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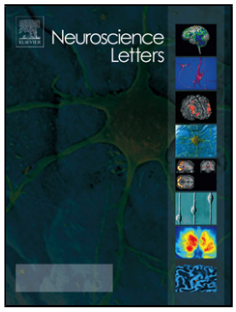

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Short-term fasting decreases excitatory synaptic inputs to ventromedial tuberoinfundibular dopaminergic neurons and attenuates their activity in male mice

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Highlights

- Whole-cell voltage-clamp techniques were applied to investigate TIDA neuron activity.
- Miniature excitatory postsynaptic currents in TIDA neurons were reduced by fasting.
- Fasting caused reduced cFos expression in TIDA neurons and increased serum PRL levels.
- Taken together, our results showed that fasting attenuated TIDA neuron activity.

Abstract

Tuberoinfundibular dopaminergic (TIDA) neurons in the arcuate nucleus (ARC) of the hypothalamus play a role in inhibiting prolactin (PRL) secretion from the anterior pituitary. PRL is involved in a variety of behaviors, including feeding. Consequently, we hypothesized that fasting might reduce the activity of TIDA neurons, which might alter PRL secretion. However, direct examinations of TIDA neuron activity are difficult. Recently, transgenic mice were generated that expressed green fluorescent protein (GFP) under the control of the rat tyrosine hydroxylase gene. We first determined that GFP in the dorsomedial ARC was a reliable marker of TIDA neurons. Then, we performed electrophysiology and immunocytochemistry in GFP-labeled TIDA neurons to examine whether different feeding conditions could change their activity. Eight-week-old male mice were fed or fasted for 24 h. After sacrifice, we prepared acutely isolated brain slices for conducting whole-cell voltage-clamp recordings. TIDA neurons were identified with fluorescence microscopy. The mean amplitude of miniature excitatory postsynaptic currents (mEPSCs) was significantly reduced in fasting mice compared to fed mice, but different feeding conditions did not affect the mean mEPSC intervals. This result

suggested that fasting reduced the number of excitatory synaptic inputs to TIDA neurons. To determine whether a reduction in excitatory synaptic inputs would cause a reduction in TIDA neuron activity, we examined the effect of 24-h fasting on c-Fos expression in the ARC. We found that fasting significantly reduced the number of Fos-positive TIDA neurons. In addition, serum PRL levels were significantly increased. Taken together, the present findings suggested that short-term fasting attenuated TIDA neuron activity.

Keywords: TIDA, PRL, miniature excitatory postsynaptic current, patch clamp, slice, cFos, mediobasal hypothalamus, fasting

Introduction

Tuberoinfundibular dopaminergic (TIDA) neurons are located in the dorsomedial arcuate nucleus (ARC), adjacent to the periventricular region of the mediobasal hypothalamus. TIDA neurons release dopamine (DA) into the portal vein [24, 27, 29, 49]. DA released from the hypothalamus was shown to inhibit prolactin (PRL) secretion from the anterior pituitary [9, 11, 23, 44]. Accordingly, TIDA neurons are thought to inhibit PRL secretion, and thus, they have neuroendocrine functions [24, 27, 29, 49].

PRL is a multifunctional hormone, involved in reproduction, metabolism, osmoregulation, and immunoregulation [11]. With respect to its metabolic function, PRL plays roles in body weight regulation, pancreatic development, insulin secretion, and adipose tissue physiology [9]. In the brain, PRL acts as a feeding stimulant [10, 38, 39, 45, 46]. In PRL receptor knockout mice, the loss of PRL signaling caused reductions in body weight [3, 12] and resistance to high fat diet–induced obesity, due to enhanced energy expenditure and increased metabolic rates [6].

As noted, PRL secretion is controlled by the tonic release of DA from TIDA neurons [24, 27, 29, 49]. Consequently, we hypothesized that fasting might attenuate TIDA neuron activity, and thereby increase PRL secretion. However, it has not been well documented whether the activity of TIDA neurons changes during different feeding states [2]. This issue has been difficult to investigate, at least in part, due to the difficulty in identifying TIDA neurons to enable direct recordings in vivo. Recently, transgenic mice were developed that expressed green fluorescent protein (GFP) under the control of the rat tyrosine hydroxylase (*TH*) gene (B6.Cg-Tg(TH-GFP)21-31) [34]. With this new tool, we investigated the activity of GFP-expressing TIDA neurons in mouse brains after short-term feeding and fasting conditions. We aimed to delineate whether changes in the excitatory synaptic inputs to TIDA neurons occurred with different feeding states.

Materials and Methods

We performed electrophysiological experiments with male C57BL/6J mice (Charles River Japan) or mice transfected with a plasmid that carried the *GFP* gene sequence under the control of the rat *TH* gene (B6.Cg-Tg(TH-GFP)21-31, RBRC02095) [34]. Mouse genotypes were confirmed by isolating genomic DNA from ear punches and amplifying the target gene with PCR (N-terminal primer: CCT GTG ACA GTG GAT GCA ATT; C-terminal primer: CTT GTA CAG CTC GTC CAT GCC GAG). Mice were fed standard rodent food and water, ad libitum, and housed in our animal center at 23±1 °C, with a 12:12 h light–dark photoperiod (lights off, 0600–1800). Experimental mice were fasted for 24 h (n = 6), and control mice were fed under normal conditions (n = 4). For immunocytochemical experiments, mice were fed normally (n = 5), fasted for 24 h (n = 3), or fasted for 24 h, then treated with leptin (1 mg/ml injected at volumes to achieve 1 μg/g body weight, n = 3; PeproTech Inc). Leptin was injected 45 min before the mice were killed. For Western blots, mice were fed (n = 4), fasted for 24 h (n = 4), or fasted for 24 h, then treated with leptin at the doses used in the immunocytochemical experiments (n = 4-5). To determine serum PRL levels, mice were fed (n = 9), fasted for 24 h (n = 9), or fasted for 24 h and treated with leptin at the same dose as above (n = 5); the blood was collected after decapitation. All experiments were performed according to the Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences (Physiological Society of Japan). The protocols were approved by the Institutional Committee for Animal Experiments. All data were analyzed with the *t*-test or one-way analysis of variance (ANOVA), followed by Fisher's least significant difference post-hoc test. Differences were considered significant at $P < 0.05$.

Electrophysiology

Mice were anesthetized with isoflurane gas, and the brains were removed. Each brain was quickly transferred into ice-cold dissection buffer (25.0 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 0.5 mM $CaCl_2$, 7.0 mM $MgCl_2$, 25.0 mM glucose, 110.0 mM choline chloride, 11.6

mM ascorbic acid, and 3.1 mM pyruvic acid) bubbled with 5% CO_2/95% O_2. Coronal brain slices were cut with a microslicer (Dosaka Pro 7) in dissection buffer and transferred to physiological solution (22–25°C, pH 7.4, bubbled with 5% CO_2/95% O_2; 118 mM NaCl, 2.5 mM KCl, 26.2 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose, 1.3 mM $MgCl_2$, and 2.5 mM $CaCl_2$). The recording chamber was perfused with physiological solution containing 0.1 mM picrotoxin and 4 M 2-chloroadenosine at 22–25 °C to reduce basal activity. Patch recording pipettes (4–6 MΩ) were filled with intracellular solution (115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 10 mM sodium phospho-creatine, and 0.6 mM EGTA at pH 7.25). Whole-cell ionic currents were recorded from TIDA neurons with an Axopatch-1D amplifier (Axon Instruments, USA). Data are expressed as the percentage of the control values. Data were analyzed with Mini Analysis software (Synaptosoft Inc), which provided nonparametric tests for statistical comparisons. Measurements of the mean amplitude and the mean interval of miniature excitatory postsynaptic currents (mEPSCs) were recorded in bath solution (118 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10 mM glucose, 1 mM $MgCl_2$, and 4 mM $CaCl_2$), which contained 0.5 µM tetrodotoxin and 0.1 mM picrotoxin. The mEPSCs were detected by setting the amplitude threshold to 3× the background noise level. The frequency and amplitude were analyzed with the Mini Analysis program.

Immunocytochemistry

We performed tyrosine-hydroxylase and GFP staining. Briefly, mice were anesthetized with isoflurane (male mice, $n = 6$; female mice, $n = 3$). Next, colchicine was dissolved in sterile saline (10 µg/µl) and injected into the lateral ventricles at a dose of 4 µg/30 g body weight. Forty-eight hours later, mice were intraperitoneally injected with an overdose of pentobarbital sodium (100 mg/kg). Then, mice were perfused through the cardiac ventricle with 4% paraformaldehyde in phosphate buffer (pH 7.5) at 4°C. Brains were removed and frozen at -80°C. Next, frozen coronal brain sections were cut (30 µm thick) with a cryostat. Sections were

incubated overnight with anti-TH monoclonal antibody (MAB318, Calbiochem, 1:1,2000). The next day, sections were incubated with biotinylated anti-mouse IgG diluted 1:200 in phosphate-buffered saline containing 1.5% NHS and 0.05% Triton X-100. Then, sections were incubated with the streptavidin–biotin–peroxidase complex (Vectastain Elite ABC Kit). Bound peroxidase was visualized by incubating the sections for 2 min in 0.05% 3.3′-diaminobenzidine with H_2O_2. Sections were mounted on glass slides, dehydrated in graded alcohol, cleared with xylene, and mounted on coverslips with Permount.

For double-staining, sections were first incubated overnight with anti-cFos rabbit polyclonal antibody (PC38, Oncogene, 1:40,000). Then, secondary antibody and reagents were added, and the bound peroxidase was visualized, as described above for TH staining. Next, the sections were incubated with anti-TH monoclonal antibody biotinylated anti-mouse IgG diluted 1:200, then with Cy3-labeled streptavidin (Amersham Pharmacia Biotech). Cells that displayed Fos immunoreactivity (Fos-IR) were double-stained for TH and Fos; they exhibited a blue–black nucleus (Fos-IR cells), surrounded by a fluorescent cytoplasm (TH-IR cells) at 200× magnification.

Biochemical analyses

Mice were killed with isoflurane anesthesia, and tissues, including the hypothalamus, were dissected [33]. Tissues were immediately frozen in liquid N_2, then mechanically homogenized in 10–20 volumes of ice-cold RIPA lysis buffer (50 mM HEPES [pH 7.4], 1% (v/v) Triton X-100, 1% (v/v) sodium deoxycholate, 0.1% (v/v) SDS, 150 mM NaCl, 10% (v/v) glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 50 mM NaF, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM benzamidine, 2 mM phenylmethysulfonyl fluoride, and 1 mM sodium vanadate). Homogenates were clarified by centrifugation (12,000 ×*g* for 20 min at 4°C). Tissue lysates (protein concentration 50 ng/μl) were resolved (30 μl/sample) with SDS-PAGE and transferred to membranes, as described previously [48]. Membranes were blocked for 1 h at room temperature with 5% bovine serum albumin in Tris-buffered saline and Tween-20, then incubated overnight

with antibodies to phosphorylated signal transducer and activator of transcription 3 (pSTAT3; 4093, Cell Signaling, 1:1,000), total STAT3 (9139, Cell Signaling, 1:1,000), pSTAT5 (9351, Cell Signaling, 1:1,000), total STAT5 (9363, Cell Signaling, 1:1,000), β-actin (3700, Cell Signaling, 1:2,000), and cytokine-inducible SH2 protein (SC15344, Santa Cruz Biotechnology, 1:1,000). Next, the membrane was incubated with horseradish peroxidase–labeled secondary antibody (1:3,000 dilution). Then, signals were visualized with Luminate™ Crescendo Western HRP substrate (Millipore). The membrane was exposed to X-ray film (RX-U, FUJIFILM) for 30–300 s. Densitometric analyses of the bands were performed with NIH ImageJ software, and the arbitrary units were normalized to appropriate controls.

Serum PRL assay

Serum PRL levels were measured with a commercially available kit (Mouse PRL ELISA Kit, CSB-E06882m; CUSABIO BIOTECH CO., LTD).

Results

Electrophysiology

Many GFP-positive cells in the dorsomedial ARC were identified with fluorescence microscopy [18, 31, 36]. These cells also exhibited TH immunoreactivity (Fig. 1A). The ratios of TH-positive cells to GFP-positive cells were 85.0% ± 2.5 in male mice (n = 6) and 84.4% ± 2.8 in female mice (n = 3; Fig. 1B), with no significant difference between the sexes (P >0.5). Only a small number of GFP-positive cells were negative for TH immunoreactivity (Fig. 1A, white arrow). These results indicated that GFP-positive cells in the dorsomedial ARC could be reliably identified as TIDA neurons [5, 14, 36, 42], as reported by Brown et al. [14].

We recorded mEPSCs in TIDA neurons in brain slices from fed (Fig. 1C) and 24-h fasted male mice (Fig. 1D). Twenty-four–hour fasting seemed to result in a reduction in the mEPSC amplitudes without affecting the firing intervals. A quantitative analysis revealed that the mean amplitude measured in brains from fed male mice (Fig. 1E, n = 4 mice and 484 events;

amplitude = 34.9 pA ± 0.86) was significantly higher than the mean amplitude measured in brains from 24-h fasted male mice (n = 6 mice and 984 events; amplitude = 29.8 pA ± 0.50; $P < 0.05$). However, the mean firing intervals of mEPSCs (Fig. 1F) did not differ significantly between fed (462 events, 869 ms ± 47) and fasted (947 events, 901 ms ± 53) male mice ($P > 0.5$). This result suggested that the number of excitatory synaptic inputs to TIDA neurons had decreased with fasting in male mice.

Immunocytochemistry

As shown in representative images of immunocytochemical experiments, 24-h fasting induced Fos expression in the ARC (Fig. 2A). In the fed condition, some Fos-IR cells were observed in the ventrolateral ARC. However, after 24-h of fasting, the number of Fos-IR cells increased in the dorsomedial ARC (Fig. 1A). This induction seemed to be attenuated with leptin treatment. A quantitative analysis confirmed that the number of Fos-IR cells in the ARC was significantly increased after 24-h fasting (Fig. 2B; ANOVA, $P < 0.0001$; post-hoc, $P < 0.05$). However, this increase in Fos induction with fasting was not affected by leptin treatment (post-hoc, $P < 0.05$). The number of TH-IR cells (TIDA neurons) did not change with the different feeding conditions (fed 22.1±1.5, fasted 24.7±0.3, fasted+leptin 26.9±2.3/TIDA area). However, we found that, among the TH-IR cells in the hypothalamus (Fig. 1A, TIDA), approximately 20–30% expressed Fos in the fed condition (Fig. 2C and 2D). Moreover, the number of Fos-positive TH-IR cells significantly decreased in 24-h fasted mice, compared to fed mice (Fig. 2D; ANOVA, $P < 0.05$; post-hoc, $P < 0.05$). Leptin treatment in 24-h fasted mice did not cause a significant change in Fos expression in TH-IR cells (Fig. 2D). This result suggested that TIDA neurons were activated under fed conditions in male mice.

Western blotting

We examined the phosphorylation of STAT3 and STAT5, which are second messengers controlled by leptin [43] and PRL binding [17, 30, 37]. As shown in Fig. 3A, 24-h of fasting did

not affect the phosphorylation of STAT3 in the hypothalamus. However, as expected, leptin treatment in 24-h fasted mice significantly increased the phosphorylation of STAT3 in the hypothalamus (ANOVA, $P < 0.05$; post-hoc, $P < 0.05$). Similarly, 24–hour fasting was not associated with a significant change in phosphorylated STAT5 in the hypothalamus (post-hoc, $P > 0.1$); but leptin treatment significantly increased STAT5 phosphorylation levels compared to the levels observed in the fed and fasted states (ANOVA, $P < 0.005$; post-hoc, $P < 0.05$). The expression of cytokine-inducible SH2 protein did not differ among the different feeding groups (ANOVA, $P > 0.5$).

Serum PRL

As shown in Fig. 3D, serum levels of PRL were significantly increased in 24-h fasted male mice (post-hoc, $P < 0.02$) and in leptin-treated 24-h fasted male mice (post-hoc, $P < 0.003$), compared to the fed male mice (ANOVA, $P < 0.007$).

Discussion

To the best of our knowledge, the present study was the first to investigate directly how fasting affected the electrical synaptic input to TIDA neurons in male mice. It is believed that presynaptic and postsynaptic functions depend, respectively, on the amplitude and frequency of quantal synaptic responses [20, 41]. According to this quantal theory, it was suggested that the amplitude of mEPSCs depend on the numbers of postsynaptic receptors, and that the frequency of mEPSCs is related to the probability of transmitter release. In the present study, we found that the amplitude of excitatory synaptic input was reduced in TIDA neurons during fasting; accordingly, this finding indicated that fasting reduced the number of transmitting receptors.

We found that TH-IR cells displayed Fos-IR under normal feeding conditions, but Fos expression was reduced with fasting. It is well documented that fasting induces c-Fos expression in the ARC of the hypothalamus [32, 50], mostly in NPY neurons [8, 19]. Thus, the present study was consistent with previous results. Furthermore, our results suggested that the

immunocytochemistry system used in the present study was an effective detection method. Consequently, our present findings inferred that we should detect an effect on PRL secretion, and accordingly, we observed an increase in PRL levels. Thus, we concluded that TIDA neuronal activity was reduced by fasting and that this reduced activity, in turn, caused an increase in PRL secretion. Alternatively, under normal feeding conditions (the fed state), TIDA neurons are active, and they tonically inhibit PRL secretion.

Previous studies have not reported consistent findings in PRL dynamics during changes in feeding conditions. Some studies reported that starvation for 3 days and refeeding for a short period of time did not affect serum PRL levels [51], and PRL levels did not change during fasting [22, 47]. In addition, the levels of DA and its metabolites were not altered in the median eminence after 3 days of fasting in male rats [2]. Among virgin female mice, TH-IR cells in the ARC showed no change in Fos expression after 24-h of fasting, compared to levels measured in fed animals, and PRL secretion was not altered [40]. Thus, serum PRL may have remained unchanged because TIDA neural activity was not altered during fasting. On the other hand, prolonged fasting was associated with reduced PRL secretion [1, 15, 35]. Fasting was shown to stimulate TIDA neuronal activity, which thereby inhibited PRL secretion via orexin A and neuropeptide Y systems; however, in that study, TIDA neuronal activity was indirectly measured [26]. It was also reported that fasting caused increases in serum PRL [7]. Thus, whether fasting stimulates, inhibits, or has no effect on PRL secretion remains unclear. In general, PRL induces feeding behavior [10, 38, 39, 45], and thus, it acts as an orexinergic factor. PRL induces the expression of neuropeptide Y through activation of the JAK2/STAT3 [43] signaling pathway and STAT5 phosphorylation [25]. Thus, the fasting-induced PRL release found in the present study seemed to be reasonable. Regardless, we do not currently have direct evidence for resolving this discrepancy.

The nutritional regulation of TIDA activity is obscure and complicated [16]. The obscurity lies, at least in part, in the complex feedback mechanism. Indeed, feeding activates NPY neurons, which activate TIDA neurons [26], which in turn, inhibit PRL secretion [9]. However, in the

brain, PRL induces feeding. Recently, an optogenetic stimulation study revealed that the activation of TIDA neurons increased food intake through the co-release of DA and GABA. That study suggested that TIDA neurons played a role as a hunger factor in energy homeostasis [52]. In addition, they showed that the effect of fasting on TIDA neurons [52] was opposite to the effect shown in the present study, in terms of Fos expression in TIDA neurons. Nevertheless, both studies showed that TIDA neurons were involved in regulating feeding behavior, and vice versa. Alternatively, TIDA neurons might participate in both DA release and feeding regulation. A recent study indicated that it is likely that another neuronal population of TIDA neurons is involved in other aspects of hypothalamic functions [13].

One might expect that fasting-induced PRL secretion would lead to the negative feedback regulation of PRL on TIDA neurons, through effects on STAT3/5 signaling. However, in the present study, we did not detect changes in either hypothalamic STAT3 or STAT5 phosphorylation during fasting. Therefore, we initially suspected that cytokine-inducible SH2 protein might be involved in the escape of TIDA neurons from PRL activation of the JAK/STAT pathway [28, 43]. Indeed, previous studies showed that cytokine-inducible SH2 protein was related to diminished sensitivity to PRL-induced phosphorylation of STAT5 [4, 21]. [21]. [4]. However, in the present study, we found that the expression of cytokine-inducible SH2 protein was not affected by feeding status or leptin treatment. Based on our current data, we concluded that cytokine-inducible SH2 protein was not involved in our finding that fasting did not affect STAT3 and STAT5 phosphorylation.

In conclusion, we showed that fasting attenuated the activity of TIDA neurons, which then increased PRL secretion.

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Figure legends

Figure 1. Activities of TIDA neurons under different feeding conditions.

(A) Representative photographs of TH-IR (*left panel*) and GFP-expressing (*right panel*) cells in the dorsomedial ARC. Scale bar = 50 μm. White arrow indicates a GFP-positive cell that did not express TH-IR. (B) There was no difference between male and female mice in the number of TH-IR cells that also expressed GFP. (C, D) Representative profiles of mEPSCs recorded in TIDA neurons identified by fluorescence microscopy, after (c) normal feeding or (D) 24-h fasting. (E, F) Quantitative analyses of (E) the mean amplitudes of mEPSCs and (F) the mean firing intervals of mEPSCs in TIDA neurons in brain slices acquired from fed (open columns) and 24-h fasted (filled columns) male mice. Columns and error bars indicate the mean ± standard error of the mean.

Figure 2. Effects of feeding state and leptin treatment on the expression of Fos in the ARC area.

(A) Representative photographs of Fos-IR cells in mouse hypothalamus in the fed, 24-h fasted, and 24-h fasted + leptin-treated states in male mice. Scale bar = 200 μm. In the area indicated with bold lines, labeled ARC, Fos-IR cells were counted (data in Figure 2B). The area indicated with bold lines, labeled TIDA, shows the tuberoinfundibular region, and this area was enlarged in Fig. 2C. (B) Quantitative analysis of the number of Fos-IR cells in the ARC under different feeding conditions. (C) Representative image of cells that are both Fos-positive (blue–black nuclei) and TH-positive (white cytoplasm, TIDA). Scale bar = 50 μm. (D) Quantitative analysis of the percentage of TH-IR (TIDA) + Fos-IR cells found in the ARC under different feeding conditions. Columns and error bars indicate the mean ± standard error of the mean.

Figure 3. Quantitative analysis of proteins expression in the hypothalamus in male mice.

Upper panels show quantitative analyses, and lower panels show representative images of immunoblots probed for the indicated proteins. Blots were prepared with lysates from brains

dissected from mice in the fed, 24-h fasted, and 24-h fasted + leptin states. Phosphorylated and total protein levels are shown for (A) STAT3 (80 kDa); (B) STAT5 (90 kDa), and (C) cytokine-inducible SH2 proteins (CIS, 32 kDa). (D) Serum levels of PRL. Columns and error bars indicate the mean ± standard error of the mean. In (D), the numbers in parentheses refer to the number of mice in each feeding group. For other panels, n = 4 or 5 mice per feeding group.

Figures: