Maternal high-sucrose consumption increases maternal and fetal glucocorticoids and decreases fetal androgens in the rat

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ABSTRACT

Maternal diet impacts the development of the fetal brain and can have long-term consequences for offspring behavior. Amounts of sucrose (table sugar) are high in modern diets, but we do not know how a maternal high-sucrose diet affects the next generation. Our previous data in rats show that a maternal high-sucrose diet (25% of calories from sucrose), which is human-relevant, alters the metabolism of the mother and has long-term effects on the offspring. Adult female offspring showed elevated corticosterone levels in the blood and brain. Adult male offspring had an increased preference for highly palatable food and were more motivated to work for a sugar reward. In this study with rats, we examined how a maternal high-sucrose diet affects steroids in the dam, placenta, and fetus at E19.5. Maternal sucrose intake increased maternal and fetal levels of glucocorticoids (11-deoxycorticosterone (DOC) and 11-dehydrocorticosterone (DHC)) and the mineralocorticoid aldosterone. In addition, maternal sucrose intake decreased levels of androgens (androstenedione and testosterone) in the placenta and fetal brain. Moreover, maternal sucrose intake reduced placenta mass. In summary, we found dramatic changes in maternal, placental, and fetal steroids that might mediate the long-term effects of maternal sucrose consumption on adult offspring neuroendocrinology and behavior.

INTRODUCTION

The consumption of sucrose (table sugar) is high across the world (Newens & Walton 2016). In the USA, many individuals obtain 25% or more of their daily calories from sucrose and other added sugars (Ricciuto *et al.* 2021). It is widely accepted that high consumption of sucrose has negative health effects, such as increased risk for metabolic disorders and cardiovascular diseases (Stanhope 2016). Importantly, high sucrose intake also increases the risk for psychiatric disorders (Jacques *et al.* 2019) and has negative effects on cognitive function (Kendig 2014). In adult mice in a semi-naturalistic environment, a high-sucrose diet (25% calories) has detrimental effects on survival in females and on reproduction in males (Ruff *et al.* 2013). In other studies of adult rodents, high amounts of sucrose in the diet impair working memory, impair motivation, and increase addictive behaviors (Wiss *et al.* 2017; Wong *et al.* 2017; Reichelt *et al.* 2019; Mizera *et al.* 2021). Thus, there are negative effects of sucrose on adult health and the adult brain.

There is very limited data on the effects of high-sucrose consumption during pregnancy on the mother and the developing fetal brain. Most research on maternal diets focuses on high-fat diets (Naef *et al.* 2008; Vucetic *et al.* 2010) or high-fat/high-sugar diets (George *et al.* 2019). In these studies, there is an increase in maternal calorie intake and obesity, and it is difficult to dissociate the effects of the diet and the effects of maternal obesity (Vucetic *et al.* 2010). With high-fat/high-sugar diet studies, it is unclear if the observed effects are caused by an increase in fat or sugar. Moreover, in studies where sucrose is administered via drinking water, food intake is typically reduced, creating group differences (confounds) in protein, fat, vitamin, and mineral intakes (Hsu *et al.* 2015).

We have developed an animal model in which we feed rat dams a high-sucrose diet (HSD) that is isocaloric to the control (CON) diet, and micro- and macro-nutrients are matched between the CON diet and HSD (**Table 1**) (Tobiansky *et al.* 2020, 2021). The amount of sucrose (26% of calories) in the HSD is a human-relevant level, and the duration of exposure is relatively long (over 12 wk). Importantly, the HSD does not affect food consumption and body mass of rat dams (Tobiansky *et al.* 2020). Therefore, group differences can be unambiguously attributed to sucrose rather than increased caloric intake or obesity.

After approximately 16 wk, this HSD has negative metabolic and physiological effects on rat dams: impaired glucose tolerance, increased liver lipids, and increased transcript levels of *Emr1* (a macrophage marker) in gonadal adipose tissue. After weaning, HSD dams have decreased corticosterone levels in the blood but not in the brain (Tobiansky *et al.* 2020). Offspring were fed standard chow after weaning. In adult offspring, we observed long-lasting neural, endocrine, and behavioral effects of maternal sucrose consumption (Tobiansky *et al.* 2021). Maternal HSD increases corticosterone in the blood and brain of female, but not male, offspring. In contrast, maternal HSD increases preference for a high-sucrose diet and high-fat diet, increases motivation to work for sugar rewards in a progressive ratio task, and decreases mRNA levels of *Cyp17a1* (an androgenic enzyme) in the nucleus accumbens (NAc) of male, but not female, offspring (Tobiansky *et al.* 2021).

These long-term effects of maternal HSD might be mediated by events during pregnancy. The placenta is created during pregnancy and has two important functions. First, it is an essential source of steroids. For example, the placenta is a source of allopregnanolone (Vacher *et al.* 2021). Second, the placenta protects the fetus from harmful substances in the mother's bloodstream but

allows nutrients and growth factors to pass to the fetus. For example, the placenta protects the fetus from high corticosterone levels in the mother by metabolizing corticosterone to 11-dehydrocorticosterone (DHC) via 11β-HSD2 (Cottrell *et al.* 2014).

In the present study, we use this model to identify potential mechanisms underlying the previously observed effects of maternal HSD on adult offspring. We focus on changes in steroid levels during pregnancy, particularly at embryonic day 19.5 (E19.5). The aim of our study was to determine the effects of a maternal HSD before and during gestation on the dam, placenta, and fetus at E19.5. We focused on changes in steroid hormones in the dam's serum, placenta (dam-derived decidua and metrial gland and embryo-derived labyrinth and basal zone), fetal blood, multiple fetal brain regions, and the amniotic fluid. We used liquid chromatography tandem mass spectrometry (LC-MS/MS), which is highly specific and sensitive and allows us to measure a panel of 11 steroids in very small samples. We also monitored maternal weight gain, pregnancy outcomes, and fetal and placental weights.

MATERIALS AND METHODS

Animals and diets

All animal procedures were approved by the Animal Care Committee of the University of British Columbia and were in accordance with the Canadian Council on Animal Care. Animals were housed in the Centre for Disease Modeling at the University of British Columbia. Adult female Long-Evans rats (postnatal day 56-62, 125-175g, N=50, Charles River Laboratories, Kingston, ON, Canada) were pair housed in ventilated Allentown cages (22°C; 45 to 60% relative humidity) on a 12h:12h light/dark cycle (lights on at 10:30 am). Rats had *ad libitum* access to standard rat chow (Teklad

Rodent Diet, catalog # 2918, Envigo, Madison, WI) for 1 wk after arrival. Rats had *ad libitum* access to water (purified by reverse osmosis and sterilized by chlorination) in plastic bottles throughout the experiment. We ran two cohorts of animals (cohort 1, N=30; cohort 2, N=20), and ran groups of 10 animals per week (groups counterbalanced).

We randomly assigned dams to either a control diet (CON) where 1% of calories were from sucrose, or a high-sucrose diet (HSD) where 26% of calories were from sucrose. The HSD is a human-relevant level of sucrose intake (Ruff *et al.* 2013). 1 wk after arrival in our facility, the female rats were gradually introduced to either the CON diet (catalogue #: D12450ki; Research Diets, Inc., New Brunswick, NJ, USA) or an isocaloric, micro- and macro-nutrient matched HSD (Research Diets, Inc.; **Table 1**). In both diets, calories were derived from 20% protein, 70% carbohydrate, and 10% fat. In the HSD, 25.6% of calories were from sucrose and 30% from corn starch, whereas in the CON diet, 1% of calories were from sucrose (contained in Vitamin Mix) and 54% from corn starch. CON and HSD females were on their respective diets for 10 wk prior to mating (**Figure 1**). Food consumption and body mass were recorded weekly. Food consumption and body mass were not regularly measured after mating to avoid unnecessary stress to the pregnant females.

Male adult Long-Evans rats (postnatal day 56, N=50 Charles River Laboratories, Kingston, ON, Canada) were pair housed for 2 wk upon arrival and always received standard rat chow except when pair housed with a female for mating. When the males were at least 10 wk old, each female was paired with a separate male. Vaginal lavages were collected from nulliparous females for estrous cycle staging. Once females entered proestrus or estrus, they were paired with a single-housed male in the evening. Vaginal lavage on the following morning was performed to detect

sperm and indicate embryonic day 0.5 (E0.5) if sperm was present. If no sperm was detected and females had progressed in their cycle, then the female was placed back in their previous cage and lavages were continued until females were in a receptive stage again. Then, mating was repeated as described before. If sperm was found, then the female was single housed and weight was monitored to confirm weight gain and pregnancy until euthanasia at E19.5 (~2 d before parturition).

Tissue collection and tissue microdissection

On E19.5, 3-5h after lights were on, rat dams were rapidly and deeply anesthetized using 5% isoflurane and rapidly decapitated. We chose E19.5 to avoid the increase in maternal glucocorticoids near parturition (E21.5) and to analyze the peak of testosterone in male fetuses from E16.5 to E20.5 (Martin *et al.* 1977; Ward *et al.* 2003). Trunk blood was collected within 2min of disturbance (1.77 ± 0.002 min) to avoid effects of stress on steroid levels (Taves *et al.* 2011). Whole blood was left on wet ice for 30 to 60 min and centrifuged at 5000g for 2min. Serum was collected and frozen on dry ice. The uterine horns were exposed and the number of fetuses and the number of resorptions were determined. Fetuses were removed from the uterus within their amniotic sacs. Amniotic fluid (20-100µl) was collected in an Eppendorf tube by cutting open the amniotic sac with fine scissors and then flash-frozen on dry ice. Then the fetus and placenta were weighed using a scale to the nearest 1 mg. Subsequently, the fetus was decapitated and trunk blood (5-20µl) was collected using a P200 pipette. Up to 8 fetuses per dam were collected for steroid analysis. Fetal blood, fetal brain (within the skull), and placenta were immediately flash frozen on dry ice and stored at -70°C. The tip of the tail for each fetus was collected and stored at -20°C for sex determination (Dhakal & Soares 2017).

Steroid extraction and analysis

Fetal brains (within the skulls) and placenta were cryosectioned (300 µm thick) at -12 °C as before using M-1 embedding matrix (Thermo Scientific) (Tobiansky *et al.* 2018). Microdissection of brain regions and placenta was performed using the Palkovits punch technique with an Integra Miltex biopsy punch (1mm diameter; Fisher Scientific) (Palkovits 1973). Punch locations are shown in **Supplementary Figure 1** for fetal brain. Brain regions microdissected were the orbital cortex (OC), medial prefrontal cortex (mPFC), nucleus accumbens (NAc), amygdala (AMY), dorsal hippocampus (dHPC), ventral hippocampus (vHPC), medial preoptic area (POA), medial hypothalamus (HYP), and ventral tegmental area (VTA). Wet weight of tissue per punch for fetal brain is 0.199 mg and, depending on the region, 2 to 12 punches were collected bilaterally. 20 punches were collected from the maternal part of the placenta ("decidua", decidua and metrial gland), as well as from the fetal part of the placenta ("labyrinth zone", labyrinth and basal zone). Wet weight of tissue per punch for placenta is 0.183 mg. 1µl of fetal blood, maternal serum and amniotic fluid were measured to weigh 1 mg.

We measured steroids in maternal serum (5µl), amniotic fluid (10µl), fetal blood (2µl), labyrinth zone (3.66mg), decidua (3.66mg), and fetal brain (0.4-2.4mg) as described in detail previously (Tobiansky *et al.* 2018; Hamden *et al.* 2021). To each sample, 50µl of deuterated internal standards were added as described previously (Tobiansky *et al.* 2020; Hamden *et al.* 2021; Jalabert *et al.* 2021). Briefly, proteins were precipitated and total steroids (bound and unbound) were extracted with 1ml HPLC-grade acetonitrile and homogenization (Hamden *et al.* 2021). To remove neutral lipids, we added 1ml HPLC-grade hexane, vortexed and centrifuged samples, and then discarded the hexane. Samples were then dried in a vacuum centrifuge (60°C for 45 min).

Steroids were resuspended in 55µl 25% HPLC-grade methanol:MilliQ water, centrifuged, and 50µl were transferred to a glass insert in an LC vial. Samples were stored at -20°C until analysis.

Blanks, quality controls, calibration curves and samples were processed at the same time. Two multiple reaction monitoring transitions were used for each analyte and one multiple reaction monitoring transition for each deuterated internal standard, as reported previously (Jalabert *et al.* 2021). Assay linearity, accuracy, precision, and matrix effects have been reported previously for progesterone, pregnenolone, corticosterone, 11-dehydrocorticosterone (DHC), androstenedione, testosterone, 17β-estradiol, and estrone (Tobiansky *et al.* 2018; Hamden *et al.* 2021; Jalabert *et al.* 2021). For allopregnanolone, aldosterone, and estriol, the transitions monitored and retention times were as follows (quantifier transition *m/z*; qualifier transition *m/z*; retention time): allopregnanolone (301.1 → 283.2; 301.1 → 135.0; 11.10min), aldosterone (361.3 → 343.3; 361.3 → 315.4; 2.83min), estriol (287.1 → 171.09; 287.1 → 144.9; 2.47min). Assay linearity, accuracy, precision, and matrix effects for various tissues were validated for these steroids (Price *et al.* 2023). The detection limit for progesterone, DHC, aldosterone, androstenedione, testosterone, estrone, and estriol was 0.05pg. The detection limit for corticosterone was 0.8pg. The detection limit for 17β-estradiol 0.4pg and the detection limit for pregnenolone and allopregnanolone was 2pg.

Data analyses

Steroid chromatograms were analyzed using Multiquant. Steroid levels were considered non-detectable if they were below the lowest point on the calibration curve. If detectable samples in a group were ≥ 20%, then non-detectable values were estimated via quantile regression imputation of left-censored missing data using the METIMP web tool (Wei *et al.* 2018). If less than 20% of samples were detectable, non-detectable values were set to 0.

When necessary, data were log-transformed prior to statistical analysis. Effects of maternal diet on steroids in maternal serum were analyzed by two-tailed independent t-tests. Effects of maternal diet and sex on fetal steroids were analyzed using two-way ANOVA. A mixed model was used when values were missing. For all analyses, α was set at ≤ 0.05. All graphs are presented using non-transformed data. Statistical analyses were done in Prism 9 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

No effects of a high-sucrose diet on food intake and body mass

12-wk-old Long-Evans female rats were put on either a HSD (26% calories from sucrose) or an isocaloric and nutrient-matched CON diet (1% calories from sucrose; **Table 1**) for 10 wk (**Figure 1**). The amount of food that the females consumed was similar between groups (**Figure 2A**; diet: $F_{(1,23)}=0.55$, $p=0.47$; time: $F_{(2.76,63.44)}=44.68$, $p<0.0001$; diet x time: $F_{(9,207)}=0.6$, $p=0.79$). Moreover, the body mass of CON diet and HSD females was similar (**Figure 2B**; diet: $F_{(1,48)}=0.01$, $p=0.91$; time: $F_{(2.5,119.8)}=411.9$, $p<0.0001$; diet x time: $F_{(10,480)}=0.72$, $p=0.71$). After 10 wk, each female was mated with an individual male. Females were kept on their respective diet throughout pregnancy.

Maternal HSD reduced placenta mass but did not affect other pregnancy outcomes

High-sucrose consumption before and during pregnancy did not significantly affect several pregnancy outcomes. Litter size tended to be higher in the HSD group (**Figure 3A**; $t_{(20)}=1.79$, $p=0.09$). The numbers of male and female fetuses tended to be higher in the HSD group (**Figure 3B**; diet: $F_{(1,20)}=3.5$, $p=0.07$; sex: $F_{(1,20)}=0.5$, $p=0.48$; diet x sex: $F_{(1,20)}=0.5$, $p=0.48$) but the

percentage of male fetuses within a litter was not significantly different (**Figure 3C**; $t_{(20)}$=1.5, p=0.15). The CON and HSD groups showed no difference in the number of resorptions (**Figure 3D**; $t_{(20)}$=0.62, p=0.54). Maternal high-sucrose consumption did not affect the weight of the fetuses (**Figure 3E**; diet: $F_{(1,30)}$=0.8, p=0.38; sex: $F_{(1,30)}$=1.8, p=0.18; diet x sex: $F_{(1,30)}$=0.1, p=0.71). However, the HSD significantly decreased placenta mass (**Figure 3F**; diet: $F_{(1,31)}$=4.7, p=0.04; sex: $F_{(1,31)}$=0.2, p=0.62; diet x sex: $F_{(1,31)}$=0.4, p=0.52) and the placenta/fetus ratio (**Figure 3G**; diet: $F_{(1,30)}$=4.2, p=0.05; sex: $F_{(1,30)}$=0.0, p=0.8; diet x sex: $F_{(1,30)}$=1.0, p=0.32).

Maternal HSD altered steroids in the maternal serum

We measured a panel of 11 steroids in maternal serum at E19.5 (**Figure 4**). In the maternal serum we found a trend for HSD to increase aldosterone levels (**Figure 5A**; $t_{(20)}$=1.87, p=0.08). 11-dehydrocorticosterone (DHC) was significantly increased in maternal serum in the HSD group (**Figure 5B**; $t_{(20)}$=2.34, p=0.03). Levels of corticosterone ($t_{(20)}$=0.91, p=0.38) and testosterone ($t_{(20)}$=1.71, p=0.1) were not significantly different between CON and HSD dams (**Figure 5C,D**). Based on these data, we analyzed maternal serum a second time and measured 11-deoxycorticosterone (DOC). DOC was significantly reduced by HSD (**Figure 5E**; $t_{(18)}$=2.1, p=0.05). Data for the other steroids that were not significantly different between CON and HSD subjects are listed in **Table 2**.

Maternal HSD altered steroids in the placenta

The panel of 11 steroids was also measured in two parts of the placenta, the region derived from the mother ("decidua", decidua and metrial gland) and the region derived from the fetus

("labyrinth zone", labyrinth and basal zone; **Figure 6**). HSD did not affect aldosterone in the decidua (**Figure 6A**; diet: $F_{(1,31)}$=0.9, p=0.35; sex: $F_{(1,31)}$=0.81, p=0.38; diet x sex: $F_{(1,31)}$=0, p=0.95), but HSD significantly increased aldosterone in the labyrinth zone (**Figure 6B**; diet: $F_{(1,31)}$=4.36, p=0.05; sex: $F_{(1,31)}$=1.08, p=0.31; diet x sex: $F_{(1,31)}$=0.42, p=0.52). Androstenedione was significantly reduced by the HSD in the decidua (apparently driven by the effects in females) (**Figure 5C**; diet: $F_{(1,31)}$=5.85, p=0.02; sex: $F_{(1,31)}$=1.5, p=0.23; diet x sex: $F_{(1,31)}$=1.89, p=0.18) and was not affected by HSD in the labyrinth zone (**Figure 6D**; diet: $F_{(1,31)}$=1.82, p=0.19; sex: $F_{(1,31)}$=9.23, p<0.01; diet x sex: $F_{(1,31)}$=3.12, p=0.09). Androstenedione levels were similar between males and females in the decidua (**Figure 6C**) but higher in males than in females in the labyrinth zone (**Figure 6D**). HSD did not significantly reduce testosterone levels in the decidua and testosterone levels were similar between males and females in the decidua (**Figure 6E**; diet: $F_{(1,31)}$=2.75, p=0.11; sex: $F_{(1,31)}$=1.87, p=0.18; diet x sex: $F_{(1,31)}$=0.13, p=0.73). Levels of testosterone were significantly higher in the labyrinth zone for males than females (**Figure 6F**; diet: $F_{(1,31)}$=5.62, p=0.02; sex: $F_{(1,31)}$=8.45, p<0.01; diet x sex: $F_{(1,31)}$=1.17, p=0.29). Importantly, HSD significantly reduced testosterone in the labyrinth zone, which again seems to be driven by females. Data from steroids in the placenta that remained unchanged by the maternal HSD are listed in **Table 3**.

Maternal HSD altered steroids in the fetus

We also analyzed 11 steroids in fetal blood, fetal brain, and amniotic fluid.

Fetal blood

Aldosterone was significantly increased in fetal blood in both sexes (**Figure 7A**; diet: $F_{(1,35)}$=9.53, p=0.004; sex: $F_{(1,35)}$=3.47, p=0.07; diet x sex: $F_{(1,35)}$=0.02, p=0.88). DHC in fetal blood was not

affected by the HSD or sex (**Figure 7B**; diet: $F_{(1,35)}$=0.29, p=0.59; sex: $F_{(1,35)}$=0.1, p=0.75; diet x sex: $F_{(1,35)}$=0, p=0.98). Androstenedione (diet: $F_{(1,35)}$=3.03, p=0.09; sex: $F_{(1,35)}$=33.23, p<0.0001; diet x sex: $F_{(1,35)}$=2.99, p=0.09) but not testosterone (diet: $F_{(1,35)}$=0.25, p=0.62; sex: $F_{(1,35)}$=121.5, p>0.0001; diet x sex: $F_{(1,35)}$=0, p=0.95) tended to be reduced by maternal HSD in fetal blood (**Figure 7B,C**). Both androgens showed robust sex differences in the fetal blood, with males having significantly higher levels of androstenedione and testosterone than females. Results for steroids in fetal blood that are not affected by maternal diet are shown in **Table 4**.

Fetal brain

We analyzed 9 microdissected regions in the fetal brain. We detected significant changes in steroids in the orbital cortex (OC) and medial prefrontal cortex (mPFC), as well as in the nucleus accumbens (NAc). There was a trend for effects in the amygdala (AMY). In detail, aldosterone was significantly increased by HSD in the OC (p=0.03), IL (p=0.05; **Table 5**), and NAc (**Figure 7E**, diet: $F_{(1,31)}$=7.96, p<0.01; sex: $F_{(1,31)}$=0.11, p=0.74; diet x sex: $F_{(1,31)}$=0.46, p=0.5) and showed a trend to be increased in the AMY (p=0.06; **Table 5**). DHC levels were not affected by sex or maternal diet (**Figure 7F**; diet: $F_{(1,31)}$=0.3, p=0.59; sex: $F_{(1,31)}$=0.29, p=0.59; diet x sex: $F_{(1,31)}$=1.32, p=0.26). Androstenedione levels in the NAc were not altered by maternal diet but were significantly higher in males than females (**Figure 6G**; diet: $F_{(1,31)}$=0.27, p=0.6; sex: $F_{(1,31)}$=17.26, p=0.0002; diet x sex: $F_{(1,31)}$=0.81, p=0.37) across multiple brain regions (**Table 5**). Testosterone levels were significantly decreased by HSD in the OC (p=0.05; **Table 5**) and NAc (**Figure 6H**; diet: $F_{(1,31)}$=4.45, p=0.04; sex: $F_{(1,31)}$=196.4, p<0.0001; diet x sex: $F_{(1,31)}$=3.87, p=0.06), and there was a trend for testosterone to be reduced in the AMY (p=0.09; **Table 5**). A detailed list of all steroid data for the different fetal brain regions is presented in **Table 5**.

Amniotic fluid

Aldosterone was significantly increased by HSD in the amniotic fluid of males and females (**Figure 7I**; diet: $F_{(1,34)}$=5.16, p=0.03; sex: $F_{(1,34)}$=0.59, p=0.45; diet x sex: $F_{(1,34)}$=0.14, p=0.71). Similar to the results in maternal serum, DHC was significantly increased in the amniotic fluid in both sexes due to maternal HSD (**Figure 7J**; diet: $F_{(1,34)}$=5.04, p=0.03; sex: $F_{(1,34)}$=0.4, p=0.53; diet x sex: $F_{(1,34)}$=0, p=0.99). Androgens in the amniotic fluid were not altered by maternal diet, but androstenedione (**Figure 7K**; diet: $F_{(1,34)}$=0.85, p=0.36; sex: $F_{(1,34)}$=23.98, p<0.0001; diet x sex: $F_{(1,34)}$=0.34, p=0.56) and testosterone (**Figure 7L**; diet: $F_{(1,34)}$=0.05, p=0.82; sex: $F_{(1,34)}$=184.5, p<0.0001; diet x sex $F_{(1,34)}$=0.59, p=0.45) were significantly higher in males than females. Data for further steroids analyzed in the amniotic fluid are listed in **Table 4**.

DISCUSSION

In this study, we investigated potential underlying mechanisms of the long-term endocrine and behavioral effects that a maternal high-sucrose diet had on the adult offspring (Tobiansky *et al.* 2021). Thus, we fed the same high-sucrose diet to female rats 10 wk prior to and during gestation and examined steroid profiles of maternal serum, placenta, fetal blood, fetal brain, and amniotic fluid at E19.5. As before, female food intake and weight gain were not affected by the HSD. However, the glucocorticoids DHC and DOC were significantly increased in maternal serum and aldosterone tended to be increased in the maternal HSD group. The mass of the placenta of male and female fetuses was significantly reduced by the maternal HSD. In the decidua, androstenedione was significantly reduced and in the labyrinth zone, testosterone was significantly reduced by the HSD. In the labyrinth zone aldosterone was also significantly increased by the maternal HSD. In male and female fetal blood aldosterone was significantly increased by maternal HSD. Aldosterone and DHC were also significantly increased in the amniotic fluid of both sexes. Testosterone was significantly reduced in several fetal brain regions in both sexes.

In the dam's serum, the HSD increased some glucocorticoids. Interestingly, corticosterone was not altered, but rather, its precursor DOC and its metabolite DHC were significantly increased in the dam's serum. In addition, the mineralocorticoid aldosterone tended to be increased in the HSD dam. The results show that glucocorticoids are elevated in the dams, but it seems the organism can manage to keep corticosterone itself low, as increased corticosterone levels could be harmful to the fetus (Cottrell *et al.* 2014; Wieczorek *et al.* 2019).

DHC and aldosterone were also significantly increased in male and female fetuses from HSD dams. Aldosterone but not DHC was also increased in the labyrinth zone of the placenta, the fetal blood, and regions of the fetal brain such as the NAc. Aldosterone has been suggested to act as the main adrenal glucocorticoid in the perinatal period in the rat (Varga *et al.* 2013). In detail, during the stress hyporesponsive period (Schmidt 2019), circulating corticosterone is very low early postnatally (P4-13) and not systemically upregulated due to a stressor to protect the developing organism (Taves *et al.* 2015). In a stressful situation, the newborn pup will increase aldosterone as a stress response, not corticosterone, which would typically increase in a stressed adult rat (Varga *et al.* 2013). The increase in aldosterone in fetal tissues that we observe here indicates that the fetus shows a stress response. Over time, the offspring might not be able to control the increase in glucocorticoids and thus we saw an increase in corticosterone in female adult offspring (Tobiansky *et al.* 2021). Interestingly, if male adult offspring from HSD dams were put on food deprivation, their corticosterone levels increased as well. This could indicate that also the males' stress axis is affected by maternal diet; however, a second hit is needed to bring the system out of balance.

Androgens were the other class of steroids that were frequently altered in the fetus due to the maternal high-sucrose diet. For example, we found interesting androgen profiles in the placenta. In the decidua, the part of the placenta formed by maternal cells, females showed very high levels of androstenedione and testosterone. In all examined fetal tissues and the labyrinth zone of the placenta, males had significantly higher androgen levels than females, except the decidua, here androgen levels were similar in males and females. There is evidence that androgens play an important role in forming the decidua, and it is known that the decidua and the placenta express androgen receptors (Gibson *et al.* 2016; Kumar *et al.* 2018). In our model, we see a

significant decrease in androstenedione in the decidua and a significant decrease in testosterone in the labyrinth zone of the placenta. This goes hand in hand with a reduction in the placental size of male and female fetuses. Here, we could speculate that autocrine or paracrine androgen signaling in the placenta regulates placental growth, as we see a reduction in placental androgens and a reduction in the size of the placenta. Future studies should address whether androgen receptor levels and steroidogenic enzymes are altered in the placenta in our model.

Testosterone was also altered in the fetal brains with a focus on the prefrontal cortex and the NAc as parts of the mesocorticolimbic system. We and others have shown before in adult rats that androgens regulate tyrosine hydroxylase (TH), a key enzyme in dopamine signaling, specifically in these regions (Adler *et al.* 1999; Kritzer 2003; Wallin-Miller *et al.* 2016; Tomm *et al.* 2022; Seib *et al.* 2023). Reducing androgens by the androgen synthesis inhibitor abiraterone acetate increased TH fibers in the mPFC (Tomm *et al.* 2022), whereas administering testosterone reduced TH fibers in the NAc (Wallin-Miller *et al.* 2016). The reduction in testosterone observed in this study could alter TH expression in the mesocorticolimbic system during development. This could lead to the observed behavioral effects on motivation and food preference in the adult male offspring (Tobiansky *et al.* 2021). The increase of aldosterone in the brain, specifically in the mesocorticolimbic system, is another mechanism that could act on the development of the brain's reward circuit. Aldosterone has been shown to act on microglia by reducing their inflammatory activity (Bast *et al.* 2018). As microglia play an important role in brain development, such as guiding migration, synapse formation, or myelination (Gildawie *et al.* 2020), their proper function could be impaired by an increase in aldosterone.

In summary, our data show that a maternal high-sucrose diet considerably affects fetal and maternal androgens, glucocorticoids, and the mineralocorticoid aldosterone. The observed changes in the placenta and the fetal brain could explain the long-lasting endocrine and behavioral effects that were observed before in the adult offspring. Future studies will examine how the altered androgen signaling could affect placental growth and function. In addition, we plan to investigate the effects of the changes in steroids in the brain on microglia physiology and TH in the mesocorticolimbic system.

DECLARATION OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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FIGURES

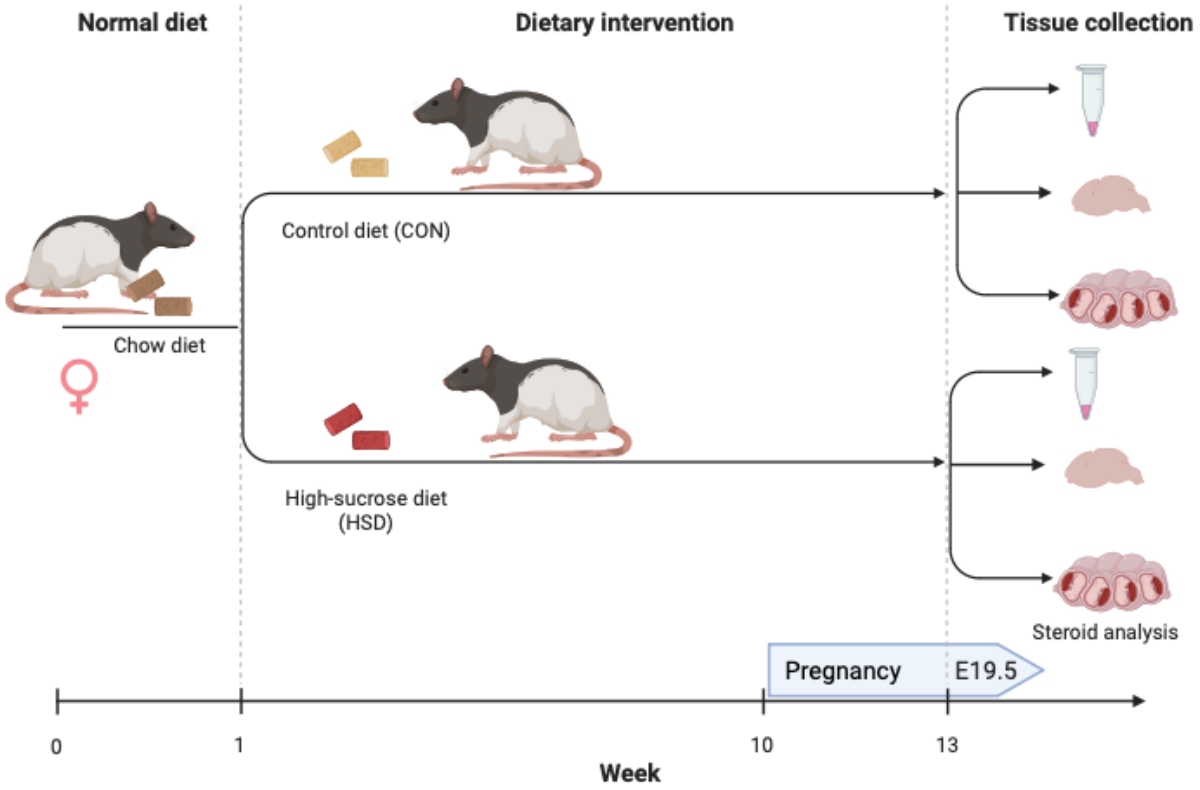

Figure 1. Experimental timeline

Adult female rats were fed a control (CON) diet or high-sucrose diet (HSD) for 10 wk. Then females were mated. At E19.5, maternal serum, fetal brains, placenta, amniotic fluid, and fetal blood were collected. Created with Biorender.com.

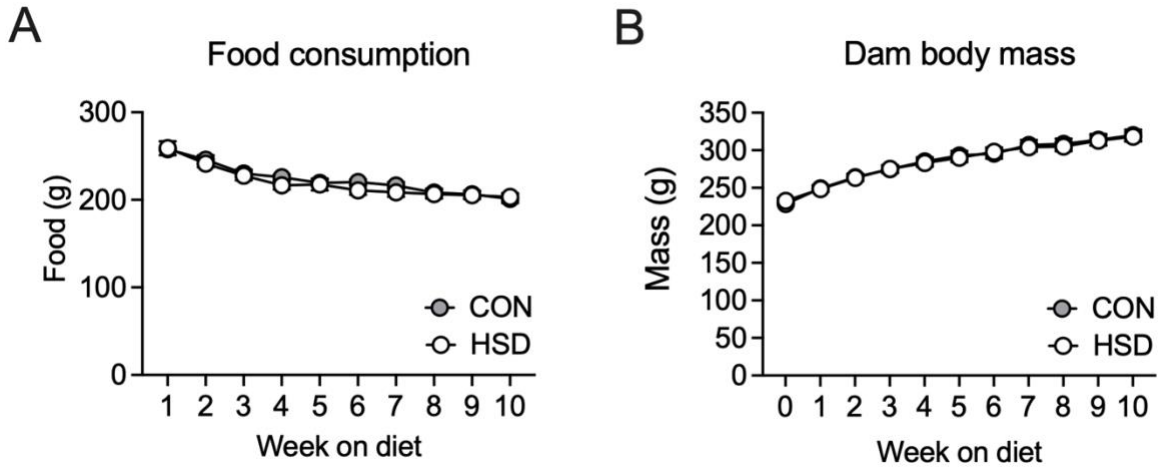

Figure 2. High-sucrose diet (HSD) does not alter food intake and body mass, relative to control (CON) diet

(A) Food intake of CON diet and HSD females pre-gestation. (B) Female weight gain pre-gestation during the first 10 wk on the CON diet and HSD.

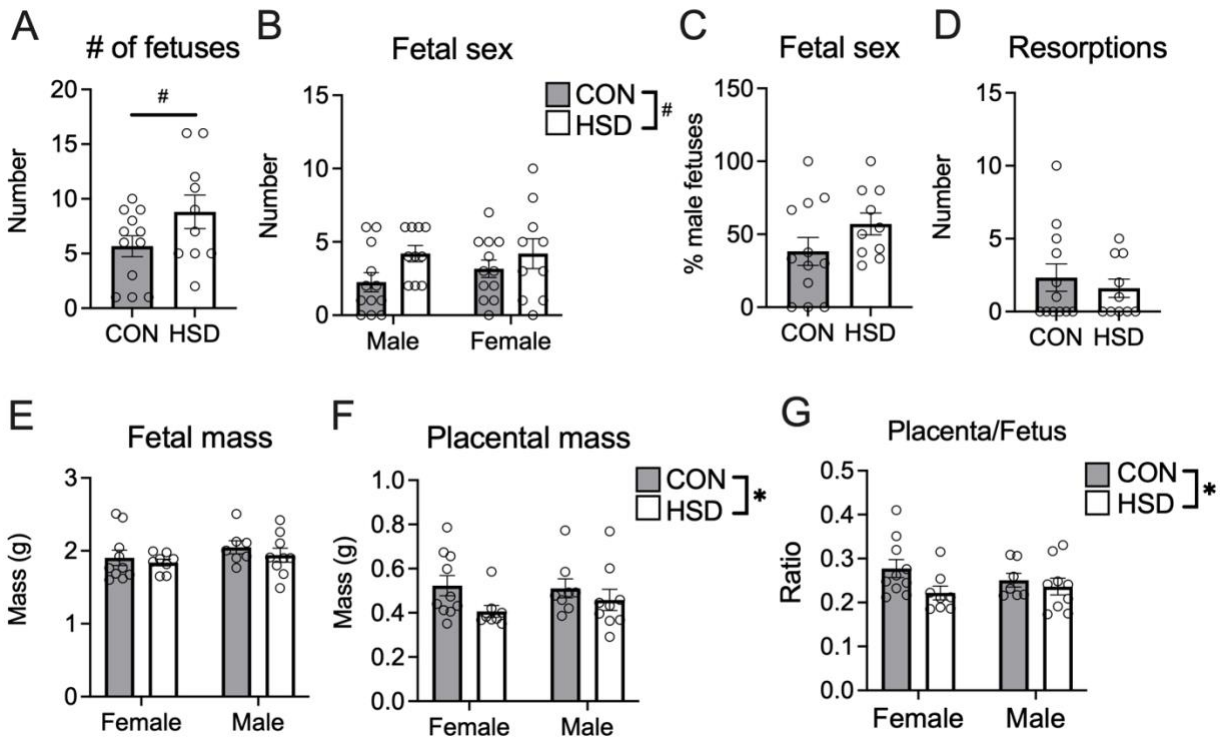

Figure 3. Maternal high sucrose consumption reduced placental mass

(A) The size of the litter tended to be increased in the HSD group as well as the (B) number of male and female fetuses. (C) The percentage of male fetuses was not affected by diet. (D) The number of resorptions and (E) fetal weight were similar between the CON and HSD groups. (F) Placental weight was reduced in the HSD group as well as the (G) placenta/fetus ratio. CON, control; HSD, high-sucrose diet. #, $p<0.1$; *, $p \leq 0.05$.

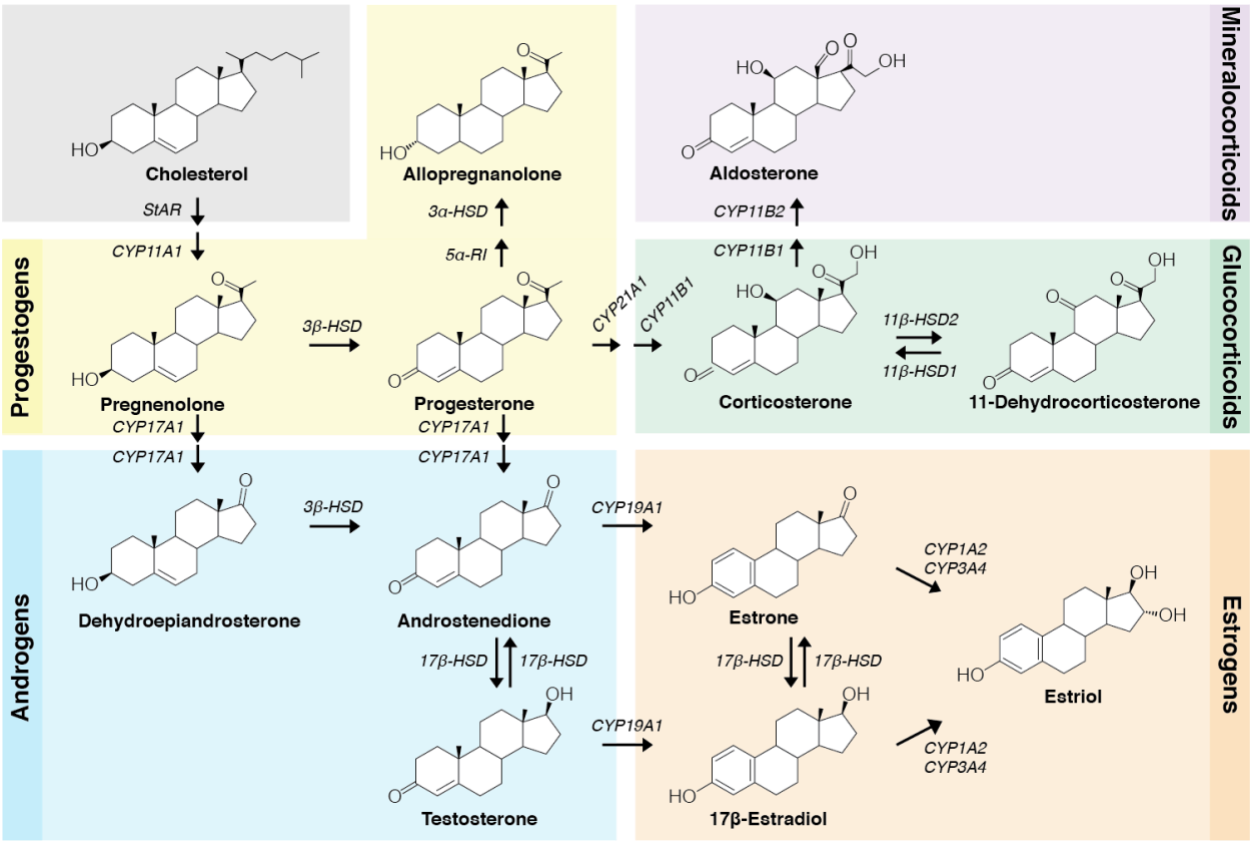

Figure 4. LC-MS/MS steroid panel

Overview of the steroidogenic pathway of the 11 steroids that were analyzed with LC-MS/MS.

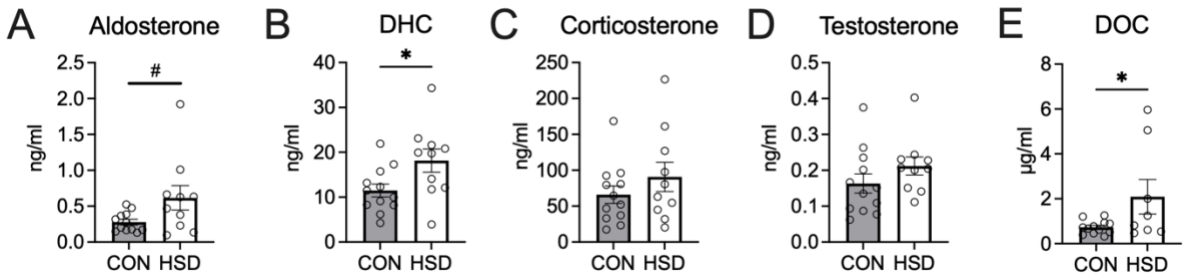

Figure 5. High sucrose intake alters maternal steroids

(A) HSD tended to increase aldosterone in maternal serum. (B) DHC was significantly increased due to maternal HSD. (C) Corticosterone and (D) testosterone were not changed by the HSD. (E) Maternal HSD significantly increased DOC in maternal serum. CON, control; HSD, high-sucrose diet; DHC, 11-dehydrocorticosterone; DOC, 11-deoxycorticosterone. $^{\#}$, $p \leq 0.1$; *, $p \leq 0.05$.

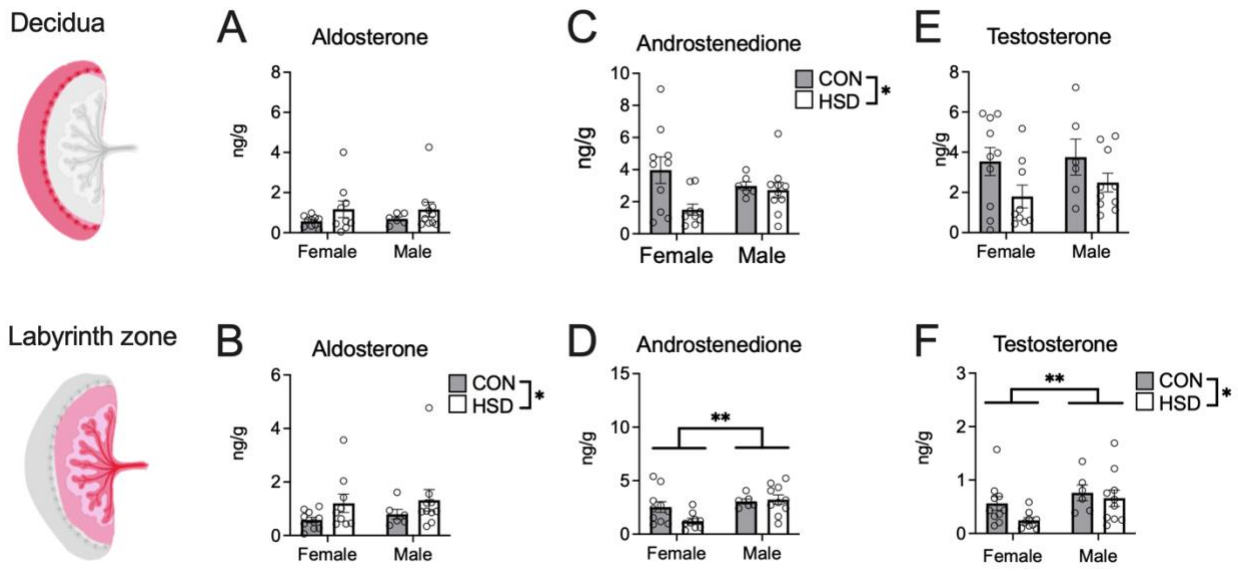

Figure 6. Placental steroids affected by maternal HSD

The upper row shows the dissection region and results for the decidua (maternal part of the placenta) and the lower row shows the dissection region and results for the fetal labyrinth zone. **(A)** Aldosterone in the decidua was not changed by the HSD. **(B)** Aldosterone in the labyrinth zone was increased by the HSD. **C** HSD significantly reduced androstenedione in the decidua. **(D)** Males had higher levels of androstenedione in the labyrinth zone than females, but levels were not altered by the HSD. **(E)** Neither diet nor sex significantly affected testosterone in the decidua. **(F)** Males had significantly higher testosterone in the labyrinth zone than females, and the HSD significantly reduced testosterone. CON, control; HSD, high-sucrose diet. *, $p \leq 0.05$; **, $p \leq 0.01$. Created with Biorender.com.

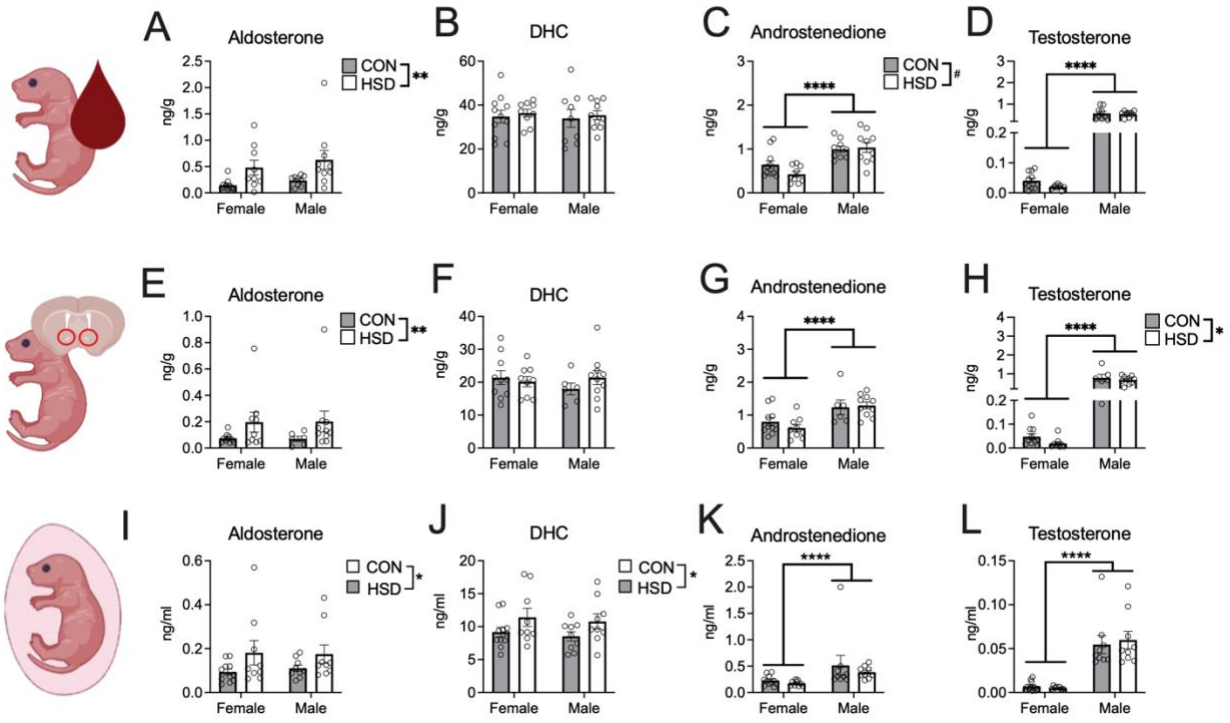

Figure 7. Chronic maternal high-sucrose intake alters fetal steroids

(A) Aldosterone was significantly increased in the fetal blood due to maternal HSD. (B) DHC in fetal blood was not altered by maternal diet or sex. (C) Androstenedione in fetal blood tended to be higher due to maternal HSD and was significantly higher in males. (D) Testosterone in fetal blood was not affected by diet, but males had significantly higher testosterone than females. (E) Aldosterone in fetal NAc was significantly increased due to maternal HSD. (F) DHC in fetal NAc was not altered by maternal diet or sex. (G) Androstenedione in NAc was not altered due to maternal HSD and males had significantly higher androstenedione than females. (H) Testosterone in NAc was significantly higher in males than females, and maternal HSD significantly reduced testosterone. Aldosterone (I) and DHC (J) were significantly increased in the amniotic fluid due to maternal HSD. Androstenedione (K) and testosterone (L) were significantly higher in male than female fetuses but not affected by maternal diet. NAc, nucleus accumbens; CON, control; HSD, high-sucrose diet; DHC, 11-dehydrocorticosterone. #, p≤0.1; *, p ≤0.05; **, p ≤0.01; ****, p ≤0.0001. Created with Biorender.com.

TABLES

Table 1. Diet composition

Diet	Control diet		High-sucrose diet	
Macronutrient	g (%)	*kcal (%)*	g (%)	*kcal (%)*
Protein	19	*20*	19	*20*
Carbohydrate	67	*70*	67	*70*
Fat	4	*10*	4	*10*
Total	90	*100*	90	*100*
kcal/g	3.8		3.8	
Ingredient	**g**	***kcal***	**g**	***kcal***
Casein	200	*800*	200	*800*
L-Cystine	3	*12*	3	*12*
Corn Starch	550	*2200*	300	*1200*
Maltodextrin 10	150	*600*	150	*600*
Sucrose	**0**	***0***	**250**	***1000***
Cellulose, BW200	50	*0*	50	*0*
Soybean Oil	25	*225*	25	*225*
Lard	20	*180*	20	*180*
Mineral Mix S10026	10	*0*	10	*0*
Dicalcium Phosphate	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*
Potassium Citrate	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*
FD&C Red Dye #40	0.025	*0*	0	*0*
FD&C Blue Dye #1	0.025	*0*	0.05	*0*
Total	**1055.05**	***4057***	**1055.05**	***4057***
Sucrose (g%)	**0.9478**		**24.6**	
Sucrose (kcal%)	**1.0**		**25.6**	

Table 2. Unchanged maternal steroids (ng/ml)

	CON	HSD
Maternal serum		
Pregnenolone	4.31±0.68	5.89±1.73
Progesterone	90.97±8.38	102.03±3.89
Allopregnanolone	11.48±1.55	13.82±1.06
Androstenedione	0.76±0.09	0.77±0.05
Estrone	0.02±0.00	0.03±0.03
Estradiol	nd	nd
Estriol	nd	nd

n=10-12/group. Data are shown as mean ± s.e.m.

Table 3. Unchanged placental steroids (ng/g)

	CON Female	HSD Female	CON Male	HSD Male
Pregnenolone				
Decidua	17,45 ± 4,88	16,49 ± 2,41	17,49 ± 3,08	12,92 ± 1,79
Labyrinth zone	13,51 ± 1,88	12,27 ± 1,82	12,90 ± 0,51	12,21 ± 1,22
Progesterone				
Decidua	16,42 ± 2,38	12,13 ± 1,71	14,27 ± 1,88	11,55 ± 0,98
Labyrinth zone	16,45 ± 2,37	12,63 ± 2,57	16,59 ± 2,46	15,51 ± 1,95
Allopregnanolone				
Decidua	36,60 ± 5,84	45,88 ± 8,61	49,47 ± 7,14	44,29 ± 6,78
Labyrinth zone	42,82 ± 5,84	45,88 ± 8,61	49,47 ± 7,14	44,29 ± 6,78
Corticosterone				
Decidua	20,10 ± 2,69	17,58 ± 2,61	24,74 ± 5,74	17,63 ± 3,79
Labyrinth zone	30,44 ± 3,83	29,89 ± 5,38	44,66 ± 10,23	29,04 ± 4,40
11-Dehydrocorticosterone				
Decidua	35,23 ± 3,16	42,37 ± 5,86	37,97 ± 6,71	40,27 ± 6,31
Labyrinth zone	57,41 ± 6,92	58,92 ± 8,47	63,70 ± 9,33	67,51 ± 7,52
Estrone				
Decidua	0,05 ± 0,02	0,05 ± 0,01	0,04 ± 0,02	0,06 ± 0,02
Labyrinth zone	0,17 ± 0,03	0,19 ± 0,04	0,21 ± 0,03	0,22 ± 0,03

n=6-10/group. Data are shown as mean ± s.e.m.

Table 4. Unchanged steroids (ng/ml) in fetal blood and amniotic fluid

	CON Female	HSD Female	CON Male	HSD Male
Fetal Blood				
Pregnenolone	4.71±1.06	3.99±1.13	3.89±0.84	3.92±0.78
Progesterone	8.71±0.84	7.41±0.67	7.18±0.56	8.15±0.73
Allopregnanolone	13.71±3.06	17.10±1.72	15.25±2.25	13.83±1.30
Corticosterone	174±11.69	175.71±8.44	160.92±14.06	155.81±14.51
Estradiol	nd	nd	nd	nd
Estrone	1.98±0.25	2.29±0.14	2.01±0.15	2.25±0.21
Estriol	nd	nd	nd	nd
Amniotic fluid				
Pregnenolone	0.50±0.12	0.67±0.16	1.69±0.88	0.50±0.08
Progesterone	0.93±0.08	0.92±0.14	1.14±0.14	1.11±0.18
Allopregnanolone	2.74±0.22	2.94±0.46	3.79±0.80	3.12±0.28
Corticosterone	49.86±4.13	55.86±7.96	60.10±5.46	57.58±9.11
Estradiol	nd	nd	nd	nd
Estrone	0.59±0.05	0.68±0.04	0.72±0.06	0.73±0.01
Estriol	nd	nd	nd	nd

n=6-10/group. Data are shown as mean ± s.e.m.

Table 5. Steroid levels (ng/g) in fetal brain regions

	CON Female	HSD Female	CON Male	HSD Male
Pregnenolone				
OC	8,97 ± 0,49	8,24 ± 0,62	8,07 ± 0,47	9,92 ± 1,03
mPFC	8,47 ± 0,91	9,63 ± 2,15	8,95 ± 0,96	10,09 ± 1,43
Nucleus accumbens	8,92 ± 0,55	8,98 ± 1,07	8,87 ± 0,66	9,94 ± 0,92
Amygdala	9,17 ± 0,83	8,30 ± 0,65	9,71 ± 1,06	10,37 ± 1,34
Dorsal Hippocampus	10,62 ± 1,10	10,73 ± 0,85	9,73 ± 0,63	11,05 ± 1,27
Ventral Hippocampus	9,21 ± 0,65	8,72 ± 0,50	8,64 ± 0,62	9,39 ± 0,90
Preoptic Area	9,18 ± 0,66	10,29 ± 0,82	9,42 ± 0,83	10,46 ± 0,89
Hypothalamus	12,32 ± 1,13	10,82 ± 0,70	10,78 ± 0,71	13,41 ± 1,28
Ventral Tegmental Area	14,33 ± 1,16	13,81 ± 0,86	13,77 ± 0,81	15,50 ± 1,70
Progesterone				
OC	18,46 ± 1,45	15,78 ± 3,02	16,17 ± 2,58	16,57 ± 1,89
mPFC	17,09 ± 1,77	17,11 ± 3,53	17,06 ± 1,71	17,22 ± 2,39
Nucleus accumbens	17,71 ± 1,60	19,98 ± 5,00	17,65 ± 2,56	18,12 ± 1,79
Amygdala	17,23 ± 1,11	15,65 ± 2,55	17,44 ± 3,04	16,28 ± 1,98
Preoptic Area	17,54 ± 1,65	17,13 ± 1,93	17,68 ± 2,55	16,61 ± 1,84
Dorsal Hippocampus	18,51 ± 1,31	19,28 ± 3,88	18,57 ± 3,11	16,21 ± 1,52
Ventral Hippocampus	17,84 ± 1,57	19,80 ± 3,69	17,80 ± 2,75	16,68 ± 1,64
Hypothalamus	16,94 ± 1,68	17,79 ± 4,01	17,34 ± 3,30	16,78 ± 2,15
Ventral Tegmental Area	18,15 ± 1,96	17,02 ± 2,92	18,66 ± 3,52	16,51 ± 2,62
Allopregnanolone				
OC	42,29 ± 10,83	49,93 ± 19,58	37,11 ± 4,47	33,02 ± 4,36
mPFC	44,49 ± 8,82	53,74 ± 13,50	53,79 ± 9,23	35,88 ± 6,90
Nucleus accumbens	33,68 ± 7,80	42,33 ± 10,74	37,24 ± 4,42	29,98 ± 3,56
Amygdala	30,14 ± 6,39	39,00 ± 12,11	34,89 ± 5,59	27,76 ± 3,42
Preoptic Area	33,20 ± 5,46	44,80 ± 10,39	41,07 ± 5,16	31,34 ± 4,75
Dorsal Hippocampus	26,76 ± 3,56	33,40 ± 6,94	32,09 ± 4,61	24,01 ± 2,68
Ventral Hippocampus	30,17 ± 4,94	37,53 ± 9,97	37,30 ± 6,41	28,95 ± 2,96
Hypothalamus	27,30 ± 2,67	32,67 ± 4,11	33,53 ± 4,51	29,27 ± 2,31
Ventral Tegmental Area	36,14 ± 5,63	47,27 ± 10,29	44,76 ± 4,88	39,68 ± 3,99

	CON Female	HSD Female	CON Male	HSD Male
Corticosterone				
OC	20,13 ± 1,13	15,16 ± 1,79	19,27 ± 3,56	16,10 ± 1,35
mPFC	19,67 ± 1,98	20,13 ± 4,52	19,56 ± 2,67	14,89 ± 2,44
Nucleus accumbens	12,12 ± 0,84	11,26 ± 1,90	13,19 ± 1,94	10,28 ± 1,25
Amygdala	16,10 ± 1,40	12,86 ± 1,63	14,48 ± 1,98	13,02 ± 1,59
Preoptic Area	14,75 ± 1,45	13,61 ± 2,84	18,10 ± 2,58	10,89 ± 1,73
Dorsal Hippocampus	25,81 ± 2,18	23,53 ± 3,07	28,32 ± 3,23	22,46 ± 2,62
Ventral Hippocampus	18,22 ± 1,91	19,44 ± 2,64	20,19 ± 2,85	18,11 ± 1,52
Hypothalamus	21,66 ± 1,71	18,60 ± 2,34	20,34 ± 3,32	16,28 ± 2,30
Ventral Tegmental Area	18,13 ± 1,75	18,63 ± 2,64	24,69 ± 4,04	17,42 ± 1,95
11-Dehydrocorticosterone				
OC	16,93 ± 1,40	14,10 ± 1,68	14,35 ± 1,40	16,80 ± 1,30
mPFC	18,61 ± 1,73	20,43 ± 3,91	18,53 ± 1,51	18,41 ± 1,88
Nucleus accumbens	21,38 ± 2,09	20,15 ± 1,55	17,95 ± 1,78	21,39 ± 2,13
Amygdala	20,08 ± 1,96	19,25 ± 2,45	16,38 ± 1,22	19,78 ± 1,95
Preoptic Area	25,35 ± 3,04	24,58 ± 3,22	23,55 ± 2,45	23,42 ± 2,46
Dorsal Hippocampus	20,79 ± 2,03	22,11 ± 2,10	21,13 ± 2,08	20,70 ± 1,92
Ventral Hippocampus	17,70 ± 1,98	24,97 ± 3,37	18,88 ± 1,05	20,53 ± 1,42
Hypothalamus	20,73 ± 2,42	21,81 ± 2,75	18,49 ± 1,78	20,66 ± 2,07
Ventral Tegmental Area	19,28 ± 1,68	22,17 ± 2,80	19,17 ± 1,86	21,16 ± 2,47
Aldosterone				
OC	**0,04 ± 0,01**	**0,08 ± 0,03**	**0,03 ± 0,01**	**0,13 ± 0,05**
mPFC	**0,01 ± 0,00**	**0,05 ± 0,02**	**0,01 ± 0,00**	**0,03 ± 0,01**
Nucleus accumbens	**0,07 ± 0,01**	**0,20 ± 0,08**	**0,07 ± 0,02**	**0,20 ± 0,08**
Amygdala	0,07 ± 0,02	0,17 ± 0,05	0,08 ± 0,03	0,19 ± 0,09
Preoptic Area	0,15 ± 0,04	0,30 ± 0,11	0,17 ± 0,04	0,36 ± 0,14
Dorsal Hippocampus	0,10 ± 0,03	0,24 ± 0,10	0,12 ± 0,03	0,26 ± 0,10
Ventral Hippocampus	0,05 ± 0,01	0,11 ± 0,04	0,04 ± 0,02	0,17 ± 0,09
Hypothalamus	0,21 ± 0,05	0,38 ± 0,15	0,22 ± 0,04	0,43 ± 0,18
Ventral Tegmental Area	0,09 ± 0,02	0,20 ± 0,08	0,11 ± 0,03	0,25 ± 0,11

	CON Female	HSD Female	CON Male	HSD Male
Androstenedione				
OC	0,85 ± 0,14	0,63 ± 0,11	1,21 ± 0,19	1,29 ± 0,11
mPFC	0,92 ± 0,21	0,64 ± 0,08	1,46 ± 0,24	1,45 ± 0,13
Nucleus accumbens	0,80 ± 0,13	0,61 ± 0,11	1,24 ± 0,22	1,29 ± 0,10
Amygdala	0,59 ± 0,09	0,48 ± 0,08	0,91 ± 0,21	1,05 ± 0,13
Preoptic Area	0,66 ± 0,14	0,48 ± 0,10	0,99 ± 0,14	0,89 ± 0,12
Dorsal Hippocampus	0,79 ± 0,09	0,58 ± 0,09	1,22 ± 0,18	1,19 ± 0,12
Ventral Hippocampus	0,84 ± 0,13	0,67 ± 0,08	1,35 ± 0,21	1,31 ± 0,12
Hypothalamus	0,80 ± 0,15	0,53 ± 0,08	1,08 ± 0,14	1,25 ± 0,13
Ventral Tegmental Area	0,93 ± 0,16	0,68 ± 0,07	1,35 ± 0,21	1,50 ± 0,16
Testosterone				
OC	**0,03 ± 0,01**	**0,02 ± 0,01**	**0,77 ± 0,21**	**0,64 ± 0,07**
mPFC	0,02 ± 0,00	0,02 ± 0,00	0,77 ± 0,15	0,67 ± 0,10
Nucleus accumbens	**0,05 ± 0,01**	**0,02 ± 0,01**	**0,79 ± 0,19**	**0,67 ± 0,07**
Amygdala	0,03 ± 0,01	0,02 ± 0,00	0,74 ± 0,23	0,61 ± 0,07
Preoptic Area	0,02 ± 0,01	0,01 ± 0,00	0,87 ± 0,18	0,67 ± 0,08
Dorsal Hippocampus	0,04 ± 0,01	0,03 ± 0,01	0,87 ± 0,24	0,66 ± 0,07
Ventral Hippocampus	0,03 ± 0,01	0,03 ± 0,01	0,88 ± 0,24	0,70 ± 0,07
Hypothalamus	0,04 ± 0,01	0,03 ± 0,00	0,83 ± 0,20	0,76 ± 0,08
Ventral Tegmental Area	0,03 ± 0,01	0,02 ± 0,01	0,92 ± 0,26	0,84 ± 0,13
Estrone				
OC	0,18 ± 0,02	0,18 ± 0,02	0,18 ± 0,04	0,20 ± 0,02
mPFC	0,18 ± 0,03	0,18 ± 0,04	0,19 ± 0,06	0,19 ± 0,02
Nucleus accumbens	0,23 ± 0,03	0,20 ± 0,02	0,22 ± 0,05	0,24 ± 0,03
Amygdala	0,40 ± 0,04	0,37 ± 0,06	0,39 ± 0,05	0,48 ± 0,07
Preoptic Area	0,59 ± 0,07	0,55 ± 0,06	0,74 ± 0,05	0,84 ± 0,07
Dorsal Hippocampus	0,28 ± 0,03	0,28 ± 0,04	0,31 ± 0,03	0,31 ± 0,03
Ventral Hippocampus	0,18 ± 0,02	0,23 ± 0,02	0,23 ± 0,03	0,24 ± 0,02
Hypothalamus	0,36 ± 0,05	0,34 ± 0,05	0,34 ± 0,02	0,42 ± 0,03
Ventral Tegmental Area	0,17 ± 0,02	0,19 ± 0,03	0,25 ± 0,02	0,24 ± 0,02

n=6-10/group. for 17β-estradiol and estriol, less than 20% of samples had detectable levels.
Main effect of diet: data shown in **bold**. Data are shown as mean ± s.e.m.

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Supplementary Material

Maternal high-sucrose consumption increases maternal and fetal glucocorticoids and decreases fetal androgens in the rat

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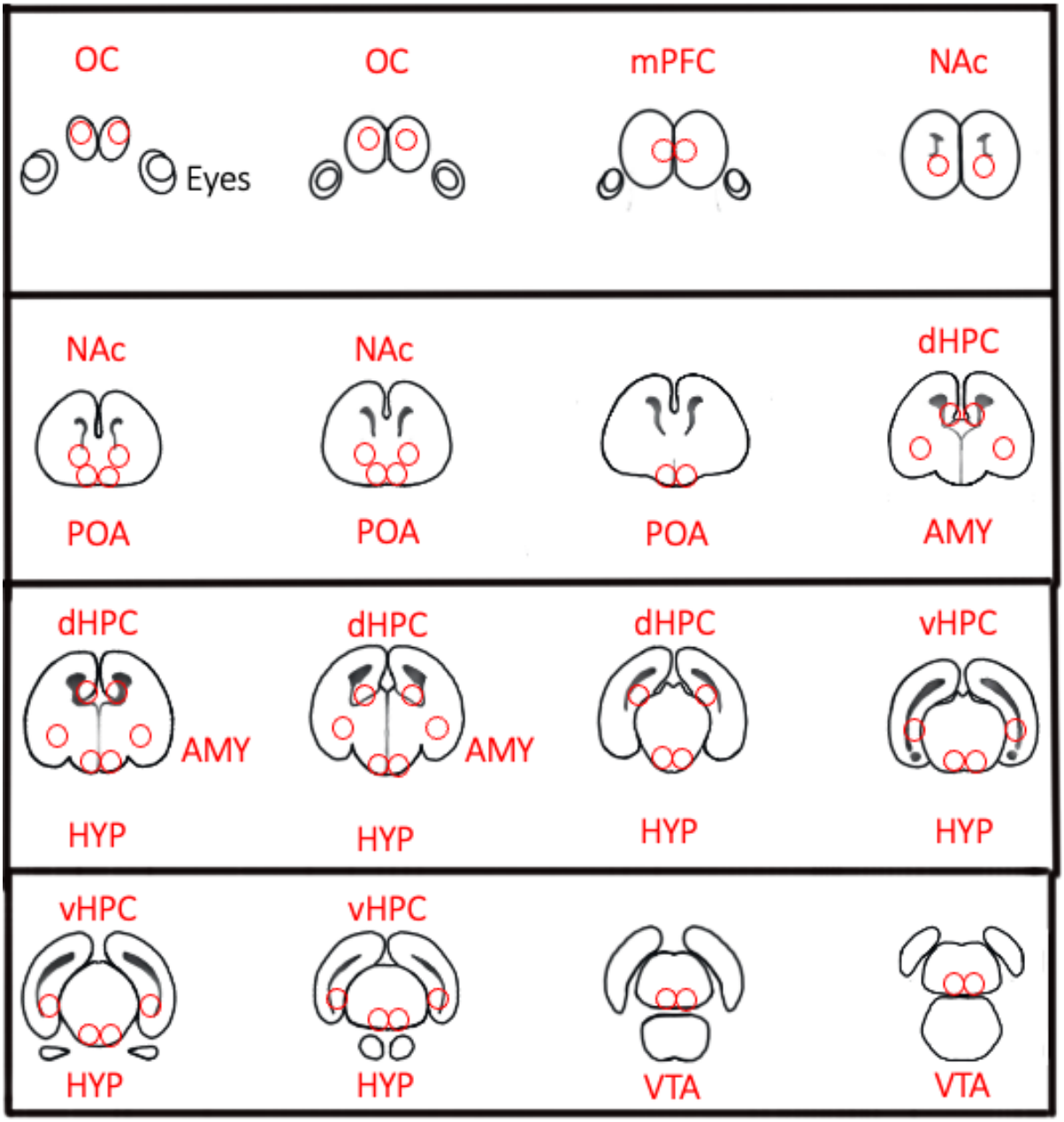

Figure S1. Schematic illustrating rostral to caudal locations for microdissection performed using 1 mm diameter Palkovits punch for LC-MS/MS steroid analysis. Section thickness 300µm. OC, orbital cortex; mPFC, infralimbic portion of the mPFC; NAc, nucleus accumbens; POA, preoptic area; AMY, amygdala; dHPC, dorsal hippocampus; vHPC, ventral hippocampus; HYP, hypothalamus; VTA, ventral tegmental area.