Developmental changes in embryonic hypothalamic neurons during prenatal fat exposure

Kinning Poon, Jessica R. Barson, Shawn E. Fagan, and Sarah F. Leibowitz

The Rockefeller University, Laboratory of Behavioral Neurobiology, New York, New York

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Poon K, Barson JR, Fagan SE, Leibowitz SF. Developmental changes in embryonic hypothalamic neurons during prenatal fat exposure. Am J Physiol Endocrinol Metab 303: E432–E441, 2012. First published June 12, 2012; doi:10.1152/ajpendo.00238.2012.—Maternal consumption of a fat-rich diet during pregnancy, which causes later overeating and weight gain in offspring, has been shown to stimulate neurogenesis and increase hypothalamic expression of orexigenic neuropeptides in these postnatal offspring. The studies here, using an in vitro model that mimics in vivo characteristics after prenatal high-fat diet (HFD) exposure, investigate whether these same peptide changes occur in embryos and if they are specific to neurons. Isolated hypothalamic neurons were compared with whole hypothalamus from embryonic day 19 (E19) embryos that were prenatally exposed to HFD and were both found to show similar increases in mRNA expression of enkephalin (ENK) and neuropeptide Y (NPY) compared with that of chow-exposed embryos, with no change in melanin-concentrating hormone, orexin, or galanin. Further examination using immunofluorescence cytochemistry revealed an increase in the number of cells expressing ENK and NPY. By plotting the fluorescence intensity of each cell as a probability density function, three different populations of neurons with low, medium, or high levels of ENK or NPY were found in both HFD and chow groups. The prenatal HFD shifted the density of neurons from the population containing low peptide levels to the population containing high peptide levels. This study indicates that neuronal culture is a useful in vitro system for studying diet effects on neuronal development and shows that prenatal HFD exposure alters the population of hypothalamic neurons containing ENK and NPY in the embryo. These changes may contribute to the increase in HFD intake and body weight observed in offspring.

prenatal high-fat diet; hypothalamus; cell culture; enkephalin; neuropeptide Y

Obesity is a growing epidemic that can have dire consequences, such as diabetes, heart disease, Alzheimer’s, and cancer (9, 13, 21, 42). The latest National Health and Nutrition Survey found 35.7% of adults and 16.5% of United States adolescents and children as young as two years of age to be obese (43). At especially high risk are the offspring of mothers who were obese or overconsumed high-calorie foods during pregnancy. In adolescence and adulthood, these offspring have altered metabolic functioning, including increased insulin resistance, which is accompanied by higher obese or overweight in energy expenditure. Nuclei of the hypothalamus that are involved in these functions include the paraventricular nucleus (PVN), arcuate nucleus (ARC), and parafornical lateral hypothalamus (PFLH). In adult models of fat intake, the neuropeptides enkephalin (ENK) and galanin (GAL) are found to increase feeding behavior when administered directly in the hypothalamus, and their endogenous mRNA expression and peptide levels are stimulated by consumption of a high-fat diet (HFD) (4, 15, 16). A similar pattern is also evident with injection and measurement of orexin (OX) and melanin-concentrating hormone (MCH) (18, 30, 46, 63). Early studies with neuropeptide Y (NPY) have also revealed robust feeding and the development of obesity with daily injections in the hypothalamus (34, 52), as well as increased endogenous expression of NPY in rats that become obese on a HFD (25).

Interestingly, similar results have been obtained in animals exposed during gestation to a HFD. Prenatal exposure to this diet has been shown to increase hypothalamic expression of ENK, GAL, OX, MCH, and NPY in the offspring at embryonic (E) day 21 or different postnatal ages (6, 12, 14, 27, 41, 59). In addition, prenatal HFD also stimulates the birth and migration of hypothalamic neurons and the density of peptide-expressing neurons as revealed in postnatal offspring (14). What has yet to be characterized is the specific nature of the developmental changes in these neurons that occur in utero in response to the HFD, an analysis that would require the use of an in vitro model.

Primary cell culture studies of the effects produced by prenatal substance exposure can provide a powerful tool for assaying mechanisms involved in fetal development. In particular, they permit one to segregate neurons from other cell types in the hypothalamus and to determine whether the effects of prenatal fat are specifically occurring in neurons. With a limited number of prenatal exposure studies adapted into culture (19, 27, 33, 45), it is important to develop and characterize a cell culture model that accurately reflects the same functioning that occurs in vivo. The objective of the present study is to use a cell culture model to determine if HFD-induced changes can be demonstrated in developing embryonic hypothalamus and also whether these changes occur specifically in neurons. In the present study that compared HFD- and chow-exposed embryos, tests were conducted on both whole tissue and primary cell culture at E19, the embryonic age that produced the most viable cultures. We found that, after prenatal HFD or chow treatment, the cultured dissociated neurons maintained the same phenotype as whole tissue. Further examination of the dissociated neurons revealed that the HFD led to an increase in the number of neurons containing ENK and NPY and a change in the population of neurons containing low or high levels of peptide. This in vitro system should allow for further studies of the mechanisms involved in the developmental changes in neurons that occur as a result of gestational HFD exposure.

Address for reprint requests and other correspondence: S. F. Leibowitz, Laboratory of Behavioral Neurobiology, The Rockefeller Univ., 1230 York Ave., New York, NY 10065 (e-mail: leibow@rockefeller.edu).
MATERIALS AND METHODS

Subjects. All animals were individually housed (22°C, with lights off at 12:00 P.M. for 12 h) in a fully accredited American Association for the Accreditation of Laboratory Animal Care facility. Dams were split into two groups; one group received solely laboratory chow (Rodent Chow 5001; LabDiet, St. Louis, MO) and water, and another received HFD and water (14, 20). Timed-pregnant E4 Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were allowed to adapt for 3 days to laboratory conditions, as previously published (14, 20). Following this acclimation period, dams in the second group were introduced to the HFD for an additional 3 days to account for neophobia, after which chow was taken away and HFD was the sole diet until E19. Daily caloric intake and weight gain of chow and HFD groups were recorded during the entire course of the pregnancy. At E19, the dams were anesthetized with 1 ml (50 mg/ml) Nembutal and killed by rapid decapitation, and the whole hypothalamus was microdissected from the embryos. The brain was sliced with the ventral side up using a no. 11 scalpel. Two coronal cuts were made 1 mm from the medial aspect of the slice (2). All procedures were approved by The Rockefeller University Animal Care and Use Committee and were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Diets. The HFD used in this study has been described in detail in previous publications (20, 35). Animals were either given a standard laboratory chow (13% fat, 58% carbohydrate, and 29% protein; 3.36 kcal/g) or HFD (50% fat, 25% carbohydrate, 25% protein, 5.02 kcal/g). The HFD consisted of 50% fat composed of 75% lard (Armour Star, Peoria, IL) and 25% vegetable oil (Crisco, Orrville, OH); 25% carbohydrate composed of 30% dextin (MP Biomedicals, Solon, OH); 30% cornstarch (ICN Pharmaceuticals, Costa Mesa, CA); and 40% sucrose (Domino Foods, Yonkers, NY); and 25% protein from casein (Bio-serv, Frenchtown, NJ). This solid diet was also supplemented with minerals (USP XIV Diet Fortification Mixture; ICN Pharmaceuticals) and vitamins (Vitamin Diet Fortification Mixture; ICN Pharmaceuticals).

Cell culture. Different embryonic ages ranging from E18 to E23 were used for viability of cells when placed in culture. The latest embryonic age that produced the most viable culture was E19; thus, this age was chosen for all cell culture experiments. Because of time constraints of the dissociation process, hypothalamic from up to 12 embryos/dam were extracted in Mg2+/Ca2+-free Hanks’ balanced salt solution (Sigma-Aldrich, St. Louis, MO). Microdissected tissue was placed in 0.05% trypsin-EDTA for 30 min at 37°C (Invitrogen, CA), and no differences were found between neurons that were cultured for 4 or 8 days. All solutions and equipment were sterilized before use.

mRNA extraction and qRT-PCR. Whole hypothalamic tissue microdissected from one embryo per dam was collected and stored in RNA later solution (Invitrogen) and placed at −80°C until extraction. A total of six to nine hypothalami were collected from each diet group. The mRNA was purified from the tissue samples using a Qiagen RNeasy kit (Qiagen, Valencia, CA). The yield was quantified with a Nano Drop ND-8000 spectrophotometer (NanoDrop, Wilmington, DE), with resulting ratios of absorbance at 260 to 280 nm of total RNA from all the animals ranging between 1.91 and 2.10, indicating high purity. Cell culture samples were collected with RNAprotect Cell Reagent (Qiagen) before mRNA extraction. The cDNA was then reverse transcribed from 1 μg of total RNA using high-capacity RNA-to-cDNA master mix (Applied Biosystems, Foster City, CA) per the manufacturer’s instructions. Minus RT controls were synthesized by replacing DNA polymerase with distilled water. Several housekeeping genes, including cyclophilin, β-actin, and GAPDH, were assessed as endogenous controls, and cyclophilin produced the most consistent and reproducible results for cDNA. The SYBR Green PCR core reagents kit (Applied Biosystems) was used for qRT-PCR and was performed in MicroAmp Optic 96-well Reaction Plates (Applied Biosystems) using 12.5 ng of cDNA template in a 25-μl reaction volume, as previously described. qRT-PCR was run on an ABI PRISM 7900 Sequence Detection system (Applied Biosystems), under the condition of 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Each sample was run in triplicate, and each run included a no-template control and negative RT control. The specificities of the PCR products were confirmed by a single band of corresponding molecular weight revealed by agarose gel electrophoresis. The levels of target gene expression were quantified to the level of cyclophilin by using the relative quantification method, where ΔΔCT = 2−ΔCT(gene)−ΔCT(cyc). Primer sequences targeting genes of interest are listed in Table 1.

Immunofluorescence. Hypothalamic neurons were dissociated and plated on cover slips and, after 4 days in culture, were fixed and processed with primary antibodies for ENK, NPY, MCH, or OX. Primary neuronal cells were cultured on poly-D-lysine (Sigma-Aldrich)-coated, no. 1.5, 18-mm round cover slips (Warner Instruments, Hamden, CT), or on no. 1.5, 18-mm round cover slips (Warner Instruments, Hamden, CT). Cells were then placed in a humidified, 5% CO2 incubator at 35°C. All plates and cover slips were coated with 25 μg/ml poly-o-lysine (Sigma-Aldrich) and rinsed with sterile distilled water 2 days before plating. Neurons were collected at day 4 or day 8 in culture, and no differences were found between neurons that were cultured for 4 or 8 days. All solutions and equipment were sterilized before use.

Table 1. Primer sequences for quantitative RT-PCR

<table>
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<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
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<tr>
<td>CYC</td>
<td>5′-GTGGTCTCTTCTGGACATGAGGCTT-3′</td>
<td>5′-CTGGTCTTTGGAAGTTTCTGCGA-3′</td>
</tr>
<tr>
<td>ENK</td>
<td>5′-GGAGTGGCTCTAAATAGCAGATG-3′</td>
<td>5′-GGTGTAATGCGAGAAATTTGG-3′</td>
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<tr>
<td>GAL</td>
<td>5′-TGCGGCAAGACTGTCAGAGATG-3′</td>
<td>5′-TGCGTGACAGGCGGTCAAA-3′</td>
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<td>MCH</td>
<td>5′-ATCGTGGTGTGGGTCTTCTGGTCG-3′</td>
<td>5′-TCGGTGGAGGGTGCTTCTGCTT-3′</td>
</tr>
<tr>
<td>NPY</td>
<td>5′-CACGAGAAAATGGCAGCAGAA-3′</td>
<td>5′-GTCAGGAGAAGAAATTGCTGC-3′</td>
</tr>
<tr>
<td>OX</td>
<td>5′-GAGATGGATGGCTCTGGGAAGTGC-3′</td>
<td>5′-GACGAGAATCTTGGTAAGAGA-3′</td>
</tr>
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Cyc, cyclophilin; ENK, enkephalin; GAL, galanin; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; OX, orexin.

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Table 2. HFD effects on dams and embryos

<table>
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<th>E13/14</th>
<th>E15/16</th>
<th>E17/18</th>
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<tbody>
<tr>
<td>Chow</td>
<td>HFD</td>
<td>Chow</td>
</tr>
<tr>
<td>ΔWeight, g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.6 ± 1.0</td>
<td>8.9 ± 1.0</td>
<td>9.0 ± 1.5</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE; n = 8 rats in each group. E, embryonic day; HFD, high-fat diet. Measurements were taken of the caloric intake and change in body weight of dams consuming HFD or chow. No statistically significant difference was found in dams or embryos between HFD or chow groups.

\[ \text{HFD effects on physiological and behavioral measures.} \]

Comparison of dams and embryos exposed to one of the two diets, HFD or chow, revealed little difference in their physiological and behavioral measures. The dams on the HFD compared with chow exhibited no significant differences in their daily food intake, or average weight gain (Table 2). Additionally, the embryos from these dams on HFD or chow also showed no significant differences in their measures of crown-to-rump body length (chow: 2.4 ± 0.04 cm; HFD: 2.3 ± 0.06 cm), body weight (chow: 1.6 ± 0.03 g; HFD: 1.5 ± 0.05 g), or brain weight (chow and HFD: 0.09 ± 0.01 g). This indicates that the pregnancy for these two groups progressed in a similar and relatively normal fashion.

\[ \text{HFD effects on peptide expression in whole embryonic hypothalamus.} \]

The ingestion of HFD during pregnancy has been shown to stimulate the expression of hypothalamic peptides in postnatal offspring at postnatal day (P) 0 and P15 (14). This experiment examined embryonic development of the peptide feeding systems during in utero exposure to HFD by examining hypothalamic tissue from E19 embryos. The mRNA expression of the different orexigenic peptides, ENK, NPY, MCH, OX, and GAL, was measured using qRT-PCR. In the embryos from HFD compared with chow dams, a significant increase in mRNA levels of ENK [t(13) = 2.50, \( t < 0.05 \)] and NPY [t(16) = 3.92, \( P < 0.05 \)] was observed (Fig. 1A). In contrast, no change was detected in the mRNA levels of MCH [t(14) = 0.15, not significant (NS)].

\[ \text{All other analyses were performed using Student's} t\text{-tests to determine significant differences between chow and HFD groups. For whole hypothalamic in vivo studies, the} \Delta C_T \text{values from the chow or HFD groups were averaged, and the percent change was calculated relative to chow. For tissue samples, 1 sample/dam in each group was used, for a total of 6–9 samples/diet group. The samples from chow or HFD were averaged, and the change in peptide expression from the HFD group was calculated relative to the chow group. For cell culture, the embryos from each dam yielded 5–12 samples, with a total of 4 or 5 dams in the chow or the HFD groups used. The} \Delta C_T \text{values from qRT-PCR data from each individual chow or HFD group were averaged, and the percent change of peptide expression relative to chow was calculated to normalize data. This percent change was then averaged across all HFD groups, and SE was calculated.} \]
HFD effects on peptide expression in hypothalamic neurons. Although hypothalamic neurochemical changes induced by dietary manipulations are generally attributed to changes in neurons, it is unclear whether these changes in the embryo caused by HFD exposure specifically reflect a direct effect on neurons. To test this, hypothalamic neurons from E19 embryos exposed to HFD or chow were dissociated and placed in culture. Neuronal structures were observed within 24 h after plating, revealing healthy and viable cultures. After 4 or 8 days in culture, neurons were harvested, and mRNA expression of the orexigenic peptides, ENK, NPY, MCH, OX, and GAL, was measured by qRT-PCR. Consistent with results from whole hypothalamus, the isolated neurons from HFD embryos compared with chow embryos exhibited a significant increase in mRNA expression of both ENK [t(11) = 0.89, NS], OX [t(15) = 0.62, NS]. The ΔΔC_T values for OX and GAL were much lower, on the order of 10^{-5}, compared with those for ENK, NPY, and MCH, which were on the order of 10^{-3} to 10^{-4}. This suggests that OX and GAL mRNA are expressed at particularly low levels at this embryonic age.

HFD effects on the number of orexigenic-expressing neurons. To determine if prenatal HFD exposure induced an increase in the number of cells expressing ENK, NPY, MCH, OX, or GAL peptide, the neurons were fluorescently labeled for visualization of individual cells (Fig. 2). In the HFD compared with chow group, there was a significant increase in the percentage of ENK-expressing cells [t(6) = 4.50, P < 0.05] and NPY-expressing neurons [t(6) = 7.08, P < 0.05] (Fig. 1B). Also, similar to whole tissue, no change in mRNA was detected for MCH [t(6) = 0.37, NS], OX [t(6) = 1.44, NS], or GAL [t(8) = 0.35, NS]. The ΔΔC_T values were also low for OX and GAL, further confirming the low mRNA levels of OX and GAL at E19.

HFD effects on peptide levels in hypothalamic neurons. The fluorescence intensity of the neurons containing ENK, NPY, MCH, or OX is a relative measure of the level of protein in each individual neuron, with higher intensity reflecting higher levels of peptide. An initial analysis, which averaged intensity data, showed ENK- and NPY-containing neurons to have higher-intensity values from HFD embryos compared with chow embryos. However, it was not entirely clear what the characteristic of each individual neuron was. To identify populations of cells that have high or low levels of peptide and determine whether any differences exist in these populations from HFD and chow embryos, the fluorescence intensity for each peptide from each cell was determined and plotted as a probability density function (see Data analysis in materials and methods). For the ENK neurons in HFD embryos, three populations were found, which corresponded to and were arbitrarily assigned as low (L), medium (M), and high (H) with fluorescence intensities of 7.2 ± 10^4, 1.4 ± 10^5, and 2.3 ± 10^5 a.u., respectively (P < 0.05; Fig. 4A). For the ENK neurons in chow embryos, in contrast, there were only two populations, corresponding to L and H, with fluorescence intensities of 8.9 ± 10^4 and 2.3 ± 10^5 a.u., respectively (P < 0.05; Fig. 4A). The percentage of neurons with low peptide levels was considerably greater in chow embryos, whereas the percentage of neurons with high peptide levels was much greater in the HFD embryos. This suggests that prenatal HFD exposure increased the number of neurons with higher levels of ENK in the hypothalamus.

A similar analysis of NPY-containing neurons revealed two distributions of unequal intensity in both the HFD and chow embryos, which reflected a total of three populations of neurons arbitrarily assigned as L, M, and H. In the chow group, neurons containing NPY consisted of L (5.8 ± 10^5 a.u.) and M (1.6 ± 10^6 a.u.; P < 0.05; Fig. 4B) intensity. This is in contrast to the NPY-containing neurons from the HFD embryos that consisted of M (1.3 ± 10^6 a.u.) and H (3.2 ± 10^6 a.u.; P < 0.05; Fig. 4B) intensity, reflecting a shift in the percentage of neurons from the L and M intensity populations to the M and H intensity populations. This suggests that the HFD not only increased levels of NPY within each neuron but, like ENK, also increased the number of neurons with these higher levels of NPY. The two distributions found with MCH neurons from HFD and chow embryos both showed similar intensity (Fig. 4C) as well as a similar number of cells in each population, indicating that there was no difference in MCH peptide levels between the two groups. This finding is in concert with the qRT-PCR data. Because of differences in antibody specificity and dilutions, the mean intensity values between ENK-, NPY-, and MCH-containing neurons could not be compared. Also, the distribution of OX neurons could not be quantified due to the low frequency and intensity of labeled neurons.

Morphology of peptide-expressing neurons. There were also differences in the morphology of the cells containing NPY, ENK, MCH, or OX at E19. The neurons containing NPY were fully differentiated, with peptide found in both the cell body and neuronal processes, and they consisted of both long projection neurons and smaller interneurons (Fig. 5, A and B). In contrast, the majority of cells containing ENK were small and undifferentiated, suggesting that ENK expression preceded cell differentiation (Fig. 5C). The peptides MCH and OX were found predominantly in the cell bodies of differentiated neurons. The HFD had no apparent impact on the morphology of these peptide-containing cells.

DISCUSSION

Prenatal HFD exposure has been shown in postnatal offspring to stimulate the expression of hypothalamic orexigenic peptides, increase the density of peptide-expressing neurons, and increase neurogenesis and migration of these neurons to the hypothalamus (14). The objective of the present study was to determine whether similar HFD-induced changes can be demonstrated in the developing embryonic hypothalami and...
also whether these changes occur specifically in neurons. To address these questions, an in vitro model of HFD exposure using isolated neurons from embryos at E19 was created and used to examine changes in peptide expression. Results revealed similar peptide changes in isolated neurons and whole hypothalamic tissue, with prenatal exposure to HFD compared with chow increasing the expression of the orexigenic peptides ENK and NPY while having no apparent effect on MCH. The expression of OX and GAL was too low to determine if any changes occurred. Furthermore, examination of these isolated hypothalamic neurons using immunofluorescence cytchemistry revealed an increase in the number of cells containing ENK and NPY from HFD embryos and also in the peptide level within each cell, with no change in MCH or OX. These results suggest three conclusions: 1) prenatal HFD exposure alters the expression and levels of ENK and NPY, indicating that the birth of these neurons in the hypothalamus is increased; 2) the effect of prenatal HFD on orexigenic peptide functioning occurs specifically in neurons; and 3) the dissociated hypothalamic neurons after prenatal treatment retain their in vivo phenotype in culture, indicating that neuronal culture is a useful in vitro system for modeling in vivo function.

Maternal weight gain in acute HFD intake. The similar weight gain in both chow and HFD dams, while surprising, can
be attributed to the relatively short-term intake of HFD. Studies that use long-term HFD intake in dams, starting from before pregnancy and continuing until after birth, result in a larger weight gain after the first week of HFD introduction (23, 27, 32, 50, 61). Analogous to short-term HFD exposure (14, 29), the first week of HFD introduction produces the same weight gain change for both groups (23, 27, 32, 50, 61). In this study, dams were given short-term HFD for 12 days and, consistent with other studies, displayed similar weight gain as the chow group.

Comparison of dissociated hypothalamic cells to whole tissue. The separation of neurons from whole tissue allows for quantification of changes that occur specifically in neurons. Although glial cells have been reported to express orexigenic peptides in the hypothalamus (28, 56), most changes in the peptides induced by dietary manipulations have generally been attributed to neurons (14, 17, 38). Our findings revealed that the dissociated hypothalamic neurons not only retained the same phenotype as whole tissue but also exhibited a degree of change that was double in magnitude. This demonstrates that the HFD-induced effects on peptides occur primarily in neurons and can be somewhat masked by the inclusion of glial cell types in whole tissue. This result strongly supports the use of cell culture as an in vitro system to supplement in vivo investigations of these peptide-containing neurons.

Prenatal HFD stimulates development of peptidergic neurons in the hypothalamus. Whereas the qRT-PCR results revealed an increase in the expression of ENK and NPY in both whole tissue and isolated neurons, this finding leaves unclear whether these changes reflect an increase in the number of cells expressing the peptides or in the level of peptide within each cell. The immunofluorescence results indicate that both of these phenomena likely occurred and contributed to the changes in the neuronal architecture of the hypothalamus. The increase in the number of ENK- and NPY-containing cells in the hypothalamus of HFD embryos suggests that the birth of these cells in the hypothalamus was enhanced, whereas the increase in levels of ENK and NPY per cell indicates that peptide function in these cells was upregulated. Furthermore, ENK from both diet groups was found primarily in nondifferentiated cells, similar to ENK cells found in the cerebellum of early postnatal rats (64). These cells are reminiscent of neurospheres, which are neuroprogenitor cells that have not yet differentiated into neurons and require growth factors to induce differentiation (47, 48, 51). Thus, while lacking the specific growth factor needed to signal differentiation into neurons, these neurospheres at E19 are still able to exhibit a change in ENK peptide that may ultimately have functional consequences. In contrast to these undifferentiated cells, the majority of the NPY neurons at E19 were fully differentiated, either into large projection neurons that could send long connections to other brain regions or small interneurons that presumably have local contacts in intact tissue as described for cortical NPY neurons at E16 (11). This is consistent with evidence showing hypothalamic NPY-rich neurons, located predominantly in the ARC, to project dorsally to the PVN and PFLH as well as locally to the ARC itself (39).

Response of neurons depends on embryonic age. The feasibility of neuronal culture as an in vitro system is reliant on the ability to detect both RNA and protein, and this in turn is dependent on the embryonic age at which these peptides begin to be expressed in the hypothalamus. The expression of NPY begins at E12–E14.5, with levels increasing up until birth and then decreasing postnatally to reach adult levels (3, 31, 55). Reflecting this early expression of NPY, the measured $\Delta C_T$ values in neurons and whole hypothalamic tissue were high, and the fluorescence intensity in the neurons was also high. Interestingly, the expression of ENK, which begins later than NPY at E17.5 (58) and yielded lower $\Delta C_T$ values and fluorescence intensity, was still significantly different between HFD and chow embryos at E19. This was not the case for the expression of OX and GAL, which begins 1–2 days later than ENK at E18–E19 (53, 57), the same age at which the hypothalamic tissue and isolated neurons were extracted from the embryos. This later time of peptide expression presumably accounts for the lack of observed change in OX and GAL, their low $\Delta C_T$ values, and the low fluorescence intensity for OX, confirming the immaturity of these two peptide systems. Consistent with published evidence showing MCH to be present in the hypothalamus relatively early in development, with peak levels detectable at E12–E13 that stabilize to adult levels by E18 (8, 49), the present study revealed high levels of MCH in both whole hypothalamic and isolated neurons. This peptide, however, showed no detectable difference between the HFD and chow embryos, clearly distinguishing it from ENK and NPY. This lack of change may be attributed to the overlapping waves of peak expression known to occur with MCH during gestation, with the development of MCH projections to different areas of the brain and spinal cord occurring at different time periods (49). Averaging MCH peptide levels across these overlapping waves may diminish any effect that HFD exposure might have at any specific time.

Functional consequences of prenatal HFD. The stimulatory effect of prenatal HFD exposure on the density of ENK cells and the intensity of ENK peptide levels in the embryo is consistent with the increase in ENK expression described in postnatal offspring at P0, P15, and P70 (14) and also in adult rats consuming a fat-rich diet (15, 16). This increase in the

<p>| Table 3. Number of peptide-positive neurons/100 cells |
|---------------------------------|----------|--------|--------|--------|</p>
<table>
<thead>
<tr>
<th>ENK</th>
<th>NPY</th>
<th>MCH</th>
<th>OX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>11 ± 1.8</td>
<td>1.6 ± 0.3</td>
<td>33 ± 1.6</td>
</tr>
<tr>
<td>HFD</td>
<td>24 ± 2.3</td>
<td>5.6 ± 0.7</td>
<td>31 ± 1.8</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE; n = 4 rats in each group.
Fig. 4. Populations of neurons expressing low (L), medium (M), and high (H) intensity. The fluorescence intensity for each cell was normalized, plotted as a histogram, and fitted to a probability density function. The area under the curve represents the density of cells within each population and is portrayed as a percentage of the total area.

A: two populations of ENK-expressing cells were found in neurons from chow embryos, representing L and H levels of peptides, and three populations were found in neurons from HFD embryos, representing L, M, and H levels of peptides. A shift from lower to higher intensity was found with HFD embryos compared with chow embryos, suggesting larger ENK levels in individual neurons.

B: two populations of NPY-expressing neurons were found, with L and M from chow embryos and M and H from HFD embryos. This shift in fluorescence intensity suggests that HFD increased the levels of NPY in each individual neuron.

C: two similar populations of neurons were found with MCH for both chow and HFD embryos. Goodness of fit was determined based on $X^2$ values corresponding to $P < 0.05$. a.u., Arbitrary units.
number of ENK cells across ages suggests that the ENK-expressing neurospheres may differentiate into neurons that survive into adulthood, possibly with long-term behavioral consequences. With evidence that central injection of ENK analogs, such as [d-Ala2]methionine enkephalinamide, preferentially stimulates the consumption of a fat-rich diet in adult rats (26, 54), it is possible that this overabundance of ENK-expressing neurons in the hypothalamus that produce a greater amount of peptide may contribute to the overconsumption of fat observed in rats exposed prenatally to a HFD (16).

The additional finding, showing a stimulatory effect of prenatal HFD on the density and intensity of NPY-expressing neurons in the embryo at E19, is consistent with other reports showing increased NPY levels in the offspring right before birth (27). This increased NPY in the embryo, which is very different from the suppressive effect produced by HFD consumption in adult animals (5, 24, 37), suggests that the neuronal changes induced in utero may have functional consequences that are of particular importance early in life, perhaps during the early postnatal period. There is evidence that, in addition to having a potent stimulatory effect on food intake in adult animals (60, 62), hypothalamic injection of NPY in pups at P2 and P15 can enhance milk intake in nursing offspring (10) and that this effect may help to prepare the pup for independent feeding during weaning (36). The present results, showing HFD exposure to increase the number of NPY neurons and cause a population shift to greater peptide levels at E19, suggests that it increases both the birth and migration of NPY neurons in the hypothalamus and the peptide levels within each neuron. This early neuronal rewiring and stronger activity of NPY may result in an improved chance of survival at a time when NPY levels are normally declining (14, 31).

In conclusion, the studies here provide further evidence that prenatal exposure to HFD results in altered developmental programming in the hypothalamus of offspring and that this effect occurs primarily in neurons. This study further shows that primary neuronal cultures retain their phenotype after extraction from whole tissue and thus can be used as an in vitro model for in vivo functioning. Further analysis allowed us to delineate the populations of neurons that expressed ENK or NPY, revealing that prenatal HFD shifts the population of neurons from expressing low levels of peptide to high levels of peptide. The characterization of these peptide-expressing neurons should allow for future in-depth studies to reveal a

Fig. 5. Morphology of neurons. NPY was found in both small interneurons (A) and large projecting neurons (B). C: majority of ENK was found in undifferentiated neurospheres. Cells were counterstained with DAPI, depicted in blue. Representative images of neurons are from HFD embryos. Arrows point to the same cell in the left and right images (DIC and fluorescence).
mechanism for prenatal HFD exposure on neuronal development of each population of cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: K.P. and S.F.L. conception and design of research; K.P., J.R.B., and S.E.F. performed experiments; K.P., J.R.B., and S.E.F. analyzed data; K.P. interpreted results of experiments; K.P., J.R.B., and S.E.F. prepared figures; K.P. drafted manuscript; K.P., J.R.B., and S.E.F. edited and revised manuscript; K.P., J.R.B., and S.E.F. approved final version of manuscript.

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