-1024; Bio-Rad).

Quantification of Fos-like immunoreactivity

Anatomical location of the brain nuclei was determined according to a rat brain atlas [10]. The number of Fos-like immunoreactive (Fos-li) cells in the NTS, PVN, ROb and RPa was counted twice bilaterally in each section under a microscope and then averaged. The slides were coded to avoid bias while counting.

Colocalization of Fos and TH, MAP2 or GFAP in the NTS and PVN was quantitated in two non-consecutive coronal sections taken from each of three OVX+E_2 rats treated with 2DG.

Statistics

Significant differences in the mean number of Fos-li cells between groups were determined by two-way ANOVA followed by the Scheffe test.

Results

Figure 1 shows the immunohistochemistry of Fos protein in the PVN, NTS, ROb and RPa in xylose or 2DG-treated rats with or without estrogen treatment. In the 2DG-treated OVX and OVX+E_2 animals, many Fos-like immunoreactivities were observed in the NTS and PVN, whereas few Fos-like immunoreactivities were observed in xylose-injected controls. In the PVN, the majority of Fos-li cells was located in the parvocellular region. Few Fos-like immunoreactivities were found in the ROb and RPa in all groups.

Figure 2 shows the mean number of Fos-li cells in the PVN, NTS, ROb and RPa in xylose or 2DG-injected groups with or without E_2 treatment. In the presence of estrogen, the mean number of Fos-li cells in the PVN and NTS was significantly higher

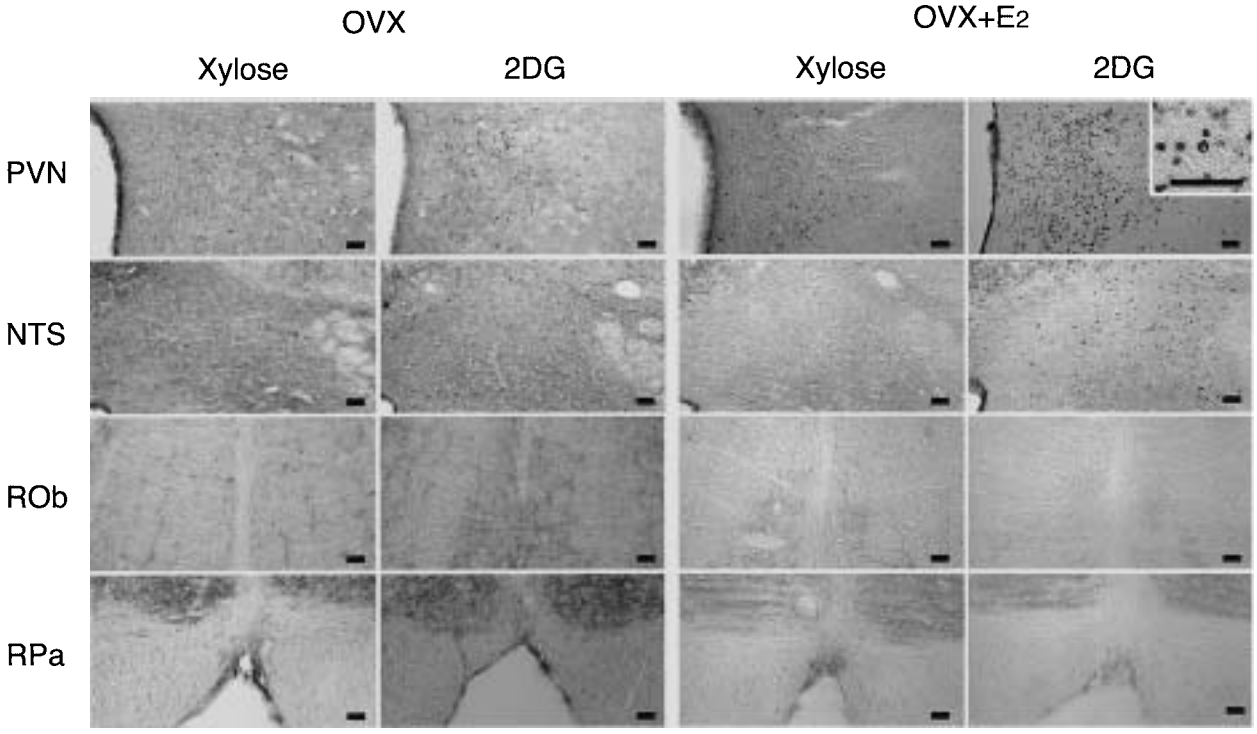

Fig. 1. Fos-like immunoreactive cells in the hypothalamic paraventricular (PVN), solitary tract (NTS), raphe obscurus (ROb) and raphe pallidus (RPa) nuclei in ovariectomized (OVX) or estradiol-primed ovariectomized (OVX+E_2) rats injected with either xylose or 2DG. The inset indicates an image with higher magnification. Scale bar = 50 μm.

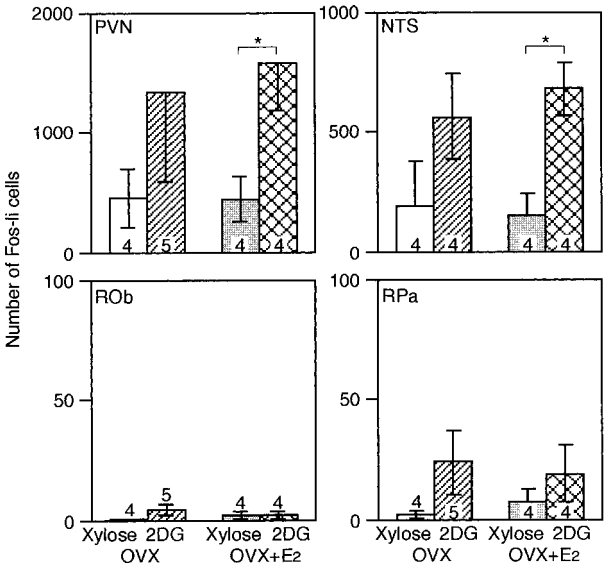

Fig. 2. Mean numbers of Fos-like immunopositive (Fos-li) cells in the hypothalamic paraventricular (PVN), solitary tract (NTS), raphe obscurus (ROb) and raphe pallidus (RPa) nuclei in OVX or OVX+E_2 rats injected with either xylose or 2DG. Numbers in each column indicate the numbers of animals used. Values are means ± SEM. *, $P<0.05$ vs. xylose-treated controls.

($P<0.05$) in the 2DG-injected group than the xylose-injected group. On the other hand, no significant difference was found in the number of Fos-li cells between the 2DG- and xylose-injected groups in the PVN and NTS in the absence of estrogen. In the ROb and RPa, no statistical difference was observed in the number of Fos-li cells between any group.

Dual immunhistochemistry indicates that most of the Fos-li cells were stained with anti-MAP2 (Fig. 3A) but not with anti-GFAP (Fig. 3B) in the PVN. Likewise, most of the Fos-li cells in the NTS were stained with anti-MAP2 (Fig. 3C) but not with anti-GFAP (Fig. 3D). Some of the Fos-li cells (8.3%) in the NTS were also stained with TH (Fig. 3E).

Discussion

In the present study, the number of Fos-li cells in the NTS and PVN significantly increased in the OVX+E_2 group after 2DG injection. These results suggest that the response to low glucose availability is mediated by the NTS and PVN. Estrogen treatment seems to enhance the 2DG-induced activation of the neural cascade, because Fos-li cells did not significantly increase in OVX animals. The present result is consistent with a previous study demonstrating Fos expression in the NTS and PVN two hours after 2DG injection in male rats [11]. The present study also revealed that most of the cells expressing Fos-li cells are neurons because the cells also had MAP2 immunoreactivities. On the other hand, no significant change in the number of Fos-li cells was found in the ROb and RPa in both OVX and OVX+E_2 groups when compared to xylose-treated controls. Thus, the raphe nuclei such as the ROb or RPa may not be involved in mediating the response to low glucose availability. Taken together, low glucose availability increases the neuronal activity in the NTS and PVN but not in the ROb and RPa. Serotonergic neurons in these raphe nuclei are more likely to sense high blood glucose availability rather than sensing the low glucose availability. Serotonergic neurons in the raphe nuclei have been known to cause satiety after food intake [12, 13] and to have GKP-like immunoreactivities [7]. These suggest that those neurons are closely involved in sensing high blood glucose availability to regulate feeding and reproduction. Further studies will be needed to clarify this issue.

The present study showed a significant increase in Fos expression one hour after 2DG injection in the NTS and PVN in OVX+E2 rats but not in the OVX group. Briski *et al.* [14] previously reported that Fos expression was induced in the NTS two hours after 2DG injection both in OVX and OVX+E_2 rats. The difference in the effect of 2DG between the two studies may be due to the different time courses of these studies. The NTS or PVN neurons could be more sensitive to glucoprivation in the presence of estrogen, and could be activated more quickly in the presence of estrogen than in its absence. A previous study showed that glucoprivic suppression of LH secretion is potentiated by estrogen [2]. The potentiation of glucoprivic suppression of LH secretion is most likely due to the increased expression of ERα in the PVN and NTS [4]. Likewise, estrogen has been known to regulate the activity of neurons by increasing their sensitivity to neural inputs [15, 16].

Fos-li cells in the PVN could be CRH neurons, because 2DG-induced Fos expression was mostly observed in the parvocellular region of the PVN, where a dense population of CRH neuronal cell bodies has been found [17]. A previous study

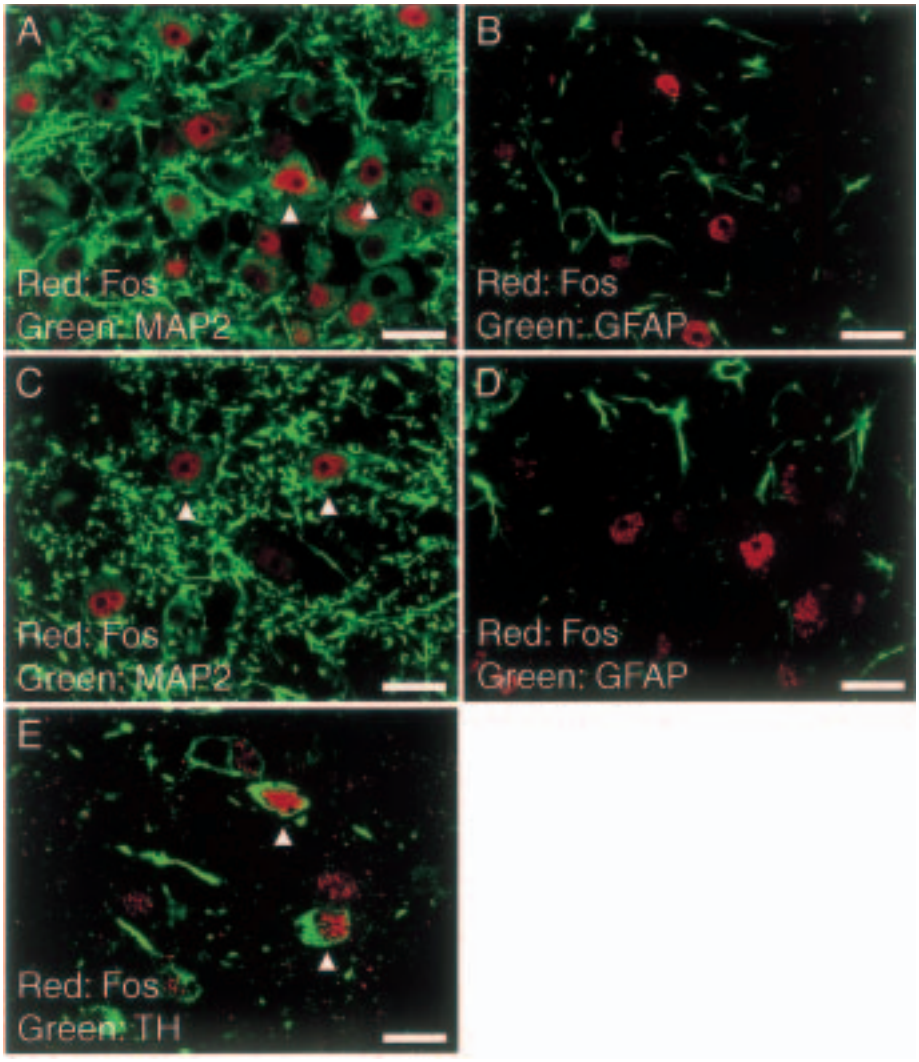

Fig. 3. Dual immunohistochemistry of Fos and either microtubule-associated protein 2 (MAP2) or glia-fibrillary acidic protein (GFAP) in the hypothalamic paraventricular (PVN, A and B) and solitary tract (NTS, C and D) nuclei; and of Fos and tyrosine hydroxylase (TH) in the NTS (E). Arrowheads indicate some dual-labeled cells. Scale bar = 20 μm.

showed that CRH mediates 2DG-induced suppression of LH secretion in estrogen-primed ovariectomized rats [18]. It has also been reported that CRH neurons show Fos expression in response to various types of stress [19–22]. In the present study, 8.3% of Fos-li cells showed TH-like immunoreactivity in the NTS after 2DG injection in OVX+E_2 group. Many noradrenergic neurons projecting to the PVN have been reported to originate in the medulla oblongata including the A2 region of the NTS [3], and to make synaptic contacts with CRH neurons in the PVN [23]. Reyes *et al.* [4] reported that many TH-like immunoreactive cells in the A2 region of the NTS show ERα immunoreactivities in 2DG-treated rats. Thus, it is plausible that estrogen may increase the sensitivity of catecholaminergic neurons in the A2 region thereby inducing Fos expression more quickly in a glucoprivic condition in the presence of estrogen than in its absence.

In summary, the present study demonstrated that 2DG-induced glucoprivation increased the number of Fos-li neurons in the NTS and PVN in an estrogen-dependent manner, but not in the raphe nuclei in the medulla oblongata. Some Fos-li neurons in the A2 region of the NTS are also

immunopositive to TH, suggesting that catecholaminergic neurons are activated by glucoprivic stimulus. We speculate that the 2DG-induced increase in the neuronal activity of catecholaminergic neurons in the A2 region is enhanced by estrogen to enable the reproductive axis to respond quickly to glucoprivic stimulus.

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