Temporal expression of estrogen receptor α in the hypothalamus and medulla oblongata during fasting: a role of noradrenergic neurons

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Abstract

Fasting-induced LH suppression is augmented by estrogen in female rats. We investigated the temporal changes in the number of estrogen receptor α (ER α)-immunoreactive (ir) cells in various brain regions in ovariectomized rats fasted for 6, 24, 30, and 48 h, commencing at 1300 h. We also determined the anatomical relationship of ER α immunoreactivity and dopamine- β -hydroxylase (DBH) neurons in the A2 region of the nucleus of the solitary tract (NTS) and the paraventricular nucleus (PVN). The number of ER α -ir cells significantly increased after 30 h from the onset of fasting in the PVN and NTS compared with the unfasted controls and was sustained until 48 h. In the A2 region of 48-h fasted rats, 46.75% DBH-ir cells expressed ER α ,

and this was significantly higher than in unfasted controls (8.16% DBH-ir cells expressed ER α). In the PVN, most ER α -ir neurons were juxtaposed with DBH-ir varicosities. These results suggest that ER α is expressed in specific brain regions at a defined time from the onset of fasting. In addition, the anatomical relationship of noradrenergic and ER α -ir neurons in the A2 region and PVN may suggest a role for estrogen in increasing the activity of noradrenergic neurons in the A2 region and enhancing sensitivity of the PVN to noradrenergic input arising from the lower brainstem and thereby augmenting the suppression of LH secretion during fasting.

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Introduction

Among the environmental factors that control mammalian reproduction, food availability plays a vital role and probably acts as a proximate regulator of reproductive performance (Bronson 1989). Indeed, repeated investigations have shown that 48-h fasting dramatically suppresses pulsatile luteinizing hormone (LH) secretion in female rats, and the suppression is largely dependent on the estrogenic milieu (Cagampang *et al.* 1991, Maeda *et al.* 1996). As such, 48-h fasting suppressed LH secretion only in intact and estradiol-treated rats but not in ovariectomized (OVX) rats (Cagampang *et al.* 1991, Maeda *et al.* 1996). Estrogen is also essential in modulating reproductive functions during seasonal shifts in reproductive activity (Goodman *et al.* 1981).

Several lines of evidence suggest a significant role for estrogen receptor (ER) in mediating reproductive events during energy-deficient conditions. In OVX Syrian hamsters, food deprivation decreases the number of ER-immunoreactive (ER-ir) cells in the ventromedial hypothalamus (VMH) and area lateral to it, and increases the number of ER-ir cells in the medial preoptic area (mPOA; Li *et al.* 1994)

and parvocellular paraventricular nucleus (PVN; Panicker *et al.* 1998). These changes in ER expression are associated with the suppression of copulatory behavior during malnutrition. In OVX rats, 48-h fasting significantly increases the number of ER α -ir cells in the PVN, periventricular nucleus (PeVN) and A2 region of nucleus of the solitary tract (NTS), with no appreciable alteration in the VMH, POA, and arcuate nucleus (ARC; Estacio *et al.* 1996a). In a similar manner, these increases in ER α expression could be associated with fasting-induced LH suppression. In addition, in prepubertal mice, 48-h underfeeding reduces ER-ir cells in the mPOA, VMH and ARC (Roemmich *et al.* 1997), which could be correlated with delayed puberty during undernutrition.

An increase in ER α expression by 48-h fasting in the PVN and A2 region of the NTS (Estacio *et al.* 1996a), could augment the binding potential of estrogen, and therefore, might explain the estrogen-dependent suppression of LH secretion during fasting. Thus, a fasting-induced increase in ER α expression may play a key role in regulating LH secretion. 2-Deoxy-D-glucose (2DG)-induced glucoprivation suppresses LH release (Nagatani *et al.* 1996) and increases ER α -ir cells in the PVN and NTS within 1 h (Reyes *et al.* 2001), suggesting that ER α

expression could be acutely stimulated. However, unlike glucoprivation, fasting generates more complex physiological signals and takes longer to suppress LH secretion compared with 2DG-induced glucoprivation (Panicker *et al.* 1998). Therefore, we determined the time course of ER α expression in the PVN and NTS from the onset of fasting, thereby identifying more fully the mechanism underlying the fasting-induced LH suppression.

Previous studies have demonstrated noradrenergic regulation of estrogen binding in the hypothalamus (Blaustein *et al.* 1986, Blaustein 1987, Blaustein & Turcotte 1987). Noradrenergic neurons arising from the lower brainstem project to the PVN (Sawchenko & Swanson 1982, Cunningham & Sawchenko 1988, Mezey & Palkovits 1991). We have demonstrated that ER α colocalized within noradrenergic cell bodies is increased by pharmacological glucoprivation (Reyes *et al.* 2001). In addition, lesioning catecholaminergic inputs to the PVN 2 weeks prior to fasting and glucoprivation precluded fasting- and glucoprivation-induced increase in ER α -ir cells in the PVN (Estacio *et al.* 2004). Hence, we hypothesized that during fasting ER α may be colocalized in the noradrenergic neurons in the A2 region of the NTS, and that noradrenergic terminals target ER α -containing neurons in the PVN. Estrogen-dependent activation of this pathway during fasting could activate the brainstem and modulate the sensitivity of the PVN to the noradrenergic input and thus be part of the cascade of events that leads to LH suppression.

In the present study, we investigated the time course of ER α expression in specific brain areas of OVX rats during fasting and characterized the anatomical relationship of ER α with noradrenergic expressing neurons in the A2 region and noradrenergic fibers in the PVN.

Materials and Methods

Animals and treatments

After monitoring at least two consecutive estrous cycles, adult female Wistar–Imamichi rats (200–240 g) were individually caged in a controlled environment (14 h light:10 h darkness, lights on at 0500 h and off at 1900 h, $24 \pm 2^\circ\text{C}$). They were allowed free access to food (CE-2, Clea Japan, Inc., Tokyo, Japan) and water unless otherwise indicated. Rats were OVX under ether anesthesia to eliminate endogenous estrogens. The antibody used in this study has been reported to recognize both occupied and unoccupied ER α (Okamura *et al.* 1992). However, one study using the same antibody shows a downregulation of ER α expression in the presence of estrogen (Okamura *et al.* 1994). If an increase in ER α expression is a key event in the enhanced LH response to fasting observed in estradiol-treated OVX rats, then this increase should occur in the presence or absence of estrogen. We chose to use the OVX rats, so that ER α expression would not be masked by the presence of estrogen.

Fasting

Rats were randomly assigned to groups ($n=4$ per group) 12 days after ovariectomy and were fasted for 6, 24, 30, and 48 h, starting at 1300 h. Feed was available for the control groups *ad libitum*.

Immunohistochemistry

At the end of each fasting period, rats were deeply anesthetized with pentobarbital sodium (50 mg/kg) and perfused intracardially with 0.05 M PBS (pH 7.5) followed by ice-cold 4% formaldehyde in 0.05 M phosphate buffer (PB). Unfasted controls were perfused at either 1300 or 1900 h. These periods were chosen because two of the fasted groups were perfused at 1300 h (24- and 48-h fasting) or 1900 h, (6- and 30-h fasting) and the feeding behavior was expected to occur around 1900 h, which is the onset of darkness. Brains were postfixed for 2–3 h in the same fixative and immersed in 30% sucrose in 0.05 M PB at 4°C . Sequential 50 μm coronal sections through the hypothalamus and medulla oblongata were prepared using a cryostat (Leica CM 1800, Leica, Nussloch, Germany) and stored at -20°C in cryoprotectant until stained.

Every fourth and third section of the hypothalamus and medulla oblongata respectively was processed for ER α immunostaining using the avidin–biotin complex (ABC) method that has been previously described (Reyes *et al.* 2001, Estacio *et al.* 2004). The AS 409 anti-rat ER α used in this study was kindly supplied by Dr Hayashi (Yokohama City University, Yokohama, Japan) and its specificity has been reported elsewhere (Okamura *et al.* 1992).

To determine whether ER α -ir cells in the A2 region are colocalized in the neurons that produce norepinephrine (NA), every sixth section (three sections per animal) from unfasted and 48-h fasted rats ($n=4$ per group) was dual-stained with rabbit anti-rat ER α and mouse monoclonal anti-dopamine- β -hydroxylase (DBH; Chemicon International, Temecula, CA, USA) using an indirect immunofluorescence technique that has been described previously (Reyes *et al.* 2001). Tissue sections were incubated in a cocktail containing anti-ER α (1:20 000) and anti-DBH (1:100) for 7 days at 4°C . ER α and DBH immunoreactivities were visualized using fluorescein isothiocyanate-conjugated donkey anti-rabbit immunoglobulin G (IgG; 1:800; Jackson Laboratories, West Grove, PA, USA) and indocarbocyanine (Cy3)-conjugated donkey anti-mouse IgG (1:800, Jackson Laboratories) for 2 h in the dark at room temperature. The sections were mounted with FluoroGuard anti-fade reagent (Bio-Rad) and observed under a confocal laser scanning microscope (MRC 1024; Bio-Rad).

To determine the anatomical relationship between noradrenergic terminals and ER α -expressing cells, sections containing the PVN were dual-stained with anti-ER α and anti-DBH using the ABC method. Sections (four sections per animal) from 48-h fasted rats ($n=4$) were immunostained for ER α and DBH. Sections were incubated in a cocktail of primary antibodies (anti-rat ER α at 1:20 000 and mouse

anti-DBH at 1:100) for 7 days at 4 °C. Following rinses with 0.1 M tris-buffered saline (TBS, pH 7.6), sections were incubated with biotinylated goat anti-rabbit and biotinylated horse anti-mouse IgG (1:400; Vector Laboratories, Burlingame, CA, USA), for 1 h. Subsequently, a 30-min incubation of ABC (Vector Laboratories) followed. Sections were then reacted with 0.05% 3,3'-diaminobenzidine and 0.05% hydrogen peroxide in 0.1 M TBS for ER α and DBH immunoreactivities. For all incubations and washes, sections were continuously agitated with a rotary shaker. DBH-ir varicosities appeared as small round structures, which were easily distinguishable from the much larger ER α -ir cell nuclei (Fig. 3C). Then, sections were treated with 1% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA, USA) in 0.1 M PB at 4 °C for 1.5 h, rinsed for 10 min in 0.1 M PB and dehydrated in an ascending series of ethanol. Osmicated sections were flat-embedded in Araldite on glass slides with silicon rubber (1 mm thick). The region of interest was examined with a microscope and photographs were taken.

Statistical analysis

Slides were coded to avoid bias while counting. ER α -, DBH- and dual-labeled cells were counted twice and the average was

calculated. ER α in the medial parvocellular PVN, A2 region of the NTS, ARC, and VMH were counted following the anatomical levels (posterior from the bregma) represented in the rat brain atlas of Paxinos & Watson (1986): PVN (2.12–1.8 mm), A2 (14.6–13.68 mm), ARC (2.12–4.16 mm), and VMH (2.12–3.60 mm). Since our previous studies show that the medial parvocellular PVN is the area where ER α -ir cells are found during fasting (Estacio *et al.* 1996a, 1996b, 2004), counting was only conducted in this subnucleus of the PVN. Statistical significance between groups was determined by one-way ANOVA. All statistical analyses were performed using the StatView program (StatView-J 5.0, SAS Institute, Inc., Cary, NC, USA) followed by *post hoc* Student–Newman–Keuls multiple comparisons test. Values were considered significant when $P < 0.05$.

Results

Fasting significantly increased the number of ER α -ir cells ($P < 0.05$) in the PVN and NTS in OVX rats 30 h after its onset as compared with unfasted controls and rats fasted for 6 and 24 h (Fig. 1). The increased expression of ER α -ir cells

Figure 1 Effects of fasting on the number of estrogen receptor α (ER α)-immunoreactive (ir) cells in the paraventricular nucleus (PVN), periventricular nucleus (PeVN), arcuate nucleus (ARC), ventromedial hypothalamus (VMH), and nucleus of the solitary tract (NTS) in ovariectomized (OVX) rats. Values are means $\pm$ S.E.M. Values with different letters are significantly different ($P < 0.05$) from each other in each time point studied (Student–Newman–Keuls multiple comparisons test after ANOVA).

($P < 0.05$) was sustained 48 h following fasting in the same nuclei compared with unfasted controls and rats fasted for 6 and 24 h. ER α expression did not change significantly at any time point in the ARC and VMH (Fig. 1).

Figure 2 presents the distribution of ER α -ir cells in the PVN (Fig. 2A–C), NTS (Fig. 2D–F), and ARC (Fig. 2G–I) of representative animals. In 30- (Fig. 2B) and 48-h (Fig. 2C)-fasted rats, ER α -ir cells were observed in the medial parvocellular PVN with more cells in the ventral and periventricular portions compared with unfasted rats (Fig. 2A). There were many ER α -ir cells distributed throughout

the A2 region of the NTS in 30-h (Fig. 2E) and 48-h-fast rats (Fig. 2F) compared with unfasted rats (Fig. 2D). A large number of ER α -ir cells were found in the ARC, but there was no difference in the distribution or number of rats that were fasted (Fig. 2H–I) or unfasted (Fig. 2G).

The numbers of ir neurons for ER α , DBH, and dual-labeled cells in the A2 region of the NTS are presented in Table 1. Immunofluorescence labeling for ER α and DBH was visualized through the A2 region of the NTS. Immunolabeling showed DBH-ir neurons in the A2 region of the NTS of both unfasted and 48-h fasted rats. The number of DBH-ir

Figure 2 Estrogen receptor α (ER α)-immunoreactive (ir) cells in the PVN (A–C), NTS (D–F), and ARC (G–I) in OVX rats. ER α -ir cells in the PVN, NTS, and ARC in OVX rats fasted for 30 h (B, E, H) and 48 h (C, F, I) respectively. Note the absence of, or few, ER α -ir cells in the PVN (A) and NTS (D) in unfasted OVX rats. Also, there was no difference in the distribution and number of ER α -ir cells in the ARC (G–I). III, third ventricle; cc, central canal. Scale bar = 100 μ m.

Table 1 Number of estrogen receptor α (ER α), dopamine- β -hydroxylase (DBH), and dual-labeled cells in the nucleus of the solitary tract of 48-h fasted and unfasted ovariectomized rats ($n=4$)

Group	ER α	DBH	ER α + DBH	Double-labeled cells (percent of DBH)
Unfast	8.25 $\pm$ 4.92 ^b	73.50 $\pm$ 8.22	6.00 $\pm$ 3.24 ^b	8.16 ^b
48-h fast	40.00 $\pm$ 6.75 ^a	63.38 $\pm$ 13.39	29.63 $\pm$ 5.81 ^a	46.75 ^a

Superscripts with different letters indicate statistical significance.

neurons were not altered by fasting, however, more DBH-ir neurons expressed ER α after 48-h fasting compared with unfasted rats ($P<0.05$, Table 1 and Fig. 3A–B). In 48-h-fasted rats, 46.75% DBH-ir cells also expressed ER α . This percentage was significantly higher than in corresponding unfasted controls (only 8.16% DBH-ir cells expressed ER α).

Our dual-immunocytochemical studies of the PVN revealed that DBH-ir varicosities, appearing as punctuate structures, were found throughout the PVN in 48-h-fasted rats. Figure 3C shows the juxtaposition of ER α -ir neurons and DBH-ir varicosities in the PVN.

Discussion

Our present findings show that fasting induced ER α in the PVN and NTS in OVX rats 30 h after its onset compared with unfasted rats. The significant increase in ER α expression was sustained until at least 48 h. Additionally, ER α and DBH are colocalized in the A2 region of the NTS, with more DBH-ir neurons expressing ER α after 48-h fasting. Our results also provide the first anatomical evidence that in the PVN, ER α -ir neurons are juxtaposed with DBH-ir varicosities.

Using immunoblot analysis, the characterization and specificity of the rabbit antiserum against rat ER α used in the present study have been previously described (Okamura *et al.* 1992). Specifically, immunocytochemistry recognizes ER α in the pituitary and neurons in specific brain regions (Okamura *et al.* 1992, 1994). While it is known that ER β immunoreactivity is expressed in the PVN and the NTS (Simonian & Herbison 1997, Shughrue *et al.* 1997), our previous reports have shown that 48-h fasting significantly increases ER α expression in these nuclei (Estacio *et al.* 1996a, 1996b, Maeda *et al.* 1996, Estacio *et al.* 2004). To our knowledge, whether ER β expression is increased during fasting has not been elucidated. Thus, the possibility of cross-immunoreactivity between ER α and ER β during 30- and 48-h fasting in the PVN and the A2 regions of the NTS requires further investigation. ER β has a specific binding affinity for estradiol and is capable of activating the estrogen-response element reporter gene construct (Kuiper *et al.* 1996). Hence, ER β has been instrumental in understanding how estrogen exerts its myriad of physiological, sometimes opposing effects (Nillson *et al.* 2001). In fact, Imamov and

colleagues (2005), using ventral prostate epithelium in mice at various postnatal periods, described the opposing actions of ER α and ER β on epithelial proliferation and differentiation in a Yin-Yang paradigm. Using the present fasting model, it would be interesting to determine if ER β mediates estrogenic signals in the PVN and the NTS that would describe the existence of the Yin-Yang paradigm or the opposing actions of ER α and ER β . Further studies are needed to address this issue.

Temporal ER α expression in the PVN and the NTS was evident 30 h after fasting and sustained until at least 48 h, confirming our previous observations that fasting for 48 h induces ER α expression in the same nuclei (Estacio *et al.* 1996a, Maeda *et al.* 1996). Similarly, 2DG-induced glucoprivation increases ER α expression in the same nuclei within an hour (Reyes *et al.* 2001). These data suggest that ER α expression in the PVN and A2 region of the NTS may play a role in enhancing the suppressive effect of fasting or glucoprivation on LH secretion. It has been demonstrated that estrogen induces *c-fos* expression in the A2 region of the NTS (Jennes *et al.* 1992) and upregulates receptor gene expression in the hypothalamus (Blaustein & Turcotte 1989, Shughrue *et al.* 1997) and A2 region (Haywood *et al.* 1999). Thus, increased ER α expression at 30-h fasting, which was sustained until at least 48-h fasting, may influence transcriptional gene expression that would direct the sequence of events culminating in intensified LH suppression by 48-h fasting.

OVX female rats were used over intact or estradiol-treated female rats for the purpose of eliminating the circulating endogenous estrogen. Although the rabbit anti-rat ER α used in this study recognizes both occupied and unoccupied ER α (Okamura *et al.* 1992), previous investigations using immunohistochemistry showed a suppressive effect of estrogen on ER expression (Okamura *et al.* 1994). Therefore, if ER α expression heralds the suppression of LH secretion in response to fasting (Maeda *et al.* 1996, Estacio *et al.* 1996a) observed in intact (Cagampang *et al.* 1990) and estradiol-treated rats (Cagampang *et al.* 1991, Maeda *et al.* 1996), there would be an increase in ER α expression in the presence or absence of estrogen. Using intact or estradiol-treated rats would certainly mask the ER α expression.

In the present study, the increased ER α expression and colocalization of ER α in DBH-ir neurons in the A2 region of the NTS in fasted rats suggest that the fasting signal

Figure 3 (A and B) Estrogen receptor α (ER α) and dopamine- β -hydroxylase (DBH)-immunoreactivities in the A2 region in the nucleus of the solitary tract in OVX rats. ER α (green) is colocalized within DBH-ir cells (red) in 48-h fasted rats. Arrows indicate dorsal (D) and lateral (L) orientation of the sections illustrated. Scale bar = 50 μ m. (C) DBH-ir varicosities (arrowheads) juxtaposed with estrogen receptor α -ir soma (asterisks) in the PVN in OVX rats. Scale bar = 375 μ m.

stimulates ER α expression in the A2 region and this may enhance the activation of A2 noradrenergic neurons projecting to the PVN during fasting. Estrogen activation of brainstem noradrenergic neurons (Jennes *et al.* 1992) and an increment in hypothalamic NA turnover in response to estrogen treatment is well established (Honma & Wuttke 1980, Wise *et al.* 1981, Demling *et al.* 1985, Liaw *et al.* 1992). Therefore, increase in ER α colocalized within the noradrenergic neurons of the A2 region during fasting suggests that estrogen could increase the activation of A2 noradrenergic neurons, thereby increasing NA transmission from the A2 region to the PVN and enhancing the suppression of LH secretion. Previously, we have illustrated that complete vagotomy restored the fasting-induced LH suppression (Cagampang *et al.* 1992a) and blocked the fasting-induced increase in ER α -ir cells in the PVN and A2 region (Estacio *et al.* 1996b). These results suggest that the afferent vagal nerve from the upper digestive tract transmits the fasting signal to the NTS, thereby mediating increased ER α expression in the PVN and A2 region.

We have demonstrated for the first time that noradrenergic varicosities in the PVN are in a close anatomical relationship with ER α -expressing cells in the rat. Anatomically, the PVN is densely innervated by NA cell bodies originating from the A1, A2, and A6 of the lower brainstem (Sawchenko & Swanson 1982, Cunningham & Sawchenko 1988, Mezey & Palkovits 1991). NA release is elevated in the PVN during fasting (Stanley *et al.* 1989). Moreover, the catecholaminergic regulation of estrogen binding in the hypothalamus has been illustrated (Blaustein *et al.* 1986, Blaustein 1987, Blaustein & Turcotte 1987). For example, the administration of α 1-noradrenergic antagonists decreases estrogen binding in female rat hypothalamus. In addition, an interaction between the NA system and [3H]estradiol-labeled cells have been previously reported (Heritage *et al.* 1977). Recently, we have demonstrated that lesioning catecholaminergic inputs to the PVN by bilateral injection of saporin-conjugated anti-DBH into the PVN 2 weeks prior to fasting and glucoprivation precluded fasting- and glucoprivation-induced increase in ER α -ir cells in the PVN (Estacio *et al.* 2004). Therefore, our present findings support the hypothesis that A2 noradrenergic neurons may directly influence ER α expression in the PVN during fasting.

Estrogen enhances LH suppression induced by microinjection of NA in the PVN (Tsukamura *et al.* 1994). The existence of noradrenergic synapses on corticotropin-releasing hormone (CRH)-containing neurons in the PVN has been demonstrated (Liposits *et al.* 1986) and this noradrenergic system facilitates synthesis and/or secretion of CRH both *in vivo* (Itoi *et al.* 1994) and *in vitro* (Tsagarakis *et al.* 1988). Furthermore, noradrenergic input to the PVN induces CRH release via the activation of the α -adrenergic receptors (Cagampang *et al.* 1992b, Maeda *et al.* 1994). Anatomical studies showed that PVN neurons project to the median eminence (Armstrong & Hatton 1980, Reyes *et al.* 2005). Since anti-CRH administered into the third ventricle reverses

fasting-induced LH suppression (Tsukamura *et al.* 1994), CRH neurons in the PVN could act at the median eminence and/or POA to inhibit gonadotropin-releasing hormone, which subsequently suppresses LH secretion (Maeda & Tsukamura 1996). The identity of the ER α -ir cells within the PVN has yet to be established, but it is tempting to speculate that the increment in ER α -ir cells in the PVN and A2 region of the NTS causes an increase in the sensitivity of the CRH-releasing system to the incoming NA signal during fasting and thereby suppresses LH release. Nevertheless, future studies would be useful to identify the neurochemical nature of ER α -containing cells expressed in the PVN during fasting.

Recently, human brain tissue sections obtained from autopsy specimens of patients having suffered from major depression/major depressive disorder or bipolar disorder demonstrated a colocalization of CRH and ER α in the PVN (Bao *et al.* 2005). Impaired appetite is one of the common features of depressed or bipolar individuals (Diagnostic and Statistical Manual IV 1994, Sugahara *et al.* 2004, Kawa *et al.* 2005, Kishi & Elmquist 2005). However, a reduced appetite is observed in most depressive patients, while an increase in appetite is seen in fewer patients (Diagnostic and Statistical Manual IV 1994). Together with the present results, it is possible that the increased ER α expression in the present experimental fasting model could somehow serve as a cellular substrate related to the impairment of appetite observed in depressed patients. This is an interesting concern for further investigation.

In conclusion, our present study provides evidence that fasting induces ER α expression in the PVN, PeVN, and NTS 30 h from its onset, which is sustained until at least 48 h. These results suggest that fasting creates complex physiological signals, some of which slowly culminate in suppression of LH secretion. Colocalization of ER α within the noradrenergic neurons in the A2 region, suggests a role for estrogen during fasting in increasing activation of the A2 noradrenergic neurons that project to the PVN. Moreover, the anatomical relationship of DBH-ir varicosities and ER α -ir cells in the PVN suggests that fasting enhances the sensitivity of the PVN to this noradrenergic signal, consequently leading to the suppression of LH secretion.

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