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Variation of NPY and AGRP MRNA expression in Syrian hamsters according to feeding times

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ABSTRACT

The purpose of this study was to determine the impact of feeding time on NPY/AgRP protein and mRNA expression in the brain during prenatal and postnatal periods. Feeding at different times of the day during pregnancy may have lasting effects on the hypothalamic circuitry in offspring energy homeostasis. In the experimental design, adult female hamsters were randomly assigned to receive three different feeding conditions: ad libitum, night-time feeding and day-time feeding groups. After the lactation period, feeding regimens were continued for the offspring born from mothers who were given nutritional regimens, until they were 30 days old. RT-PCR for NPY/AgRP mRNA expression and ELISA analysis for protein levels were performed on hypothalamus tissues of hamsters at 10, 20 and 30 days of postnatal stage. There was no difference between the groups in terms of the daily measured food consumption of the offspring's. Body weights were significantly decreased in both night-time and day-time feeding groups compared to the ad libitum group ($p<0.05$). The highest increase in mRNA expression of NPY/AgRP was seen in the samples taken at 10. day of lactation in the night-time and day-time feeding groups of the offspring treated with three different feeding regimens. When the feeding regimes were compared, it was observed that the protein and mRNA expression of both NPY and AGRP increased the most in the offspring groups in which the feeding was restricted night –time only. Early stages of development have shown that maternal factors have significantly affected the offspring NPY and AgRP mRNA expression and protein levels. These results show that the metabolic regulation of energy balance may change with maternal factors during the very early stages of development.

Keywords: *Maternal Transfer; NPY/AgRP Neurons; Mesocricetus Auratus; RT-PCR; ELISA*

INTRODUCTION

Nutrition is the most basic need for the survival and growth of the organism. Changes in nutrition very early in development can have long-term adverse effects on the health and growth of offspring's [1]. Changes in the mother's diet during pregnancy have been reported to be associated with obesity in the offspring's adulthood [2]. Obesity is a rapidly increasing metabolic disease worldwide [3]. It is known that obesity poses a risk for cardiovascular diseases, type 2 diabetes, hypertension, metabolic syndrome, some hormone-dependent cancers, and various diseases [4-6]. Although various factors (genes, neuroendocrine, and environment) affect obesity, the most important environmental factor is nutrition [7].

The hypothalamus plays a central role in perceiving nutritional signals while controlling energy use and homeostasis that affect development [8]. Feeding behavior and body weight are regulated by the hypothalamic neurons Neuropeptide Y (NPY) and agouti-related peptide (AgRP) [9]. NPY, the most potent orexigenic peptide in mammals, causes potent increases in food intake and body weight when administered centrally. It has been shown to cause obesity when administered chronically [10-12]. Studies in rats have shown that mRNA expression of hypothalamic NPY is increased in fetuses and adult offspring as a result of changing maternal diet [13, 14]. Maternal feeding is thought to

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cause hyperphagia by increasing NPY and AgRP by affecting the hypothalamic circuits that regulate the offspring's food intake. In response to maternal nutrient restriction, the offspring display an increased fasting/fed gene expression [8]. In maternally malnourished offspring, NPY increase inhibition of anorexigenic neurons.

Maternal transfer, defined as the transfer of information from mother to offspring in mammals, is a physiological event that occurs through the placenta or during lactation [15]. Being pregnant is a very demanding physiological process in which the mother must fulfill all the needs of the unborn child. The life span, growth, and body composition of the offspring are known to be influenced by environmental maternal factors, such as nutrition and photoperiod during pregnancy. Due to unfavorable conditions encountered by the mother during the development of the offspring, it is born with a low birth weight. One of the most significant signals that is transmitted maternally in photoperiodic animals is light information. It has been shown by previous studies that photoperiod information is of great importance in transferring from mother to fetus and that it affects the developing fetus [16, 17]. It should be investigated how and by which mechanisms food restriction affects nutritional physiology in the early period and how metabolic programming plays a role in the development of diseases such as obesity in the long term. In this context, it is not fully understood to what extent the nutrition taken at different times of the day during pregnancy plays a role in this mechanism. Nutrient restriction at different times of the day may have molecular and physiological effects on the development of offspring.

Photoperiodic organisms show changes in many important physiological mechanisms such as energy metabolism, body weight, and reproduction with the stimulation of light information [18, 19]. The Syrian hamster (*Mesocricetus auratus*) is an experimentally preferred model organism due to its photoperiodic character that responds effectively to daylight [18]. Body weight and fat content are controlled by the photoperiod in Syrian hamsters [20]. Therefore, obesity is not observed in these animals. However, the body weight regulation mechanisms in *M. auratus* have not been clearly elucidated yet. There aren't enough studies on how the mother's diet changes during pregnancy under various photoperiodic conditions are transferred to her offspring and how that affects the expression of the relevant neuropeptide genes. The purpose of this study is to see how the relationship between fetal development and nutrient intake affects NPY/AgRP mRNA expression in offspring by restricting maternal nutrition to the light or dark phases.

MATERIAL METHOD

Animals and Diets

Female adult *M. auratus* and its offsprings were used in the experiments. Our study was carried out with the decision of the ethics committee numbered 2019/07-03. For the experiment, adult female hamsters at least 3 months old were selected from the colony in the long photoperiod (14L) with their weights closest to each other (100-110 g). The study was carried out at the Canakkale Onsekiz Mart University, Experimental Research Center (COMUDAM) under a long photoperiod of 14 L (14 hours light, 10 hours dark; lights off between 21.00-07.00 hours). All lighting is provided by automatic programmable timers under controlled 200 lux white fluorescent light. Adult female hamsters were mated with adult male hamsters before starting the experiment, and females found to be pregnant by the vaginal smear method were separated and placed in cages individually. Three different feeding groups were applied to female hamsters during their pregnancy and lactation. These nutritional groups; ad libitum (hamsters received food ad libitum throughout the day; n:4 pregnant animals), night restriction (hamsters fed only in the dark phase; n:4 pregnant animals) and day-restriction (hamsters fed only in the light phase; n:4 pregnant animals). Animals in each feeding group were decapitated on the specified days (10th day, 20th day and 30th day) after birth and tissue and blood samples were taken. Except for the group that was decapitated only on the 10th day (n=8/group), the 20th (n=8/group) and 30th day (n=8/group) groups were placed in separate cages after separation from their mothers and continued to be fed with the mother's diet program. On the 20th and 30th days, the pups were decapitated and their tissues and blood were taken.

Food Consumption

After the lactation period, offspring in all groups had access to a known amount of food (~50 g/d) daily. In the ad libitum group, the food was placed in the cages before the lights were turned off (21.00 h), and the remaining

food was calculated at the same time the next day. In the night-time feeding group, food was placed in the cages before the lights were turned off (21.00 h), and the remaining food was calculated immediately after the lights were turned on (07.00 h). In the day-time feeding group, food was placed in the cages immediately after the lights were turned on (07.00 h), and the remaining food was calculated before the lights were turned off (21.00 h). While the foods were calculated, the pieces of food that fell into the cage and the pieces of food that the offspring kept in their mouths were also collected and calculated. Food intake was measured every day but was taken as the average of two day intervals.

Body Weight Measurement

The body weights of the offspring were measured just before the hypothalamic tissues were collected (10, 20, and 30 days). Pieces of food in their mouths (except those in the lactation period) were checked before the hamster offspring's were individually placed on the digital scale. All measurements were made between 12:00 and 13:00 (mid day).

Collection of Tissue Samples

Decapitation was carried out in all animals between 12:00 and 13:00. After decapitation, the hypothalamus region was quickly removed with microdissection scissors under a stereo dissection microscope (Stemi DV 4 SPOT-ZEISS, USA) in 0.9% NaCl solution, which allows the brain to maintain the viability of the tissue. Tissues taken for each group were placed in eppendorf and immediately frozen in liquid nitrogen. Tissues were stored at -80°C until RNA and protein isolation.

Total RNA Isolation

The Trizol method was applied to ensure high-quality total RNA extraction by removing the hypothalamus samples from -80˚C (Rio et al., 2010). According to the protocol, 1 ml of TRIzol™ Reagent (Sigma-Aldrich, USA) was added to 50 mg tissue from each sample and homogenized under cold conditions, and total RNA was obtained after chloroform, isopropyl alcohol, and ethyl alcohol steps. The concentrations and purity of the RNAs obtained were measured by micro drop (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific™, Finland).

cDNA Synthesis

After equalizing the Total RNA concentration, cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo-Fisher) according to the protocol. 10 min at 25˚C, 60 min at 37˚C, 5 min at 95˚C, and 25 min at 4˚C in a thermal cycler (Eppendorf 6333 Nexus MasterCycler Thermal Cycler- Eppendorf, USA) as per the protocol modified.

Gene Expression

In this study, the cDNAs obtained were used as a reference for the determination of gene expression levels. The regulation levels of *Npy* and *Agrp* genes were calculated using the 2-ΔΔCt (Livak and Schmittgen, 2001) method in the RT-qPCR (Applied Biosystems™ 7500 Fast -Thermo Fisher Scientific, USA) with BrightGreen qPCR Master Mix (ABM, Canada). *Actb* gene region (housekeeping gene) was used as a normalizer in experimental applications. Primer designs for individual genes were made with the Primer 3Plus program (Biomers, Germany) and are given in **Table 1**. In RT-qPCR, the reaction conditions were at 95 ˚C for 10 minutes and 40 cycles after the first denaturation step; Denaturation at 95 ˚C for 5 s, binding at 57 ˚C for 30 s, and elongation at 72 ˚C for 60 s. Melting curves were taken during RT-qPCR studies and a single peak was observed in each sample (Figure 1).

Table 1. Primer sequences of target and endogenous genes

Genes	Primer	Sequence(5'3')	Length	Tm°C	Accession no
NPY	Foward	AGAACAAGGCTTGAAGA	17 bp	59°C	
	Reverse	CAGGATGAGACGAGATG	17 bp		XM_005087180
AGRP	Foward	AATTCCCAGGTCTAAGTC	18 bp	59°C	
	Reverse	GATTCTGTGGATCTAGCA	18 bp		XM_005076290
ACTB	Foward	TGAAGATCAAGATCATTG	18 bp	57°C	
	Reverse	CTCATCGTACTCCTGCTT	18 bp		NM_001281595

Accession no; NCBI Genbank Access Number

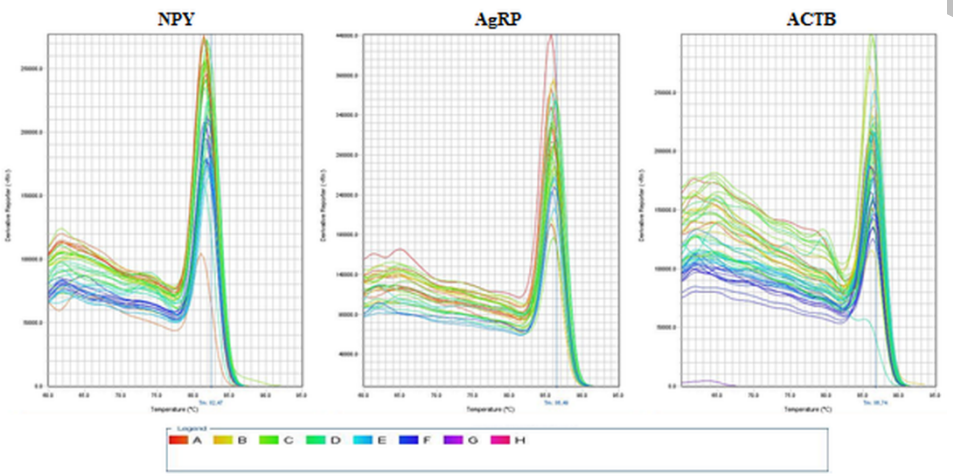

Figure 1. Melting curve image of the Real-Time PCR reaction of the experimental groups. A/B/C/D/E/F/G/H represent samples in each row on the plate.

Protein Analysis

After total RNA extraction, protein purification was performed from the pellet at the bottom of the ependorfs containing TRIzol. The obtained protein samples were stored at -20 °C until ELISA analysis. Protein amounts were equalized before using the ELISA method. Protein amounts of target genes were made with the ELISA kit (Hamster Neuropeptide Y/Agouti-related peptide, Bioassay Technology Laboratory Kit) according to the manufacturer's instructions. Protein standards are given ready-made in the kit. Samples were measured with a spectrophotometer (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific™, Finland) at 450 nm.

Statistical Analysis

The data obtained were analyzed using the IBM SPSS Statistics 21 software package. Body weight, food consumption, mRNA expression levels of NPY/AGRP, and protein amounts were subjected to a two-way ANOVA to compare group means. The differences between groups were determined using the post-hoc multiple comparison test Duncan. Data within groups were expressed as mean ± standard error (SE). The significance level ( ) was set at 95% ($p<0.05$). The graphs were created using the SigmaPlot 14.0 software.

RESULTS

Food Consumption

The food consumption of hamsters after the lactation period is shown in Figure 2. There was no significant difference in the amount of food consumed by the hamsters ($p>0.05$). The amount of food consumed by animals due to growth increased in all groups ($p<0.05$).

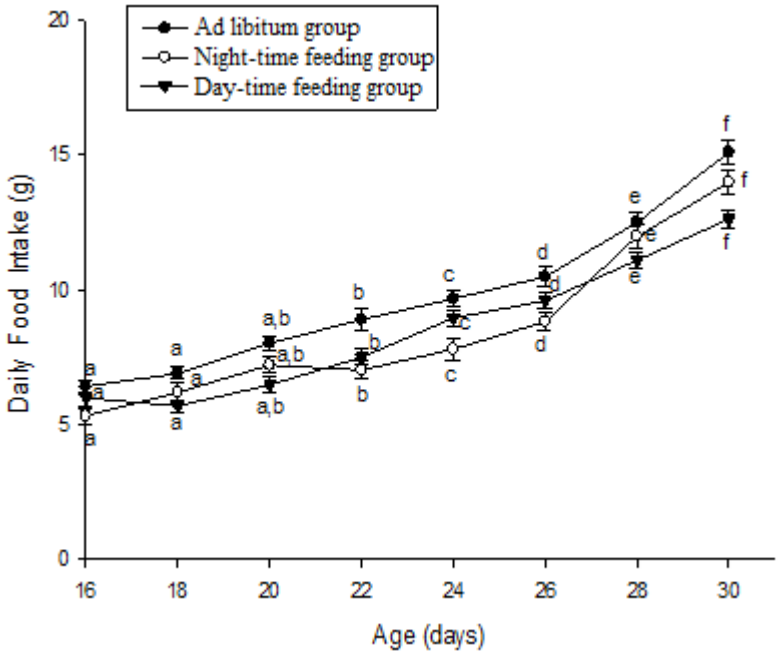

Figure 2. Effects of feeding times on food consumption in *M. auratus* offspring after lactation. Values are expressed as mean ± SE. Different letters indicate statistical significance (Dunn's post hoc test, $p<0.05$).

Body Weights

It was found that the offspring's body weights decreased in the night-time and day-time feeding groups when compared to the ad libitum group ($p<0.05$) (Figure 3). Body weights in the day-time and night-time feeding groups did not differ significantly ($p>0.05$).

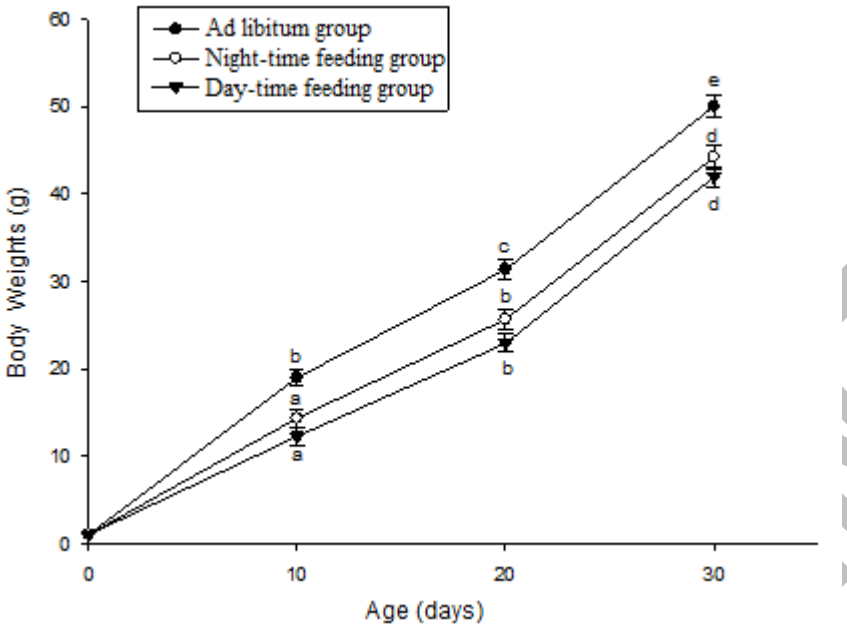

Figure 3. Effects of feeding times on body weight of the offspring Syrian hamsters (*Mesocricetus auratus*). From birth to 30 days, the body weights of the offspring were measured every ten days. Values are expressed as mean ± SE. Different letters indicate statistical significance (Dunn's post hoc test, $p<0.05$).

Gene Expression

The mRNA expression of NPY and AgRP in offspring treated with different feeding regimens are shown in Figures 4 and 5. The mRNA expression of NPY was highest in the night-restricted group at day 10 and day 20 (Figure 4). ($p<0.001$). In the night-time feeding group, NPY mRNA expression on the 30th day decreased compared to the values in the lactation period, but increased when compared to the ad libitum group ($p<0.05$). The mRNA expression of NPY was increased in the day-time feeding groups compared to the al libitum group ($p<0.05$). mRNA expression of AgRP increased more in the night-time feeding groups both during and after lactation compared to the ad libitum group (Figure 5) ($p<0.001$). The highest mRNA expression of AgRP was observed at day 10 in the day-time feeding group ($p<0.01$).

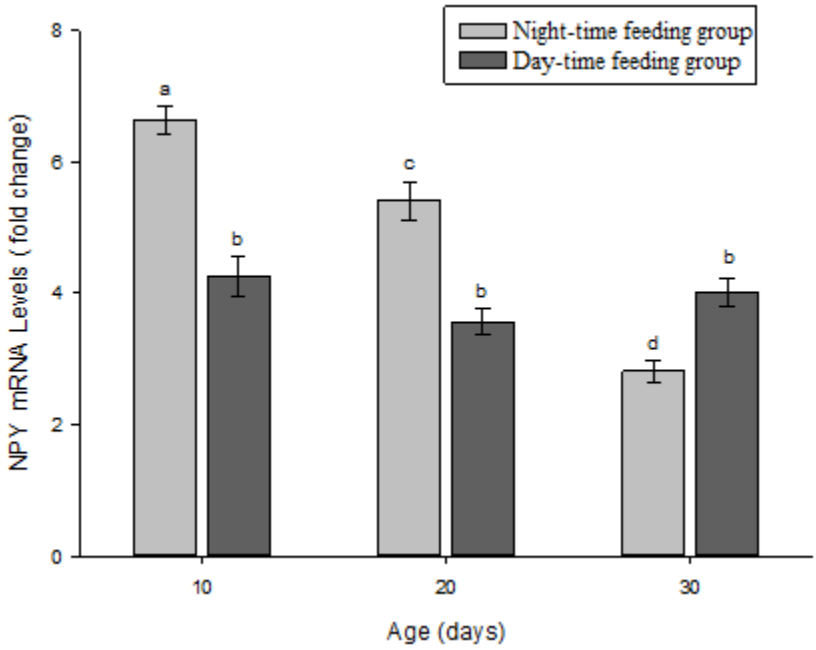

Figure 4. NPY mRNA expression of offspring Syrian hamsters *(Mesocricetus auratus)* relative to the ad libitum group. The NPY mRNA expression profiles of three different feeding regimens at 10, 20, and 30 days from birth are shown. Values are given as mean ±SE. Different letters indicate statistical significance (Dunn's post hoc test, $p<0.05$).

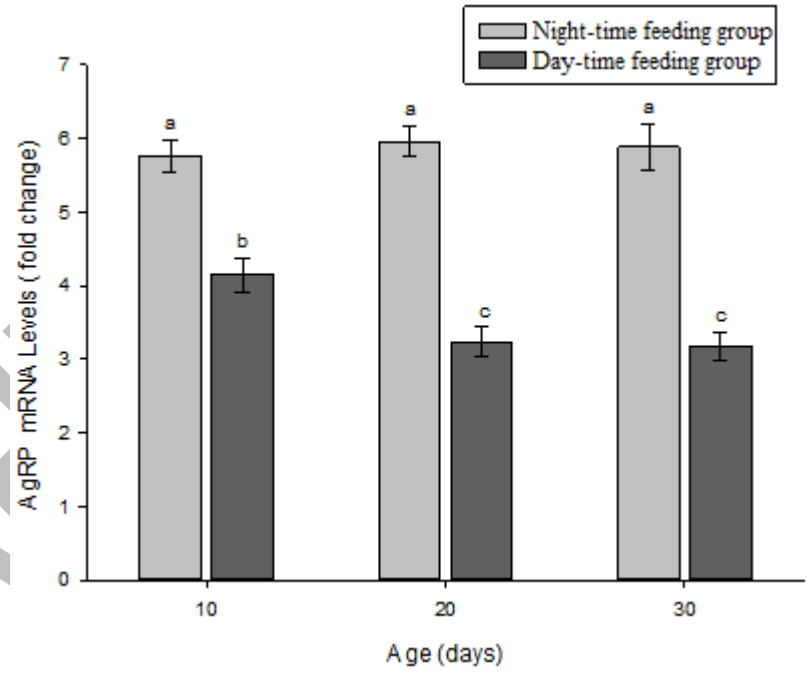

Figure 5. AgRP mRNA expression of the offspring Syrian hamsters *(Mesocricetus auratus)* relative to the ad libitum group. The AgRP mRNA expression profiles of three different feeding regimens at 10, 20, and 30 days from birth are shown. Values are given as mean ± SE. Different letters indicate statistical significance (Dunn's post hoc test, $p<0.05$).

Protein Levels

The level of NPY and AgRP proteins in the hypothalamus of offspring treated with three different feeding regimens are shown in Figures 6 and 7. It has been shown that there is a significant difference in the level of NYP protein on the 10th and 20th days only in the groups with night-time feeding ($p<0.001$). The increase in the protein level of AgRP was determined the most in the night-time feeding groups ($p<0.001$). Among the groups with day-time feeding, the 10-day group had the highest expression levels of AgRP ($p<0.001$). When the three different feeding regimens were compared, the night-time feeding groups had higher NPY and AgRP protein content ($p<0.05$).

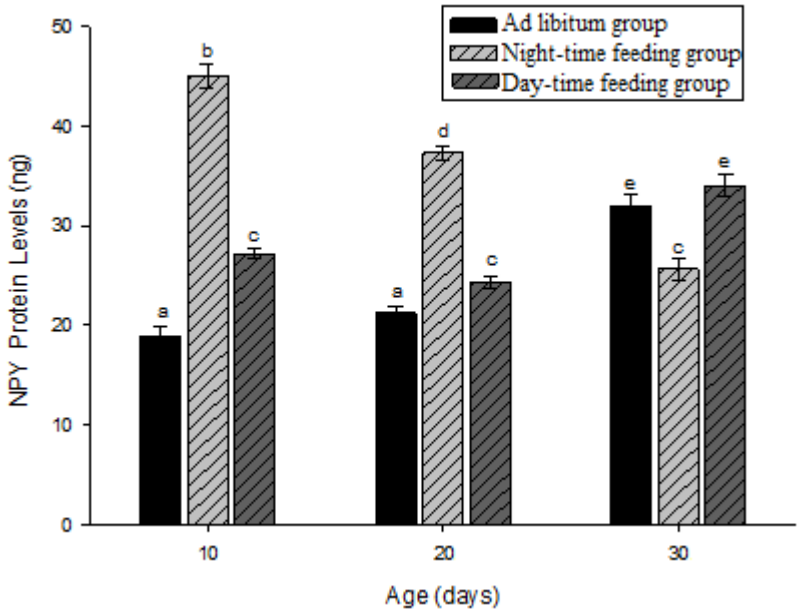

Figure 6. NPY protein levels of the offspring exposed to different feeding regimens. Values are shown as mean ± SE. Different letters indicate statistical significance (Dunn's post hoc test, $p<0.05$).

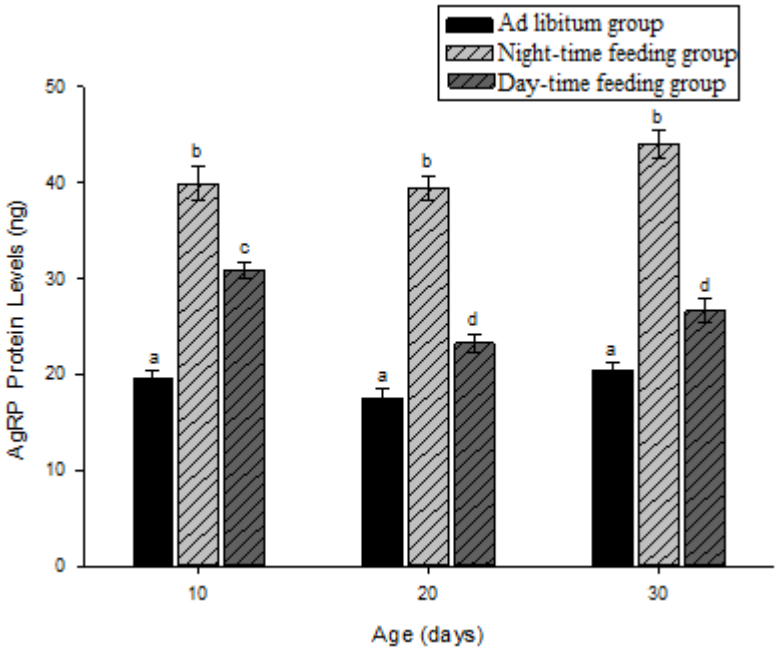

Figure 7. AgRP protein levels of offspring exposed to different feeding regimens. Values are shown as mean ± SE. Different letters indicate statistical significance (Dunn's post hoc test, $p<0.05$).

DISCUSSION

According to epidemiological research on both humans and animals, nutrition plays a critical role in the metabolic regulation of energy balance beginning very early in development [21, 22]. During pregnancy and lactation, a nutrient restriction may cause permanent effects on the metabolic mechanisms of the offspring and change the obesity tendency in adult life [23, 24]. It was determined in this study how a crucial maternal factor, such as nutrition, affects the gene expression of hypothalamic neuropeptides NPY/AgRP in the brains of the offspring and how it changes from the early stages of development to the 30th day.

When compared to the ad libitum group, it was found that the body weights of the offspring in the night-time and day-time feeding groups decreased. It has also been shown in previous studies that the offspring subjected to maternal nutrient restriction during critical periods such as pregnancy and lactation have low body weights [25, 26]. This can be explained by the fact that NPY reduces body weight and bone formation in order to reduce the energy needed in fasting conditions in energy-requiring situations such as pregnancy and breastfeeding [1]. In our study, it was determined that maternal factors (eg nutrition) caused significant differences in NPY/AgRP mRNA expression and protein level in offspring. When dietary regimens were compared, the mRNA expression and protein content of NPY/AgRP increased significantly in the 10th day (lactation period) group, where food intake was restricted especially to night-time only. In previous studies, exposure to maternal diet during lactation increased the protein and mRNA expression levels of NPY/AgRP in offspring [27-29]. The NPY/AgRP mRNA expression and protein content of the offspring appear to be significantly impacted by changes in nutrition during the very early stages of development. The difference in the expression of neuropeptide genes during and after lactation is especially stunning.

NPY mRNA expression decreased after lactation, whereas AgRP expression increased in the night and day food restriction groups of offspring whose feeding regimens were maintained. This pattern of NPY and AgRP mRNA levels has been linked to both short- and long-term fasting in the literature. In the study, it was found that only the AgRP level was significant during long-term fasting, while NPY levels and AgRP levels were both significant during short-term fasting [30]. In a different study, food restriction was implemented for 90 days starting at birth, and it was found that while AgRP increased significantly, NPY did not significantly increase [31]. A food restriction was reported to have had no effect on the expression of NPY in Syrian hamsters, but a significant increase in AgRP [32]. The rapid onset of food intake requires NPY, whereas the chronic onset of food intake requires AgRP. AgRP neuropeptide inhibits the anorexigenic effect of □-MSH when fasting because it binds specifically to the melanocortin 4 receptor (MC4R). It promotes the overexpression of AgRP by acting as an antagonist on MC4R [33, 34]. AgRP neurons have a critical role in directing food intake. Long-term increases in food intake are thought to be mediated by AgRP signaling through MC4R [35].

When the feeding schedules were compared, the nighttime food restricted group showed the highest levels of NPY and AgRP mRNA expression. This can be explained by the fact that Syrian hamsters are nocturnal, and they consume food at night. Additionally, the circadian rhythm is impacted by the presence or absence of food [36, 37]. The circadian rhythm and the control of energy homeostasis are intrinsically linked. Leptin, ghrelin, and insulin, among other dietary hormones that control the activity of NPY and AgRP neurons, circulate at unstable levels [38]. These hormones follow the light-dark cycle or nutritional cycles [39]. In light of this, restricting food intake to the day or night may have an impact on circadian rhythms.

CONCLUSION

The mRNA expression of NPY/AgRP, the offspring's hypothalamic neuropeptides, was examined in this study up to day 30 of development to determine the effects of an important maternal factor, such as nutrition. The results show that dietary changes during the very early stages of development have a significant impact on the expression of NPY/AgRP mRNA in the offspring. Especially, the difference in the expression of neuropeptide genes during and after lactation is remarkable. According to our research, the lactation period is when feeding the young at various times of the day has the greatest impact. Because this was the time when NPY/AgRP protein levels and mRNA expression both increased. The risk of metabolic disorders in the offspring is known to be increased by the mother's improper feeding schedule or undernutrition. Our findings may offer a fresh perspective on energy metabolism, reshape the process of metabolic disease, and identify new targets for early disease prevention and treatment using

nutritional approaches in the early stages of life. Maternal factors that act throughout pregnancy influence the neuronal development of the offspring and may regulate the programming of the expression of nutritional neuropeptide genes throughout life. Our study was limited to the long photoperiod, and it will be necessary to conduct additional research that will consider other photoperiods, as well as factors related to pregnancy, in addition to the timing of feeding.

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