THE EFFECTS OF LEPTIN ANTAGONIST ON HYPOTHALAMIC NEUROPEPTIDE Y CIRCUITRY IN INFANTILE MALE LAMBS

A Senior Scholars Thesis

by

MICHELLE BEDE BAUGH

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

May 2012

Major: Animal Science
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Approved by:

Research Advisor: Marcel Amstalden
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ABSTRACT

The Effects of Leptin Antagonist on Hypothalamic Neuropeptide Y Circuitry in Infantile Male Lambs. (May 2012)

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It is proposed that leptin signaling during infantile development is critical for structural organization of hypothalamic circuitry involved in the control of food intake and energy expenditure. The present study tested the hypothesis that disruption of leptin signaling alters the neuropeptide Y (NPY) circuitry in the hypothalamus of infantile lambs.

Twelve neonatal Dorset X Finn male lambs were assigned randomly to one of three experimental groups (n = 4/group): 1) Early Antagonist (EA), 2) Late Antagonist (LA) and 3) Control (CN). Lambs in the EA group were injected i.v. with 0.23 mg/Kg^{0.75} of pegylated super ovine leptin antagonist (PEG-SOLA) twice daily from birth to postnatal Day 14. Concurrently, lambs in the LA and CN groups were injected with an equivalent volume of physiological saline. From postnatal Day 30 to 36, lambs in the LA group were injected i.v. with 0.46 mg/Kg^{0.75} of PEG-SOLA twice daily, while lambs in the CN and EA groups were injected with physiological saline. On postnatal Day 40, lambs were euthanized, and a block of tissue containing the hypothalamus was collected and processed for detection of NPY by immunocytochemistry. The distribution of NPY
fibers in tissue sections was observed using bright-field microscopy and images representing various regions of the hypothalamus were captured using a 10X objective. The area covered by NPY-immunoreactive fibers was determined in regions of interest to represent the density of NPY fibers within each hypothalamic area analyzed. The mean density of NPY fibers did not differ significantly among the three treatment groups in any of the areas studied; however, the area covered by NPY fibers in the paraventricular nucleus tended to be greater in lambs treated with leptin antagonist early during the infantile period than in the control and late antagonist groups. Results of this study indicate that short-term disruption of leptin signaling during the infantile period does not clearly alter the density of NPY fibers in the hypothalamus of lambs. Additional studies should investigate whether other functional aspects of the NPY system (e.g., NPY synthesis and projections to target cells) are disrupted by leptin antagonist.
DEDICATION

This manuscript is dedicated to my parents, Stephen and Christine Bedenbaugh, and my brother, Robby Bedenbaugh, who have always been there to offer advice and support.
ACKNOWLEDGMENTS

The author expresses her sincere appreciation to Dr. Marcel Amstalden for his continuous support and advice throughout the process of completing this thesis. Thanks also to Sarah Sharpton and Bruna Alves for always offering advice and helping out with lab work. Special thanks to Dr. Yves Boisclair and Jose Ramos-Nieves, Cornell University, for their collaborative efforts in designing and conducting the animal-related experiments and tissue collection, and to Dr. Arieh Gertler, The Hebrew University of Jerusalem, for providing the leptin antagonist.
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CHAPTER I

INTRODUCTION

The recent increased prevalence of childhood obesity in the United States has been a major health concern. Currently, approximately 33% of children between the ages of 6 and 11 are considered overweight or obese [1], and becoming overweight at early ages predisposes children to adulthood obesity, diabetes and cardiovascular disease. Therefore, understanding the mechanisms by which excessive weight gain during the juvenile period predisposes individuals to disease later in life may help in the design of novel strategies to improve human health and well-being.

Increased adiposity has been associated with increased circulating concentrations of the adipocyte-derived hormone leptin [2, 3, 4]. Leptin administration decreases food intake and increases energy expenditure [5, 6]. A major pathway by which leptin seems to regulate metabolic function involves neuropeptide Y (NPY) neurons located in the arcuate nucleus (ARC) of the hypothalamus. Feed restriction increases NPY expression in the ARC [7] and NPY stimulates food intake [8, 9]. Administration of leptin decreases expression of the NPY gene in the ARC [10], likely by direct actions on NPY neurons [11]. Leptin may also exert its effects by regulating structural components of neuronal circuitries within the hypothalamus. Circulating concentrations of leptin

This thesis follows the style of Domestic Animal Endocrinology.
increase during the early postnatal period in rodents [12], and there is evidence that this elevation in leptin plays a role in regulating the development of neural projections within the ARC involved in the control of food intake [13]. There is also evidence for early postnatal increases in circulating leptin in sheep [14]. However, maternal obesity during gestation, a condition that predisposes offspring to increased appetite and weight gain [15], ablates the early postnatal increase in circulating leptin [16]. In addition, postnatal nutrition influences concentrations of leptin in circulation [17]. It is proposed that during the infantile and juvenile periods, leptin promotes structural organization of hypothalamic circuitries important for programming neuroendocrine functions in lambs. Therefore, we hypothesized that disruption of the leptin signaling alters the structural organization of the NPY circuitry in the hypothalamus of infantile lambs. To test this hypothesis, an antagonist of leptin was administered to neonatal and infantile lambs and neuronal projections containing NPY were investigated in hypothalamic regions known to regulate various physiological functions, including the control of metabolism and food intake.
CHAPTER II
MATERIALS AND METHODS

Animals and experimental procedures

All animal-related procedures were performed at Cornell University with the approval of the Cornell University Institutional Animal Care and Use Committee. Neonatal Dorset X Finn male lambs were used for this study. At birth, lambs were retrieved before first suckling, towel-dried, weighed and fed pooled cow colostrum to an amount equivalent to 10% of their birth weight within the first 30 minutes of life. Only lambs weighing approximately 3.5 to 4 kg at birth were used. The lambs were then transferred to a controlled-environment facility that was maintained at a temperature of 25°C and a photoperiod of 12/12 hours of light/dark cycles. Lambs were housed individually in stainless steel cages (75cm width x 80cm length x 80cm height) for 17 days. After this initial period, lambs were then transferred to pens where they were housed until the completion of the experiment on postnatal day (PD) 40. During the experimental period, lambs were offered ad libitum access to a milk replacer prepared with 20% wt/wt (18.9 dry matter [DM] wt/wt) milk powder containing 28% fat and 30% protein on a DM basis. Milk replacer was offered cold (4°C) at 8 am in clean plastic containers with latex nipple units, and it was allowed to equilibrate to room temperature. Buckets were re-filled at 4 pm and 8 pm when necessary.
Lambs were assigned randomly to one of the three treatment groups; 1) Early Antagonist (EA; n = 4): lambs were injected with pegylated super ovine leptin antagonist (PEG-SOLA; 0.23 mg/kg^{0.75}) intravenously (i.v.) twice a day from birth to PD 14 and an equivalent volume of physiological saline from PD 15 to 36; 2) Late antagonist (LA; n = 4): lambs were injected with physiological saline i.v., twice a day from birth to PD 29 and with PEG-SOLA (0.46 mg/kg^{0.75}) from PD 30 to 35; and 3) Control (CN; n = 4): lambs were injected with physiological saline i.v. twice a day from birth to PD 36. At PD 40, lambs were administered a dose of 10,000 U heparin i.v. and anaesthetized with an overdose of propofol (8mg/kg BW, i.v.) 5 minutes later. After confirmation that animals were deeply anesthetized (respiratory depression and loss of eye-lid reflexes), lambs were decapitated. The heads were perfused via both carotid arteries with heparinized saline (10U/ml) for 5 minutes and then with 3 liters of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3) containing 0.1% sodium nitrite. The cranium was dissected, and the brain was removed. A block of tissue containing the septum, the preoptic area and the hypothalamus was dissected. The tissue block was stored in 4% paraformaldehyde at 4°C overnight and then transferred to a 0.1 M PB solution containing 30% sucrose until the tissue had sunk, indicating sucrose infiltration. Tissue blocks were shipped to Texas A&M University for further processing.
**Tissue processing and immunocytochemistry**

Tissue blocks were cut in coronal sections of 50 µm using a freezing microtome. The sections were then stored at -20 °C in a cryopreservative solution until processed for immunocytochemistry. One series of sections 200 µm apart was used for this study. Immunocytochemistry for detection of NPY was performed at room temperature with gentle shaking. Free-floating tissue sections were washed in 0.1 M PB containing 0.9% NaCl (phosphate-buffered saline; PBS) for at least 4 hours to aid in removal of cryopreservative. After this initial washing, the sections were washed in PBS four times for 5 minutes to ensure that the cryopreservative had been removed. The sections were then incubated in PBS containing 1% hydrogen peroxide for 10 minutes to remove endogenous peroxidase activity. After further washing, the sections were incubated in a solution of PBS containing 0.4% Triton-X100 (PBSTX) and 4% normal goat serum (NGS) for at least 1 hour. The sections were then incubated in a solution containing rabbit anti-NPY antiserum (1:250,000 dilution; Sigma Aldrich) in PBSTX, and 4% NGS for 16 hours. After incubation with the primary antibody, the sections were washed and then incubated in a solution containing biotinylated goat anti-rabbit IgG (1:400 dilution; Vector Laboratories), PBSTX, and 4% NGS for 1 hour. The sections were washed and then incubated in a solution containing streptavidin horseradish-peroxidase conjugate (Vectastin Elite ABC, Vector Laboratories; 1:600 dilution) for 1 hour. Sections were washed and incubated in a solution containing 3,3-diaminobenzidine (DAB; 0.2 mg/ml) with hydrogen peroxide (0.012%; Sigma) in PBS for 10 minutes. The sections were
washed, mounted on slides and covered with coverslip using DPX (VWR International) for analysis.

**Morphological analysis**

Neuropeptide Y-immunoreactive fibers were identified by microscopy and visualized by the presence of dark-brown DAB staining using a dark and bright field microscope (Nikon 80i Eclipse; Nikon Inc., Melville, NY, USA). The location of NPY fiber immunoreactivity was investigated in a rostral to caudal continuum from the septum and preoptic regions through the hypothalamus. Anatomical distribution of NPY fibers within these regions were in accordance to those described previously for the sheep brain [18, 19] and included the preoptic area (POA), the periventricular nucleus (PeV), the paraventricular nucleus (PVN), the arcuate nucleus (ARC), the ventral medial hypothalamus (VMH), the dorsal medial hypothalamus (DMH), the premammillary ventral nucleus (PMV), the median eminence (ME), and the lateral hypothalamus (LH).

**Density of NPY fibers**

For each region of the brain examined, four sections within the POA (including two sections at the level of the organum vasculosum of the lamina terminalis [OVLT]), three through the PeV, four through the PVN, two through the rostral ARC, four through the medial ARC, two through the caudal ARC, three through the VMH, three through the DMH, three through the PMV, three through the ME, and three through the LH were selected. Sections were selected to represent comparable levels through all areas
examined from each lamb. Images of each region were captured using a 10X objective and a digital camera (DS-Qi1; Nikon) attached to the microscope. The density of fibers was determined by establishing the area covered by immunoreactive product in a region of interest (ROI) within each image collected. The NIS-Elements software (Nikon) was used for image analysis. A threshold signal was established and applied to all images before the number of pixels was determined in the standardized ROI. The number of pixels associated with NPY immunoreactivity within each ROI was recorded and transformed to proportion of the total area of the ROI to represent the density of NPY fibers in the various areas analyzed.

**Statistical analysis**

The proportional area covered by NPY fibers in each region was normalized using the arcsine-square root transformation and analyzed by Analysis of Variance (ANOVA) using the general linear models (GLM) procedure of the Statistical Analysis System (SAS). Treatment was used as source of variation.
CHAPTER III

RESULTS

Distribution of immunoreactive NPY fibers in the POA and hypothalamus

Immunoreactive NPY fibers were observed to be widely distributed from the septum and POA, including at the level of the OVLT, to the caudal portions of the hypothalamus at PD 40 in male lambs. Overall, the highest density of fibers was found in the ME, with fibers extending towards the external (secretory) zone. A high density of fibers was also observed in the ARC and PVN (Fig. 1). In the more rostral aspect of the PVN, NPY fibers were first present ventrally projecting along the third ventricle. In the more caudal portions of the PVN, the fibers were distributed throughout the nucleus, forming a characteristic wing-shape adjacent to the third ventricle (Fig. 1A). Within the POA, PMV, VMH, and PeV, the density of NPY fibers was moderate. In the more rostral sections of the POA, the majority of fibers were clustered around the OVLT. In the more caudal sections of the POA, fibers were concentrated at the base of the brain, adjacent to the third ventricle. Similar to the distribution in the PVN, NPY fibers in the PeV were observed distributed throughout the extent of and adjacent to the third ventricle. NPY fibers in the DMH and LH were observed only sparsely distributed. Table 1 summarizes the relative distribution of NPY fibers in the POA and hypothalamus.
Table 1

Distribution and density of NPY-immunoreactive fibers in the preoptic area and hypothalamus of 40 day-old male lambs.

<table>
<thead>
<tr>
<th>Area</th>
<th>Fiber Density&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>ARC</td>
<td>++++</td>
</tr>
<tr>
<td>DMH</td>
<td>+</td>
</tr>
<tr>
<td>LH</td>
<td>+</td>
</tr>
<tr>
<td>ME</td>
<td>++++</td>
</tr>
<tr>
<td>PeV</td>
<td>++</td>
</tr>
<tr>
<td>PMV</td>
<td>++</td>
</tr>
<tr>
<td>POA</td>
<td>++</td>
</tr>
<tr>
<td>PVN</td>
<td>+++</td>
</tr>
<tr>
<td>VMH</td>
<td>++</td>
</tr>
</tbody>
</table>

<sup>a</sup> ++++, dense; +++, high; ++, moderate; +, low.

Figure 1. Low power photomicrograph illustrating NPY-immunoreactive fibers in the paraventricular nucleus (PVN; A) and arcuate nucleus (ARC; B). Note the wing-shape distribution of fibers in the PVN, and the high density of fibers in the infundibular process of the ARC and ME. VMH, ventromedial hypothalamus; pt, pars tuberalis of the adenohypophysis; 3v, third ventricle. Scale bars = 500 µm.
Density of NPY-immunoreactive fibers in the POA and hypothalamus

The proportion of area covered by NPY immunoreactivity varied among the hypothalamic regions, but no significant differences among treatment groups were observed within any of the regions analyzed. However, there was a trend for the proportion of area covered by NPY fibers to be greater in the PVN and ARC of lambs treated with leptin antagonist between birth and PD 14 than in control lambs and lambs treated with leptin antagonist between PD 30 and 35 (Table 2).
Table 2

Mean proportion of area covered by NPY-immunoreactive fibers in the POA and hypothalamus of 40-day old male lambs (n = 4/group) treated with saline (CN), leptin antagonist between birth and PD 14 (EA), and leptin antagonist between PD 30 and 35 (LA).

<table>
<thead>
<tr>
<th>Area</th>
<th>Control</th>
<th>Early Antagonist</th>
<th>Late Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arcuate nucleus</td>
<td>8.9±1.2</td>
<td>9.3±0.7</td>
<td>7.5±0.9</td>
</tr>
<tr>
<td>Dorsomedial hypothalamus</td>
<td>3.1±0.3</td>
<td>2.3±0.4</td>
<td>2.2±0.1</td>
</tr>
<tr>
<td>Lateral hypothalamus</td>
<td>1.0±0.2</td>
<td>1.2±0.2</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Median eminence</td>
<td>11.3±1.5</td>
<td>10.6±0.8</td>
<td>12.3±0.8</td>
</tr>
<tr>
<td>Periventricular nucleus</td>
<td>4.4±0.8</td>
<td>3.5±0.5</td>
<td>3.0±0.3</td>
</tr>
<tr>
<td>Premammillary ventral nucleus</td>
<td>5.5±0.6</td>
<td>4.0±1.1</td>
<td>4.1±0.4</td>
</tr>
<tr>
<td>Preoptic area</td>
<td>5.4±0.8</td>
<td>5.6±0.7</td>
<td>4.6±0.5</td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>5.7±0.9</td>
<td>8.0±1.2</td>
<td>5.6±0.4</td>
</tr>
<tr>
<td>Ventromedial hypothalamus</td>
<td>3.2±0.2</td>
<td>3.0±0.3</td>
<td>2.5±0.3</td>
</tr>
</tbody>
</table>
CHAPTER IV
DISCUSSION AND CONCLUSIONS

The involvement of the leptin-NPY pathway on the control of food intake and energy expenditure has been well characterized [20]. In addition, a role for leptin on promoting neurite outgrowth in the hypothalamus during the infantile period in mice has been demonstrated [13]. In the current study, we investigated whether disruption of leptin signaling could influence development of NPY neuronal projections in the hypothalamus. Although no effects of treatment with ovine leptin antagonist during the neonate and infantile periods were observed, there was a trend for increased density of NPY fibers in the PVN of lambs treated with leptin antagonist soon after birth. This structural alteration may represent an important functional response of the hypothalamus to leptin insufficiency during the early postnatal period.

Bouret et al. [13] demonstrated that hypothalamic explants collected from neonate mice and treated with leptin exhibited increased neurite outgrowth in the ARC compared to control explants. A surge of leptin occurs during the early postnatal period in mice. Although it is unclear whether a consistent surge of leptin occurs in neonate/infantile lambs [17], it is believed that leptin is necessary during this period for appropriate development of neuronal projections that control metabolic functions. Indeed, leptin treatment in leptin-deficient (ob/ob) mice restores the pattern of neuronal projections in the PVN [13]. In addition, these projections to the PVN contain agouti-related protein. Because agouti-related protein colocalizes with NPY in ARC neurons, it is predicted that
the increased density of agouti-related protein-containing projections in the PVN is resulted from an increased number of neuronal projections that originated from NPY/agouti-related protein neurons in the ARC. Therefore, this observation indicates that leptin is necessary for normal development of neuronal projections from the ARC to the PVN.

Injection of NPY directly into the PVN in rats increases food intake [8]. Because NPY receptors are linked to inhibition of cAMP formation via activation of the $G_{ai}$ signaling pathway [21], it is plausible to suggest that NPY-inhibition of PVN neurons is involved in stimulation of food intake and decrease in energy expenditure. Therefore, increased NPY fiber projection in PVN neurons may facilitate NPY inhibition of PVN neurons. This structural change in the hypothalamus may lead to increased feed consumption and weight gain, and decreased energy expenditure, later in life (juvenile/adulthood periods) when nutrient requirements for growth are diminished and food is abundant. Whether this structural-functional change leads to alterations in metabolism remains to be determined.

The trend toward increased density of NPY fibers in the PVN of lambs treated with the leptin antagonist early during the neonate period may indicate a critical role for leptin on supporting neuronal development during this period. In mice, ARC projections to the PVN, DMH and LH form during the first two weeks of life [22]. During this time, leptin does not have a significant effect on food intake, but it alters development of neuronal
projections in the hypothalamus [22]. The lack of differences in NPY fiber density in the PVN of lambs treated with leptin antagonist later during the infantile period (postnatal days 30 to 36) observed in the current study indicates that PVN projections are already established or less sensitive to perturbations in leptin signaling.

In contrast to a previous study in which leptin deficiency in \((ob/ob)\) mice is associated with reduction in fiber density in the PVN [13], we observed a trend toward increased NPY fiber density in PVN of lambs treated with leptin antagonist during the infantile period. These contradictory observations may result from distinct temporal roles of leptin on development of hypothalamic functions. In the \((ob/ob)\) mouse model, leptin deficiency occurs during fetal and postnatal development. Thus, mechanisms to compensate for the absence of leptin signaling may have occurred. In our study in lambs, perturbation in leptin signaling was produced only during a short period after birth.

With the exception of a trend for differences in NPY fibers in the PVN, no detectable changes in the density of NPY fibers were observed in any of the other regions investigated in this study. We acknowledge that the procedure used in the current study to detect NPY fibers (immunocytochemistry) may have limited sensitivity in the detection of all components of NPY cells. For example, NPY soma in the ARC is difficult to detect by immunocytochemistry due to rapid axonal transport of vesicles containing the peptide. Pretreatment with colchicine, a compound that arrests axonal
transport, improves detection of NPY-immunoreactivity in the soma of ARC neurons [23]. In addition, NPY gene expression, NPY synthesis and NPY release in those regions were not determined in our study. Therefore, it is possible that disruption of leptin signaling as used in the current study may regulate distinct aspects of NPY neuron function not investigated in this study. Moreover, only four lambs per group were used in the current study and inclusion of additional animals into each group is warranted to increase statistical power of analysis and for more appropriate conclusions.

In conclusion, it is evident that both leptin and NPY interact for the development and organization of neuronal circuitries within the hypothalamus. However, the results from this study indicate that short-term disruption of leptin signaling in infantile lambs does not clearly influence the density of NPY fibers in the hypothalamus. Inclusion of additional animals in the study will increase the power for appropriate analysis. In addition, further studies are needed to investigate if disruption of leptin signaling affects other biological functions of the NPY neuron.
REFERENCES


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