

The effect of food deprivation and experimental diabetes on orexin and NPY mRNA levels

Irné Swart, J. Michael Overton, Thomas A. Houpt*

Program in Neuroscience, The Florida State University, Tallahassee, FL 32306, USA

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Abstract

Although exogenous orexin can induce feeding, reports of increased orexin gene expression after caloric manipulations have been inconsistent. We hypothesized that orexin gene expression is increased only by extreme negative energy balance challenges. We measured hypothalamic orexin and NPY mRNA by *in situ* hybridization and orexin-A immunoreactivity in rats after food deprivation, streptozotocin-induced diabetes, and combined deprivation and diabetes. Neither food deprivation, nor diabetes, nor the combination affected orexin mRNA levels, although orexin-A immunoreactivity was increased by diabetes. NPY mRNA levels were increased by either treatment. These results suggest that increased orexin gene expression is not a consistent correlate of negative energy balance challenges. © 2001 Elsevier Science Inc. All rights reserved.

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1. Introduction

Orexin A and B (also known as hypocretin 1 and 2) are two neuropeptides expressed exclusively in lateral hypothalamus (LHA) and perifornical area [15,21]. These are areas involved in the control of food intake and body weight [1], and are sensitive to changes in blood glucose and insulin levels [16–18]. While orexin expressing neurons are ideally located to potentially integrate a variety of nutritional signals and through their projections effect key feeding regulatory targets, a specific role for endogenous orexins as physiologic mediators of food intake has not been clearly established.

Central administration of orexin A or B has been reported to stimulate feeding in a dose dependent manner [6,20,22]. Additionally, orexin gene expression [5,11,21] and peptide content [13] have been reported to be increased in the LHA in response to food deprivation in male rats. However, several authors have reported failure of the orexins to consistently produce an orexigenic effect when administered centrally [7,9,10,12]. In addition, chronic food restriction has been shown not to affect orexin gene expres-

sion in both rats (50% restriction) [5] and C57Bl/6J mice (60% restriction) [25].

These inconsistencies led us to hypothesize that orexin gene expression in the LHA could be increased only by extreme (or combined) negative energy balance challenges. Therefore, we examined orexin mRNA levels by *in situ* hybridization, and orexin-A immunoreactivity in the LHA by immunohistochemistry in rats after food deprivation, experimental diabetes, and combined food deprivation and diabetes. Both conditions are associated with loss of body weight and hyperphagia. Furthermore, there is evidence that orexin neurons may be sensitive to glycemic fluctuations [5,8,14]: food deprivation results in hypoglycemia and experimental diabetes induces sustained hyperglycemia. As a positive control, we measured hypothalamic NPY mRNA levels because NPY gene expression is consistently increased in response to food deprivation [2,3] and experimental diabetes [19,24].

2. Materials and methods

Male Sprague Dawley rats (11 weeks of age, 300–400 g, Charles River) were singly housed under controlled light-dark conditions (12h/12h, Ta = 22–24°C). Rats were assigned to one of four groups ($n = 6/\text{group}$): control-ad

* Corresponding author. Tel.: +1-850-644-4907; fax: +1-850-644-0989.

E-mail address: haupt@neuro.fsu.edu (T.A. Houpt).

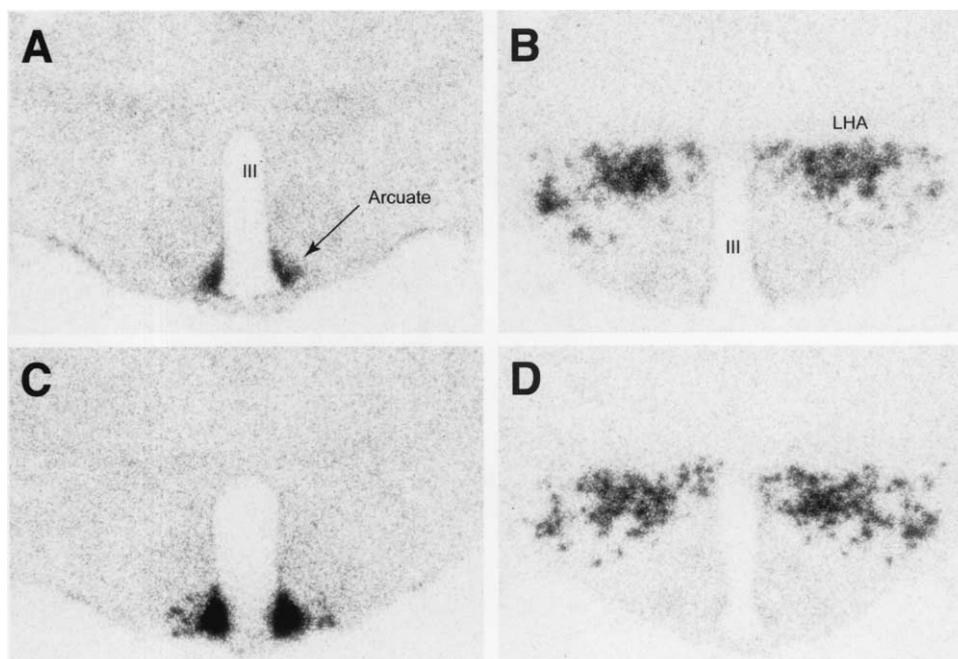


Fig. 1. Representative photomicrographs of ppNPY (A, C) and pp-orexin (B, D) mRNA in arcuate nucleus and lateral hypothalamus respectively in diabetic ad libitum fed (A, B) and 48 h food-deprived (C, D) rats. III –third ventricle, LHA –lateral hypothalamus.

libitum fed (C-al), control-food deprived (C-fd), diabetic-ad libitum fed (D-al), diabetic-food deprived (D-fd). Insulin deficient diabetes was induced by intraperitoneal (i.p.) streptozotocin (STZ) injection at a dose of 45 mg/kg body weight (in 0.01M sodium citrate; pH 4.5). This dose induced sustained hyperglycemia within 48 h (blood glucose > 200 mg/dl measured from tail prick sample; Glucometer, Encore). Control rats (C-al and C-fd) received an ip injection of sodium citrate buffer. Rats (control and diabetic groups) were then maintained and allowed ad libitum access to food and water for 18 days. Subsequently, the C-fd and D-fd groups were food-deprived (but not water deprived) for 48 h beginning at 7am. Following food deprivation, all four groups were perfused between 7 am and 11 am.

Rats were overdosed with pentobarbital and transcardially perfused with 4% phosphate-buffered paraformaldehyde. Brains were rapidly dissected, blocked, post fixed and cryoprotected in 30% sucrose. Brains were cut on a sliding freezing microtome through the rostral caudal extent of the hypothalamus (Bregma -1.80 to -3.80, Paxinos and Watson). Alternate 40 micron sections were processed either for in situ hybridization or orexin-A immunohistochemistry.

For in situ hybridization, free floating tissue sections were hybridized in 60% formamide, 0.02 M Tris pH7.4, 1 mM EDTA, 10% dextran sulfate, 0.8% Ficoll, 0.8% PVP, 0.8% BSA, 2× SSC, 0.1M dithiothreitol (DTT), and 1.6 mg/mL herring sperm DNA for 16–20h at 37°C with radiolabeled probe ($\sim 1.0 \times 10^7$ cpm/ml). Hybridization was performed with either tail labeled prepro-orexin (pp-orexin) antisense oligonucleotide (bases 2–49), or tail labeled preproNPY (ppNPY) antisense oligonucleotide (bases 59–88).

Sections were then sequentially rinsed in 2× SSC, 1× SSC and 0.5× SSC for 10 min at 37°C. The tissue sections were mounted and exposed to autoradiographic film (β -max, Amersham) for 2–3 days. Tissue sections from control (C-al, C-fd) and diabetic (D-al, D-fd) rats were hybridized within the same vial, and exposed to film together to ensure that in situ hybridization was carried out on representative members of each experimental group at the same time under identical conditions, allowing direct comparison of mRNA expression. mRNA levels and area of tissue hybridization were quantified by computer densitometry (Zeiss Stemi-2000 stereoscope attached to a Dage-MTI CCD 72 camera and Macintosh image analysis system) (e.g. Fig. 1). The mean background pixel density (1 to 256) and variance were measured in a single section for each rat in a region of unlabeled hypothalamus. Specific hybridization for NPY and orexin within images encompassing the arcuate nucleus and LHA respectively was defined as all pixels two standard deviations above the mean background. The mean level of mRNA expression in each rat was calculated across the three sections with the greatest area of specific hybridization.

For immunohistochemistry, free floating tissue sections were permeabilized with 0.2% Triton X-100, then incubated overnight at room temperature with a polyclonal anti-orexin-A peptide rabbit antibody diluted 1:2000 (Phoenix Pharmaceuticals Inc.). Orexin-A staining was visualized by ABC amplification diaminobenzodine reaction. Staining intensity of orexin-A positive cell bodies in 8–12 tissue sections per group was quantified by densitometry. The lateral hypothalamus was defined as a rectangular area 720 × 540 microns at Bregma -1.80 to -3.80, bordered by the third

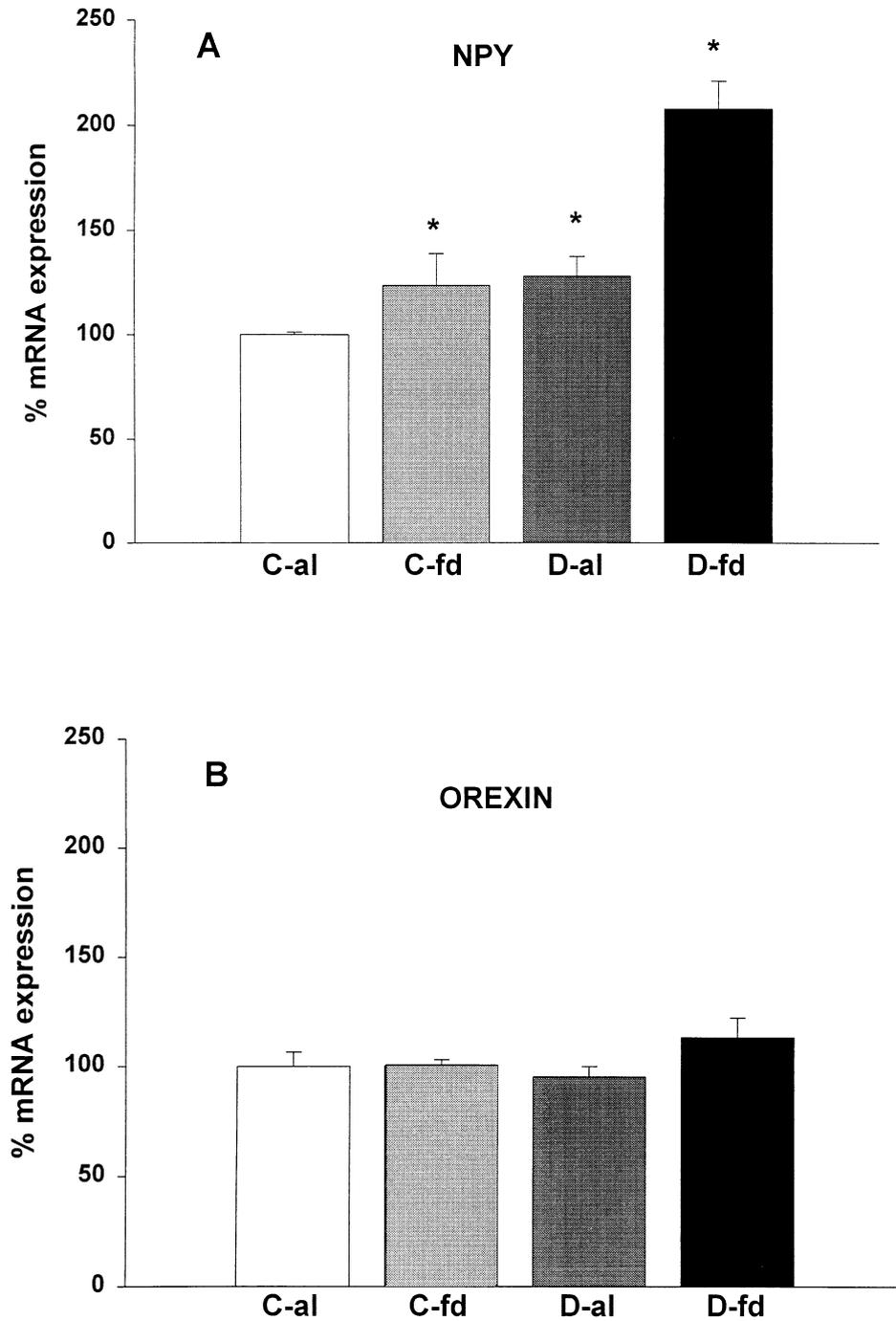


Fig. 2. ppNPY mRNA levels in the arcuate nucleus (A) and pp-orexin mRNA levels in the LHA (B) of ad libitum fed control (C-al), food-deprived control (C-fd), ad libitum fed diabetic (D-al), food-deprived diabetic (D-fd) rats. mRNA levels are expressed as % mean value of C-al group. * $P < 0.05$ versus C-al.

ventricle medially and the fornix ventrally (although the LHA was not precisely defined, orexin expression is limited to this general area).

3. Results

Two-way ANOVA revealed that both food deprivation ($F [1,20] = 16.5$, $P < 0.001$) and experimental diabetes (F

$[1,20] = 26.4$, $P < 0.001$) significantly increased hypothalamic ppNPY mRNA levels (Fig. 2A). There was also a marginal interaction between the effects of food deprivation and diabetes ($F [1,20] = 4.2$, $P = 0.06$) on ppNPY mRNA levels. Neither food deprivation, nor experimental diabetes, nor the combination had a significant effect on pp-orexin mRNA levels (Fig. 2B).

Experimental diabetes, independent of food deprivation,

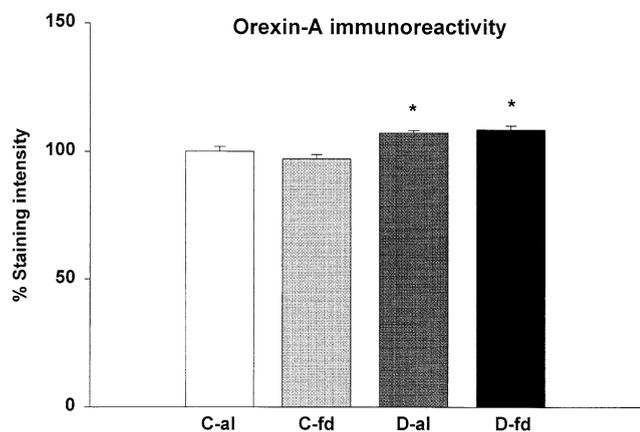


Fig. 3. Orexin-A immunoreactive staining intensity in the LHA of ad libitum fed control (C-al), food-deprived control (C-fd), ad libitum fed diabetic (D-al), food-deprived diabetic (D-fd) rats. Staining intensity is expressed as % mean value of C-al group. * $P < 0.05$ versus C-al.

significantly increased orexin-A positive immunoreactivity in the LHA ($F [1,20] = 32.9$, $P < 0.001$) (Fig. 3). Food deprivation had no effect on orexin-A immunoreactivity in the LHA.

4. Discussion

Consistent with previous reports [2,19] both food deprivation and experimental diabetes significantly increased hypothalamic ppNPY mRNA levels. We predicted that if the orexins are, similar to NPY, important physiologic mediators of energy balance regulation, and orexin gene expression is regulated in a manner similar to NPY gene expression, the extreme negative energy balance challenge produced by combined food deprivation and experimental diabetes would increase orexin gene expression. However, neither food deprivation, nor experimental diabetes, nor the combination had a significant effect on pp-orexin mRNA levels.

The lack of effect of diabetes on orexin gene expression has been previously reported [4,5], however, the lack of effect of food deprivation is in contrast to previous reports [5,11,21]. This suggests that an increase in orexin gene expression is not a consistent correlate of energy balance challenges. Indeed, in a recent report 60 h of food deprivation failed to affect orexin mRNA levels in mice as measured by riboprobe in situ hybridization [23]. While species difference or technique utilized (in situ hybridization versus Northern blot analysis) could potentially account for these inconsistencies, our data supports a growing body of evidence in both rats [5] and mice [23,25] that orexin gene expression is not consistently regulated by negative energy balance challenges.

Food deprivation had no effect on orexin-A immunoreactivity in the LHA. A previous report suggested a trend

toward an increase in hypothalamic orexin-A content following 48 h food deprivation [13], though these changes did not reach significance. Of interest is that experimental diabetes, independent of food deprivation, significantly increased orexin-A positive immunoreactivity in the LHA. This finding suggests that while orexin mRNA is not regulated, orexin peptide content is increased by stimuli associated with experimental diabetes. Because food deprivation had no effect on orexin-A immunoreactivity, factors common to experimental diabetes and food deprivation (e.g. decreased plasma leptin and insulin) may not influence orexin gene expression. Therefore, the sustained hyperglycemia unique to diabetes may be the responsible factor. Previous studies have shown that orexin neurons may be sensitive to glycemic fluctuations: orexin neurons are activated [14] and mRNA levels increased [8] by insulin induced hypoglycemia, while peptide content is not affected [8].

In summary, orexin gene expression is not regulated similar to hypothalamic NPY gene expression by food deprivation and/or experimental diabetes. These results add to the list of inconsistencies relating the orexins with physiological mediators of food intake.

Acknowledgments

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