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## Programmed Hyperphagia in Offspring of Obese Dams: Altered Expression of Hypothalamic Nutrient Sensors, Neurogenic Factors and Epigenetic Modulators

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### Abstract

Maternal overnutrition results in programmed offspring obesity, mediated in part, by hyperphagia. This is remarkably similar to the effects of maternal undernutrition on offspring hyperphagia and obesity. In view of the marked differences in the energy environment of the over and under-nutrition exposures, we studied the expression of select epigenetic modifiers associated with energy imbalance including neurogenic factors and appetite/satiety neuropeptides which are indicative of neurogenic differentiation. HF offspring were exposed to maternal overnutrition (high fat diet; HF) during pregnancy and lactation. We determined the protein expression of energy sensors (mTOR, pAMPK), epigenetic factors (DNA methylase, DNMT1; histone deacetylase, SIRT1/HDAC1), neurogenic factors (Hes1, Mash1, Ngn3) and appetite/satiety neuropeptides (AgRP/POMC) in newborn hypothalamus and adult arcuate nucleus (ARC). Despite maternal obesity, male offspring born to obese dams had similar body weight at birth as Controls. However, when nursed by the same dams, male offspring of obese dams exhibited marked adiposity. At 1 day of age, HF newborn males had significantly decreased energy sensors, DNMT1 including Hes1 and Mash1, which may impact neuroprogenitor cell proliferation and differentiation. This is consistent with increased AgRP in HF newborns. At 6 months of age, HF adult males had significantly increased energy sensors and decreased histone deacetylases. In addition, the persistent decreased Hes1, Mash1 as well as Ngn3 are consistent with increased AgRP and decreased POMC. Thus, altered energy sensors and epigenetic responses which modulate gene expression and adult neuronal differentiation may contribute to hyperphagia and obesity in HF male offspring.

### Keywords

Energy sensors; epigenetic factors; bHLH genes

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## INTRODUCTION

The epidemic of obesity continues to plague the United States<sup>1</sup> and much of the industrialized world.<sup>2</sup> Less industrialized countries, which previously experienced significant undernutrition, have evidenced a dramatic demographic transition from stunting to childhood and adult obesity.<sup>3</sup> Although postulates have included an increase in food availability, a western high fat diet, and reduced work-related energy expenditure, these etiologies are unlikely to have accounted for this dramatic increase in the past 30 years. In fact, even if one should attribute the obesity epidemic to these environmental changes, it further indicates an intrinsic dysregulation of central energy balance regulation.<sup>4:5</sup> Questions arising are whether some humans are simply unable to regulate their food intake in response to reduced energy requirements and plentiful food,<sup>6:7</sup> or alternatively, whether they have an intrinsically dysfunctional food intake-regulatory system which is manifested under these conditions?

Early studies of Barker and colleagues indicated that maternal undernutrition results in offspring with a marked predisposition to the development of metabolic syndrome, including obesity, hypertension, and diabetes.<sup>8</sup> Our laboratory and others have confirmed in animal models that maternal undernutrition is associated with low birth weight offspring, which consistently develop adult obesity and metabolic syndrome.<sup>9-11</sup> We and others have further demonstrated that low birth weight offspring have enhanced food intake contributing, in part, to the development of obesity,<sup>12:13</sup> and *in vivo* studies have confirmed reduced food-intake response to anorexigenic factors such as leptin and sibutramine.<sup>14</sup> Low birth weight offspring have altered energy sensing, specifically SIRT1 within the hypothalamic arcuate nucleus (ARC), a putative site of appetite regulation.<sup>15</sup> Our studies utilizing both *in vivo* models and fetal/newborn neuroprogenitor cell (NPC) culture, have indicated that these altered energy sensors effect neurogenic factors which reduce NPC proliferation, induce premature NPC differentiation, and ultimately result in an increase of hypothalamic eating-stimulatory (NPY; neuropeptide Y and AgRP; agouti-related protein) versus eating-inhibitory (POMC; pro-opiomelanocortin) neurons and peptide expression.<sup>15:16:41</sup>

With the 30 year obesity epidemic, the population shift has resulted in a more than twofold increase in the proportion of overweight and obese women presenting for prenatal care.<sup>17</sup> Epidemiologic studies of this maternal cohort have also suggested an increased risk of offspring obesity in progeny.<sup>18</sup> Although offspring socioeconomic and nutritive factors may well contribute to the human condition, animal studies by our laboratory and others have further demonstrated the programming effects of a maternal high fat (HF) diet and obese environment on programming of offspring obesity.<sup>19-22</sup> In male, but not female, offspring of HF fed dams, food intake was significantly increased in parallel to weight gain.<sup>19</sup>

Thus, both maternal undernutrition and maternal overnutrition result in offspring predisposed or programmed for the development of adult obesity, mediated in both groups, in part, by hyperphagia. In view of the marked differences in the energy environment of the under- and overnutrition exposures, we sought to examine energy sensors within the hypothalamus (one day old) and specifically within the hypothalamic arcuate nucleus (ARC;

adult). We further examined the expression of select epigenetic modifiers associated with energy imbalance, neurogenic factors and feeding-related neuropeptides which are indicative of neurogenic differentiation.

## METHODS

The animals used in the present study are from a larger study that includes additional groups of high fat exposure solely during pregnancy or lactation. The phenotypic characteristics including long-term offspring body weight gain, food intake and select metabolic parameters have been published previously.<sup>19</sup>

### Maternal Diet

Studies were approved by the Animal Research Committee of the Los Angeles Biomedical Research Institute at Harbor UCLA and are in accordance with the American Association of Accreditation of Laboratory Care and National Institutes of Health Guidelines. The rat model of maternal obesity was created using a high fat diet prior to and through pregnancy and lactation as previously described.<sup>19</sup> Briefly, Sprague Dawley (Charles River Laboratories, Inc., Hollister CA) were housed in a facility with constant temperature and humidity and on a controlled 12 hour light - 12 hour dark cycle. Beginning as weanlings, female rats were fed a high fat (HF; 60% kcal fat, Research Purified Diet 1249258Y1, New Brunswick, NJ; N=6) or Control (10% kcal fat, Research Purified Diet 12450B58Y2, New Brunswick, NJ; N=6) diet. At 11 weeks of age the rats were mated and continued on their respective diets during pregnancy and lactation. Animals gave birth spontaneously and pups were culled to eight per litter (4 males and 4 females) to normalize rearing.

### Offspring

Excess pups (beyond the 4 males and 4 females) were sacrificed at postnatal day one and hypothalami from two male pups per litter were pooled. The eight remaining pups were nursed by HF dams and weaned to a control 10% kcal diet at three weeks of age. At six months of age six males (one per litter) were sacrificed and the arcuate nucleus dissected. Of note, obese HF females did not show hyperphagia and hence were not studied. At each age, 6 litters were studied per group.

### Hypothalamic/ARC Dissection

For mediobasal hypothalamus, semi-spheres adjunct to two sides of hypothalamus were cut, the dorsal part removed, and the ventral part (~2 mm) used. The ARC was microdissected using the fornix and third ventricle as landmarks (the area adjacent to the bottom of the third ventricle is dissected parallel to the border of the ventricle).

### Western Blot

Westerns were performed as previously reported by our group.<sup>23</sup> Sample lysates (lysis buffer with protease and phosphatase inhibitors) together with positive control lysate or available recombinant protein was used for each gel. Specificity of antibody binding was verified by mixing available blocking peptide with lysate samples. For detection of pAMPK, NaF (50mM) was added to all buffers which had been demonstrated to be an effective

phosphoserine and phosphothreonyl protein phosphatase inhibitor, and the value is expressed as a ratio of pAMPK/AMPK.<sup>24</sup> Blots were applied with SuperSignal West Pico Chemiluminescence Substrate (Pierce) to produce chemiluminescence which was visualized by exposing blots to X-ray film (HyBlot CL™ Autoradiography Film, Denville Scientific, Inc.). Densities of target protein bands were determined by a densitometer (Alpha digidoc 1000, Alpha Innotech Corporation, CA, USA) and normalized against GAPDH (37kd, 1:10,000, MAB374, Millipore, Billerica, MA). Antibodies were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) unless otherwise specified: AgRP (1:500, 14kd, sc-50299); AMPK (1:1000, 63kd, sc-19128); DNMT1 (1:500, 184 kd, sc-10221); HDAC1 (1:500, 60 kd, sc-81598); Hes1 (1:500, 35kd, sc-25392); LSD1 (1:1000, 110kd, ab62582, Abcam, Cambridge, MA); Mash1 (1:500, 30kd, sc-13222); mTOR (1:1000, 220 kd, sc-8319); Ngn3 (1:1000, 23kd, ab38548, Abcam, Cambridge, MA); POMC (1:500, 30kd, sc-20148); SIRT1 (1:2000, 120 kd, sc-5322).

### Data Analysis

NCSS statistical software was used for data analysis. Differences between HF and the Control males were compared using unpaired t-test.

## RESULTS

### Phenotype

As a consequence of the maternal HF diet, dams were significantly heavier than controls at the time of mating ( $301\pm 9$  vs  $233\pm 7$  g;  $p<0.001$ ), at term pregnancy (e20:  $430\pm 15$  vs  $330\pm 12$  g;  $p<0.001$ ) and end of lactation ( $345\pm 7$  vs  $309\pm 5$  g;  $p<0.001$ ). Despite maternal obesity, male offspring born to obese dams had similar body weight at birth as Control ( $7.3\pm 0.1$  vs  $7.4\pm 0.2$ g). However, when nursed by the same dams, adult male offspring of obese dams demonstrated marked increased body weight ( $659\pm 17$  vs  $866\pm 27$  g;  $p<0.001$ ) and adiposity (i.e., percent body fat as measured by DEXA;  $28.1\pm 1.7$  vs  $15.6\pm 1.3$  %,  $p<0.001$ ). The phenotype of these offspring that include increased adiposity and hyperphagia has been previously reported. Importantly, despite the pups born to HF dams and nursed by Control dams and pups born to Control dams and nursed by HF dams of both sexes gained more weight than the Controls, only male pups born to HF dams and nursed by HF dams exhibited significant hyperphagia.<sup>19</sup>

### Energy Sensors and Epigenetic Factors

At 1 day of age, HF males had significantly decreased expression of hypothalamic energy sensors mTOR (0.7-fold) and pAMPK (0.8-fold). In addition, hypothalamic expression of DNA methylase DNMT1 (0.6-fold) was also reduced as compared to Control males. However, hypothalamic expression of histone demethylase LSD1 and histone deacetylase SIRT1 and HDAC1 was similar in HF and Control males (Fig. 1).

At 6 months of age, HF adult males showed significantly increased ARC expression of mTOR (1.3-fold), pAMPK (1.5-fold) and LSD1 (1.4-fold) though normalized expression of DNMT1. Furthermore, ARC expression of SIRT1 (0.8-fold) and HDAC1 (0.7-fold) was significantly suppressed in HF as compared to Control males (Fig. 2).

### Expression of Feeding-Related Neuropeptides

At 1 day of age, HF males had significantly decreased expression of hypothalamic bHLH neurogenic factors Hes1 (0.6-fold) and Mash1 (0.6-fold), though similar Ngn3 as Control. Moreover, HF males exhibited increased hypothalamic expression of neuropeptide AgRP (1.4-fold) with unchanged POMC (Fig. 3).

At 6 months of age, HF adult males showed sustained reduction in ARC Hes1 and Mash1 (0.6-fold) expression and, in addition, demonstrated reduced expression of Ngn3 (0.5-fold). ARC AgRP continued to be increased (1.5-fold) though there was now decreased POMC expression (0.5-fold) in HF as compared to Control males (Fig. 4).

## DISCUSSION

The results of the present study indicate the hypothalamic programming effects of maternal obesity/HF diet on offspring hypothalamic energy regulation. Exposure to an overnourished fetal/newborn environment potentiates development and expression of orexigenic peptide and perhaps neurons, likely promoting hyperphagia and obesity.

As reported previously,<sup>19</sup> a maternal HF diet prior to and during pregnancy and lactation results in male and female rat offspring with similar body weights at birth but marked obesity at six months of age, despite weaning to control diet. Programmed obesity results in part from nursing by high-fat fed dams, as the HF pups are heavier at weaning.<sup>19</sup> The 30% difference in body weight at three weeks of age between Control and HF offspring is sustained till six months of age, indicating additional postweaning mechanisms of obesity. Accordingly, we have previously demonstrated that the male HF offspring have significant hyperphagia through at least 24 weeks of age. Notably, Control males exposed to maternal HF during nursing period alone and all female HF offspring are obese though not hyperphagic,<sup>19</sup> suggesting additional energy regulatory mechanisms.

The hypothalamic ARC nucleus is a central regulator of energy homeostasis, specifically the control of eating. Within the ARC are primarily medial eating-stimulatory NPY/AgRP neurons and primarily lateral eating-inhibitory POMC neurons. Both neuronal populations project to second order centers (e.g., paraventricular nucleus) where they affect food intake. In the adult, ARC AgRP and POMC neuronal activity are regulated by fed versus fasting states, hormones (e.g., leptin, insulin, ghrelin), and the levels of activation of specific energy sensors.<sup>25–27</sup> Specifically, SIRT1, AMPK and mTOR respond to changes in NAD/NADH and AMP/ATP and influence neuronal AgRP and POMC neuronal activity.<sup>28–30</sup>

As compared with energy effects on ARC activity in adult life, altered hypothalamic energy levels and sensor-mediated responses may influence expression of putative neuroproliferative and neurodifferentiation factors and hence ARC development during fetal life. Using hypothalamic NPC from newborn rat, we have previously confirmed direct impact of SIRT1 on NPC proliferation and differentiation by both SIRT1 siRNA and exposure to SIRT1 pharmacologic inhibitor and activator.<sup>15</sup> In the present study, despite evidence of maternal overnutrition during fetal life, one day old HF newborns demonstrated normal hypothalamic SIRT1 expression. Nutrition influences on SIRT1 expression remains

conflicting, with studies demonstrating that fasting both decreases and increases hypothalamic SIRT1.<sup>29;31;32</sup> Previously, we have demonstrated that maternal undernutrition during the second half of pregnancy increased SIRT1 expression in one day old newborns.<sup>16</sup> Accordingly, it was unexpected to find no change in hypothalamic SIRT1 expression in pups of overnourished dams. Several mechanisms may explain this finding. Firstly, obese mice fed a high fat/high sucrose diet did not display feeding-induced changes in hypothalamic SIRT1.<sup>33</sup> It is possible that the maternal HF diet and thus fetal exposure may have similarly altered newborn SIRT1 responses. Secondly, SIRT1 expression may have been altered in the ARC nucleus, though this may not have been detected by sampling the whole hypothalamus (due to ARC size) in one day old newborns. Also, it is likely that it is the SIRT1 activity is altered. Thirdly, newborn pups were of normal not increased weight, suggesting that despite the maternal overnutrition, fetal energy sensors may be less impacted.

At six months of age, ARC SIRT1 expression was suppressed in HF males, consistent with the enhanced energy status associated with offspring obesity.<sup>19;34</sup> SIRT1 is an NAD(+)-dependent histone deacetylase (HDAC). Whereas histone acetylation at specific loci correlates with open chromatin state permitting gene transcription, deacetylation effectively suppresses gene transcription. In contrast to SIRT1, an NAD(+)-dependent enzyme, HDAC1 is a zinc-dependent enzyme. Notably SIRT1 interacts physically with HDAC1, and SIRT1 and HDAC1 may co-localize.<sup>35</sup> Furthermore, SIRT1 deacetylates HDAC1, stimulating its activity.<sup>35</sup> Thus, it is not unexpected to find decreased ARC HDAC1 coincident with reduced SIRT1. Although we did not find evidence of suppressed HDAC1 expression in whole hypothalamus of one day old newborns, it remains possible that ARC or subventricular NPCs<sup>36</sup> may be selectively impacted, or that HDAC1 expression is suppressed during early neonatal life. HDAC1 has significant effects during neurogenesis impacting both neuroproliferation and neurodifferentiation, including that of subventricular neural cells<sup>37;38</sup> which may ultimately form the ARC nucleus. In the present study, whether similar histone mediated suppression of gene transcription occurs in the HF offspring, is not known.

In regard to epigenetic modifications, one day old HF newborns demonstrated a reduction in hypothalamic DNMT1, but similar expression in the ARC as the Controls at 6 months of age. DNMT1 is responsible for maintaining DNA methylation following development, and may impact the expression of genes sensitive to promoter CpG methylation levels. There have been limited studies of DNMT1 expression in regard to programmed obesity, though Xia *et al*<sup>39</sup> demonstrated reduced DNMT1 associated with the leptin promoter at 4 and 8 weeks following HF diet-induced obesity. Whether the DNMT1 reduction was a result of the nutrient environment or a consequence of obesity itself was not investigated. In the present study, the reduction in hypothalamic DNMT1 in the HF newborns would imply enhanced expression of select genes. Whereas ARC DNMT1 expression was normalized at 6 months of age, the HF offspring demonstrated increased LSD1, which would have a similar effect as reduced DNMT1 (i.e., reduced methylation). The mechanism by which DNMT1 and LSD1 expression are regulated in response to a maternal HF diet and offspring

hyperphagia and obesity are unknown. Moreover, the activity of these factors which ultimately regulate gene expression needs to be confirmed.

mTOR is a highly conserved serine-threonine kinase that controls multiple critical cell growth functions via phosphorylation of intracellular signals. AMPK is a protein kinase which elicits cellular response to acquire energy sources and reduce energy expenditure, including inhibition of mTOR signaling.<sup>40</sup> In response to adequate cellular energy, sensed via AMP/ATP ratio, mTOR kinase activity increases and AMPK activity decreases.<sup>40</sup> In the present study, mTOR and AMPK expression levels responded in similar fashion. One day old HF newborns demonstrated reduced hypothalamus mTOR and pAMPK expression while at 6 months of age, HF offspring ARC mTOR and pAMPK expression were increased, consistent with an “orexigenic state.” In prior studies we demonstrated that offspring of undernourished dams had elevated pAMPK.<sup>41</sup> Notably, hypothalamic AMPK responds differently as compared to peripheral AMPK. Whereas AMPK activation in the hypothalamus promotes energy intake, pAMPK promotes energy consumption in the peripheral tissues (liver, skeleton muscle).<sup>42</sup>

ARC development is mediated via a series of bHLH genes which regulate neurogenesis.<sup>43</sup> These genes include repressors of differentiation (e.g., Hes1) which maintain the periventricular NPC population, and activators (e.g., Mash1, Neurogenin3; Ngn3) which accelerate neurogenesis.<sup>44</sup> Ultimately, neuroprogenitor proliferation is followed by differentiation, first to neurons or glia, and subsequently neuronal differentiation to subtype specific cells. AMPK levels modulate NPC proliferation, as NPC stimulation by the AMPK activator (AICAR) inhibits proliferation, which is rescued in part by AMPK inhibition (compound C).<sup>45</sup> In contrast to AMPK effects on NPC proliferation, SIRT1 epigenetically induces NPC differentiation, promoting an astrocytic cell fate.<sup>46</sup> Specifically, SIRT1 deacetylation of the Hes1 histone inhibits Hes1 expression and suppresses the proneuronal transcription factors Mash1 and Ngn.<sup>46;47</sup> Once differentiated to neurons, both Ngn3 and Mash1 promote the development of POMC and inhibition of NPY neurons (Figure 5).<sup>48</sup>

HF offspring demonstrated reduced Hes1 and Mash1 expression at one day of age. Although the reduction in pAMPK may have facilitated NPC proliferation, the reduction in Hes1 suggests an enhanced differentiation of hypothalamic NPC cells, which may have reduced the neuronal stem cell population. Mash1 is required for normal development of POMC neurons.<sup>48</sup> Thus, we propose that the reduction in Mash1 expression during this critical period contributed to the increased hypothalamic AgRP/POMC expression ratio in adult offspring and suggest a relative underdevelopment of anorexigenic neurons. Assuming that the peptide expression reflects the relative activity of these neuronal populations, an increased AgRP/POMC ratio would explain the previously observed HF hyperphagia.<sup>19</sup> At 6 months of age, ARC Mash1 and Ngn3 remained significantly suppressed in HF offspring. Postnatal rewiring of ARC circuits has been demonstrated in response to leptin<sup>49</sup> and neurogenesis continues to occur in the adult hypothalamus.<sup>50</sup> Consequently, the persistent suppression of ARC Mash1 and Ngn3 may influence adult neuronal differentiation and is consistent with increased AgRP and reduced POMC expression. Whether the Mash1 and Ngn3 responses are entirely a result of the fetal/newborn programmed environment or a consequence, in part, of subsequent offspring obesity has not been determined.



Prior studies of the maternal nutritional impact on ARC neuronal cell types have confirmed the potential for maternal-obesity induced offspring obesity. Maternal obesity/overnutrition programs offspring hyperphagia via appetite/satiety gene expression.<sup>19;51;52</sup> Specifically, maternal obesity/HF diet induces increased orexigenic neurons in offspring hypothalamus<sup>52</sup> whilst post-weaning HF diet permanently disrupts hypothalamic pathways.<sup>53</sup>

Together with prior studies demonstrating the obese phenotype of adult offspring born to HF fed dams, these results demonstrate that maternal and thus fetal nutrient environments program the offspring hypothalamic ARC, resulting in enhanced activity in AgRP neurons relative to POMC neurons and hyperphagia. Although appetite neuronal circuitry is complex, and ARC rewiring is possible, if not likely, the early manifestations of obesity as early as 3 weeks of age<sup>19</sup> may further contribute to altered energy sensors and epigenetic responses which modulate gene expression and adult neuronal differentiation. Although the present studies have focused upon the hypothalamus and specifically the ARC, we speculate that NPCs populating diverse brain regions may be impacted by the maternal nutrient environment. Interventions are required prior to or, at the latest, during pregnancy, as newborn or infant interventions may be unsuccessful in the prevention of offspring obesity programming.

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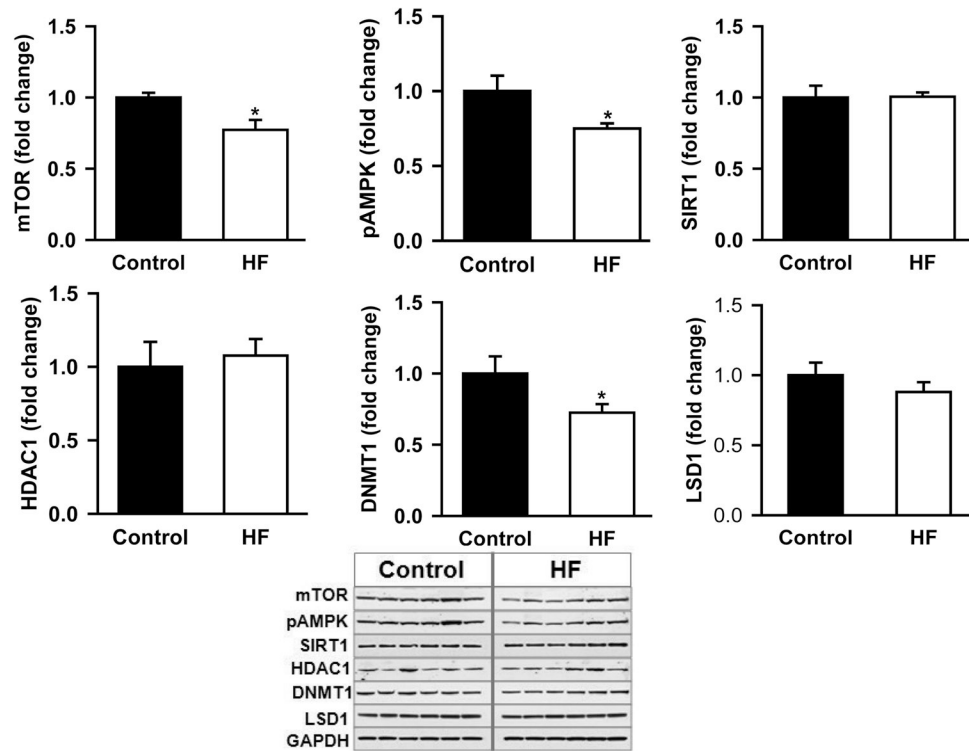
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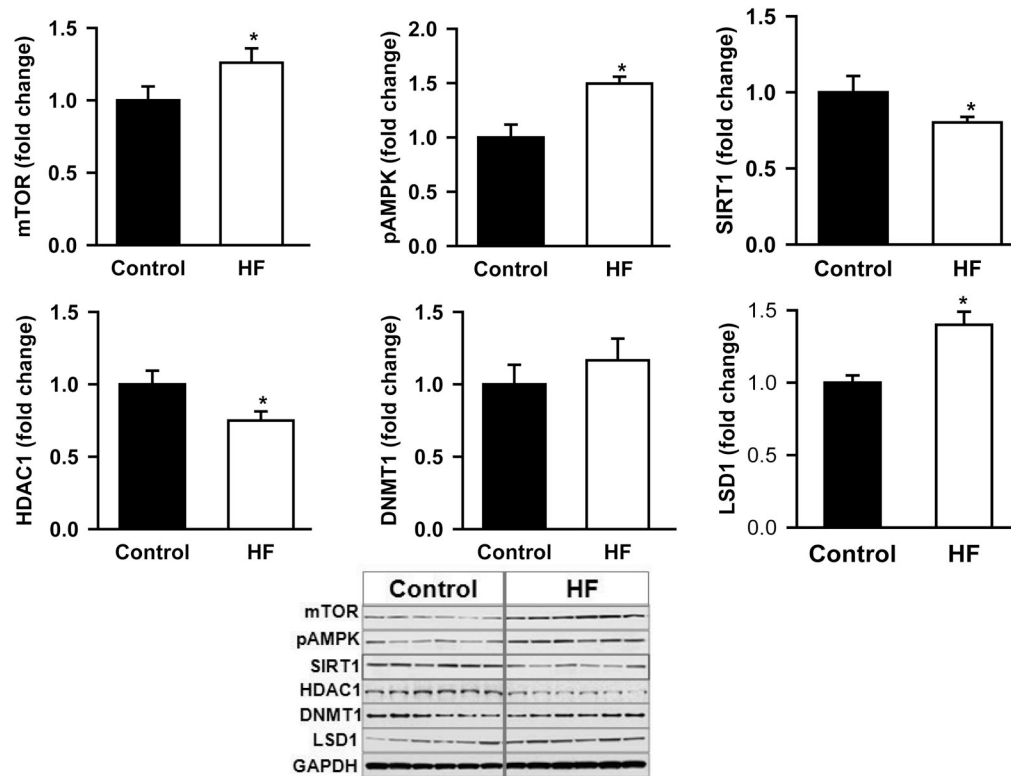
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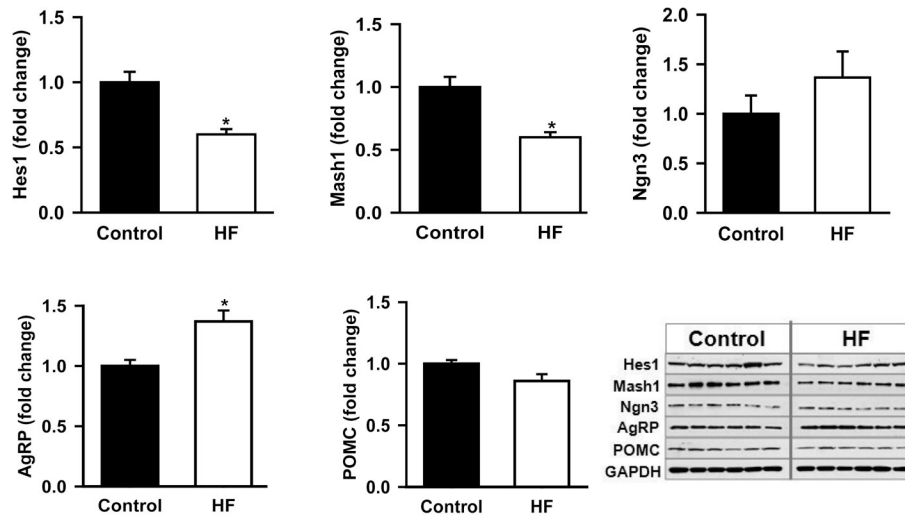
**Figure 1. Newborn Hypothalamic Tissue Protein Expression of Energy Sensors and Epigenetic Factors**

Hypothalamic protein expression of mTOR, pAMPK/AMPK, SIRT1, HDAC1, DNMT1 and LSD1 from 1 day old Control (■) and HF (□) males. \* $p < 0.05$  vs Control; N=6 male pups from 6 litters were studied in each group.



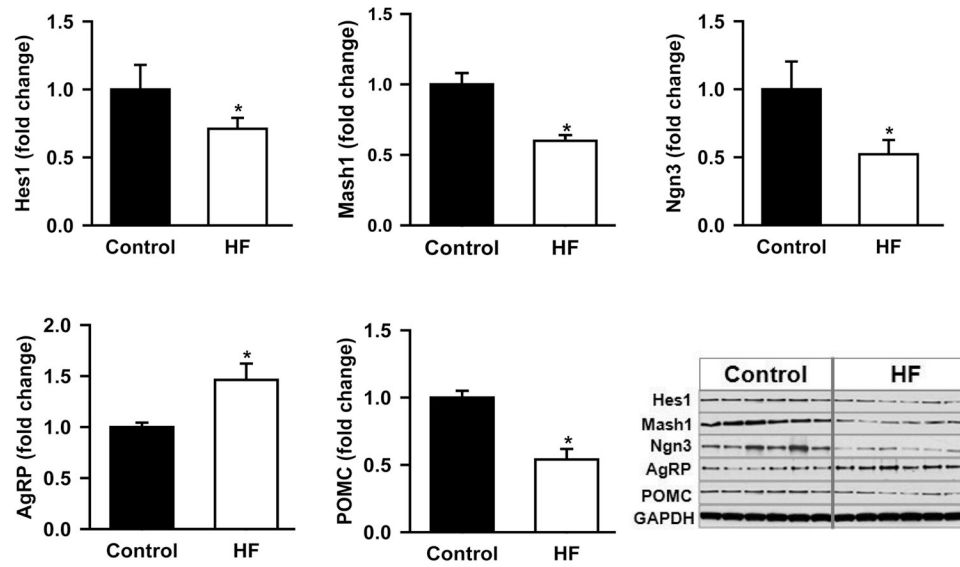
**Figure 2. Adult Hypothalamic Arcuate Nucleus Tissue Protein Expression of Energy Sensors and Epigenetic Factors**

ARC protein expression of mTOR, pAMPK/AMPK, SIRT1, HDAC1, DNMT1 and LSD1 from 6 month old Control (■) and HF (□) males. \* $p < 0.05$  vs Control; N=6 male pups from 6 litters were studied in each group.



**Figure 3. Newborn Hypothalamic Tissue Protein Expression of Neurogenic Factors and Neuropeptides**

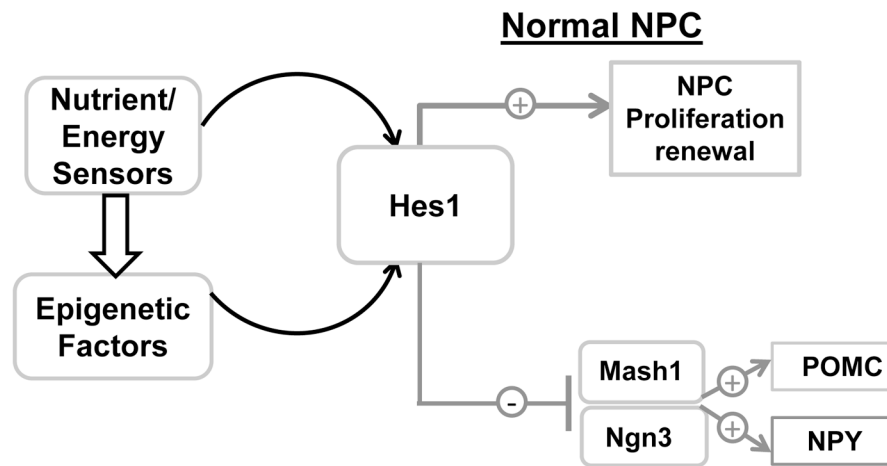
Hypothalamic protein expression of Hes1, Mash1, Ngn3, AgRP and POMC from 1 day old Control (■) and HF (□) males. \* $p < 0.05$  vs Control; N=6 male pups from 6 litters were studied in each group.



**Figure 4. Adult Hypothalamic Arcuate Nucleus Tissue Protein Expression of Neurogenic Factors and Neuropeptides**

ARC protein expression of Hes1, Mash1, Ngn3, AgRP and POMC from 6 month old Control (■) and HF (□) males. \* $p < 0.05$  vs Control; N=6 male pups from 6 litters were studied in each group.





**Figure 5. Regulation of NPC Proliferation and Differentiation**

Nutrient/energy sensors (SIRT1, mTOR, AMPK) can impact epigenetic factors (SIRT1, HDAC1, DNMT1), both of which can alter neuroproliferative factor (Hes1). Increased Hes1 promotes NPC proliferation and inhibits neurogenic factors (Mash1, and Ngn3). Both neurogenic factors are required for the development of POMC neurons. Therefore, increased Mash1/Ngn3 promotes POMC while inhibiting NPY expression.