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Direct and indirect modulation of neuropeptide Y gene expression in response to hypoglycemia in rat arcuate nucleus

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Abstract Expression of neuropeptide Y (*Npy*) heteronuclear (hn) RNA, an indicator of gene transcription, was significantly increased in the arcuate nucleus of rats 30 min after insulin injection. *Npy* hnRNA levels were also increased significantly in response to hypoglycemia in rats in which the hypothalamus was deafferentated, although the absolute levels were significantly lower than in sham-operated rats. Direct effects of lowering glucose levels on *Npy* gene expression were also confirmed in hypothalamic organotypic cultures. Thus, *Npy* gene transcription in the arcuate nucleus increases rapidly in response to hypoglycemia, and both direct and indirect inputs are involved in the rapid upregulation.

Keywords: Hypoglycemia; Neuropeptide Y; Hypothalamus; Gene transcription

1. Introduction

Several lines of evidence indicate that glucoprivation activates neurons expressing neuropeptide Y (NPY) in the arcuate nucleus, one of the most potent stimulants for food intake in the central nervous systems [1]. Peripheral injection of insulin increased *Npy* mRNA expression in the hypothalamus in mice [2], and the injection of 2-deoxy-D-glucose (2DG) which blocks glucose utilization [3] also increased NPY expression in the arcuate nucleus in rats [4,5]. Central injection of 2DG reportedly induced *c-fos* mRNA expression, an immediately early gene which has been used as an indicator for neuronal activities [6], in the NPY neurons in the arcuate nucleus as early as 30 min after injection [7]. While these data suggest that NPY neurons in the arcuate nucleus are activated immediately in response to glucoprivation, it is unclear what degree of glucopenic stimuli is required to activate the NPY neurons in the arcuate nucleus.

Ritter and colleagues showed that ablation of hindbrain catecholaminergic neurons that project to the arcuate nucleus abolished increases in *Npy* mRNA expression by glucopenic stimuli [8], suggesting that glucoprivation activates the NPY neurons in the arcuate nucleus indirectly through catecholaminergic neurons in the hindbrain. On the other hand, hypothalamic neurons have been shown to directly sense changes in glucose concentrations in vitro [9–11], and previous studies demonstrated that the disruption of glucose sensing in neurons could lead to impaired glucose homeostasis in vivo [12]. A subpopulation of these glucosensing neurons, called glucose-inhibited (GI) neurons, increase their firing rates when ambient glucose levels decrease [9,11], and NPY neurons in the arcuate nucleus reportedly contain GI neurons [13,14]. These data suggest another possibility that the NPY neurons in the arcuate nucleus might directly respond to changes in glucose levels in vivo as well.

The current study was undertaken to better understand how NPY neurons in the arcuate nucleus respond to changes in blood glucose levels. To assess changes in gene transcription, we measured heteronuclear (hn) RNA, the first transcript which has been used as a sensitive indicator of gene transcription [15,16], with intronic in situ hybridization.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats [250–300 g bodyweight (BW); Chubu Science Materials, Nagoya, Japan] were housed individually in plastic cages under controlled conditions (23.0 ± 0.5 °C, lights on from 0900 to 2100), and had access to standard chow and water ad libitum until experiments. All rats were handled for five days before experiments. All procedures were performed in accordance with the institutional guidelines for animal care at Nagoya University Graduate School of Medicine and approved by the Animal Experimentation Committee.

2.2. Insulin injection

Either 1, 1.5, 2, 2.5 or 3 IU/kg insulin (Humalin R; Eli Lilly Japan, Kobe, Japan) dissolved in isotonic saline or vehicle was injected (2% BW) intraperitoneally (ip) to rats which had been fasted overnight. Rats were decapitated before and 30, 60 and 120 min after injection.

2.3. Glucose injection

Rats which had been fasted overnight were injected (2% BW) ip with either 5% glucose or isotonic saline (control). Rats were decapitated before and 30, 60 and 120 min after injection.

2.4. 2DG injection

Either 750 mg/kg 2DG (Sigma, St. Louis, MO) dissolved in isotonic saline or vehicle was injected (2% BW) ip in rats which had access to food ad libitum before injection. Rats were decapitated 30 min after injection.

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2.5. Deafferentation of hypothalamus

Deafferentation was performed according to Halasz's method with minor modifications [17]. Rats were anesthetized with an ip injection of pentobarbital (50 mg/kg) for stereotaxic surgery. The coordinates of the hypothalamus deafferentation were 2.8 mm posterior to the bregma and 9.4 mm ventral to the surface of the skull. A knife 3.5 mm in height with a 2 mm radius was lowered in the midline with the blade pointing laterally, and rotated.

2.6. Blood glucose measurement

Blood glucose levels were immediately measured with a glucose analyzer Antsense (Horiba Ltd., Kyoto, Japan).

2.7. Glucagon, epinephrine, and norepinephrine measurement

Plasma glucagon concentrations were determined using radioimmunoassay kits (TFB Inc., Tokyo, Japan), while plasma epinephrine and norepinephrine concentrations were analyzed by high-performance liquid chromatography.

2.8. Slice-explant culture procedure

Hypothalamic slice-explant cultures were performed as described previously [18]. To see whether changing medium glucose concentrations could affect *Npy* gene expression in the arcuate nucleus, the medium containing 100 mg/dl glucose was changed to that containing various concentrations of glucose (12.5–200 mg/dl). The slices were incubated for 30 min in each medium while 10^{-8} M dexamethasone (Sigma) and 10^{-6} M tetrodotoxin (TTX; Sankyo, Tokyo, Japan), which blocks action potentials [19], were added to the medium for 24 h. To see time course changes in *Npy* hnRNA expression induced by lowering glucose concentrations in the medium, the slices were incubated for 30, 60 or 120 min in the medium containing 12.5 mg/dl glucose while the control slices were incubated in the medium containing 100 mg/dl glucose for 120 min. When the medium was changed to that containing various concentrations of glucose, the medium was also added onto the surface of slices so that they were immersed in the medium and glucose concentrations in the slices would be changed immediately.

2.9. In situ hybridization

Prehybridization, hybridization and posthybridization procedures were performed as described previously [18]. Sections hybridized with *Npy* mRNA probes were dipped in nuclear Kodak NTB2 emulsion (Kodak, Rochester, NY) and exposed for 24 h. To assist cellular localization of the hybridized signals, emulsion-dipped sections were stained with cresyl violet. Any neuronal cross sections with grains of more than three-fold the background density was considered labeled.

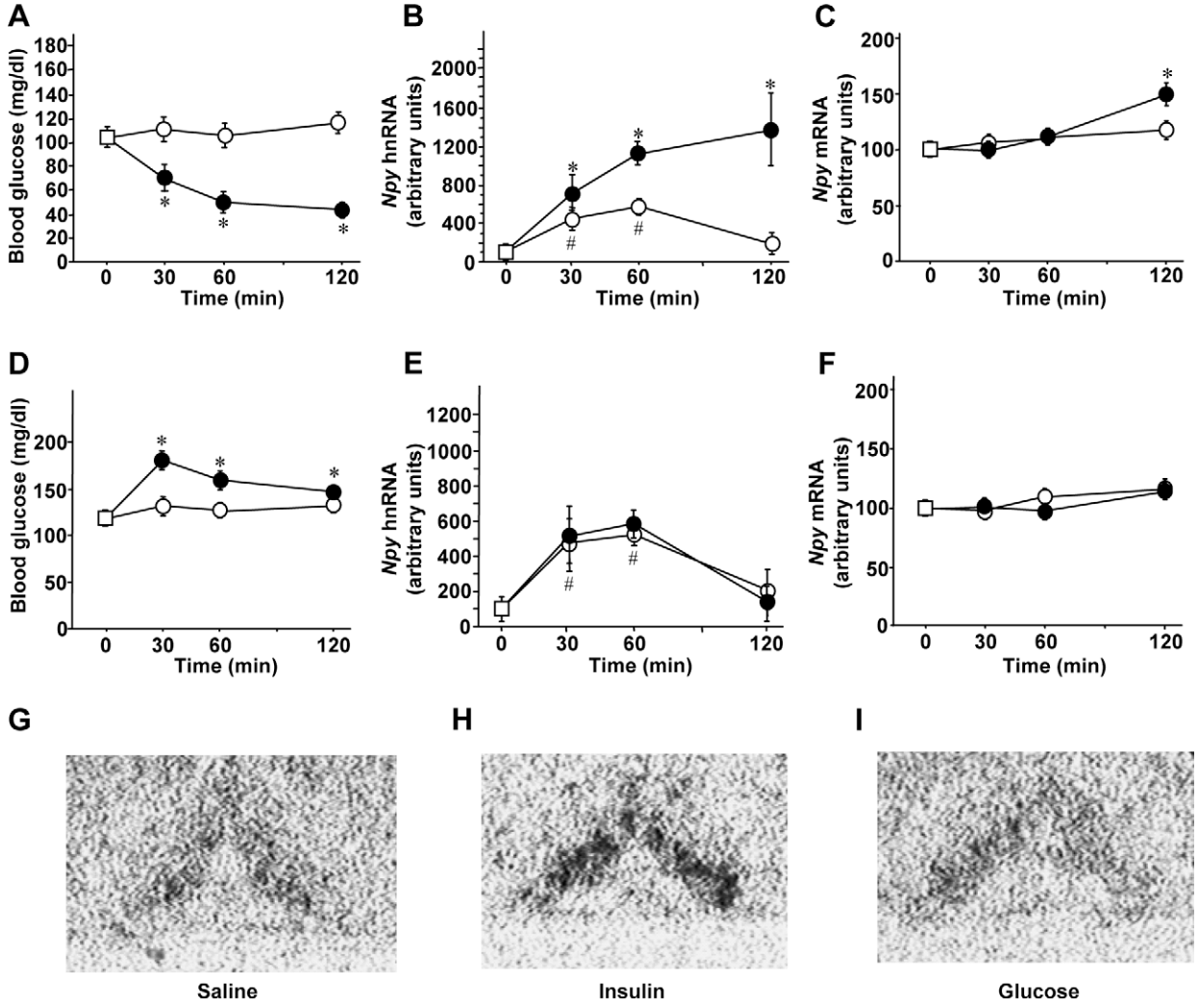

Fig. 1. Effects of decreases or increases in blood glucose levels on *Npy* gene expression in the arcuate nucleus. Isotonic saline (control, white circle) or 3 IU/kg insulin (black circle) was injected ip, and changes in levels of blood glucose (A) *Npy* hnRNA (B) and mRNA (C) were examined before (square) and 30, 60 and 120 min after injection. Isotonic saline (control, white circle) or glucose (black circle) was injected ip, and changes in levels of blood glucose (D), *Npy* hnRNA (E) and mRNA (F) were examined before (square) and 30, 60 and 120 min after injection. Representative autoradiographs of *Npy* hnRNA expression 120 min after injection are shown (G, H, I). $^{*}P < 0.05$ vs. values in control. $^{\#}P < 0.05$ vs. values at 0 min.

2.10. Statistics

Statistical significance of the differences between groups was calculated by one-way ANOVA followed by Fisher's protected least significant difference test. Results are expressed as means ± S.E., and differences were considered significant at $P < 0.05$. The mean expression levels of *Npy* hnRNA or mRNA in non-treated rats are expressed as 100 arbitrary units. The number of rats in each group was eight unless indicated otherwise.

3. Results

3.1. Effects of decreased or increased blood glucose levels on Npy gene expression in arcuate nucleus

Blood glucose levels were significantly decreased in rats injected with insulin (3 IU/kg), whereas the levels were significantly increased in rats injected with glucose compared to those injected with saline (Fig. 1A and D). *Npy* hnRNA expression levels in the arcuate nucleus were significantly increased in rats injected with saline compared to the basal levels at time 0 (Fig. 1B and E). After insulin injection, the *Npy* hnRNA expression levels were significantly increased as early as 30 min and progressively increased until 120 min (Fig. 1B). The injection of 2DG resulted in significant increases in blood glucose levels compared to saline injection (2DG: 397 ± 13; saline: 159 ± 1 mg/dl, $P < 0.01$), and significantly increased *Npy* hnRNA expression (2DG: 232 ± 27; saline: 100 ± 30 arbitrary units, $P < 0.01$). On the other hand, there were no significant differences in the levels of *Npy* hnRNA expression between glucose and saline groups at any time point examined (Fig. 1E). *Npy* mRNA expression levels in the arcuate nucleus were significantly increased only at 120 min after insulin injection (Fig. 1C and F). Representative autoradiographs demonstrating the effects of changing glucose concentrations on *Npy* hnRNA expression at 120 min are shown in Fig. 1G–I.

3.2. Threshold of blood glucose levels for activating Npy gene transcription in arcuate nucleus

While any concentration of insulin decreased blood glucose levels significantly compared to control injected with saline at 30 min, *Npy* hnRNA levels were significantly increased only in rats injected with 2.5 IU/kg and 3 IU/kg insulin, in which blood glucose levels were 74 ± 7 mg/dl and 68 ± 5 mg/dl, respectively (Fig. 2).

3.3. Effects of decreased blood glucose levels on Npy gene expression in arcuate nucleus in deafferentated hypothalamus

The deafferentation of the hypothalamus was confirmed histologically at the end of the experiments (Fig. 3A). As shown in

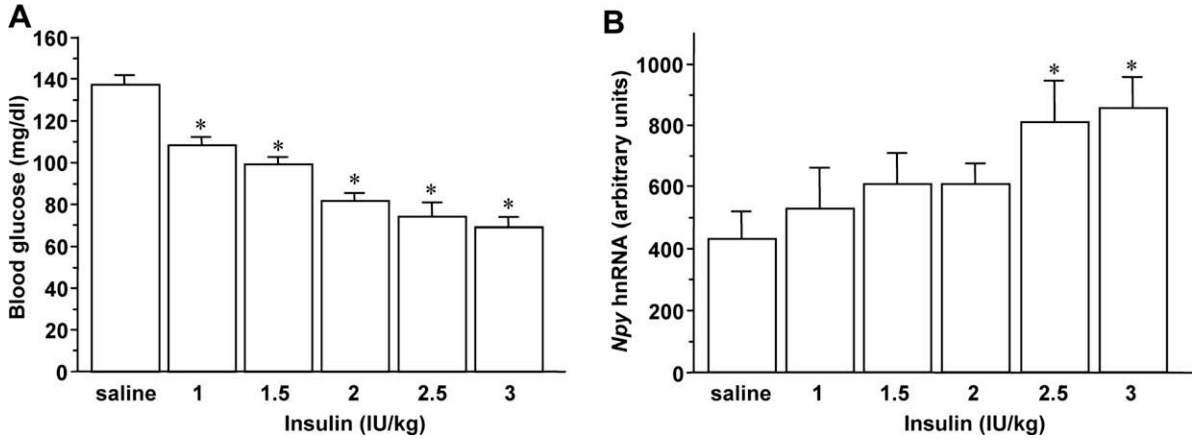

Fig. 2. Correlation between decreases in blood glucose levels and *Npy* gene expression in the arcuate nucleus. Isotonic saline (control) or various doses of insulin dissolved in isotonic saline were injected ip, and changes in levels of blood glucose (A) and *Npy* hnRNA (B) were examined 30 min after injection. $^*P < 0.05$ vs. values in control.

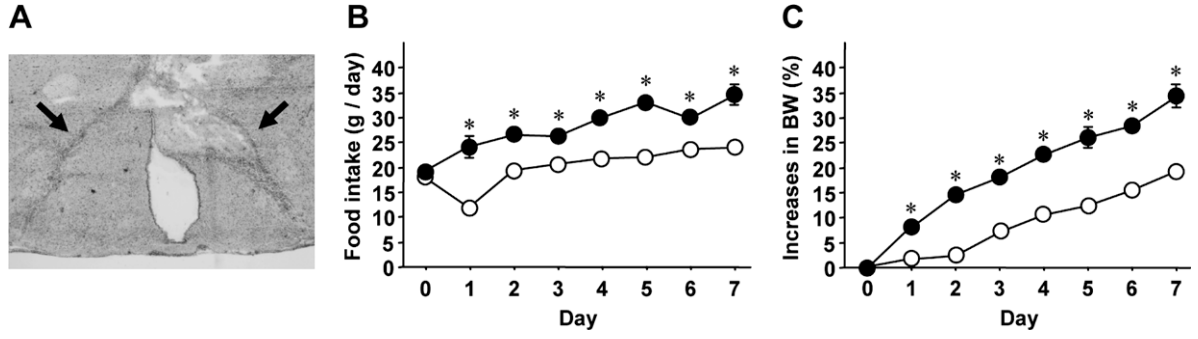

Fig. 3. Changes in food intake and BW after deafferentation of hypothalamus. Deafferentation was confirmed at the end of experiments, and arrows indicate Halasz knife cut in a brain section −2.80 mm of bregma stained with cresyl violet (A). After operation on day 1, daily food intake (B) and BW (C) were monitored in both sham-operated (white circle) and deafferentated (black circle) rats ($n = 40$). $^*P < 0.05$ vs. sham group.

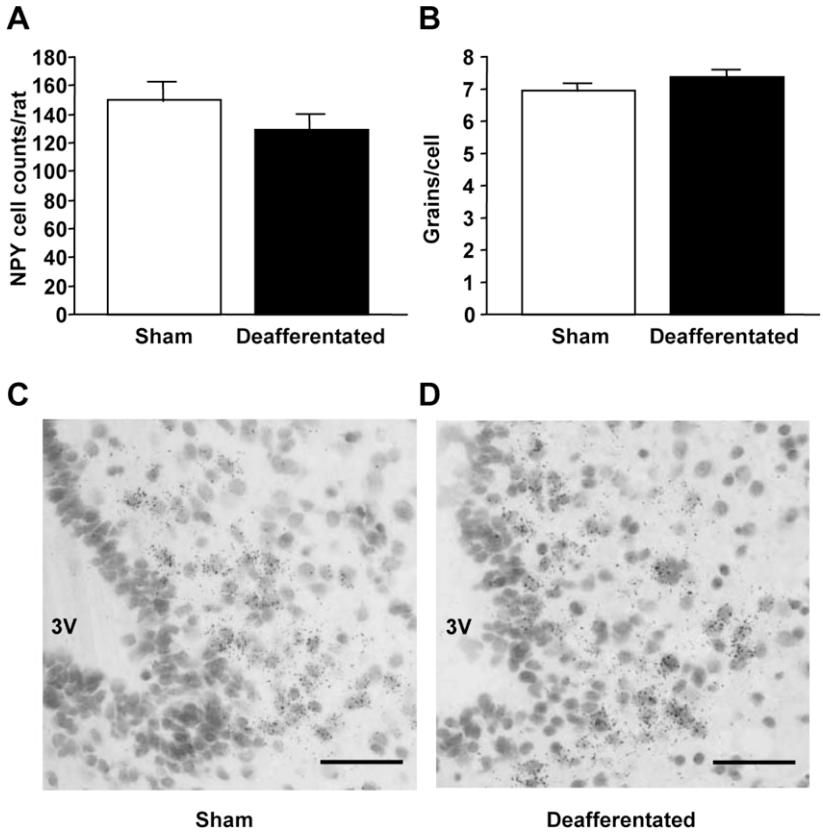

Fig. 4. Expression of *Npy* mRNA in the arculate nucleus in deafferentated and sham-operated rats. Neither cell numbers expressing *Npy* mRNA (A) nor grains per cell (B) were significantly affected by deafferentation. Representative photographs showing *Npy* mRNA expression in sham-operated (Sham) (C) and deafferentated (D) rats are shown. Scale bars indicate 50 μm. 3 V; third ventricle.

Fig. 3B and C, daily food intake and BW were significantly increased in deafferentated rats compared to sham-operated (sham) rats. Analyses with in situ hybridization using *Npy* mRNA probes demonstrated that neither cell numbers expressing *Npy* mRNA nor transcripts per cell were affected by the deafferentation (Fig. 4), indicating that deafferentation did not decrease cell numbers or viability. Administration of insulin (3 IU/kg) decreased blood glucose concentrations to similar levels in deafferentated and sham groups at 30 min (Fig. 5A and B). However, the levels were significantly lower at 120 min in the deafferentated group compared to the sham group (Fig. 5A and B). The plasma levels of epinephrine and glucagon, but not norepinephrine, were significantly increased in the sham rats injected with insulin compared to those in the sham rats injected with saline (Table 1). On the other hand, only the plasma levels of glucagon were significantly increased in the deafferentated rats after insulin injection, and the levels of epinephrine and glucagon were significantly lower in the deafferentated rats injected with insulin compared to those in the sham rats (Table 1). The *Npy* hnRNA expression levels were significantly increased in rats injected with saline compared to the basal levels at 30 min in the sham group but not in the deafferentated group (Fig. 5C and D). *Npy* hnRNA expression levels were increased 30 min and 120 min after injection of insulin in both deafferentated and sham groups compared to each control injected with saline, although the absolute values were significantly lower in the deafferentated group compared to the sham group at both time points (Fig. 5C and D).

3.4. Effects of changing glucose concentrations in medium on Npy gene expression in arcuate nucleus in hypothalamic organotypic cultures

There were no significant differences in *Npy* hnRNA expression levels in the arcuate nucleus between hypothalamic explants exposed to 200 mg/dl, 100 mg/dl and 50 mg/dl glucose (Fig. 6A). On the other hand, the *Npy* hnRNA expression levels in the explants exposed to 25 mg/dl or 12.5 mg/dl glucose were significantly increased compared to those exposed to 100 mg/dl glucose (Fig. 6A). The time course study demonstrated that *Npy* hnRNA levels continued to be elevated in medium containing 12.5 mg/dl glucose for 120 min compared to those in medium containing 100 mg/dl glucose (Fig. 6B). Representative autoradiographs showing the effects of decreasing glucose concentrations in the medium on *Npy* hnRNA expression in the hypothalamic cultures are shown in Fig. 6C and D.

4. Discussion

While GI neurons are defined as those that decrease their firing rates when glucose levels rise and these neurons respond in minutes to changes in ambient glucose levels in vitro [9,11], the physiological relevance of such rapid regulation of neuronal activities remains unclear. In this study, we first examined whether changes in blood glucose levels rapidly affected NPY neurons in vivo by examining the *Npy* hnRNA expression.

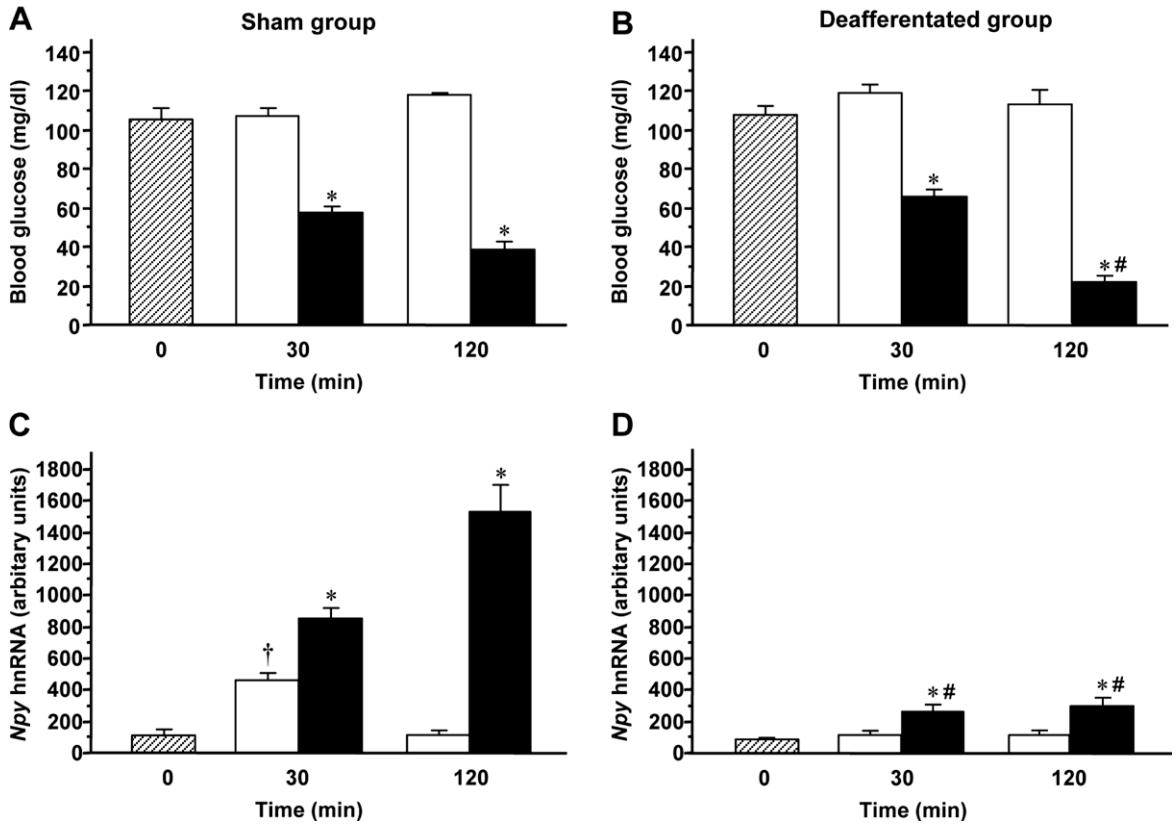

Fig. 5. Effects of decreased blood glucose levels on *Npy* gene expression in the arcuate nucleus in sham-operated and deafferentated rats. Isotonic saline (control, white bar) or 3 IU/kg insulin (black bar) was injected ip, and changes in levels of blood glucose (A, B) and *Npy* hnRNA expression (C, D) were examined before (striped bar) and 30 or 120 min after injection in both sham-operated (sham) (A, C) and deafferentated (B, D) rats. $^{*}P < 0.05$ vs. values in control. $^{\#}P < 0.05$ vs. values at each time point in sham group. $^{\dagger}P < 0.05$ vs. values at 0 min.

Table 1
Plasma levels of epinephrine, norepinephrine and glucagon 120 min after injection of either saline or insulin

		Epinephrine (ng/ml)	Norepinephrine (ng/ml)	Glucagon (pg/ml)
Sham rats	Saline	5.4 ± 0.8	1.9 ± 0.3	33 ± 4
	Insulin	10.1 ± 1.2[a]	2.7 ± 0.3	358 ± 59[a]
Deafferentated rats	Saline	5.6 ± 0.9	2.1 ± 0.3	44 ± 8
	Insulin	6.9 ± 0.8[b]	2.6 ± 0.2	184 ± 27[a,b]

[a] $P < 0.05$ vs. values in rats injected with saline in each group.
[b] $P < 0.05$ vs. values in rats injected with insulin in sham group.

The advantage of examining hnRNA rather than mRNA expression is that changes in hnRNA levels are more dynamic due to its short half-life and the relatively low levels of basal expression, so that even subtle changes in transcriptional activities which were not clear at mRNA levels could be detected [15,16]. By using this sensitive indicator, we have shown that *Npy* gene transcription in the arcuate nucleus responded within 30 min to decreases, but not to increases, in blood glucose levels in vivo. Our data also demonstrated that glucoprivation by 2DG injection increased *Npy* hnRNA levels at 30 min, suggesting that changes in *Npy* gene expression by insulin injection were caused by glucoprivation rather than insulin per se. The effects of changes in blood glucose levels on *Npy* gene transcription are consistent with a recent in vitro study demonstrating that NPY neurons in the arcuate nucleus respond to changes in glucose below but not above 5 mM [14]. Furthermore, we here provided the first evidence of the threshold of blood glucose levels to increase *Npy* gene transcription, which indicates that a moderate reduction in blood glucose levels is enough to activate the NPY neurons.

The rats in which the hypothalamus was deafferentated were: (1) hyperphagic and obese and (2) the responses of counterregulatory hormones to hypoglycemia were blunted. The first finding may be explained by the fact that the deafferentated areas involved not only NPY neurons in the arcuate nucleus but also other neurons which have been implicated in the regulation of food intake and energy metabolism, such as proopiomelanocortin neurons in the arcuate nucleus, CRH neurons in the paraventricular nucleus and those in the ventromedial hypothalamic nucleus [20,21]. The latter finding may probably

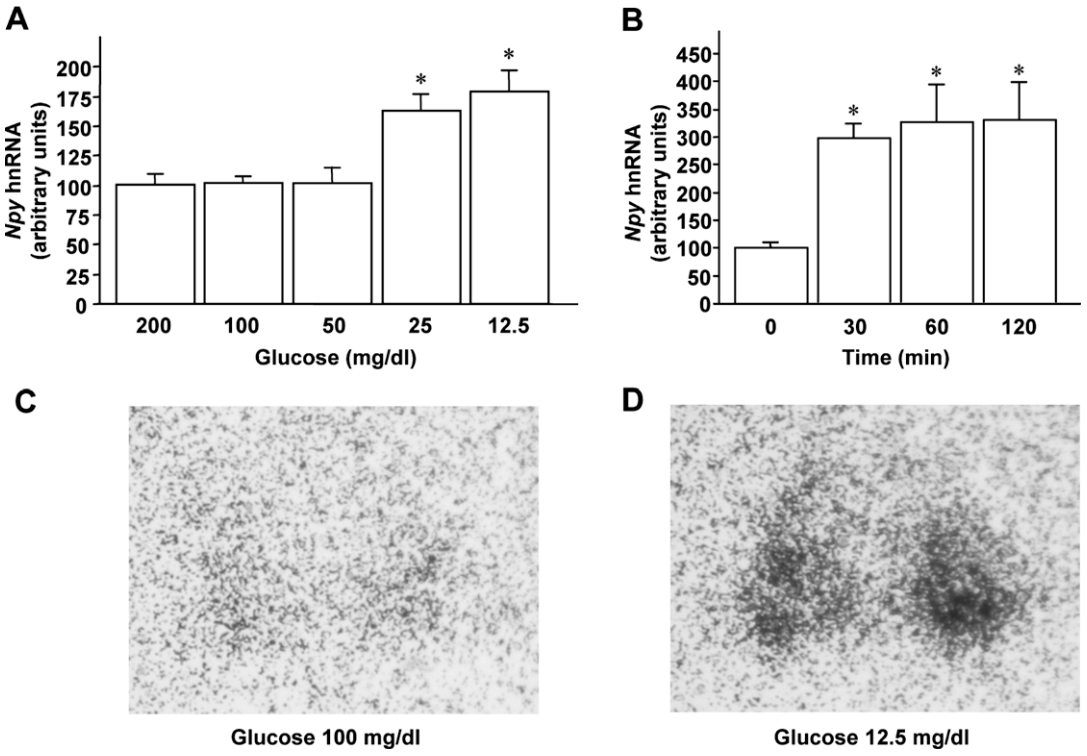

Fig. 6. Effects of changing glucose concentrations in medium on *Npy* gene expression in the arcuate nucleus in hypothalamic organotypic cultures. *Npy* hnRNA expression was examined 30 min after exposure of hypothalamic slices to medium containing 200, 100, 50, 25 or 12.5 mg/dl glucose (A). Time course changes are shown in *Npy* hnRNA expression in hypothalamic slices exposed to medium containing 12.5 mg/dl glucose (B). Representative autoradiographs showing the effects of decreasing glucose concentrations in medium on *Npy* hnRNA expression are demonstrated (C, D). The mean expression levels of *Npy* hnRNA cultured in the medium containing 100 mg/dl glucose are expressed as 100 arbitrary units ($n = 10$). $^{*}P < 0.05$ vs. values in 100 mg/dl glucose medium (A) or those at 0 min (B).

be explained by the fact that the ventromedial hypothalamic nucleus, which has been shown to be implicated in the regulation of the release of glucagon, epinephrine and norepinephrine in response to hypoglycemia [22], was involved in the area cut with the Halasz's knife. The deafferentation of NPY neurons in the arcuate nucleus might also be responsible for the blunted release of counterregulatory hormones, although a previous study reported that the response of glucagon release to hypoglycemia was intact in *Npy* knockout mice [2]. Despite the more severe hypoglycemia 120 min after insulin injection, the increases in *Npy* gene transcription were only mild in the deafferentated rats compared to sham rats (Fig. 5C and D). This is consistent with a previous study in which ablation of hindbrain catecholaminergic neurons that project to the arcuate nucleus abolished the increase in *Npy* mRNA expression by glucopenic stimuli [8], and suggests that signals mediating *Npy* gene expression in response to hypoglycemia were mostly indirect. On the other hand, our results also demonstrated that *Npy* hnRNA expression levels increased two-fold in deafferentated rats 30 min after insulin injection, as in sham-operated rats (Fig. 5C and D). These data suggest that *Npy* gene transcription could also be upregulated directly by glucopenia at this early time point.

It is reported that glucose levels in CSF and in the interstitial fluid in hypothalamus were about 50% [23] and 20–30% [24,25] of blood glucose levels, respectively. The glucose levels in the central nervous systems reportedly followed changes in blood glucose levels rapidly in vivo [23,24], and NPY neurons have been shown to respond to drastic decreases in glucose levels in the medium in vitro [13,14]. In this study, we clarified that the threshold of glucose levels which directly increase the *Npy* gene transcription is around 25 mg/dl, which is 30–40% of the blood glucose levels (around 70 mg/dl) which activate the NPY neurons in vivo, suggesting that decreases in glucose levels in the central nervous system following hypoglycemia could indeed stimulate *Npy* gene transcription directly.

The arcuate nucleus has been implicated in the regulation of stress responses [26], and several stressors such as restraint stress and foot shock reportedly increased *Npy* mRNA expression in the arcuate nucleus [27,28]. The ip injection of isotonic saline is shown to increase *c-fos* expression in several brain areas which have been implicated in stress response [29], suggesting that ip injection per se is a stressor. Interestingly, the significant increase in *Npy* hnRNA after ip injection of saline was completely abolished in the deafferentated rats in the present study. These data suggest that the stress accompanied by ip injection was neuronally conveyed to the NPY neurons in the arcuate nucleus.

In conclusion, our data demonstrated that *Npy* gene transcription in the arcuate nucleus rapidly increased in response to decreases in blood glucose levels through direct and indirect inputs.

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