

Evidence of Altered Neuropeptide Y Content and Neuropeptide Y₁ Receptor Gene Expression in the Hypothalamus of Pregnant Transgenic Mice

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Alterations in the density of neuropeptide Y (NPY)-immunoreactive fibers and of NPY₁ receptor gene expression in the hypothalamus of *Y₁R/LacZ* transgenic pregnant mice were investigated. In the paraventricular nucleus of mice on the 18th d of pregnancy NPY immunoreactivity was significantly decreased, and NPY₁ receptor gene expression, as measured by histochemical staining of β -galactosidase and *in situ* hybridization of NPY₁ receptor mRNA, was significantly increased compared with those in estrous mice. Conversely, pregnant transgenic mice displayed a significant induction of

NPY immunoreactivity and a reduction of NPY₁ receptor gene expression in the ventromedial nucleus. A significant increase in *Y₁R/LacZ* transgene expression and NPY₁ receptor mRNA, but no changes in NPY immunoreactivity were observed in the arcuate nucleus of mice on the 18th d of pregnancy. These results suggest that the elevated expression of NPY in the ventromedial nucleus may contribute to the state of leptin resistance that occurs during pregnancy. (Endocrinology 144: 4826–4830, 2003)

LEPTIN, THE PRODUCT of the *ob* gene, is a hormone secreted by adipocytes that reduces appetite and stimulates metabolism. Mutations in the *ob* gene or mutations in the leptin receptor *db* gene are associated with massive obesity (1, 2). Leptin interacts with hypothalamic leptin receptor-positive neurons, mostly located in the tuberal region, to influence multiple orexigenic and anorexigenic peptidergic pathways involved in the regulation of appetite and energy expenditure (3, 4).

Neuropeptide Y (NPY) is one of the most potent orexigenic agents known (5, 6). Within the hypothalamus, NPY is mainly synthesized in neurons whose cell bodies lie in the arcuate nucleus (ARC) and send projections to several areas, such as paraventricular (PVN), perifornical hypothalamus, and dorsomedial and ventromedial (VMH) hypothalamic nuclei, that are involved in the control of food intake (7). Evidence indicates that multiple hypothalamic sites are responsive to the appetite-stimulatory effect of NPY. For instance, administration of NPY into the PVN or the perifornical hypothalamus elicits a robust orexigenic effect (8, 9). Moreover, injection of NPY into the VMH potentiates feeding (10, 11), whereas the localized injection of NPY antibody inhibits food intake (12). Among the five NPY receptors identified to date, both the Y₁ and the Y₅ receptor subtypes appear to be involved in food intake and energy balance, although their specific roles remain to be determined (13).

Although hypothalamic NPY is not the only downstream regulator of body adiposity that responds to leptin, the NPY system is strongly influenced by systemic levels of leptin (5, 6). In the ARC, NPY neurons coexpress leptin receptor

mRNA (14). Leptin deficiency in the fasting state or in leptin function-deficient models, such as *ob/ob* and *db/db* mice, increases NPY mRNA in the ARC, whereas leptin administration decreases arcuate NPY mRNA (15). Thus, reduced leptin signaling in the hypothalamus may contribute to hyperphagia and obesity through modifications of the NPY orexigenic pathway.

Pregnancy is a hypermetabolic state with a great increase in maternal body fat and weight, hyperlipidemia, resistance to insulin, and numerous neuroendocrine changes. Pregnancy is associated with a positive energy balance, primarily due to an increase in food intake to prevent depletion of maternal energy stores. During pregnancy, leptin serum levels increase in humans and rodents, an effect that would normally be expected to decrease food consumption (16, 17). This paradoxical increase in leptin levels suggests that pregnancy may be a physiological state of leptin resistance in the hypothalamus similar to the leptin resistance in obesity.

Despite the well recognized link between NPY and leptin and its stimulating effect on feeding behavior, the involvement of NPY in mediating the increase in food intake during pregnancy has not been yet investigated. To determine the status of the hypothalamic NPY system during pregnancy, we used a transgenic mouse model, carrying the Y₁ receptor (Y₁R) gene promoter linked to the *LacZ* reporter gene (*Y₁R/LacZ* mice) (18), to measure Y₁R gene expression and NPY immunoreactivity at three hypothalamic sites reported to regulate NPY-induced food intake and energy balance (PVN, ARC, and VMH).

Materials and Methods

Adult female *Y₁R/LacZ* mice from transgenic line 62 (18) (25–30 g) from our breeding colony were used for the experiments. Animal care

Abbreviations: AOL, Area of interest; ARC, arcuate nucleus; NPY, neuropeptide Y; PVN, paraventricular nuclei; VMH, ventromedial hypothalamic nuclei; Y₁R, Y₁ receptor.

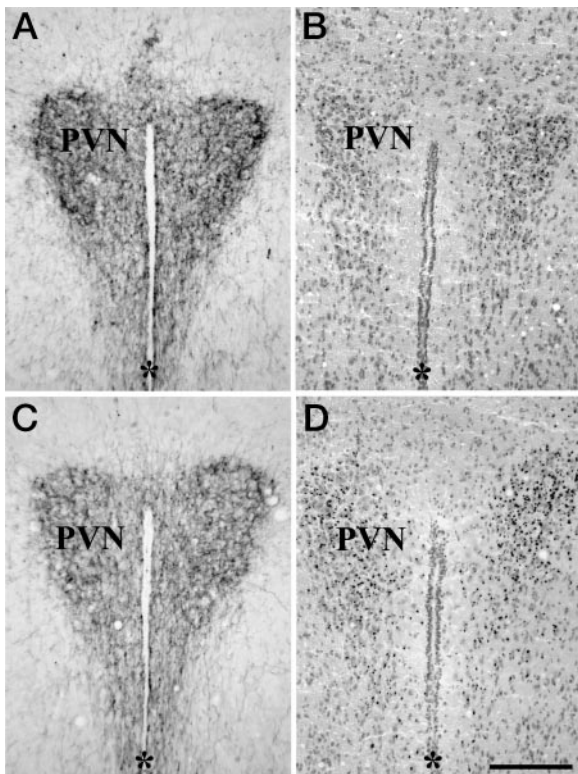


FIG. 1. Coronal sections illustrating different immunoreactivities for NPY (A and C) and different expressions of the $Y_1R/LacZ$ transgene (B and D) in the PVN of female mice during the estrous phase (A and B) and on d 18 of pregnancy (C and D). *, Third ventricle. Scale bar, 400 μ m.

was in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC), and the experimental protocol was approved by the animal investigation committee of the Ministero dell'Istruzione, dell'Università e della Ricerca.

The stage of the estrous cycle was determined from daily vaginal smears. For the induction of pregnancy, females were caged with fertile males on the evening of proestrus, and mating was verified by the detection of a vaginal plug on the morning of the next day, designated d 1 of pregnancy.

β -Galactosidase histochemistry

Females mice on d 18 of pregnancy and in the estrous stage were killed by cervical dislocation. Brains were quickly dissected and frozen on dry ice. Quantitation of $Y_1R/LacZ$ transgene expression was performed by computer-assisted morphometric analysis of β -galactosidase histochemical staining of coronal brain sections (25 μ m) according to Zammaretti *et al.* (19). Identification of brain structures was based on the atlas of the mouse brain (20). Three standardized sections of comparable levels of the PVN (around bregma, $-0.70/-0.82$ mm), ARC (around bregma, -1.70 mm), and VMH (around bregma, -1.70 mm) were examined. Selected sections were placed on a Leitz Diaplan microscope and analyzed with NIH Image software (version 1.62, W. Raysband, NIH, Bethesda, MD). Sections were digitized first with the use of a green filter to define the area of interest (AOI) by drawing a line along the boundary of the selected nuclei. The same sections were then digitized with a red filter, dots were selected, the corresponding image was converted to binary, and the number of dots and the extension of the AOI were automatically recorded to obtain the density of transgene expression (dots per square micrometer).

In situ hybridization for Y_1R mRNA

For *in situ* hybridization, coronal brain sections (12 μ m thick) were processed according to the protocol reported by Wisden and Morris (21).

To strongly increase the signal four different 40-/45-mer oligonucleotide probes complementary to different regions of the murine Y_1R mRNA were simultaneously used (22). The oligonucleotides were 3' end labeled with [35 S]deoxy-ATP (Amersham Pharmacia Biotech, Arlington Heights, IL) to a specific activity of 200,000 dpm/ μ l, added to the hybridization solution, and incubated in 150 μ l overnight at 42 C. Sections were then washed, dehydrated in alcohol, air-dried, and exposed to x-ray film (Kodak BioMax MR-1 film, Eastman Kodak Co., Rochester, NY) at room temperature for 10 d. Autoradiograms were placed on a stereomicroscope (Stemi 2000-C, Nikon, Milano, Italy) and analyzed by means of the software NIH Image (22). Calibration of the system was performed using a 12-point scale carbon-14 radioactive standard (Amersham Pharmacia Biotech). Values of specific binding in the AOI (density of binding in the measured region minus nonspecific binding in adjacent regions of the section) from seven or eight adjacent sections were measured, averaged, and expressed in nanocuries per animal.

NPY immunohistochemistry

For NPY immunohistochemistry, the animals were irreversibly anesthetized with chloral hydrate (300 mg/kg, ip) and transcardially perfused with 20 ml heparinized saline solution, followed by 20 ml fixative (4% paraformaldehyde in 0.1 M PBS, pH 7.2). Brains were dissected, postfixed overnight in the same fixative at 4 C, rinsed in PBS, placed overnight at 4 C in 0.1 M PBS containing 30% sucrose, and frozen on dry ice. Coronal sections (25 μ m) were immunostained by the indirect biotin-avidin system using a rabbit anti-NPY IgG (1:6000; a gift from H. Vaudry, Rouen, France) diluted in 0.1 M PBS containing 0.1% normal goat serum and 0.02% Triton X-100. The production and the controls of specificity of the antibody against NPY have been previously described (23). One alternate set of sections was treated for β -galactosidase histochemistry. Sections were observed with a light microscope (Diaplan, Leitz, Milan, Italy), selected with the same criteria reported for histochemistry, and

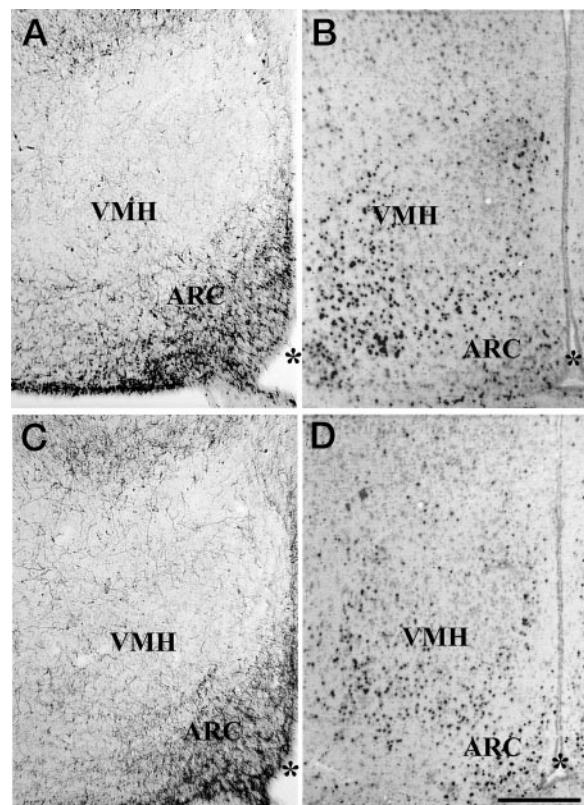


FIG. 2. Coronal sections illustrating different immunoreactivities for NPY (A and C) and different expressions of the $Y_1R/LacZ$ transgene (B and D) in the ARC and VMH of female mice during the estrous phase (A and B) and on d 18 of pregnancy (C and D). *, Third ventricle. Scale bar, 400 μ m.

digitized. The density of NPY-immunoreactive staining in PVN, VMH, and ARC was measured on three sections per nucleus per animal with NIH Image software by calculating the fractional area (percentages of pixels) covered by these structures (see Ref. 24 for further details). The AOI was selected on the adjacent section stained for β -galactosidase by following the boundaries of the nuclei.

Statistical analysis

Quantitative results were analyzed by *t* test; differences were considered significant at $P < 0.05$.

Results

Images representative of those subjected to computer-assisted quantitation of NPY immunoreactivity and β -galactosidase histochemical staining of coronal sections of PVN, ARC, and VMH hypothalamic nuclei from mice in estrus and on the 18th d of pregnancy are shown in Figs. 1 and 2. Images typical of those subjected to computer-assisted quantitation of Y_1 R mRNA *in situ* hybridization of PVN, ARC, and VMH from pregnant or estrus mice are shown in Fig. 3.

Computerized quantitative analysis demonstrated that NPY immunoreactivity was significantly decreased and that both β -galactosidase activity and Y_1 R mRNA expression were increased in the PVN of pregnant Y_1 R/*LacZ* mice compared with those in estrus mice (Fig. 4 and Table 1). In the ARC, NPY immunoreactivity was not altered by pregnancy, whereas a significant increase in Y_1 R/*LacZ* transgene expression and Y_1 R mRNA was observed in this nucleus.

Conversely, a significant induction of NPY-immunoreactive fibers was measured in the VMH of mice on the 18th d of pregnancy that was accompanied by the reduction of both β -galactosidase activity and optical density of Y_1 R mRNA (Fig. 4 and Table 1).

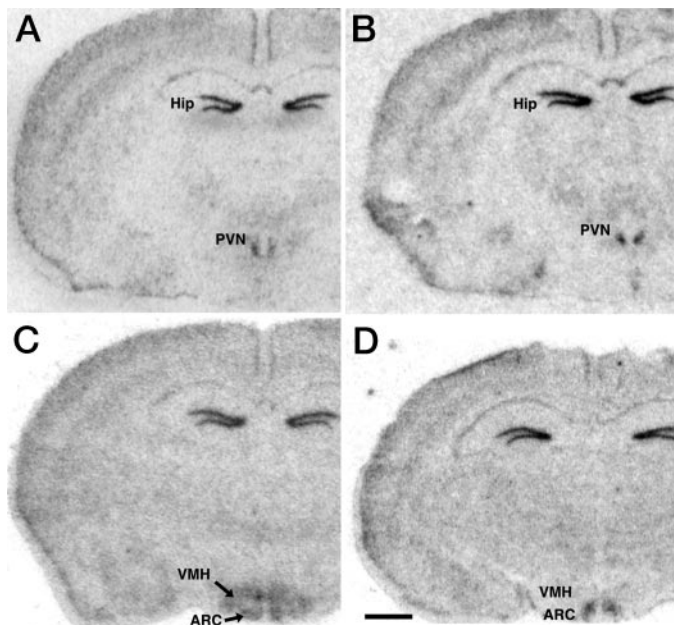


FIG. 3. Autoradiograms of female mice in estrus (A and C) and on d 18 of pregnancy (B and D) illustrating the effects of pregnancy on Y_1 R mRNA expression in mice hypothalamus. Original sections were hybridized with four oligonucleotide probes and exposed to x-ray film for 10 d. Hip, Hippocampus. Scale bar, 1 mm.

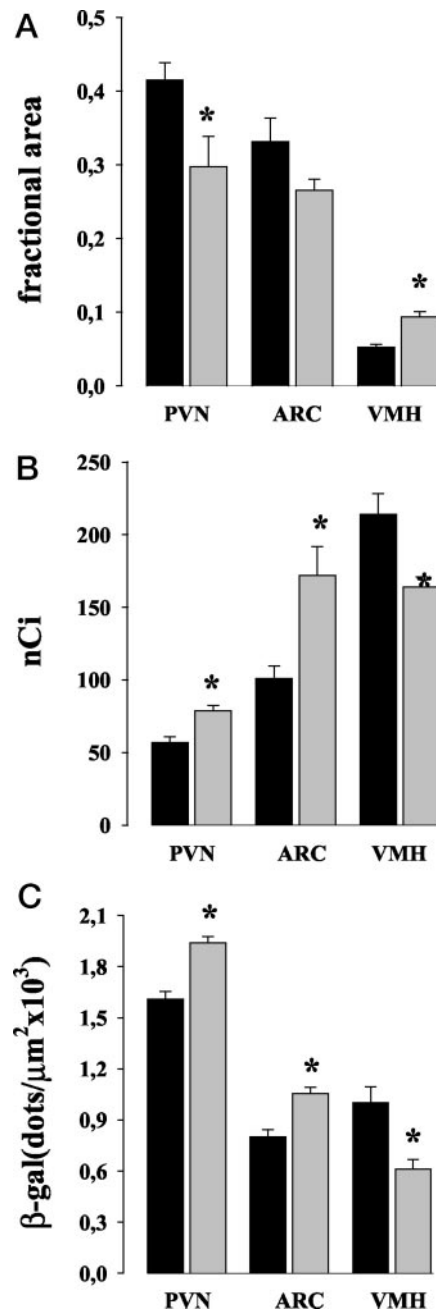


FIG. 4. Quantitative analysis of NPY-immunoreactive fibers (A), Y_1 R mRNA *in situ* hybridization (B), and β -galactosidase histochemical staining (C) of coronal sections of PVN, ARC, and VMH from female mice in estrus (■) or on d 18 of pregnancy (▒). Data are expressed as the fractional area (NPY immunoreactivity), nanocuries (Y_1 R *in situ* hybridization), or the density of blue dots (β -galactosidase) and are the mean \pm SEM. The number of animals and statistical analysis are reported in Table 1. *, $P < 0.05$.

Discussion

In pregnant mice both circulating leptin concentrations and food intake are elevated, suggesting an ineffectiveness of leptin to reduce food intake (17). This pregnancy-induced leptin resistance appears to be associated in part with a dramatic increase in plasma leptin binding activity that limits the access of leptin to target hypothalamic neurons and in

TABLE 1. Number of animals and *t* values corresponding to the quantitative analysis reported in Fig. 4

Nucleus	Fractional area			P	nCi			P	Density of dots		
	P	E	<i>t</i>		P	E	<i>t</i>		P	E	<i>t</i>
PVN	n = 6	n = 5	2.623	n = 8	n = 7	–3.476	n = 8	n = 8	2.195		
VMH	n = 6	n = 5	–5.495	n = 7	n = 5	3.058	n = 7	n = 5	3.130		
ARC	n = 5	n = 5	1.674	n = 5	n = 5	–3.303	n = 7	n = 8	–3.775		

part to a down-regulation of the long form of the leptin receptor (Ob-Rb) at the hypothalamic level.

NPY is known to be one of the most potent orexigenic peptides. The hypothalamic NPY system originating mainly from the ARC is strongly influenced by the systemic levels of leptin (5, 6, 13–15). An increase in the level of this hormone normally leads to a reduction of NPY mRNA in the ARC and of NPY levels in the ARC, PVN, and dorsomedial nuclei in the manner of a negative feedback loop.

In the present study we show that during pregnancy there are no changes in ARC NPY levels, but there is a significant decrease in PVN NPY immunoreactivity that, in turn, up-regulates Y₁R gene expression. Both Y₁R/*LacZ* gene expression and Y₁R mRNA endogenous levels are significantly increased on d 18 of pregnancy, suggesting that these changes may occur through the activation of transcriptional mechanisms.

Our data also demonstrate a profound induction of NPY content in the VMH of pregnant mice accompanied by a significant decrease in Y₁R gene expression. These data can be interpreted as a factor possibly contributing to the increase in food intake during pregnancy. Although the PVN is one of the hypothalamic sites particularly involved in the NPY effects on feeding behavior, both Y₁R and Y₅R subtypes are also found in the VMH (25). Injections of NPY into the VMH lead to food intake (10, 11). NPY was found to inhibit over one fifth of spontaneously active VMH neurons, and this inhibition was potentiated by overfeeding (26).

Depending on the genetic or nutritional state, the various hypothalamic nuclei seem to be differently involved in changes in the peptide content or the expression of NPY receptors. For example, in *ob/ob* mice the marked elevation of ARC NPY mRNA induced by functional leptin deficiency is combined with down-regulated expression of Y₅R in the ARC and VMH (27). Conversely, increased NPY levels in the VMH with a concurrent decrease in ARC NPY mRNA expression have been observed in some leptin resistance models, including tubby or diet-induced obese mice (28, 29). With regard to this, we could speculate that the increase in NPY immunoreactivity in the VMH of pregnant mice and the associated down-regulation of Y₁R synthesis in our study may reflect a compensatory response to the prolonged decrease in PVN NPY contributing to the hyperphagia observed during pregnancy.

VMH is a key nucleus for regulating neuroendocrine and behavioral functions during pregnancy and after parturition and contains estrogen receptors (30). Thus, a direct effect of steroids on Y₁R gene expression in the VMH cannot be ruled out. However, the possibility that the decrease in Y₁R gene expression is due to homologous down-regulation appears more likely, given that it is associated with an increase in the density of NPY-immunoreactive fibers.

In conclusion, here we provide the first evidence, to our knowledge, that the physiological state of leptin resistance observed in pregnancy modulates the NPY orexigenic pathway. We suggest that NPY–Y₁ transmission might participate in the regulation of food intake during pregnancy, and that the target cells mediating this response reside at least in part in the VMH nucleus.

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