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RELATIONSHIP BETWEEN HYPOTHALAMIC NEUROPEPTIDE Y AND FOOD INTAKE IN THE LACTATING LABORATORY MOUSE, *Mus musculus*

J. K. Kagya-Agyemang[1, 2], C. Hambly[1], M. E. Sharon[1] and J. R. Speakman[1]
[1]School of Biological Sciences, University of Aberdeen, Aberdeen, U.K
[2]Department of Animal Science Education, University of Education, Winneba, Mampong-Ashanti, Ghana
E-Mail: jkagyaagyemang@yahoo.com

ABSTRACT

The laboratory mouse (strain MF1) was used as a model animal to study the hypothalamic mechanisms underlying food intake in lactating animals. Sixty female mice exposed to 21°C were fed high fat (HF), medium fat (MF) and low fat (LF) diets *ad libitum* between days 4 and 18 of lactation. Also fifteen (15) non-reproductive females exposed to 21°C but used as controls were fed *ad libitum* on the same diets (HF, MF; and LF) for 14 days. Fifteen (15) lactating/reproductive females each from HF, MF and LF-fed groups and 5 non-reproductive controls each from HF, MF and LF-fed groups were weighed and killed by CO_2 inhalation on day 18 of lactation. Brains from both the reproductive and non-reproductive females were processed for studies on hypothalamic neuropeptides. Results indicated that lactation decreased pro-opiomelanocortin (POMC) and increased neuropeptide Y (NPY) and agouti-related peptide (AgRP) gene expression determined by *in situ* hybridisation in the hypothalamic arcuate nucleus (ARC). There was no significant change in suppressor of cytokine signalling-3 (SOCS-3) expression ($P>0.05$) in the ARC during lactation. Activated NPY and AgRP orexigenic pathways and attenuated anorexigenic POMC pathways in the hypothalamus probably promoted the hyperphagia of lactation in the MF1 mice. Since NPY and AgRP increased ($P<0.05$) while POMC decreased ($P>0.05$) but SOCS-3 was unchanged probably indicate that the MF1 mice were sensitive to the action of leptin.

Keywords: neuropeptide y **(**NPY), agouti-related peptide (AgRP), pro-opiomelanocortin (POMC), suppressor of cytokine signalling-3 (SOCS-3), leptin.

INTRODUCTION

Reproduction comprises a period of high energy demand so it is the appropriate period for studying the limits in energy intake. Lactation is a physiological state characterized by a large energy demand due to milk production that increases greatly the nutrient needs of an animal (Wade and Schneider, 1992; Barber *et al*., 1997). The energy demand far exceeds that present in a non-lactating animal (Denis *et al*., 2003; Król *et al*., 2007). To meet the increased energy demand, food intake is increased several-fold during lactation (Ota and Yokoyama, 1967; Barber *et al*., 1997), but factors regulating the hyperphagia of lactation are not well understood. In addition, the increased food intake in most mammals is not sufficient to meet the metabolic demands, resulting in a state of negative energy balance during early lactation when body lipid reserves are mobilized (Barber *et al*., 1997; Sorenson *et al.*, 2002).

The major appetite and energy balance centres in the rodent consist of two distinct populations of neurons located in the arcuate (ARC) and ventromedial nuclei (VMT) of the hypothalamus (Kalra *et al.*, 1999). One regulatory pathway consists of neurons co-expressing neuropeptide Y (NPY) and agouti related peptide (AgRP), potent stimulators of food intake, while an adjacent set of neurons co-express pro-opiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART), which suppress food intake (Williams *et al*., 2001). These cells respond to peripheral hormones that signal both the long and short term energy status of the animal. Leptin, secreted by, and in proportion to, white adipose tissue (WTO) enters the brain via the short forms of the leptin receptor (Ob-Ra and Ob-Rc; Bjorbaek *et al*., 1999). It then signals the status of energy stores by activating POMC/CART neurons and inhibiting NPY/AgRP neurons (Elias *et al*., 1999) via the long form of leptin receptor (Ob-Rb; Mercer *et al*., 1996), resulting in the inhibition of feeding and an increase in energy expenditure.

Leptin levels in lactation are generally reduced in rats (Brogan *et al*., 2000; Denis *et al*., 2003) but others found no change (Lopez-Soriano *et al*., 1999). In mice, Mistry and Romsos (2002) observed increased leptin levels during lactation. Brogan *et al*. (2000) reported that leptin receptor levels increased in the supraoptic nucleus (containing oxytocin and vasopressin expressing neurons), but were decreased in the ventromedial nucleus. Leptin induces expression of suppressor of cytokine signalling-3 (SOCS-3), a protein that acts as a feedback inhibitor of leptin signalling (Denis *et al*., 2004). SOCS-3 is expressed in both POMC and NPY neurons (Elias *et al*., 1999). The hypothalamic expression of SOCS-3 in ARC is involved in the modulation of the strength of the leptin signal to facilitate seasonal cycles in body mass and adiposity in the Siberian hamster (*Phodopus sungorus*) and voles (*Microtus agrestis*) (Mercer and Tups, 2003; Król *et al*., 2006).

The orexigenic neuropeptides NPY and AgRP, which are both key downstream effectors of the leptin signal in the brain, are generally elevated in ARC, PVN and dorsomedial nucleus (DMH), and in the median

eminence of lactating rats (Garcia *et al*., 2003; Crowley *et al*., 2004). Both Smith (1993) and Brogan *et al*. (2000) reported that POMC is down regulated in the ARC during lactation in the rat.

The idea that reduced leptin levels do not play the sole role in signalling increasing food intake during lactation was corroborated by Mistry and Romsos (2002) and Stocker *et al*. (2004), when they artificially increased the leptin levels in lactating mice using osmotic pumps. Food intake at peak lactation decreased below that of mice with saline-filled pump but the animals consumed more food than the non-lactating animals, indicating the infused leptin did not completely inhibit food intake in lactating animals. This suggests the involvement of other signalling systems in lactation hyperphagia.

The present study examined the relationship between hypothalamic neuropeptide Y (NPY) and food intake in the lactating laboratory mouse.

MATERIALS AND METHODS

Animals and experimental protocol

All studies involving animals were licensed under the Animals (Scientific procedures) Act of 1986 and received approval from the University of Aberdeen's Ethical Review Committee. Seventy five virgin female mice (*Mus musculus* L.: out bred MF1) aged 8-9 weeks old (Harlan UK Limited, England) were used in this study. The animals were individually housed in "shoe-box"cages (44 cm x 12 cm x 13 cm) with sawdust under a controlled 12 h light: 12 h darkness cycle at 21ºC (range 20 to 22 ºC). Rat and mouse breeder and grower diet made up of 15.60 kJ/g gross energy, 18.80% crude protein, 60.30% carbohydrate, 3.40% crude oil, 3.7% crude fibre and 3.80% ash - all values calculated to nominal 10% moisture content, Special Diets Services, BP Nutrition, Witham, UK) and water were supplied *ad libitum*. Baseline measurement of body mass was used to randomly allocate 60 mice into three (3) treatment groups with 20 animals per treatment group. The completely randomized design (CRD) was used in the study and each treatment group had two (2) replicates with 10 animals per replicate. The females were paired with males for 11 days. The remaining 15 mice were also randomly allocated into three (3) treatment groups with 5 animals per treatment group and they went through the protocol as non-reproductive females.

The body mass, litter size and litter mass were recorded (± 0.01 g) every day from day 1 to the end of lactation (day 18). On days 2 to 3 of lactation, mothers from each treatment group were presented with either high fat (HF), medium fat (MF) or low fat (LF) diet while still supplied with rat and mouse breeder and grower diet *ad libitum*. The diets used were HF, MF and LF diets (Research Diets, New Brunswick, NJ, U.S.A.) (Table-1). From day 4 onwards, the animals were switched from the rat and mouse breeder and grower diet to HF, MF or LF diet exclusively. Maternal food intake was recorded between days 5-18 of lactation. Mice were weaned on day 18 of lactation. Each group of 5 non-reproductive females was fed *ad libitum* on the same diets. Their body masses were recorded for 18 days but their food intake was recorded between days 5-18 of diet manipulation.

Table-1. Composition of experimental diets.

Ingredients (g/kg diet)	**High fat**	**Medium fat**	**Low fat**
Casein	200	200	200
L-cystine	3	3	3
Corn starch	0	72.8	315
Maltodextrin	125	100	35
Sucrose	68.8	172.8	350
Cellulose	50	50	50
Soya oil	25	25	25
Lard	245	177.5	20
Mineral mix	10	10	10
Dicalcium phosphate	13	13	13
Calcium carbonate	5.5	5.5	5.5
Potassium citrate	16.5	16.5	16.5
Vitamin mix	10	10	10
Choline bitartrate	2	2	2
Gross energy (kJ g^{-1})	19.90	19.89	19.89

Source: Research Diets, New Brunswick, NJ, U.S.A.

Tissue collection and preparation

Forty five (45) lactating/reproductive mice (HF, N=15, MF, N=15, LF, N=15) were chosen at random and these together with 15 non-reproductive females (HF, N=5, MF, N=5, LF, N=5) were weighed and killed by CO_2 inhalation on day 18 of lactation. Brains were removed immediately, frozen in isopentane, chilled over dry ice and stored at -80ºC. Brains were mounted onto a chuck using OCT compound (Raymond Lamb, UK) and 20 µm sections cut on a cryostat (Leica, CM3050S, Milton Keynes, UK). Coronal hypothalamic portions of mouse brains were cut throughout the extent of arcuate nucleus (ARC) and mounted on each gelatine-and poly-L-lysine-coated slide and stored at -80°C.

Hypothalamic gene expression during lactation in the laboratory mouse

Complementary fragments of a number of energy balance related neuropeptides and receptors were amplified from mouse hypothalamic cDNA by polymerase chain reaction (PCR). PCR reactions were all performed by 1 min steps of denaturation at 94°C, annealing 53 to 60°C, elongation 72°C, over 33 to 36 cycles. The PCR products of expected size were cloned into the pCR-Script Amp SK (+) vector (Stratagene, CA, USA) or the pGMET Easy Vector (Promega, Southampton, UK) and transformed into competent cells. All sequences were verified by Eurofins Sequencing Services (Ebersberg, Germany) and antisense and sense riboprobes were tested for specificity and background binding. For riboprobe synthesis, the plasmid DNA of interest was isolated from bacterial cultures, incubated at 37ºC overnight using the QIAprep Spin Miniprep kit (Qiagen, Germany). DNA concentration was determined using the Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, USA) and the DNA linearised at 37ºC with restriction enzymes (Promega, Southampton, UK) specific for the plasmid. The linearised DNA was purified using QIAquick Purification Kit. The following RNA polymerase/restriction enzyme pairings were used to generate the antisense riboprobes: T7/SacI was used to transcribe neuropeptide Y (NPY), pro-opiomelanocortin (POMC) and agouti-related peptide (AgRP) and T7/SalI to transcribe suppressor of cytokine signalling-3 (SOCS3).

***In situ* hybridisation**

For labeling with sense and antisense probes, adjacent sections were fixed in 4% paraformaldehyde (Sigma-Aldrich, UK) in 0.1 mol^{-1} phosphate buffer saline (PB) at room temperature and washed in 2 x 0.1 M PB. Sections were immersed in 0.1 mM triethanolamine (TEA) (Sigma-Aldrich, UK) and acetylated in 0.1 mM TEA/ 0.25% acetic anhydride (Sigma-Aldrich, UK). Sections were washed in 0.1 M PB and dehydrated using increasing concentrations of 50%, 70%, 90% and 100% ethanol and dried under vacuum. Using ^{35}S-labelled riboprobes (Perkin Elmer, UK) at a concentration of $1x10^6$ c.p.m ml^{-1}, slides were hybridised overnight at 58ºC. Post-hybridisation, cover slips were removed by washing slides in 4 x SSC at 60ºC. Unhybridised RNA was digested with incubation in RNase. Sections were desalted in a series of SSC solutions: 2 x SSC, 1 x SSC, 0.5 x SSC and 0.1 x SSC. Sections were dehydrated for a second time in increasing concentrations of 50%, 70%, 90% and 100% ethanol. The slides were air-dried and exposed to Kodak Bio Max Film (Sigma, UK) for 5 days. Autoradiographs of sections including micro scale standards were scanned on Umax Power Look II (UMAX Data System, Fremont, CA, USA), and gene expression was taken as the optical density in the area of interest using Image J software system (WinZip Computing Inc., USA).

Statistical analysis

Data are represented as means ± S.E.M. and analysed by general linear modelling (GLM) using one way analysis of variance. All statistical analyses were performed using Minitab for Windows (version 14; Minitab Inc., State College, PA, USA; Ryan *et al*., 1985). $P<0.05$ was considered statistically significant.

RESULTS

Maternal body mass

The body masses of HF, MF and LF-fed reproductive females at peak lactation (days 12-17) were significantly higher ($P<0.001$) than that of the HF, MF and LF-fed non-reproductive females. Tukey pair wise comparisons indicate that the body mass of HF, MF and LF-fed reproductive females was not different ($P>0.05$) but this was significantly higher ($P<0.05$) than that of HF, MF and LF-fed non-reproductive females. The body mass of the reproductive females averaged 44.27±1.46 g, 44.38±0.42 g, and 45.03±0.74 g for HF, MF and LF-fed reproductive females while that of the non-reproductive females averaged 27.70±1.06 g, 26.09±0.47 g, and 26.75±0.89 g, respectively at peak lactation (Figure-1).

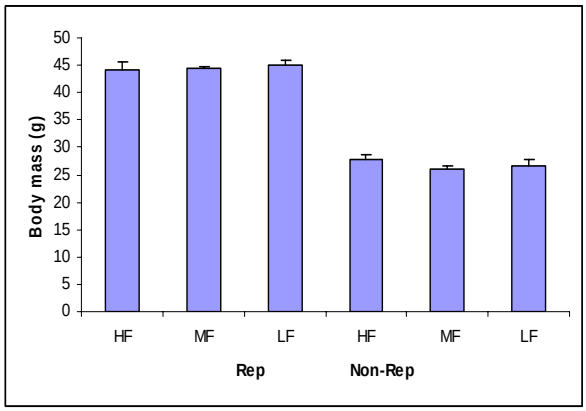

Figure-1. Mean body mass of reproductive (Rep) and non-reproductive (Non-Rep) female mice. Values are means ± SE.

Maternal energy intake

The food intake of both reproductive and non-reproductive females during days 12-17 of lactation is shown in Figure-2. The reproductive mice consumed more food ($P<0.001$) than the non-reproductive ones. The peak lactation (days12-17) energy intake of the reproductive females and non-reproductive females was significantly different ($P<0.001$), showing that the reproductive mice consumed more energy at peak lactation than the non-reproductive mice. The energy intake of HF and MF-fed reproductive females was not different ($P>0.05$), but these were significantly higher ($P<0.05$) than that of LF-fed reproductive females. The energy intake of the LF-fed reproductive females was significantly higher ($P<0.001$) than that of the non-reproductive females (Figure-3). The peak lactation energy intake of the HF, MF and LF-fed reproductive mice averaged 306.52±10.45 kJ day^{-1}, 340.52±5.52 kJ day^{-1} and 266.67±1.82 kJ day^{-1} while that of the non-reproductive mice averaged 88.25±3.96 kJ day^{-1}, 58.87±2.66 kJ day^{-1} and 53.07±2.73kJ day^{-1}, respectively for HF, MF and LF-fed females.

There was a highly significant effect of diet ($P<0.001$) on maternal energy intake at peak lactation. All Tukey pair wise comparisons among levels of diet showed that the peak lactation energy intake of HF-fed females was significantly higher ($P<0.05$) than that of LF-fed females (between days 12-17), but the energy intake of HF and MF-fed females was not different ($P>0.05$). Again, there was a highly significant effect of reproductive state ($P<0.001$). Tukey pairwise comparisons showed that peak lactation energy intake of the reproductive females was significantly higher ($P<0.05$) than that of non-reproductive females. Furthermore, there was a highly significant interaction effect on diet and reproductive state ($P<0.001$). Comparisons among levels of diet showed that the influence of HF diet on reproductive state at peak lactation was significantly higher ($P<0.05$) than LF diet. However, HF and MF diets were not significantly ($P>0.05$) different, but MF diet was significantly different ($P<0.05$) from LF diet.

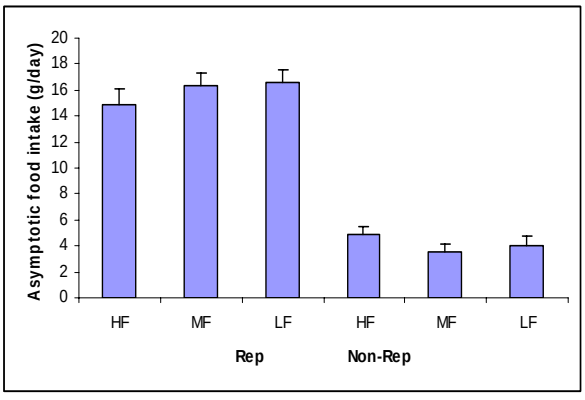

Figure-2. Mean food intake of reproductive (Rep) and non-reproductive (Non-Rep) female mice. Values are means ± SE.

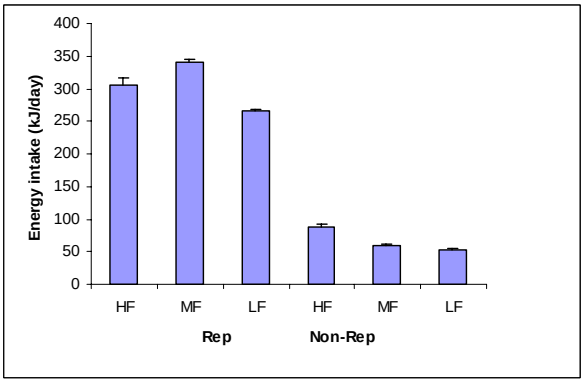

Figure-3. Daily digestible energy intake of reproductive (Rep) and non-reproductive female mice. Values are means ± SE.

Hypothalamic peptides and receptor gene expression

The probes for all the four peptides studied were hybridised to arcuate (ARC), ventromedial (VMH) and dorsomedial (DMH) nuclei but gene expression outside the ARC was variable in the case of NPY, AgRP, POMC and SOCS-3.

There was a significant effect of diet ($P<0.05$) on NPY gene expression in the ARC. Again, there was a highly significant effect of reproductive state ($P<0.001$). Tukey pair wise comparisons among levels of reproductive state showed that the NPY gene expression in the reproductive females was significantly higher ($P<0.05$) than that of the non-reproductive females (Figure-4). There was no significant interaction effect on diet and reproductive state ($P>0.05$). However, there was a significant effect of diet ($P<0.05$) on AgRP gene expression in the ARC. Tukey pair wise comparisons among levels of diet showed that the AgRP gene expression in the LF-fed females was significantly higher ($P<0.05$) than that of HF and MF-fed females but the gene expression in the HF and MF-fed females was not different ($P>0.05$). Again, there was a significant effect of reproductive state ($P<0.05$). Tukey pair wise comparisons among levels of reproductive state showed that AgRP gene expression in the reproductive females was significantly higher ($P<0.05$) than that of non-reproductive females (Figure-5). However, there was no significant interaction effect on diet and reproductive state ($P>0.05$). Diet had no significant effect ($P>0.05$) on POMC gene expression in the ARC. However, there was a highly significant effect of reproductive state ($P<0.001$). Tukey pair wise comparisons among levels of reproductive state showed that POMC gene expression in reproductive females was significantly reduced ($P<0.05$) when compared with that of non-reproductive females (Figure-6). There was no significant interaction effect on diet and reproductive state ($P>0.05$). SOCS-3 gene expression in the ARC showed no significant effect of diet ($P>0.05$), reproductive state ($P>0.05$) (Figure-7) and interaction effect on diet and reproductive state ($P>0.05$).

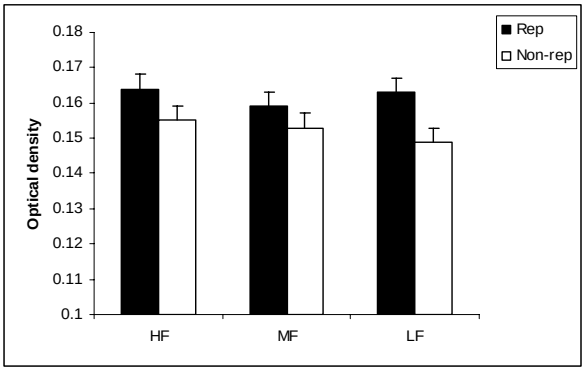

Figure-4. Hypothalamic arcuate nucleus (ARC) gene expression of neuropeptide Y (NPY) in reproductive females (solid bars) and non- reproductive females (open bars). Values are means ± SE.

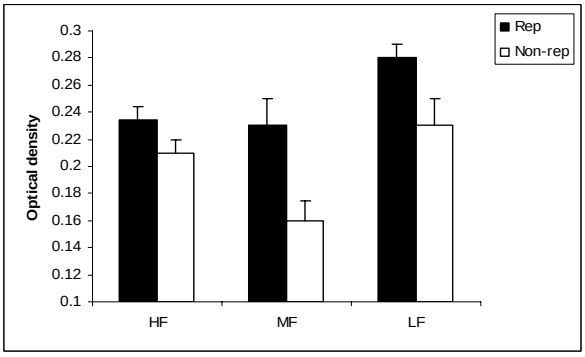

Figure-5. Hypothalamic arcuate nucleus (ARC) gene expression of agouti-related peptide (AgRP) in reproductive females (solid bars) and non-reproductive females (open bars). Values are means ± SE.

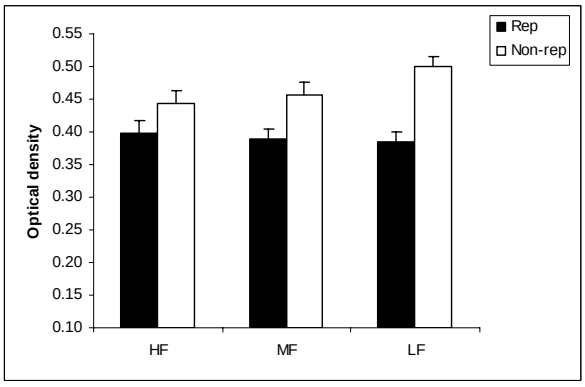

Figure-6. Hypothalamic arcuate nucleus (ARC) gene expression of pro-opiomelanocortin (POMC) in reproductive females (solid bars) and non-reproductive females (open bars). Values are means ± SE.

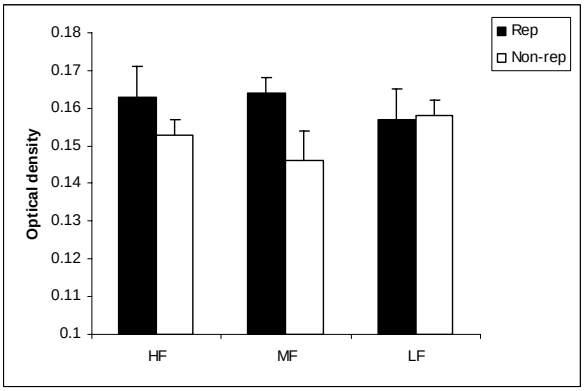

Figure-7. Hypothalamic arcuate nucleus (ARC) gene expression of suppressor of cytokine signalling-3 (SOCS-3) in reproductive females (solid bars) and non- reproductive females (open bars). Values are means ± SE.

DISCUSSIONS

The female mice fed HF, MF and LF diets continued to increase in body mass until the end of lactation and therefore recorded higher body masses when compared with the non-reproductive mice (Figure-1). They also consumed more food (Figure-2) and more energy (Figure-3) at peak lactation than the non-reproductive mice. This finding is consistent with the report that lactation is a physiological state in which the energy expenditure attributed to milk production is met by a large increase in food intake compared with non-reproductive animals (Johnson *et al*., 2001; Król *et al*., 2007). Associated with the hyperphagia of lactation, previous studies suggested that the expression of orexigenic peptides NPY and AgRP was elevated (Chen *et al*., 1999; Chen *et al*., 2004) whereas the anorectic peptide, POMC, was reduced (Smith, 1993). Reduced leptin increases the expression of anorexigenic neuropeptide POMC and CART and decreases expression of the orexigenic neuropeptides NPY and AgRP in the hypothalamus (Schwartz *et al*., 2000). Similar to previous reports, NPY (Figure-4) and AgRP (Figure-5) were elevated and POMC (Figure-6) was significantly reduced in the present study. These findings are consistent with the report that NPY and AgRP, which are both key downstream effectors of the leptin signal in the brain, are generally elevated in the ARC of lactating rats (Garcia *et al*., 2003; Crowley *et al*., 2004) while POMC is down regulated in the ARC during lactation in the rat (Smith, 1993 and Brogan *et al*., 2000). Other studies have also reported a rise in NPY (Ciofi, *et al*., 1991; Sorensen, *et al*., 2002) and AgRP gene expression (Chen *et al*., 1999) and a fall in POMC gene expression (Smith, 1993; Pape and Tramu, 1996).

The lack of change in SOCS-3 expression in the MF1 mice (Figure-7) is quite difficult to explain. However, it appears that other factors other than leptin based signalling also drive hyperphagia of lactation (Sorensen *et al*., 2002). Suckling stimulus is necessary for

hyperphagia in rats (Flemming, 1976; Woodside *et al*., 2000), but the mechanism is unresolved. Suckling increases the secretion of both prolactin and oxytocin but oxytocin decreases food intake in rats (Benelli *et al*., 1991). It has been suggested that prolactin released from the pituitary in response to suckling stimulation acts centrally to stimulate food intake during lactation by either reducing the activity of POMC neurons or increasing that of the NPY/AgRP system (Chen and Smith, 2004; Kokay and Grattan, 2005).

CONCLUSIONS

In the present study, NPY and AgRP gene expression increased while POMC was reduced but SOCS-3 was unchanged in the lactating mice fed on HF, MF and LF diets at 21°C. This probably shows that the MF1 mice are sensitive to leptin action. However, since SOCS-3 gene expression was unchanged indicates that the mechanisms driving hyperphagia of lactation in the MF1 mice are not well understood. It is possible that the suckling-induced prolactin release in MF1 mice might act centrally to stimulate food intake but this requires further investigation.

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