Amylin receptor insensitivity impairs hypothalamic POMC neuron differentiation in the male offspring of maternal high-fat diet-fed mice

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ABSTRACT

Objective: Amylin was found to regulate glucose and lipid metabolism by acting on the arcuate nucleus of the hypothalamus (ARC). Maternal high-fat diet (HFD) induces sex-specific metabolic diseases mediated by the ARC in offspring. This study was performed to explore 1) the effect of maternal HFD-induced alterations in amylin on the differentiation of hypothalamic neurons and metabolic disorders in male offspring and 2) the specific molecular mechanism underlying the regulation of amylin and its receptor in response to maternal HFD.

Methods: Maternal HFD and gestational hyper-amylin mice models were established to explore the role of hypothalamic amylin and receptor activity-modifying protein 3 (Ramp3) in regulating offspring metabolism. RNA pull-down, mass spectrometry, RNA immunoprecipitation, and RNA decay assays were performed to investigate the mechanism underlying the influence of maternal HFD on Ramp3 deficiency in the fetal hypothalamus.

Results: Male offspring with maternal HFD grew heavier and developed metabolic disorders, whereas female offspring with maternal HFD showed a slight increase in body weight and did not develop metabolic disorders compared to those exposed to maternal normal chow diet (NCD). Male offspring exposed to a maternal HFD had hyperamylinemia from birth until adulthood, which was inconsistent with offspring exposed to maternal NCD. Hyperamylinemia in the maternal HFD-exposed male offspring might be attributed to amylin accumulation following Ramp3 deficiency in the fetal hypothalamus. After Ramp3 knockdown in hypothalamic neural stem cells (htNSCs), amylin was found to fail to promote the differentiation of anorexigenic alpha-melanocyte-stimulating hormone-propiomelanocortin (α-MSH-POMC) neurons but not orexigenic agouti-related protein-neuropeptide Y (AgRP-Npy) neurons. An investigation of the mechanism involved showed that IGF2BP1 could specifically bind to Ramp3 in htNSCs and maintain its mRNA stability. Downregulation of IGF2BP1 in htNSCs in the HFD group could decrease Ramp3 expression and lead to an impairment of α-MSH-POMC neuron differentiation.

Conclusions: These findings suggest that gestational exposure to HFD decreases the expression of IGF2BP1 in the hypothalami of male offspring and destabilizes Ramp3 mRNA, which leads to amylin resistance. The subsequent impairment of POMC neuron differentiation induces sex-specific metabolic disorders in adulthood.

Keywords Amylin; Ramp3; POMC neurons; Maternal high-fat diet; mRNA stability

1. INTRODUCTION

Intrauterine development is a challenging stage of development that affects the growth and health of future generations [1]. Maternal high-fat diet (HFD)-induced obesity and metabolic syndromes in adulthood have attracted widespread attention. Studies have shown that maternal obesity during pregnancy is closely related to macrosomia or large-for-gestational-age babies, and it can cause fetal overgrowth and an increased susceptibility to the future development of type 2 diabetes and cardiovascular diseases [2—4].

In recent years, studies have suggested that HFD during gestation could change the structure and function of the hypothalamus in offspring starting in the embryonic development period [5]. Dysregulation of hypothalamic neurons has been shown to be involved in the...
increased risk of metabolic diseases in offspring from mothers on a HFD during pregnancy [6]. The arcuate nucleus (ARC) of the hypothalamus is a critical area involved in the control of energy balance and food intake [7]. First-order neurons of the ARC that express neuropeptide Y (NPY) and agouti-related protein (AgRP) provide orexigenic signals. However, other neurons that express proopiomelanocortin (POMC) provide anorexigenic signals, sense energy stores within the body, and connect to effector neurons that modulate caloric intake and energy expenditure [8]. Furthermore, the hypothalamus is also the core of the peripheral regulation of glucose and lipid metabolism [9]. NPY/AgRP neurons directly regulate glucose uptake and insulin sensitivity in muscle [10], while POMC neurons directly affect fat lipolysis [11]. The development of the hypothalamus in rodents covers a long-term period that includes the entire fetal phase and 3 weeks after birth [12–14]. Maternal HFD feeding during pregnancy alters the hypothalamic programing of appetite regulation and may promote appetite in offspring, which would increase their long-term susceptibility to metabolic dysfunction [5,15].

Amylin has been reported to be an important neurotrophic factor in the hypothalamus that controls energy metabolism and food intake [16]. The amylin receptor is composed of the core calcitonin receptor (CTR), which heterodimerizes with one or several receptor activity-modifying proteins (RAMP1, 2, and 3). The presence of RAMPs leads to different affinities for the peptide amylin [17]. RAMP2 is not significant in specific amylin binding, whereas RAMP3 shows the highest affinity for amylin [18]. Reports have indicated that altered expression of RAMP3 is associated with the regulation of hypothalamic neurons [19]. However, few studies have focused on the hypothalamic expression of RAMP3 or its regulation of hypothalamic neurons in response to maternal HFD.

The present study was designed to investigate the role of amylin and RAMP3 in the differentiation of hypothalamic neurons in offspring from mothers fed a HFD during gestation. We further employed this model to delineate the specific molecular mechanism underlying the regulation of RAMP3 in response to a maternal HFD.

2. MATERIALS AND METHODS

2.1. Animal Care

All animal experiments were approved by the Institute of Animal Care and Use Committees (IACUC) at Shanghai Research Center for Model Organisms (2017-0009-1). Mice were housed under specific pathogen-free conditions (12/12-h light/dark cycle) with a room temperature of 24 °C. Water and food were available ad libitum.

To establish the maternal HFD models, 8-week-old virgin C57BL/6J female mice were mated with normal male mice. Pregnancy was dated by the observation of a vaginal plug (day 0.5). Pregnant female mice were randomly assigned to a normal chow diet (NCD, 10% calories from fat, Research Diet, NJ, USA) or HFD (60% calories from fat, Xietong Pharmaceutical Bioengineering, Jiangsu, China) or maternal HFD during pregnancy before injection in the GTT and at 0, 15, 30, 60, and 90 min in the ITT; the glucose levels were measured with an automatic glucometer (Roche Diagnostics, IN, USA). The area under the curve (AUC) was calculated as an index of glucose and insulin tolerance.

2.2. In vivo metabolic testing

Intraperitoneal glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed at 4 and 16 weeks after birth. Glucose (2 g/kg body weight, Sigma—Aldrich, St Louis, MO, USA) and insulin (0.8 U/kg body weight, Novo Nordisk, Denmark) were administered to unrestrained conscious mice after a 16- and 4-h fast, respectively. Blood samples were obtained from the tail vein at 0, 15, 30, 60, and 120 min after injection in the GTT and at 0, 15, 30, 60, and 90 min in the ITT; the glucose levels were measured with an automatic glucometer (Roche Diagnostics, IN, USA). The area under the curve (AUC) was calculated as described (21).

2.4. Isolation of hypothalamic neural stem cells and in vitro differentiation of hypothalamic neurons

The isolation and culture of embryonic fetal hypothalamic neural stem cells (htNSCs) were conducted according to Hassan et al. [22]. Briefly, pregnant mice at E16.5 were euthanized by avertin (400 mg/kg body weight, Sigma—Aldrich), and then fetal mice were dissected from the uterus and euthanized by decapitation. The fetal ARC region of the hypothalamus was separated using a stereomicroscope and digested with TrypLE Express (Thermo Fisher, CA, USA) with gentle shaking. The cells were then suspended in growth medium and seeded in ultralow adhesion 6-well plates (Corning, NY, USA) at a density of 10^5 cells/well.

2.5. Immunofluorescence analysis

Mice under anesthesia were transcardially perfused with saline and 4% paraformaldehyde, and the brains were removed and embedded in optimal cutting temperature compound. A 20-μm brain section was produced using a cryostat (Leica, Wetzlar, Germany). For the immunocytochemistry assay, cells were attached to poly-L-ornithine - (Sigma—Aldrich) and laminin (Roche Diagnostics, IN, USA)-coated coverslips at 10^5 cells/well overnight. The cells were differentiated from days 2—35 in the differentiation medium, which was changed once every other day via a half volume. The media ingredients are shown in Table S3.
each image using ImageJ software (ImageJ 1.53c; National Institutes of Health) according to Vogt et al. [16], and the value reflects the total number of pixels in a given region of the image and is proportional to the total density of labeled fibers in this region.

2.6. Western blots

The tissue or cell proteins were extracted as previously described [24]. Samples were separated on a 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel and then transferred to polyvinylidene fluoride membranes (Pall, NY, USA) and exposed to a primary antibody (listed in Table S2). Protein bands were visualized using enhanced chemiluminescence detection reagent.

2.7. Quantitative reverse transcription polymerase chain reaction (RT-PCR) and mRNA decay analyses

Total RNA was extracted from cells or tissues using the RNAiso reagent (TaKaRa, Dalian, China) and reverse-transcribed by applying the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa). Quantitative real-time PCR assays were carried out using SYBR Green Fast qPCR Mix (TaKaRa). The primer sequences used in this study are listed in Table S3.

For the mRNA decay analyses, htNSCs knocked down with shRNA lentivirus were directly harvested or treated with actinomycin D (Sigma–Aldrich) and then harvested at 30, 60, 90, and 120 min. Total RNA extraction and qPCR were conducted as described above,
and 28S rRNA was used as a reference control in the mRNA decay assay.

2.8. RNA interference
To generate shRNA of the candidate genes and control shRNA lentivirus, HEK293T cells were transfected with pLKO.1 shRNA or the control vector along with pREV, pGag/Pol/PRE, and pVSVG. After transfection, the cells were cultured in medium with 20% fetal bovine serum (FBS). The culture medium containing lentivirus particles was centrifuged, and lentiviruses in the supernatant were used for infection. The shRNA sequences are shown in Table S4.

2.9. Overexpression of candidate genes and construction of lentivirus
Overexpression of candidate genes and construction of lentivirus were performed as previously described [24]. The coding region sequence of RAMP3 and insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1) were cloned into the p3 × FLAG-myc-CMV-24 and pLVX-puro vector and then co-transfected with pVSVG and pPAX2 plasmids into HEK293 cells to produce lentiviruses overexpressing RAMP3 and IGF2BP1. All constructs were verified by sequencing.

2.10. RNA pull-down assay
All processes were performed in RNase-free conditions. Ramp3 cDNA was cloned into a pEASY-T3 vector (Transgene, Beijing, China). Double-stranded cDNA templates, including sense and antisense Ramp3 cDNA, were linearized using PCR and provided with T7 promoter (primer sequences are listed in Table S5). Biotin-labeled RNA probes were transcribed in vitro by T7 RNA Polymerase according to the manufacturer’s instructions (Lucigen, WI, USA). Brain tissues were lysed, incubated with streptavidin magnetic beads (Thermo Fisher), and coated with biotin-labeled sense probes. A biotin-labeled antisense probe sample was used as a negative control. The retrieved proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) for mass spectrometry or western blotting.
2.11. Mass spectrometry
The eluted proteins from the Ramp3 mRNA pull-down assay were identified using a gel-based liquid chromatography-tandem mass spectrometry approach according to Tuli et al. [25]. The data processing was conducted using Mascot software.

2.12. RNA immunoprecipitation
Hypothalamic tissues were lysed in hypotonic buffer supplemented with RNase inhibitor and protease inhibitor before centrifugation. Lysates were incubated with magnetic beads (Invitrogen) coated with the indicated antibodies (listed in Table S2). The coprecipitated RNAs were
extracted as described above and detected by PCR. Proteins isolated from the beads were detected by immunoblotting.

2.13. Statistical analysis
Statistical analyses were conducted using GraphPad Prism. All data are shown as the mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by the least significant difference post hoc test and two-tailed Student’s t-test as described in the table and figure legends. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Maternal HFD increases body weight and impairs glucose and lipid metabolism in male offspring
We found that the body weights of the male offspring from mothers fed the HFD during gestation (HFD offspring) on E16.5 and the day of birth were significantly higher than those of male offspring from mothers fed the NCD (NCD offspring) (Figure 1B–E). However, there were no differences in the body weights of the female offspring between the two groups (Figure S1A, B). After weaning, all offspring were fed the NCD until adulthood. HFD-exposed male offspring displayed significantly increased body weight gain until 16 weeks of age compared to the NCD-exposed male offspring (Figure 1F), while only a slight difference in body weight gain between the two groups was observed in the female offspring after 10 weeks of age (Figure S1C). Moreover, we observed the food intake of the offspring of the two groups and found that the HFD-exposed male offspring tended to eat more after weaning than the NCD-exposed male offspring (Figure 1G). However, the female offspring from both groups showed a similar food intake (Figure S1D).

The hormonal and metabolic parameters of the male and female offspring in both the juvenile and adult periods are summarized in Table 1 and Table S6, respectively. The HFD-exposed male offspring at 4 weeks of age showed higher levels of total cholesterol and amylin than the NCD-exposed male offspring. At 16 weeks of age, the total cholesterol, amylin, insulin, HOMA-IR index, triglyceride, and leptin levels were significantly higher in the HFD-exposed male offspring than in the NCD-exposed male offspring. The female offspring had no significant differences in these hormone and metabolic parameters between the HFD and NCD groups at 4 weeks of age or 16 weeks of age.

We performed a GTT and an ITT to examine the glucose tolerance and insulin sensitivity, respectively. Although both male and female offspring at 4 weeks of age did not show differences in the AUC of the GTT and ITT between the two groups (Figure 1H,J, Figure S1E, G), the HFD-exposed male offspring at 16 weeks of age showed significantly increased AUCs of GTT and ITT (Figure 1I,K). However, this phenotype was not found in the HFD-exposed female offspring (Figure S1F, I).

3.2. Maternal HFD leads to decreased POMC neurons and increased NPY neurons in the hypothalamus of adult offspring
The hypothalamus is critical for the regulation of energy expenditure and hormonal homeostasis; thus, the two hypothalamic neurons POMC and NPY were examined in the offspring from both groups. The results from the immunofluorescence assay showed that the synaptic density of NPY neurons in the hypothalamus of the HFD-exposed male offspring in adulthood (16 weeks old) was significantly increased, while the synaptic density of POMC neurons was greatly decreased in the hypothalamus of the HFD-exposed male offspring in adulthood (Figure 2A, B). Quantitative PCR showed that the Npy mRNA levels in NPY neurons were significantly increased and the Pomc mRNA levels in POMC neurons were significantly decreased in the HFD-exposed male offspring at 16 weeks of age (Figure 2C). Consistent protein levels of NPY and POMC were also confirmed by an immunoblotting assay (Figure 2D–F). However, the female offspring did not show significant differences in the synaptic density of POMC and NPY neurons between the two groups (Figure S2).

3.3. Effect of the embryonic amylin level during pregnancy on the metabolic program and hypothalamic neurons of male offspring
The serum amylin levels were significantly higher in the HFD-exposed male offspring than the NCD-exposed male offspring from juvenile to adult offspring (Table 1); therefore, we examined whether the amylin levels might be increased in the HFD fetuses. We found that the serum amylin levels of the HFD-exposed male offspring at E16.5 were significantly higher than those of the NCD-exposed fetuses. Moreover, the amylin levels in the placenta were also higher in the HFD group than the NCD group (Figure 3A, B).

We then established gestational hyper-amylin models to determine the physiological effects of amylin during pregnancy on the metabolic program and hypothalamic neurons of the male offspring as illustrated in Figure 3C. The serum amylin levels of male offspring at E16.5 were detected, and the results confirmed that the model was established successfully (Figure 3D). We found that the body weight gains were significantly lower for male offspring treated with amylin during pregnancy than for the control offspring that were treated with saline from 5 to 16 weeks old (Figure 3E). Compared to the those in the saline control group, the levels of total cholesterol and triglyceride in the amylin group remained similar at 4 weeks of age and slightly decreased at 16 weeks of age (Figure 3F, G). Compared to that of the saline control groups, neither glucose tolerance nor insulin tolerance was affected in the amylin group at 4 weeks of age, while offspring

| Table 1 — Hormonal and metabolic parameters of male offspring. |
|-----------------|-----------------|-----------------|
|                  | NCD (n = 8)     | HFD (n = 8)     | P value |
| Glucose (mg/dL) | 88.28 ± 4.82    | 91.68 ± 4.26    | 0.61    |
| Insulin (ng/mL) | 0.92 ± 0.45     | 1.16 ± 0.32     | 0.67    |
| HOMA-IR         | 4.67 ± 2.63     | 5.48 ± 1.65     | 0.8     |
| Total cholesterol (mg/dL) | 105.24 ± 8.49 | 140.29 ± 11.89 | 0.03    |
| Triglycerides (mg/dL) | 102.34 ± 5.87 | 113.31 ± 7.01  | 0.25    |
| Leptin (ng/mL)  | 2.20 ± 0.22     | 2.74 ± 0.41     | 0.27    |
| Ghrelin (ng/mL) | 0.26 ± 0.06     | 0.36 ± 0.05     | 0.21    |
| Amylin (ng/mL)  | 1.73 ± 0.32     | 3.02 ± 0.47     | 0.04    |

NCD, normal chow diet; HFD, high-fat diet; HOMA-IR, homeostasis model assessment of insulin resistance. N refers to the number of offspring from each separate litter.
from the amylin group were more sensitive to the glucose and insulin at 16 weeks of age (Figure 3H–K). Furthermore, compared to those in the saline control group, the mRNA and protein levels of POMC were greatly increased in the amylin group. No differences were found in the mRNA and protein levels of NPY between both groups (Figure 3L–O). These results suggest that under physiological conditions, amylin may play an important role in maintaining normal metabolism in mice.

3.4. Maternal HFD leads to insensitivity to amylin-related signaling activation in fetal hypothalamic neural stem cells

Next, we sought to determine why the amylin levels, which are associated with increased body weight gain and decreased POMC mRNA and protein levels, increased in the HFD-exposed male offspring as shown in Figure 2. We speculated that maternal HFD might impair the responses of cells to amylin signaling, which might lead to excessive amylin secretion to maintain homeostasis and subsequent alteration in the hypothalamus in adulthood.

To verify this hypothesis, we analyzed a series of genes related to the amylin receptor by qPCR in the hypothalamus of male fetuses at E16.5 in both the HFD and NCD groups. The amylin receptor is composed of the core CTR, which heterodimerizes with one or several RAMP(s), including RAMP1, 2, and 3. We found that Ramp3 mRNA was substantially decreased in the fetal hypothalamus in the HFD group (Figure 4A). A decreased protein level of Ramp3 was also confirmed (Figure 4B). Therefore, we speculated that the downregulation of Ramp3 might be associated with the insensitivity to amylin in the fetal hypothalamus. We isolated fetal hNSCs from E16.5 male offspring (Figure 4C) and detected the post-receptor signaling pathways of the hypothalamus. Our findings demonstrated that amylin could increase signal transducer and activator of transcription 3 (STAT3) and extracellular regulated protein kinases (ERK) phosphorylation in fetal hNSCs; however, the amylin-induced phosphorylation levels of STAT3 and ERK were much lower in the HFD group than the NCD group (Figure 4D–F).

3.5. Maternal HFD induces Ramp3 mRNA instability in fetal hypothalamic neural stem cells

We also found that although the mRNA and protein levels of Ramp3 were significantly reduced in the HFD group, the precursor mRNA level of Ramp3 remained similar between the HFD and NCD groups (Figure 5A). We hypothesized that direct binding proteins may regulate the mRNA expression of Ramp3. Hence, we performed a RNA pull-down assay to identify the interactome of Ramp3 mRNA and found that one prominent target protein selectively pulled down by biotin-labeled sense probes against Ramp3 mRNA, and it was identified as IGFBP1 and found at approximately 70 kDa in both the NCD and HFD hNSCs (Figure 5B, C). Information of mass spectrometry shown in Table S7). Consistently, Ramp3 mRNA was coimmunoprecipitated by an anti-IGFBP1 antibody by RNA immunoprecipitation assays in hNSCs from both the NCD and HFD groups (Figure 5D). Importantly, the protein expression of IGFBP1 was also confirmed to be downregulated in the HFD group (Figure 5E, F). The protein level of IGFBP1 remained unchanged after we knocked down or overexpressed Ramp3 (Figure 5G, H). However, expression of Ramp3 was greatly downregulated following knockdown of IGFBP1 in hNSCs but remained similar following the overexpression of IGFBP1 (Figure 5I, J). These results validated IGFBP1 as a negative regulator of Ramp3 in hNSCs. Moreover, we also examined the expression of Ramp3 pre-mRNA and mRNA and found that the Ramp3 mRNA levels were significantly reduced in hNSCs after IGFBP1 knockdown, whereas the Ramp3 pre-mRNA levels remained unaffected (Figure 5K). To confirm the role of IGFBP1 in stabilizing Ramp3 mRNA, RNA decay analyses were performed in hNSCs treated with actinomycin D, and mRNA degradation was analyzed by qPCR. The
knockdown of IGF2BP1 led to the accelerated decay of Ramp3 mRNA compared to cells transfected with negative control shRNA (Figure 5L). These results indicate that downregulation of IGF2BP1 and RAMP3 in the hypothalamus may be involved in the mechanisms of maternal HFD-induced obesity in male offspring.

3.6. Downregulation of RAMP3 impairs neural differentiation of POMC neurons rather than NPY neurons

To identify whether RAMP3 serves a function in regulating the differentiation of hypothalamic neurons, including POMC-α-MSH- and NPY-AgRP-positive neurons, RNA interference-based "loss-of-function" studies were performed in htNSCs. We first improved an in vitro culture system to generate hypothalamic neurons from mouse fetal htNSCs that express hypothalamic neuron markers, including POMC-α-MSH and NPY-AgRP (Figure 6A). In vitro differentiation assays were then conducted after interfering with the expression of Ramp3. Amylin significantly promoted the differentiation of POMC-α-MSH neurons in htNSCs treated with control shRNA, while this effect of amylin on cell differentiation was significantly decreased after htNSCs were treated with Ramp3-specific shRNA (Figure 6B, C). Notably, treatment of htNSCs with Ramp3-specific shRNA and amylin did not alter the differentiation of NPY-AgRP neurons; however, interleukin-6 (IL-6) significantly improved the differentiation of NPY-AgRP neurons independent of treating NSCs with RAMP3-specific shRNA or amylin (Figure 6D, E). Together, these findings suggest that the maternal HFD-induced upregulation of NPY neurons in male offspring was attributed to the elevated serum IL-6 level during the embryonic period (Figure S3). They were also in agreement with the findings that gestational amylin exposure affected the expression of POMC rather than NPY neurons in Figure 3. As indicated above, RAMP3 could be stabilized by IGF2BP1 in htNSCs; thus, the in vitro differentiation efficiency of POMC-α-MSH neurons was also detected after knocking down IGF2BP1. As shown in Figure 6F, G, the amylin-induced differentiation efficiency of POMC-α-MSH neurons was obviously reduced in the knockdown group transfected with IGF2BP1-specific shRNA compared to that in the control group transfected with control shRNA.

4. DISCUSSION

In this study, we have provided novel insights into the mechanism of maternal HFD-induced obesity and metabolic disorders in male offspring, as indicated in Figure 7. Maternal HFD during pregnancy can downregulate IGF2BP1 in the hypothalamus of fetal mice, which may reduce the binding of IGF2BP1 to Ramp3 mRNA and decrease its stability, thereby resulting in the impaired ability of hypothalamic neural stem cells to differentiate into POMC neurons. These effects...
eventually change the feeding behavior and metabolic function of male offspring in adulthood. It is one of the key mechanisms underlying the maternal HFD-induced increased birth weight, body weight gain, and glucose and lipid metabolism disorder in adulthood in male offspring. Although several studies have focused on the effects of a maternal HFD provided during the pre-pregnancy and pregnancy periods on the long-term health of offspring [26–29], to the best of our knowledge, most of these studies did not directly control for the effects of breastfeeding the offspring. Samuelsson et al. revealed that a maternal HFD significantly increased the levels of leptin in their milk, and an excessive intake of exogenous leptin during lactation has been shown to impair epigenetic reprogramming of the central appetite, which might lead to obesity and other metabolic disorders [30,31]. In 2014, Vogt et al. confirmed that providing a HFD during lactation can impair hypothalamic neurocircuit formation and lead to islet dysfunction in the offspring [16]. Other studies have also indicated that an altered transcriptome profile of the breast tissue induced by a maternal HFD can affect the milk components, thereby impairing the growth development and glucose and lipid metabolism of the offspring [32,33]. Thus, in this study, the provision of a HFD was strictly limited during the period of pregnancy based on the observation of the vaginal plug to the birth of offspring, and the offspring were breastfed with milk from lactating NCD foster mothers. Thus, exposure to a maternal HFD during pregnancy could still cause the acceleration in growth and obesity in male offspring as well as metabolic dysfunction in adulthood. However, only allowing a HFD during pregnancy might be unlikely to occur among humans. The model used in this study mainly focused on the effect of HFD on the metabolism of offspring during this specific period of pregnancy, with the aim of eliminating the interference of other periods including the pre-pregnancy and lactation periods. As other studies have reported, the phenotypes of obesity and metabolic dysfunction following maternal HFD feeding showed a sex-specific effect and were mainly observed in the male offspring [27,33,34]. An epidemiological study has identified significant sex
difference in the prevalence of these metabolic diseases, with women under 50 years of age presenting a decreased risk of obesity-related metabolic disorders and post-menopausal women showing an increased risk of metabolic disorders, including glucose and lipid metabolism [35]. In addition, the prevalence of obesity and its related metabolic disorder was higher in male adolescents than female adolescents [36,37]. Shi et al. believed that the protective effect of the obesity-related metabolic disorder might be attributed to sex hormones, especially estrogen [38]. Estradiol has been reported to augment the effects of satiating hormones, thereby increasing the anorexia behavioral [39,40]. Thus, the lack of a protective effect from estrogen might be explained by the obesity and metabolic dysfunction in male offspring born from mothers fed a HFD in our study. An elevated level of amylin was found in the HFD group at the embryonic stage in this study. As a neurotrophic factor, amylin can pass through the blood-brain barrier and act on the hypothalamic feeding regulation center, which could suppress appetite, inhibit gastric emptying, and lower the blood glucose levels [17]. However, previous studies have not investigated the effect of embryonic exposure to high levels of amylin on the growth and glucose and lipid metabolism during the embryonic stage; however, whether this effect will influence the ability of neural differentiation must be determined. In our study, amylin significantly promoted the differentiation of POMC/α-MSH neurons in normal hNSCs that inhibited feeding. This phenotype is also consistent with amylin’s physiological function of lowering blood glucose and inhibiting gastrointestinal emptying [17,43]. However, amylin’s sensitizing effect on the differentiation of POMC/α-MSH neurons was greatly inhibited after interfering with the expression of RAMP3 in hNSCs. These in vitro findings were partially consistent with the in vivo experiments by Lutz et al. that treating wild-type mice with amylin could increase the number of POMC/α-MSH neurons via increasing p-ERK signaling, while this effect will be suppressed in RAMP1 and -3 knockout mice [44]. Despite this documented evidence, the lack of in vivo experiments on RAMP3 knockout mice is one of the limitations in addressing underlying molecular impairments on neural differentiation and metabolic disturbances in this study. Although the differentiation of POMC/α-MSH neurons was suppressed after knocking down RAMP3 in hNSCs, the levels of the differentiation ability failed to return to the baseline level. This phenotype might be attributed to the following reasons: 1) RAMP3 expression could not be completely suppressed via knockdown with shRNA, and 2) the expression of other amylin receptor activity-modifying proteins which might be involved in amylin-induced differentiation of POMC/α-MSH neurons might not have been knocked in this study. Furthermore, we also found that amylin acts preferentially on POMC/α-MSH neurons compared to NPY/AgRP neurons as indicated in Figure 6D, E. The regulation of POMC/α-MSH neuronal formation is a direct process that activates the STAT3 and ERK signaling pathway following amylin sensitization, whereas the regulation of NPY/AgRP is an IL-6-dependent process mediated by microglia [43,45]. In our study, upregulation of NPY/AgRP neurons might be associated with the elevated IL-6 level in male offspring due to exposure to maternal HFD feeding, and this effect is RAMP3-independent in hNSCs. According to our findings, we believe that the downregulation of RAMP3 is the main cause of amylin receptor dysfunction and the culprit of an abnormal metabolic state in male offspring caused by maternal HFD feeding during pregnancy. Thus, it is critical to identify the key mechanism that downregulates RAMP3 under HFD conditions.

Figure 7: Proposed mechanism of this study. The proposed mechanism of maternal HFD-induced obesity and metabolic disorders in male offspring hypothesizes that 1) maternal HFD during pregnancy decreases IGF2BP1 in the hypothalamic neural stem cells of fetal mice, reduces the binding of IGF2BP1 to Ramp3 mRNA, and destabilizes Ramp3 mRNA; 2) decreased RAMP3 results in amylin resistance indicated by the insensitivity of post-receptor signaling activation of amylin; and 3) amylin resistance impairs the ability of hypothalamic neural stem cells to differentiate into POMC neurons. CTR, calcitonin receptor; ERK, extracellular regulated protein kinases; hNSCs, hypothalamic neural stem cells; IGF2BP1, insulin-like growth factor 2 mRNA binding protein 1; POMC, proopiomelanocortin; Ramp3, receptor activity-modifying protein 3; and STAT3, signal transducer and activator of transcription 3.
Our findings indicate that the mRNA expression of Ramp3 was reduced in the HFD group while the pre-mRNA expression Ramp3 showed nonsignificant changes between the HFD and NCD groups. Thus, regulating the mRNA processing of Ramp3 in hNSCs is an important step. After screening the RNA of the proteins that interact with Ramp3 mRNA via RNA pull-down assays, we found that IGF2BP1 was an important binding protein in the fetal hypothalamus. Remarkably, the levels of RAMP3 were decreased upon knockdown of IGF2BP1 and remained stable after overexpression of IGF2BP1 in hNSCs. Conversely, the levels of IGF2BP1 remained similar after altering the RAMP3 levels. Thus, a functional relationship of IGF2BP1 targeting Ramp3 was confirmed, and IGF2BP1 knockdown substantially decreased the stability of Ramp3 mRNA following actinomycin D treatment. Notably, the decrease in IGF2BP1 was corroborated in the hypothalamus of the male offspring after maternal HFD feeding, indicating that a lack of IGF2BP1 decreases the Ramp3 mRNA stability. Several studies have confirmed that IGF2BP1 contains a unique combination of two RNA recognition motifs and four K homology domains, which could bind to specific mRNAs and influence their transcript fate [46]. In addition to regulating the translation of target mRNAs, IGF2BP1 has been reported to contribute to the stabilization of IGF2BP-RNA complexes and regulate the mRNA stability [47,48]. Recently, IGF2BP1 was also reported to be an m6A reader to recognize m6A-modified mRNAs and promote stability in posttranscriptional gene regulation [49]. More importantly, the lack of IGF2BP1 in hNSCs will impair the amylin-induced differentiation of POMC/C211-MSH neurons as shown in Figure 6F, G, which is consistent with the results after knocking down RAMP3 in Figure 6B, C. These findings might also indicate the importance of IGF2BP1 in regulating Ramp3 mRNA stability and the subsequent differentiation of POMC/C211-MSH neurons in the hNSCs of male offspring following maternal HFD feeding.

AUTHOR CONTRIBUTIONS

HFH conceived the study and participated in its design. CL and JJX were responsible for the animal study and writing the manuscript. HTH carried out the molecular experiments. CJY participated in the cell culture experiments. CL and JJX wrote the manuscript. YTW and JZS supervised the study and critically revised the manuscript. All authors substantially contributed to the revision of the manuscript.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2020.101135.

ABBREVIATIONS

AgRP agouti-related protein
ARC arcuate nucleus
AUC areas under the curve
CTR calcitonin receptor
ERK extracellular regulated protein kinases
GTT glucose tolerance test
HFD high-fat diet
hNSCs hypothalamic neural stem cells
IGF2BP1 insulin-like growth factor 2 mRNA binding protein 1
ITT insulin tolerance test
NCD normal chow diet
NPY neuropeptide Y
POMC proopiomelanocortin
RAMP receptor activity-modifying protein
STAT3 signal transducer and activator of transcription 3

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