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Chronic Consumption of a High Fat Diet During Pregnancy Causes Perturbations in the Serotonergic System and Increased Anxiety-like Behavior in Nonhuman Primate Offspring

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Abstract

Childhood obesity is associated with increased risk of behavioral/psychological disorders including depression, anxiety, poor learning, and attention deficient disorder. As the majority of women of child-bearing age are overweight or obese and consume a diet high in dietary fat, it is critical to examine the consequences of maternal overnutrition on the development of brain circuitry that regulates offspring behavior. Using a nonhuman primate (NHP) model of diet-induced obesity, we found that maternal high-fat diet (HFD) consumption caused perturbations in the central serotonergic system of fetal offspring. In addition, female infants from HFD fed mothers exhibited increased anxiety in response to threatening novel objects. These findings have important clinical implications as they demonstrate that exposure to maternal HFD consumption during gestation, independent of obesity, increases the risk of developing behavioral disorders such as anxiety.

Keywords

Maternal obesity; high-fat diet; anxiety; serotonin; nonhuman primate; development

Introduction

Childhood obesity is not only associated with increased risk of metabolic diseases, but also with a range of behavioral/psychological disorders, including depression (Rofey et al., 2009), anxiety (Rofey et al., 2009), poor learning (Cserjesi et al., 2007), and attention deficient disorder (Waring and Lapane, 2008). However, it is difficult to separate cause and effect with psychological diagnosis as there is evidence that the stigma associated with obesity increases the risk for behavioral disorders (Griffiths and Page, 2008). Furthermore, the relative contribution of early programming events caused by maternal obesity, diabetes and diet on behavioral and metabolic disorders in offspring remains unclear. Abnormalities in the serotonergic system are clearly linked to behavioral disorders, including anxiety and depression (Kiyohara and Yoshimasu, 2009), and this system is a regulator of energy homeostasis (Tecott, 2007) and is affected by energy status and diet (Hassanain and Levin, 2002). Moreover, serotonergic drugs are broadly used to treat numerous behavioral disorders.

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Recently our group used a nonhuman primate (NHP) model of diet-induced obesity to demonstrate that consumption of a high fat diet (HFD) during pregnancy causes lipotoxicity within the developing fetus resulting in high levels of circulating inflammatory cytokines and evidence of fatty liver disease (McCurdy et al., 2009). HFD offspring displayed accelerated growth during nursing and had twice the body fat by 6 months of age. Considering that over 50% of women of child-bearing age are overweight or obese (King, 2006), and that the typical American diet is high in dietary fat, it is critical to examine the consequences on development of critical brain circuitry that regulates offspring behavior. The NHP is a uniquely qualified model as they have similar development to humans and exhibit complex social behavior. These studies used the NHP model of maternal overnutrition to examine the consequences of maternal obesity and HFD consumption on development of the serotonergic system in fetal offspring as well anxiety-like and aggressive behavior in infant offspring.

Materials and Methods

Animals

All animal procedures were approved by the ONPRC Institutional Animal Care and Use Committee and conformed to NIH guidelines on the ethical use of animals. A complete characterization of the maternal and fetal phenotype has been previously reported (McCurdy et al., 2009).

Adult females—Briefly, weight (7-9 kg) and age (5-7 years of age) matched adult female Japanese macaques (*Macaca fuscata*), were fed a control (CTR) or HFD for up to 4 years. The CTR diet (Purina Mills Co., St Louis, MO) provided 13% of calories from fat and the HFD (Test Diet, 5A1F, Purina Mills Co., St Louis, MO) provided 32% of calories from fat and was supplemented with calorically-dense treats. The HFD represents a typical Western diet in regards to the saturated fat content. Monkeys were housed in indoor/outdoor pens in groups of approximately 10-12 individuals (male: female ratio 2:9-10). Monkeys had *ad libitum* access to food and water. During monthly health checks, animals were examined for pregnancy by palpation and pregnancies were confirmed by ultrasound (allowing estimate of gestational age).

Fetal Offspring—Fetuses were collected by caesarean section on gestational day 130 (G130) (early 3rd trimester; full term is 175 days) from CTR and HFD groups after 2-4 years on their respective diets. Fetal brains (CTR, $n=5$, HFD 2/3/4, $n=6$) were collected, perfused with 4% paraformaldehyde fixative and blocked as previously described (Grayson et al., 2006).

Juvenile Offspring—Full term offspring were maintained on their mothers' diet. Infants and mothers were left undisturbed for the first 30 days after birth. On post-natal day 30 the offspring were weighed and DEXA scanned to examine body composition. The mother and offspring were then left undisturbed until behavior testing on post-natal day 130. For the CTR group 4 male and 4 female offspring were examined and for the HFD group 12 male and 11 female offspring were examined.

In situ Hybridization (ISH)

Fetal hypothalamic and midbrain blocks were sectioned at 35 μ m using a freezing microtome and collected in 1:24 series. Sections were stored in ethylene glycol cryoprotectant at -20°C until use. For ISH, 1:8 series of sections were slide-mounted in potassium phosphate buffer saline (KPBS) (pH 7.4) and vacuum-dessicated overnight. cRNA probes were transcribed from cDNA clones (kindly provided by CL Bethea) (Pecins-Thompson et al., 1998), Tryptophan hydroxylase 2 (TPH2) – 300 base pairs (bp), serotonin transporter (SERT) – 253bp, and serotonin 1A receptor subtype (5-HT_{1A}R) – 431bp and transcribed in the presence of 100% P³³ labeled UTP (Perkin Elmer, Waltham MA; Table 1). Standard ISH methods were used

(Grayson et al., 2006). For visualization, probe-labeled sections were exposed to film (Biomax MR, Kodak) for 2 (SERT) or 5 (TPH2, 5-HT_{1A}R) days. Autoradiographic images were captured using a CoolSnap HQ camera (Photometrics, Westchester, PA) and Metamorph Software (Universal Imaging Corp). Integrated morphometry analysis was used to measure total density by multiplying total area by optical density of the three levels of the midbrain, rostral (approximately bregma -17.78 ± 1 mm), medial (bregma -19.75 ± 1 mm) and caudal (bregma -23.40 ± 1 mm) (reported in relative units). 3-4 matched sections were analyzed for each level in each animal.

Fluorescent Immunohistochemistry

Two experiments were performed using standard fluorescent immunohistochemistry methods (Grayson et al., 2010): 1) serotonin (5-HT)-*ir* projections in sections containing the arcuate nucleus of the hypothalamus (ARH) to determine the terminal field density and 2) TPH2-*ir* in raphe-containing sections to determine the relative numbers of TPH2-containing neurons. Sections were washed in KPBS and blocked in 2% donkey serum in 0.4% triton X-100/KPBS. Rabbit anti-5-HT (Lot Number 057K4753, #S5545, 1:5000, Sigma-Aldrich) and rabbit anti-TPH2 (Lot Number 1068-102, #NB100-74555, 1:2500, Novus Biologicals) primary antibodies were used. The specificity of these antibodies has been validated by preabsorption studies (Sakowski et al., 2006; Dai et al., 2008). Primary antibodies were diluted with 2% donkey serum in 0.4% triton-X-100/KPBS and incubated with the tissue overnight at 4°C. After incubation, the tissue was rinsed in KPBS, incubated in FITC or rhodamine-labeled donkey anti-rabbit or mouse IgG (Jackson Immunoresearch, West Grove, PA; 1:200) in 0.4% triton-X-100/KPBS for 1 hr. Sections were wet mounted and coverslipped with glycerol-based mountant and stored at 4°C prior to analysis.

Image Analysis

Confocal laser microscopy, as previously described (Grove et al., 2000), was used to capture immunofluorescent images. Images were captured with 25x oil objective (NA = 0.75). For fluorescence intensity measurements of 5-HT in the ARH, a series of optical planes at 0.5 μ m intervals along the z-axis of the section were scanned for each fluorescent signal and stored as a stack of 1024 \times 1024 pixel images, processed with MetaMorph and presented as maximum projections totaling 5 μ m. Two fields of view per section (right/left) in three equally spaced anatomically-matched sections per animal were imaged and analyzed (CTR/HFD $n=3$). Total immunoreactive fluorescent intensity was measured using the same conditions and threshold parameters for all images. Total gray value was measured for each field. Analysis was performed by individuals blind with respect to group. The Metamorph Imaging System was used to process the images, and brightness and contrast levels of the digital images were adjusted with Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Cell counting

Immunohistochemical images were captured under fluorescent illumination using a Photometrics CoolSNAP HQ camera (Roper Scientific, Tucson, AZ) connected to a Nikon microscope (E800) with a Plan Apo 4X objective. Total numbers of immunoreactive cells were counted in all raphe-containing sections.

Behavioral Testing

Behavior tests commonly used to assess anxious, fearful and inhibited behavior in young children and NHP were utilized (i.e. The Human Intruder and Novel Objects Tests; (File 2001; Goldsmith and Rieser-Danner 1990, Kalin et al. 1991)). On post-natal day 130, each infant and mother were removed from their home pen and placed in a cage located in an adjacent room at 900 h. Ten minutes prior to the test, the mother was sedated with ketamine HCL (5

mg/kg IM Fort Dodge Animal Health) and the mother and infant were transported to the behavioral testing suite in a covered transfer box. All behavior tests were initiated and completed between 900 and 1200h. Upon arrival at the behavioral suite, the infant was hand caught and placed in a standard primate cage in a separate room from its mother. The infant's behavior throughout the tests was videotaped from an adjacent room through a one way mirror.

Human Intruder test—This test reliably assess individual differences in primate stress response and anxiety (Williamson et al., 2003) by assessing behavior in three stress-inducing conditions. This test began with a 10 minute acclimation period, after which a human intruder (unfamiliar to the monkey) entered the testing room and stood 0.3 m from the cage with their facial profile (a non-threatening social stimulus) to the monkey for 2 minutes (Profile period). The intruder exited the room leaving the monkey alone for a 2 minute control period. The human intruder then re-entered the room and made continuous direct eye contact (a threatening social stimulus) with the monkey for 2 minutes (Stare period) before exiting. Behaviors scored included vocalizations, exploration, movement and response to the human intruder.

Novel Objects Test—This test was designed to assess response to a variety of potentially threatening and nonthreatening novel objects. Novel object tests have been used to assess anxiety-like behavior in a variety of species (Belzung and Le Pape, 1994). Latency to examine novelty has been shown to be heritable and stable over time (Williamson et al., 2003) and has been pharmacologically validated (Belzung and Le Pape, 1994). Moreover, evidence in children indicates that decreased latency to explore novel objects or situations is associated with early-onset anxiety (Schwartz et al., 1999).

This test began two minutes after the end of the Stare period. The infant was presented with a series of 4 novel objects. For each object, the human intruder entered the room taking care to avoid direct eye contact, quickly introduced the object, removed the previous object (except for the novel fruit, which was left in the cage), and immediately left the room (within 5 seconds). Each object was left with the infant for 5 minutes. The first object to be presented to the infants was a piece of novel fruit (kiwi), which was placed in the cage. After the kiwi, the human intruder then placed a potentially threatening novel object (commercially available toy, Mr. Potato Head, selected because it has large eyes). Only the eyes and feet were attached to Mr. Potato Head's body to emphasize the eyes. The next object was a colorful hanging bird toy (a non-threatening novel object) which was hung on the cage. Lastly, a realistic rubber snake (similar to natural Japanese macaque predators) and a piece of apple (a familiar food) was placed on the tray in such a way that the monkey had to reach across the snake to access the apple. This was done to examine the monkey's willingness to overcome a threatening stimulus (the snake) to get to a desirable object (apple). When the test was complete the infant was hand caught and placed in the transfer cage with its mother, and both monkeys were returned to their home pen.

The videotapes were scored by an observer blind to maternal diet using The Observer XT software (Noldus Information Technology, Wageningen, The Netherlands). The latency (seconds) to intentionally touch each item was scored, as well as behaviors such as movement, exploration, vocalizations, and other responses to the novel objects. We defined latencies to touch the novel object greater than two standard deviations (S.D.) above the mean as an anxious response.

Cerebrospinal Fluid Assays

Cerebrospinal fluid (CSF) was collected from juvenile offspring at 13 months of age. Samples were snap frozen in liquid nitrogen and stored in -80°C until time of assay. 5-HT and 5-Hydroxyindoleacetic acid (5-HIAA) ELISAs were performed by the ONPRC Endocrine

Services Core using commercially available kits (5-HT: #RE59121 and 5-HIAA: #RE59131, IBL Transatlantic, Toronto, Ontario, CAN) in 50 μ l of monkey CSF according to the manufacturer's specifications (CTR $n=6$, HFD $n=11$).

Statistical Analysis

For all analyses, normality and homogeneity of variance were initially tested. An independent t-test was utilized to compare TPH2, 5-HT_{1A}R, and SERT mRNA and SERT immunoreactivity in the rostral and caudal dorsal raphe and CSF 5-HT of CTR and HFD offspring. The latency to touch the novel objects and the number of vocalizations during the acclimation were nonparametric, thus a Mann-Whitney U test was utilized to compare CTR and HFD offspring. A univariate ANOVA was utilized to examine the amount of time spent being active or sedentary. Data are presented as mean \pm SEM. Alpha values are considered significant with $p \leq 0.05$. Statistical analyses were conducted using the SPSS software package, version 16.0 (SPSS Inc., Chicago, Illinois).

Results

The 5-HT System of Fetal Offspring

TPH2 (the rate limiting enzyme for central 5-HT synthesis) mRNA expression was increased by more than 2-fold in the rostral dorsal raphe (rDR) region of fetal HFD offspring (CTR: 3.23 ± 1.06 , HFD: 12.88 ± 3.69 relative units; $t=-2.51$, $df=14$, $p=0.03$; Fig. 1a). However, TPH2 expression in the caudal DR (cDR) was similar in HFD and CTR fetuses (CTR: 1.81 ± 0.39 , HFD: 2.12 ± 0.62 relative units; $t=-0.44$, $df=14$, $p=0.66$). The increase in TPH2 mRNA was due to increased expression per cell as the number of TPH2-ir cell bodies was similar between groups (CTR: 287 ± 28.99 , HFD: 270.67 ± 67.71 relative units, $t=0.22$, $df=4$, $p=0.84$). Activity of the inhibitory 5-HT_{1A} autoreceptor (5-HT_{1A}R) is critical for controlling midbrain 5-HT production. Expression of the 5-HT_{1A}R was up-regulated in the rDR (CTR: 0.44 ± 0.29 , HFD: 11.66 ± 4.36 relative units, $t=-2.57$, $df=10$, $p=0.028$; Fig. 1b), but not in the cDR (CTR: 2.23 ± 1.18 , HFD: 2.96 ± 1.35 relative units, $t=-0.40$, $df=8$, $p=0.70$) of HFD fetuses. Expression of SERT was unchanged in either the rDR (CTR: 3.73 ± 1.03 , HFD: 3.03 ± 0.77 relative units, $t=0.55$, $df=8$, $p=0.56$; Fig. 1c) or cDR (CTR: 3.80 ± 0.90 , HFD: 3.16 ± 0.84 relative units, $t=0.52$, $df=9$, $p=0.61$) in HFD fetal offspring. Hypothalamic 5-HT immunoreactivity (-ir) and SERT-ir were similar in fetuses from CTR and HFD fed mothers (data not shown) indicating that serotonergic projections are unaffected by treatment. The disturbances in the expression of TPH2 and 5-HT_{1A}R were independent of maternal obesity or metabolic abnormalities, as the differences were consistent among all HFD offspring, whether the mother was obese/insulin resistant or lean and insulin sensitive (see (McCurdy et al., 2009)). Furthermore, changes were not sex dependent (data not shown).

Behavior of Juvenile Offspring

Female ($p=0.01$), but not male HFD offspring ($p=0.36$) exhibited increased latency to touch a potentially threatening novel object (Mr. Potato Head) as compared to CTR offspring (Fig. 2a). Overall, 50% of female HFD offspring had a latency greater than 2 S.D. above the group mean (i.e. were anxious in response to this stimulus) while no CTR offspring or male HFD offspring exhibited increased latency. HFD female offspring also took longer to touch a rubber snake (a threatening object; $p=0.04$; Fig. 2b) compared to CTR female offspring. While there was no significant difference in the latency of female HFD offspring to touch a non-threatening novel object (the hanging bird toy; $p=0.16$; Fig. 2c), 4 female offspring from HFD mothers exhibited anxious behavior towards the toy, as opposed to none of the CTR offspring. Female HFD offspring exhibited no differences in latency to touch either a novel (kiwi $p=0.28$; Fig. 2d) or familiar food item located near a potential threat (apple; $p=0.85$; data not shown). Male HFD and CTR offspring did not differ in their latencies to inspect the rubber snake ($p=0.74$),

hanging toy ($p=0.65$), and kiwi ($p=0.95$; Fig. 2b-d). Interestingly, the same animals exhibit anxious behavior across the novel object tests. Monkeys that showed increased latency to touch one novel object also showed increased latency to touch the other novel objects. For example, latency to touch Mr. Potato Head correlated with latency to touch the snake ($r_s=0.69$, $p<0.0001$), hanging toy ($r_s=0.51$, $p=0.003$) and kiwi ($r_s=0.56$, $p=0.001$).

Male HFD offspring exhibited a non-significant increase in aggressive behavior towards the snake and unfamiliar human intruder compared to CTR offspring and female HFD offspring. During the stare period none of the CTR offspring threatened the human intruder, whereas 42% of HFD male offspring threatened the intruder. Overall, 78% (18 out of 23) of HFD offspring displayed some sort of anxious and/or aggressive behavior, while 11% (1 out of 9) of CTR offspring displayed such behavior during the tests. In contrast, there was no difference in the amount of time spent being active or sedentary () or in the number of vocalizations ($p=0.11$; data not shown) during the acclimation period between CTR and HFD offspring. While we were unable to examine the brain 5-HT system at the time of the behavior tests, at 13 months of age CSF 5-HT was decreased in HFD offspring ($t=2.67$, $df=15$, $p=0.02$).

Discussion

As exposure to inflammatory cytokines and early stress are known to cause disturbances in development of the serotonergic system (Ishikawa et al., 2007), the 5-HT system of fetuses were examined. Maternal HFD consumption resulted in an increase in gene expression of the rate limiting enzyme for serotonin synthesis, TPH2, and an increase in the 5-HT_{1A}R inhibitory autoreceptor expression in the rostral raphe which is the primary source of hypothalamic 5-HT (Willoughby and Blessing, 1987). In contrast the serotonergic system in the caudal raphe which primarily projects to higher brain regions (Willoughby and Blessing, 1987) was similar in HFD and CTR fetuses. Maternal HFD consumption did not change SERT expression or 5-HT immunoreactivity in the hypothalamus indicating that serotonergic projections were unaffected by treatment. Together these data clearly indicate that maternal HFD consumption results in perturbations in the fetal serotonergic system.

The increased 5-HT_{1A}R expression in the rDR in fetal offspring and decreased CSF 5-HT in the juvenile HFD offspring suggests that the serotonergic system is suppressed in offspring from HFD consuming mothers. The increased expression of TPH2 is likely a compensatory response to the increased expression of 5-HT_{1A}Rs within the DR. An increased expression of TPH2 mRNA and protein in the DR has also been reported in depressed patients (Bach-Mizrachi et al., 2008; Lowry et al., 2008) who also have decreased CSF 5-HT (Mann and Malone, 1997). These studies also speculate that the increased TPH2 mRNA in the DR is a homeostatic response to deficient 5-HT neurotransmission. Alternatively it is possible that the increase in TPH2 expression in the DR of fetal HFD offspring is a response to exposure to a stressful prenatal environment as acute stress upregulates TPH2 expression in the DR (Shishkina et al., 2007) and that the prolonged exposure to stress during the post-natal period leads to death of 5-HT neurons and decreased serotonergic tone (Mizoguchi et al., 2008; Bambico et al., 2009).

Maternal HFD consumption resulted in behavioral changes in female offspring with 55% displaying increased anxiety in the novel object tests. Male offspring from HFD mothers did not exhibit anxiety, but did exhibit increased aggression. Overall, the majority of HFD offspring (78%) displayed some sort of aberrant behavior (anxious and/or aggressive) during the test while only 11% of CTR offspring displayed such behavior. As there were no difference in the amount of time spent being active or sedentary during the acclimation period we hypothesize that the maternal HFD consumption had a specific effect on anxiety-like behavior. The observed increase in anxious and aggressive behavior is consistent with decreased serotonergic

tone (Lesch et al., 1996). It is interesting that though both male and female offspring exhibit perturbations in the 5-HT system, but that only female offspring exhibit increased anxiety. This is consistent with evidence in humans which suggests that females are more prone to anxiety than males and that the association between obesity and anxiety is stronger in women than men (Desai et al., 2009). As animal models have demonstrated that reduced 5-HT tone during early development also leads to impaired neuronal development (Khozhai and Otellin, 2006), learning deficits (Mazer et al., 1997) and enhanced stress response (McCormack et al., 2009) future studies need to examine the impact of maternal HFD consumption on neuronal development, stress response and cognition.

While further work is needed to determine if abnormalities in the central serotonergic system and behavior persist beyond weaning and into adulthood, these data have important clinical implications as they suggest that in primates exposure to maternal HFD consumption can increase the risk of early development of behavioral disorders such as anxiety. Moreover, as perturbation in the serotonergic system underlies a number of behavioral disorders, developmental changes in the central serotonergic circuitry could explain the comorbidity of obesity with anxiety (Rofey et al., 2009), depression (Rofey et al., 2009) and attention deficit hyperactivity disorder (Waring and Lapane, 2008).

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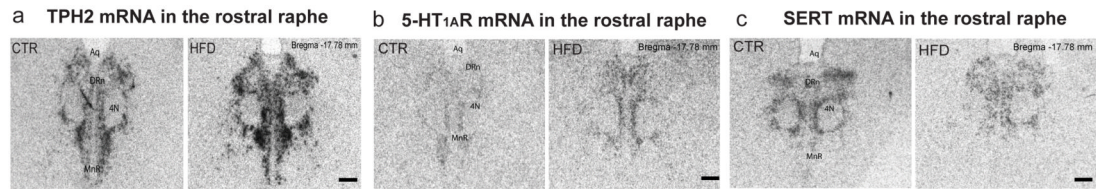


Figure 1. Maternal High Fat Diet Consumption Causes Perturbations in the Serotonergic System of Fetal Offspring

HFD offspring displayed (a) increased TPH2 mRNA in the rostral raphe ($p=0.03$, $n=8$); (b) increased 5-HT_{1A}R mRNA ($p=0.03$, $n=6$); and (c) no difference in SERT mRNA ($p=0.60$, $n=5$) as compared to CTR offspring. . Abbreviations: Aq aqueduct, Drn dorsal raphe nucleus, 4N trochlear nerve, MnR median raphe nucleus.

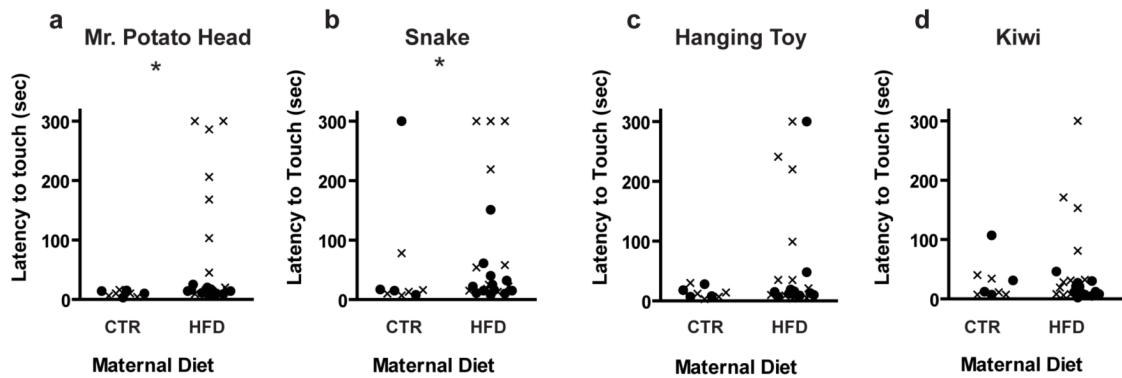


Figure 2. Maternal High Fat Diet Consumption Increased Anxiety-Like Behavior in Female Offspring

Female offspring from HFD fed mothers display increased latency to touch (a) Mr. Potato Head ($p=0.01$), and (b) the Rubber Snake ($p=0.04$) as compared to female CTR offspring. There was no difference in the latency to touch (c) the Hanging Bird Toy ($p=0.16$) or (d) the kiwi fruit ($p=0.32$) between female HFD and CTR offspring. Male HFD and CTR offspring had a similar response to (a) Mr. Potato Head ($p=0.41$), (b) the Rubber Snake ($p=0.75$), (c) the Hanging Bird Toy ($p=0.66$) and (d) the kiwi fruit ($p=0.95$). Data are presented such that each point represents an animal ●=male offspring and x=female offspring. * indicates $P<0.05$ when comparing female CTR and HFD offspring. For the CTR group $n=4$ male and 4 female offspring and for the HFD group $n=12$ male and 11 female offspring.