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Prenatal Cafeteria Diet Primes Anxiety-like Behavior Associated to Defects in Volume and Diffusion in the Fimbria-fornix of Mice Offspring

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Abstract—Prenatal exposure to high-energy diets primes brain alterations that increase the risk of developing behavioral and cognitive failures. Alterations in the structure and connectivity of brain involved in learning and memory performance are found in adult obese murine models and in humans. However, the role of prenatal exposure to high-energy diets in the modulation of the brain's structure and function during cognitive decline remains unknown. We used female C57BL6 mice (n = 10) exposed to a high-energy diets (Cafeteria diet (CAF)) or Chow diet for 9 weeks (before, during and after pregnancy) to characterize their effect on brain structural organization and learning and memory performance in the offspring at two-month-old (n = 17). Memory and learning performance were evaluated using the Y-maze test including forced and spontaneous alternation, novel object recognition (NORT), open field and Barnes maze tests. We found no alterations in the short- or long-time spatial memory performance in male offspring prenatally exposed to CAF diet when compared to the control, but they increased time spent in the edges resembling anxiety-like behavior. By using deformation-based morphometry and diffusion tensor imaging analysis we found that male offspring exposed to CAF diet showed increased volume in primary somatosensory cortex and a reduced volume of fimbria-fornix, which correlate with alterations in its white matter integrity. Biological modeling revealed that prenatal exposure to CAF diet predicts low volume in the fimbria-fornix, which was associated with anxiety in the offspring. The findings suggest that prenatal exposure to high-energy diets prime brain structural alterations related to anxiety in the offspring.

Key words: Prenatal programming, high-energy-diet, magnetic resonance imaging, myelin, cognitive behavior.

INTRODUCTION

Exposure to external stimuli during prenatal period provides a time-dependent modulation of physiological outcomes after birth, known as fetal programming (Gawlińska et al., 2021). Accordingly, exposure to an adverse or hostile environment during critical developmental stages such as maternal obesity, smoking, metabolic/endocrine failures, infections, physical inactivity and nutrition regulate long-term susceptibility to diseases (Gawlińska et al., 2021). For instance, evidence from both animal and human studies showed that maternal obesity or exposure to high-energy diets is linked to an elevated risk of diabetes, hypertension, and also cognitive and behavioral alterations in the offspring including depression-like (de la Garza et al., 2019; Trujillo-Villarreal et al., 2021), addiction-like (Camacho, 2017; Cruz-Carrillo et al., 2020) and asocial-like behaviors (Seremak-Mrozikiewicz et al., 2014; Tabachnik et al., 2017; George et al., 2019; Cruz-Carrillo et al., 2020; Liu et al., 2021; Maldonado-Ruiz et al., 2022).

Cognitive impairment results from a complex interaction of environmental, demographic, socioeconomic, genetic and lifestyle contributors through life (Dominguez & Barbagallo, 2018). The most important independent predictor of cognitive decline is age. However, changes in diet or nutrition during prenatal programming have been documented as a relevant modulator of cognition (Gerstein et al., 2013; Dominguez & Barbagallo, 2018). Prenatal exposure to high-energy diets primes cognitive impairment, affecting learning and

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memory processes in the offspring after life (Sarker & Peleg-Raibstein, 2019; Liu et al., 2021). Some reports documented that offspring exposure to high-energy diets developed hippocampal-dependent behavioral abnormalities that code for defects in spatial learning and memory performance during the Morris water and the Barnes maze tests (Miller & Spencer, 2014; Peleg-Raibstein, 2021). In fact, in a murine model of diet-induced obesity, the male offspring of obese parents showed defective learning performance (Gerstein et al., 2013). Accordingly, variations in energy consumption during prenatal stages disrupts hippocampus function and cognitive processing, learning, and memory performance (Gerstein et al., 2013). However, previous studies documented mixed results showing no influence of prenatal diets on cognitive performance whereas an enhanced or worsened cognitive performance was documented by other researchers (Bilbo & Tsang, 2010; Page et al., 2014; Robb et al., 2017). This evidence suggests the detrimental effect of high-energy diets during prenatal programming in modulating positive or negative cognitive performance in the offspring, potentially by affecting hippocampus-dependent function (Jacka et al., 2015).

The effect of prenatal exposure to high-energy diets on behavior is starting to be decoded. We reported that prenatal exposure to cafeteria diet (a high-energy diet formula) promoted structural brain alterations including volume reduction in the nucleus accumbens (NAc), hippocampus, and prefrontal cortex (Trujillo-Villarreal et al., 2021). Notably, structural brain alterations were positively associated to pathological behavior in the offspring such as autism-like and depression-like behaviors (Trujillo-Villarreal et al., 2021; Maldonado-Ruiz et al., 2022). While still under investigation, a positive energy balance such as happens during obesity or prenatal exposure to high-energy diets is also able to promote structural brain abnormalities in infants and in adult individuals (Ward et al., 2005; Verstynen et al., 2012). For instance, obese individuals experienced a decrease in total brain volume, regardless of age and morbidity (Ward et al., 2005; Verstynen et al., 2012). Also, gray matter atrophy in the temporal, frontal, and occipital cortices, and a reduction of white matter integrity in the brain were reported in obese human individuals (Gunstad et al., 2008; Shefer et al., 2013). In fact, an increase in body mass index during the pre-pregnancy or during gestation stages altered white matter microstructure in the child's brain (Verdejo-Román et al., 2019; Hasebe et al., 2021; Norr et al., 2021) or predisposes to decreased volume in the left hippocampus in adulthood (Jacka et al., 2015), respectively. However, the contribution of prenatal exposure to high-energy diets on structural brain conformational and activity coding for aberrant behavior after birth has not been documented.

In the current study, we characterized the effect of exposure to high-energy diets during prenatal stage on the macro and microstructural alterations in the brain using structural and diffusion MRI analysis, and their association to learning and memory performance in the offspring.

EXPERIMENTAL PROCEDURES

Animals and housing

All the experiments were performed using two-month-old, wild-type male C57bl6 mice. Animals were handled according to the NIH guide for the care and use of laboratory animals (NIH Publications No. 80–23, revised in 1996). We followed the Basel Declaration to implement the ethical principles of Replacement, Reduction and Refinement of experimental animal models. Our study was approved by the local Animal Care Committee (BI20-00006) at the Universidad Autónoma de Nuevo León, México. Mice were housed individually in Plexiglas-style cages, maintained at 20–23 °C in a temperature-controlled room with a 12-h light/dark cycle. Water was available *ad libitum* in the home cage. Food availability is described below.

Diets

We used a standard chow diet formula composed of 57% carbohydrates, 13% lipids, 30% proteins, and 290 mg of sodium, caloric density = 3.35 kcal/g (LabDiet, St. Louis, MO 63144, 5001, Cat. D12450B). Cafeteria (CAF) diet was made of liquid chocolate, biscuits, bacon, fried potatoes, standard diet, and pork pâté at a 1:1:1:1:1:1:2 ratio respectively, with a composition of 39% carbohydrates, 49% lipids, 12% proteins, and 290 mg of sodium, caloric density = 3.72 kcal/g, as reported previously (Camacho, 2017; Cardenas-Perez et al., 2018; Maldonado-Ruiz et al., 2019; Cruz-Carrillo et al., 2020; Trujillo-Villarreal et al., 2021).

Maternal programming model

Upon arrival, 10-week-old females (n = 10) and 12-week-old males (n = 5) C57BL/6 mice were group-housed for at least 10 days to acclimatize, with ad libitum access to standard Chow diet and water. Females were randomized into two different dietary groups: standard Chow diet (Control, n = 5) and CAF diet (n = 5). After randomization, female mice were fed for 9 weeks, including 3 weeks of pre-pregnancy, pregnancy, and lactation. Females were mated with 12–14 weeks old CB57BL/6 males, 30–35 g (2 females/male), for two days. Mating day was determined by observation of vaginal plug (see below Fig. 1A for experimental design). Pregnant mice were transferred to individual cages and were kept on their assigned diet. By postnatal day 21st, all male offspring were exposed to a control diet until 2 months of age, and the behavioral phenotyping was performed (see below Fig. 1B for experimental design). Only male offspring were further studied due to potential hormone sensitive-behavioral effects in females. To follow the ethical principles of Replacement, Reduction and Refinement of experimental animal models, we allocated all female offspring to a second experimental behavioral protocol which is currently under investigation.

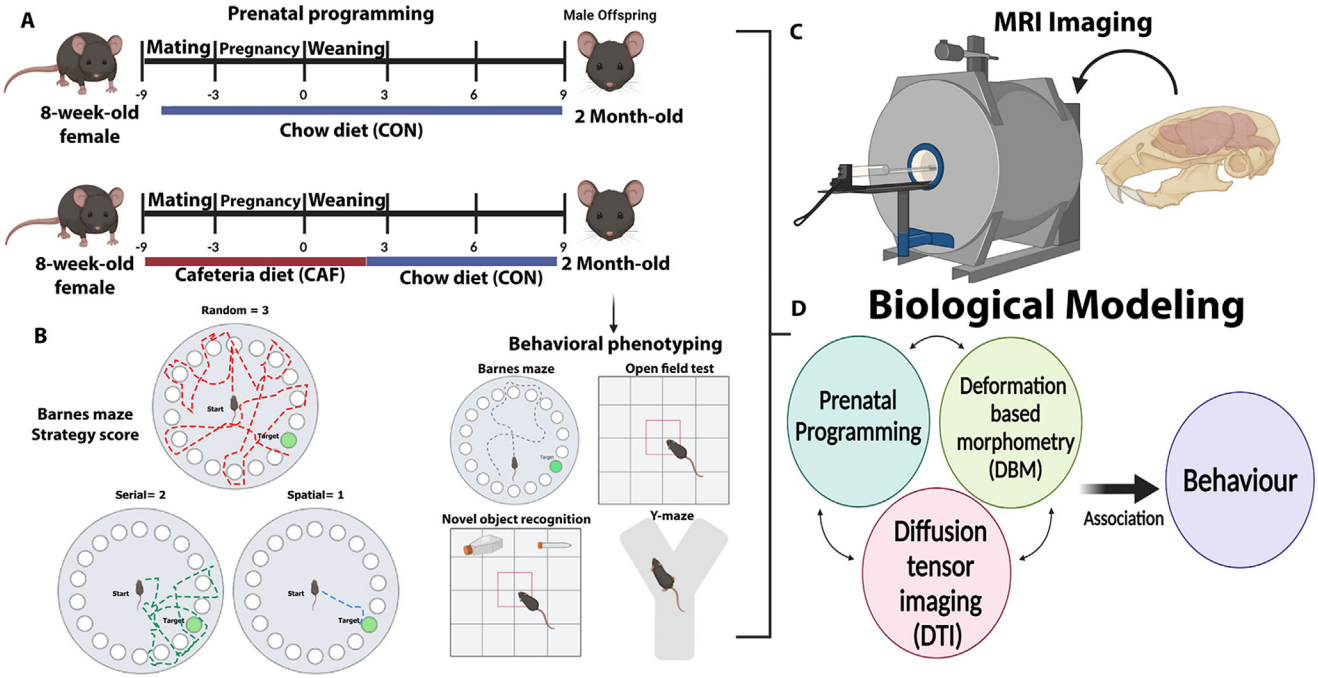

Fig. 1. Prenatal programming model and behavioral phenotyping. For prenatal programming, female C57BL6 were fed with chow (CON, $n = 11$) or CAF ($n = 7$) diets for 9 weeks (pre-pregnancy, pregnancy, and lactation) offspring were fed with chow diet after weaning at postnatal day 21 **(A)**. At 2 months of age male mice were tested in a behavioral test battery consisting of forced alternation, spontaneous alternation (Y-maze), Barnes maze, NORT and open field test. Search strategies employed by mice in the Barnes maze are shown, sorted from highly spatial (bottom-right) to non-spatial (top): Spatial search, Serial search and Random Search and each score given **(B)**. Magnetic resonance imaging (MRI) was performed to analyze macrostructural (Deformation based morphometry, DBM) and microstructural (diffusion tensor imaging, DTI) brain alterations in the offspring **(D)**. Biological modeling integrating the effect of prenatal diet exposure, DBM and DTI metrics predicting behavioral outcomes was performed **(E)**.

Behavioral phenotyping in the offspring

The behavioral test battery was performed in the offspring of male mice exposed to Chow ($n = 11$) or CAF ($n = 7$) diet, respectively, and both were exposed to four memory tests in the following order: Y- maze forced alternation, open field, NORT, Barnes maze and Y-maze spontaneous alternation (Fig. 1B). We followed a specific order to conduct the tests so that the mice could have several resting days between the tasks and to avoid carryover effects from prior tests. Visual cues were placed on the walls of the testing area for all tests except the NORT. All mice were habituated to the testing room for 1-hour before each test day. All behavioral tests were conducted by the same experimenter to avoid extra stress on the animals; the experimenter was blinded from the groups. During the trials, mice were not able to see the experimenter, as they were separated by a wall. Also, to avoid any metabolic stress in the mice we omitted tests involving food restriction (Wolf et al., 2016).

Y-maze

Forced alternation. We conducted the test using a symmetrical Y-maze, each arm was 35 cm long, 5 cm wide, and 10 cm high, and the wall at the end of each arm was marked with a different visual cue (Fig. 1B). We followed the protocol reported by Andrea Wolf and collaborators (Wolf et al., 2016) with slight modifications. In brief, the test consisted of a 15-min sample trial (T1) followed by a 15-min retrieval trial (T2). In T1, the mouse was placed into the end of the start arm, facing the wall and away from the center. The mouse was then allowed to explore two arms of the Y-maze, while entry into the third arm was blocked by a barrier. After the sample trial, the mouse was returned to its home cage for a 30 min inter-trial interval. In T2, the barrier in arm 3 was removed, the mouse was again placed into the start arm, and then allowed to access all three arms of the maze. After each animal and between T1 and T2, the maze was wiped with 70% ethanol to prevent odor cues. Time in Novel Arm (seconds) was counted as for how many seconds the mouse spent exploring the novel arm.

Spontaneous alternation. Using the previously described Y-maze each arm was designated as 'A', 'B' & 'C'. The mouse was placed it into one arm of the Y-maze, facing the center and allowed to explore the arms of the Y-maze. An arm entry occurs when all 4 paws of the mouse cross the threshold of the central zone and into the arm and the animal's snout is oriented toward the end of the arm. A spontaneous alternation occurs when a mouse enters a different arm of the maze in each of 3 consecutive arm entries (Miedel et al., 2017). Spontaneous alternation percentage is then calculated with the following formula:

$$Spontaneous\ Alternation\ \% = \frac{\#\ spontaneous\ alternations}{total\ number\ of\ entries - 2} \times 100$$

Novel object recognition test (NORT)

The NORT was conducted as previously reported (Bevins & Besheer, 2006; Fig. 1B). We used identical square arenas (40 × 40 cm) surrounded by walls (35 cm high). In brief, the test was performed as a three-step schedule which consisted of a habituation phase of 15 minutes where the mouse explored freely the empty box, a familiarization step which consisted in placing the two identical objects in the open field and finally, the recognition test where the two familiar objects were placed, one with the triplicate copy (to ensure that there are no residual olfactory cues on the previously used object) and the other by a novel object. After each trial, the objects and boxes were cleaned with 70% ethanol to eliminate odor cues. Exploration time of the novel object was measured in seconds; mice with less than 7 s of total object interaction in either trial were excluded from the analysis as reported (Kalueff et al., 2006). Time exploring the objects was measured as following:

Object Interaction [%] for the familiar object was calculated as (familiar object interaction × 100)/(familiar object interaction + novel object interaction) and object interaction [%] for the novel object was calculated as (novel object interaction × 100)/(familiar object interaction + novel object interaction).

$$\frac{Novel\ object\ interaction \times 100}{Familiar\ object\ interaction + Nobel\ object\ interaction}$$

Barnes maze

The Barnes maze test was performed as we previously reported (Cárdenas-Tueme et al., 2022). Briefly, the test consisted of an adaptation phase, spatial acquisition and two probe trials (short and long-term memory) as we will describe: In the adaptation period mice were guided gently to the scape box and remained there for 2 min. Then, during the spatial acquisition, mice performed 4 trials per day for 4 days, with an inter-trial interval of 15 minutes. Briefly, mice were placed in the middle of the maze within a cylindrical black start chamber. After 10 s elapsed, the chamber was lifted the mouse was allowed to explore the maze freely for 3 minutes. During these 3 minutes the number of total errors and primary latency were measured by the experimenter. Finally, the short reference memory test was performed on day 5, 24 h after the last training day, and the long reference memory test was conducted 7 days after the last training which would be day 12. During these trials, mice were given 30 seconds to end the test and total number of errors and latency to escape were measured by the experimenter.

We analyzed the respective search tactics employed by the mice to identify the target hole to determine the qualitative aspects of spatial learning. The random search approach entails a disorganized maze search with several center crossings. The serial method involves the mouse running to the edge and visiting subsequent holes in a clockwise or counterclockwise way. The spatial method is the most efficient search technique, and it entails going straight to the target hole or another nearby hole before going to the target. The overall strategy score for one rat was generated as follows: random = 3, serial = 2, and spatial = 1, and this score was evaluated every day as previously reported (Zhu et al., 2016).

Open field test

Subjects were subjected to the open field test as described (Maldonado-Ruiz et al., 2022). The Open Field chamber is a clear polycarbonate square arena of 50 × 50 × 50 cm. Offspring (male and female mice) were handled by the base of their tails and placed in one of the four corners of the open field arena. Subjects were allowed to explore the arena for 5 minutes. After the 5-minute test, mice were returned to their home cages and the open field area was cleaned using 70% ethyl alcohol. Activity was video recorded for 5 minutes using an automatic motion sensor system (OMNIALVA, Inc). We quantified the total distance traveled, the percentage of inactivity, time spent in the center and on the edges of the arena.

Behavioral diagnosis during the open field test included: 1. Duration of time in which the mice crossed one of the red lines with all four paws into the central square. 2. Duration of time the mice spent in the edges of the arena. 3. Duration of time the mice spent immobile.

Ex-vivo fixation and MRI analysis by deformation based morphometry (DBM)

Following the behavioral phenotyping, mice (Chow, n = 11 and CAF, n = 7) were sacrificed by an intraperitoneal injection of 1 mL pentobarbital (PiSA Agropecuaria) overdose. A dermal dissection was performed from the abdominal region to the upper part of the thoracic cage, exposing the heart. Then, the left ventricle of the heart was perforated from its apex and a cut was made in the right atrium, to open the circulatory system. 0.1 M PBS + Heparin + Prohance wash solution (4 mM) was perfused using an infusion pump (Fisher Scientific GP1000) at a flow rate of 1 mL/min. Subsequently, the washing solution was changed to the fixing solution including 4% paraformaldehyde in PBS 0.1 M (PFA) + 4 mM Prohance for 25 min, following the same flow as previously reported (Cárdenas-Tueme et al., 2022). The brain protected by the skull was collected and samples were stored at 4 °C in 4% PFA Prohance 4 mM for 24 hours and then the solution was changed to a storage solution containing 0.1 M PBS with 0.02% sodium azide and 4 mM Prohance until MRI analysis (2 months).

For MRI acquisition, the skulls were submerged and fixed inside plastic tubes filled with Fomblin (a chemically inert perfluoropolyether fluorocarbon; Solvay Solexis, Inc.). Imaging was performed in a 16 cm bore 7 T Bruker scanner (Pharmascan 70/16) using a Paravision 7 system and a Tx/Rx volume coil for mice

with a 72 mm inner diameter. We then acquired 2 sequences: A T1-weighted FLASH sequence with three-dimensional spatial encoding (TR/TE = 31.56/8.64 s, flip angle = 20°, averages = 1) with 0.069 mm isometric resolution. Diffusion-weighted images (DWI) were acquired using segmented echo-planar images with three-dimensional spatial encoding (TR/TE = 250/19.24 s, flip angle = 90°, averages = 1, number of segments = 64 spatial, resolution: 0.135 × 12.42 × 0.200 mm^3). Diffusion encoding was applied in 32 unique directions with b = 1000 s/mm^2, as well as 8 volumes without diffusion sensitization (i.e., b = 0 s/mm^2).

Image data processing

Structural analysis was performed by converting DICOM to MINC format, and then preprocessed using an in-house pipeline based on MINC-Tools (https://github.com/CoBrALab/minc-toolkit-extras/blob/master/mouse-preprocessing-v5.sh) and the pydpiper pipeline (https://github.com/Mouse-Imaging-Centre/pydpiper). All analyses were performed using pydpiper version 1.8 (Friedel et al., 2014), R studio version 3.6.3 (Rstudio, 2020), and the RMINC version 1.5.2.2 (Lerch et al., 2017) and tidyverse version 1.3.1 (Wickham et al., 2019) packages. All T1-weighted images were preprocessed using an in-house pipeline based on MINC-Tools and ANTs, which performed the following steps: center image, full mask and N4Bias Field Correction. We then used an image registration-based approach to assess anatomical differences between groups. Image registration finds a smooth spatial transformation that best aligns one image to another such that corresponding anatomical features are superimposed. We used an automated intensity-based group-wise registration approach (Lerch et al., 2017) to align all brains in the study into a common coordinate system, yielding an average image of the 45 T1w scans. The nonlinear deformations (warps) that align each of the images to the template then become a summary of how they differ. To assess volume differences between groups, we performed DBM as it provides a continuous voxel by voxel definition of volume changes (expansion/contraction) related to their diet group. Deformations were then mapped from the individual scans back to the average image. The final deformation fields were computed with a greedy symmetric diffeomorphic registration (the SyN algorithm in ANTS) (Avants et al., 2008; Bird et al., 2009) then inverted and blurred with a 0.1 mm FWHM Gaussian smoothing kernel. The Jacobian determinants of these deformations were extracted, giving a measure of local volume expansion/contraction at every voxel in the brain. Log-transformed Jacobian relative determinants (blurred 0.2 mm) were used to assess differences between groups because they better estimate a normal distribution. DWI data sets were denoised (Cordero-Grande et al., 2019) and corrected for motion and eddy-current-induced distortions using linear transformations (12 degrees of freedom). The MRtrix 3.0 software package was used to estimate the diffusion tensor (Tournier et al., 2019), from which we obtained the corresponding eigenvalues ($\lambda1$, $\lambda2$, and $\lambda3$). From these, we created quantitative maps of FA, apparent diffusion coefficient (ADC), axial diffusivity (i.e., $\lambda1$; D||), and radial diffusivity ([$\lambda2$ + $\lambda3$]/2; D⊥). Diffusion tensor imaging (DTI) parameters were analyzed using the principal diffusivity maps (aided by the non-diffusion weighted images) to manually outline regions of interest (ROIs), fimbria, corpus callosum, fornix, and internal capsule.

Statistical analyses

Data are presented as mean ± SEM for all data. All statistical analyses including testing the normality of data distribution were performed using GraphPad Prism 7.01 and IBM SPSS statistics version 22 software and a corrected p value <0.05 was considered as significant. All results were tested for normality using Shapiro-Wilk test. Significant differences in cognitive performance during behavioral phenotyping data are shown as the mean ± SEM and significant differences at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. The statistical analysis on DBM was performed using the log-transformed Jacobian determinants as the dependent variable, "diet group" as the independent variable. We compared the two groups using a general lineal model and analyses were corrected for multiple comparisons using the false-discovery rate (FDR) at 10%. Furthermore, we extracted the jacobian values from significant peaks at the fimbria-fornix region and primary somatosensory cortex. These analyses were performed in R studio version 3.6.3. We compared the FA, AD, ADC and RD results from each region of interest for CON and CAF groups using t-test. Data are presented as mean ± SEM for all data.

RESULTS

Effect of prenatal programming by CAF diet on cognitive behavior in the offspring

We tested whether prenatal exposure to CAF diet affects body weight in the young male offspring. We found a significant increase in body weight at 2 months of age, compared to male offspring born from Chow-fed dams ($t[16] = 3.516$, $p = 0.0029$) (Fig. 2A). Offspring exposed to CAF diet did not show significant differences during the open field test when compared with the control group ($t[16] = 0.8646$, $p = 0.400$) (Fig. 2B). Precisely, the total distance traveled in the control offspring was 35943 mm whereas the offspring exposed to CAF diet traveled 39042 mm (Fig. 2B).

Anxiety-like behavior

Individual behavioral phenotyping was performed in the offspring of subjects exposed to Chow or CAF diet, respectively, to determine the effect of ageing on cognitive decline. We used the open field test to diagnose anxiety by quantifying time spent in the edges as rodents typically prefer not to be in the center, supporting an anxiety-like behavior. We found that offspring exposed to CAF diet significantly spent more time on the edges of the open field ($t[16] = 2.132$, $p = 0.0489$) (Fig. 2C). This result confirms an anxiety-like behavior in the male offspring of dams exposed to a high-energy diet during pregnancy and lactation.

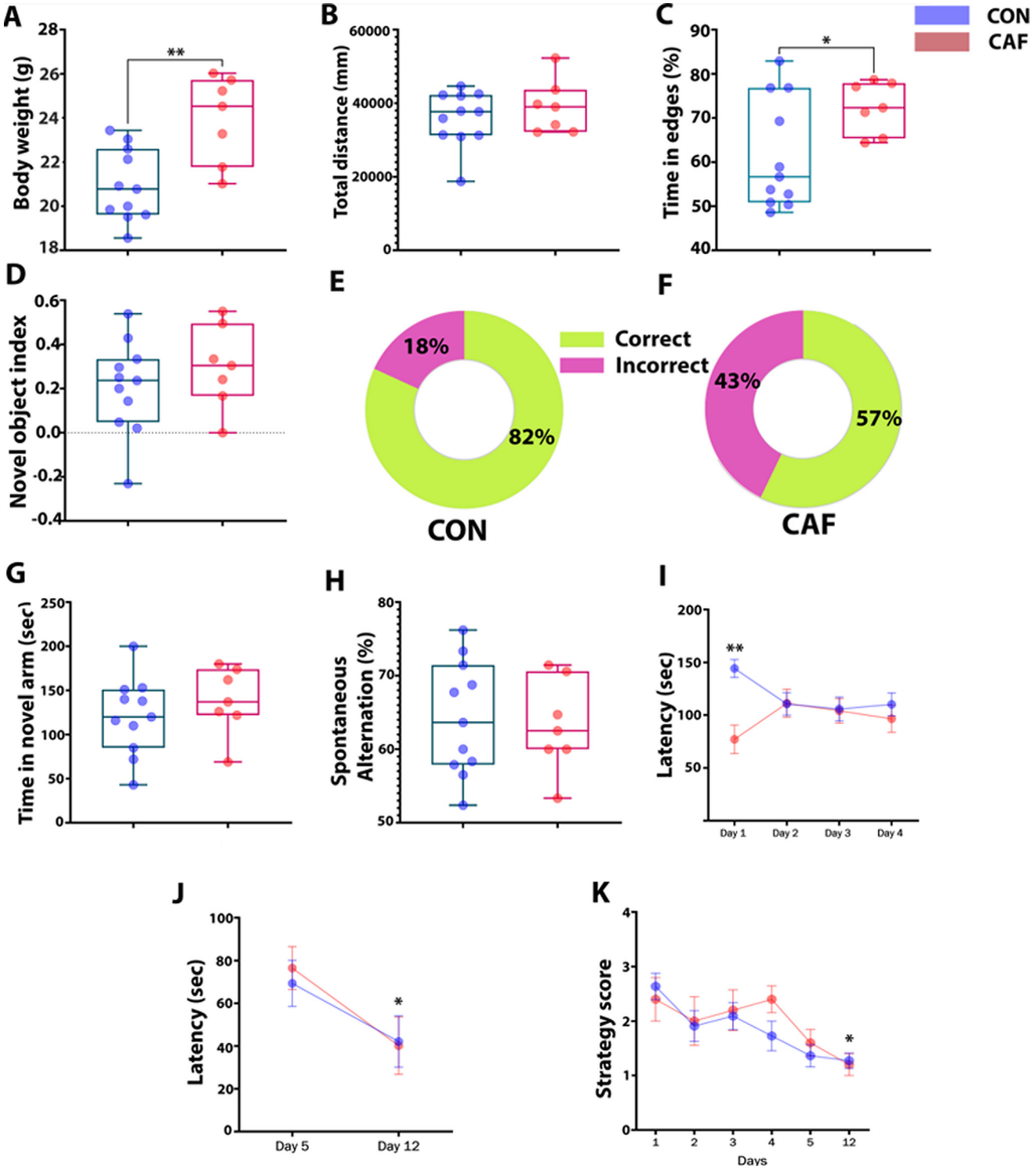

Fig. 2. Prenatal exposure to high-energy-diets affects long-term-memory performance in the offspring. (A) Female C57BL6 were fed with chow (CON) or CAF diets as described in Fig. 1, and offspring were analyzed using behavioral tests. **(A)** Offspring exposed to CAF diet showed significantly higher weight gain when compared with control group. **(B, C)** Comparison of total distance traveled, and time spend in on the edges expressed in percentage in the center of the open-field test. **(D)** Time spent with the and novel object is represented as % of object interaction. **(E, F)** Results show the percentage of alternation between groups, was evaluated according to the arm chosen as the first option in the Y maze. **(G)** Time spent in the novel arm was conducted 15 min after the familiarization session. Results show time spent (s) in the novel arm between groups. **(H)** Spontaneous alternation Y-maze by alternating number of arm visits compared to control group. **(I)** Results show latency to exit (s) until the exit was found during 4 days of training. **(J)** Results show latency to exit (s) until the exit was found, for both STM and LTM. K) Strategy score obtained during Barnes maze trials that the spatial score (1), serial score (2) and random score (3). Data are expressed as mean ± SEM and statistical differences were depicted followed "*t*" test analysis. CAF ($n = 7$) CON ($n = 11$). *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$ vs the control group. Abbreviations: CAF: cafeteria; CON: Control; LTM: long-term-memory; STM: shorth-term-memory.

Forced alternation

To evaluate the effect of prenatal CAF diet exposure on the spatial working memory of the offspring, we used Y-maze–based forced alternation test. First, we found that 80.82% of the offspring exposed to Chow diet during prenatal programming recognized the blocked arm as novel and first choice compared with only 57.14% of subjects born from dams exposed to CAF diet, however, it did not show a significant association (Fig. 2E, F). Also, we found no significant differences in the offspring exposed to CAF diet in the time spent in the novel arm when compared with control subjects (t[16] = 0.8896, p = 0.3869) (Fig. 2G). These results confirm that prenatal exposure to CAF diet alters recognition performance and but does not alter the short time spatial memory performance in the male offspring.

Spontaneous alternation

To measure spatial memory using y-maze and exploratory activity of animals in a short time frame, the percentage of spontaneous alternation was calculated. We found no significant differences in the offspring exposed to CAF diet when compared with subjects exposed to Chow diet, (t [16] = 0.2783, p = 0.7843; Fig. 2H), confirming no alterations on short time spatial memory performance caused by high-energy diets.

Novel object recognition

The NORT assesses recognition memory in mice based on rodents' natural tendency to explore a novel object for a longer period of time when compared with a familiar one. No significant differences were found in the offspring exposed to CAF or Chow diet during the prenatal stage, displaying the same interaction index of exploratory activity during the test (t[16] = 0.9501; p = 0.3562; Fig. 2D).

Barnes maze

To assess spatial memory and learning performance during short- and long-term schedules we used the classical Barnes maze test. As described in Materials and Methods, offspring were trained and the total number of errors before reaching the exit, as well as latency to enter the scape-box on the platform, were scored. We found a significant decrease in latency to reach the exit on the first day of training in the offspring exposed to CAF diet when compared to control subjects (p = 0.0023) (Fig. 2I). However, no major changes in the latency to enter the scape-box were identified from training day 1 to 4 between groups (Fig. 2I).

Short-term memory (STM) in the offspring exposed to CAF or Chow diet was evaluated one day after the four-day training sessions. We did not find major significant differences between both groups by day 5 (Fig. 2J). Spatial memory and learning performance during the long-term schedule (LTM) were evaluated at day 12 post-training. We found no differences in the memory and learning performance in the offspring exposed to Chow diet during the prenatal stage (Fig. 2J). Notably, offspring exposed to CAF diet showed impairment in spatial memory and learning performance during the long-term schedule (day 12) when compared with its own performance at day 5 (p = 0.0433) (Fig. 2J).

Finally, outcomes of search strategies are displayed in Fig. 1K. Quantification of all the potential trajectories in the offspring born from dams exposed to CAF or Chow diet exhibited a significant decrease of random strategy by the test days (Fig. 2K). These results confirm that prenatal exposure to CAF diet does not compromise spatial searching and memory in the offspring at 2 months of age.

High-energy diets program brain macrostructural alterations in the offspring

We performed a global MRI analysis using DBM to selectively characterize regional volume-related macrostructural brain alterations in the offspring exposed to CAF or Chow diet during development. Globally, the pairwise analysis showed significant local differences in brain volume in the offspring exposed to CAF diet during the prenatal stage compared with subjects exposed to Chow diet (Fig. 3). Using the 40-micron DSUR atlas (Mouse Brain Atlases) Coordinates Atlas, we identified two significant punctual structural changes, displaying lower volume in the fimbria/fornix region (p = < 0.0001) (Fig. 3A) and higher volume in the primary somatosensory cortex (p = < 0.0001) (Fig. 3B) of subjects exposed to CAF diet when compared with those exposed to Chow diet. A simple linear regression was calculated to predict Jacobians contribution of diet (CAF vs Chow) on changes in primary somatosensory cortex or fimbria-fornix volume. A significant regression equation was found to predict the primary somatosensory cortex ($F_{(1,\ 16)}$ = 67.74, ***p = 3.83e−07) and to predict fimbria-fornix ($F_{(1,\ 16)}$ = 50.9, ***p = 2.37e−06), with an adjusted R^2 of 0.7459. Predicted primary somatosensory cortex is equal to − 0.14110 (CON) + 0.17766 (CAF) Jacobian value, whereas predicted fimbria-fornix is equal to +0.09919 (CON) − 0.12310 (CAF) Jacobian value. Selectively, prenatal exposure to CAF diet increased 0.12766 Jacobians in the primary somatosensory cortex whereas decreased 0.12310 Jacobians in the fimbria-fornix in the offspring. Volumetric changes in brain regions comparing CON vs CAF value were depicted in the Table 1.

Brain macrostructural alterations in specific ROIs and their connectivity are diet-dependent

Next, we performed a DTI analysis to reveal macrostructural abnormalities in the ROIs found by the DBM analysis in the offspring exposed to CAF diet during the prenatal stage. In Fig. 4A, diffusion tensors are visualized as ellipses colored according to their orientation, overlaid on the corresponding fractional

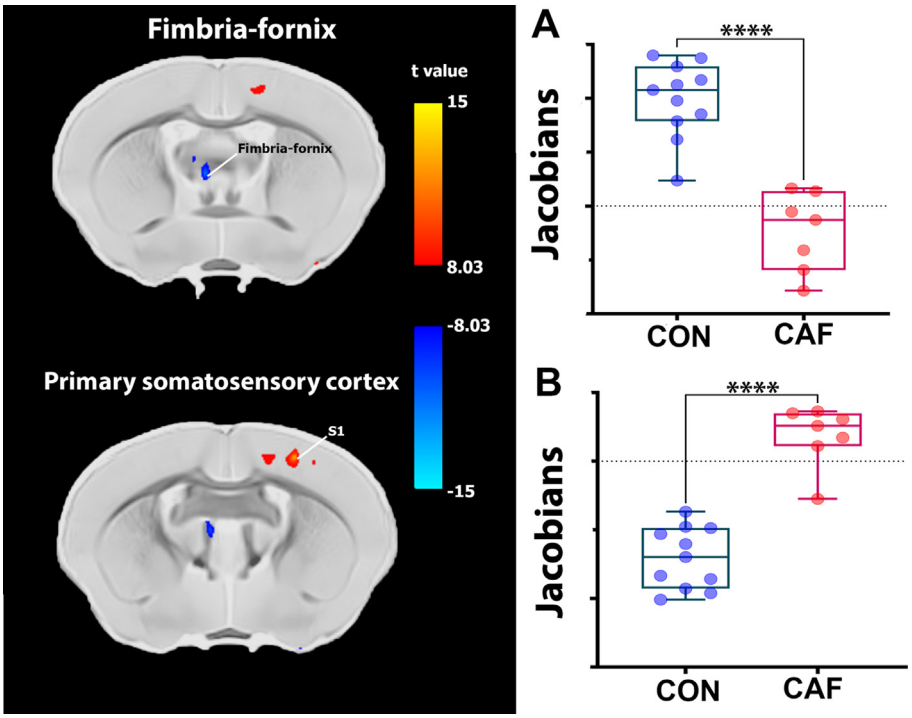

Fig. 3. Prenatal exposure to high-energy-diets promoted volume brain changes in the offspring. Female C57BL6 were fed with chow (CON) or CAF diets as described in Fig. 1, and offspring were analyzed using MRI analysis. **(A)** DBM of brain volume comparison. MRI images of DBM data of CAF offspring vs control offspring. ROI = fimbria-fornix and primary somatosensory cortex (Left), boxplot of relative volume peak. Results are significant at FDR 10%. Boxplots show the relative volume (y axis = Jacobians) in each group (x axis). Data are expressed as mean ± SEM and statistical differences were depicted followed "*t*" test analysis. *p = 0.03, **p = 0.002, ***p = 0.0002. CAF (n = 7) CON (n = 11). Abbreviations: CAF: cafeteria; CON: Control; DBM: Deformation based morphometry; MRI: magnetic resonance imaging; ROI: region of interest analyses.

anisotropy map. Control animals showed significantly more elongated diffusion tensors (indicating high fractional anisotropy), as compared to the tensors in the offspring exposed to CAF diet (showing low fractional anisotropy) (Fig. 4A). Paired to this image we presented the boxplots corresponding to the dMRI metrics in the fornix-fimbria ROI which provide the peak obtained by DBM analysis. Notably, we found significant lower fractional anisotropy value (t(16) = 2.695, *p = 0.0166) (Fig. 4B), with a concomitant increase of axial diffusivity (t(16) = 2.440, *p = 0.0267) (Fig. 4C), radial diffusivity (t(16) = 2.150, *p = 0.0472) (Fig. 4D) and apparent diffusion coefficient (t(16) = 2.504, *p = 0.0235) (Fig. 4E) values in the offspring prenatally exposed to CAF diet when compared to subjects exposed to Chow diet. Conversely, we did not find major changes at the primary somatosensory cortex in ROI using the dMRI metrics (Fig. 4F–J). Additional changes in brain regions identified by the DTI analysis according to their p value were depicted in Table 2.

Decreased volume dMRI metrics in the fimbria-fornix predict behavior in offspring exposed to CAF diet

We performed a multiple linear regression to predict the association between diet (CAF vs Chow), volume changes and dMRI metrics in the fimbria-fornix to behavioral outcomes in the offspring (Fig. 5A, B). According to our mathematical model, we tested several interactions between the behavioral phenotyping tests (Y- maze forced alternation, Open field, NORT, Barnes maze or Y-maze spontaneous alternation) and fractional anisotropy values. Based on this, the time spent in the novel arm in the Y-maze test during the forced alternation test and the time spent in the edges in the open field test were displayed as the two major interactions to perform the analysis. Our multiple regression analysis identified the interaction between dMRI metrics and fimbria-fornix volume and behavior in the offspring exposed to CAF diet. Accordingly, dMRI metrics and fimbria-fornix volume found in the offspring prenatally exposed to CAF diet significantly predicts higher time spent in the edges during the open field tests (Fig. 5A), suggesting a higher anxiety score. Conversely, offspring exposed to Chow diet showed a negative interaction, displaying larger fimbria-fornix volume, a decrease in apparent diffusion coefficient value and less anxiety score (Fig. 5A). Multiple regression analysis displayed an adjusted R^2 = 0.6116 (Table S1). Finally, a lower fimbria-fornix volume and a major apparent diffusion coefficient value showed a non-significant effect to predict forced alternation outcomes in the offspring exposed to CAF diet during prenatal programming (Fig. 5B). Adjusted multiple regression analysis confirmed R^2 = 0.1193 (Table S1).

DISCUSSION

Prenatal exposure to high-energy diets primes behavioral alterations in the offspring at early stages of life. Neurobiological traits coding for aberrant behaviors

Table 1. Volumetric changes in brain regions comparing CON vs CAF

Region	X	Y	Z	t-value
Primary somatosensory cortex	1.769999839	−2.709999703	2.120000049	10.92
Fimbria-fornix	−0.430000111	−2.949999698	−0.1599999	−8.86

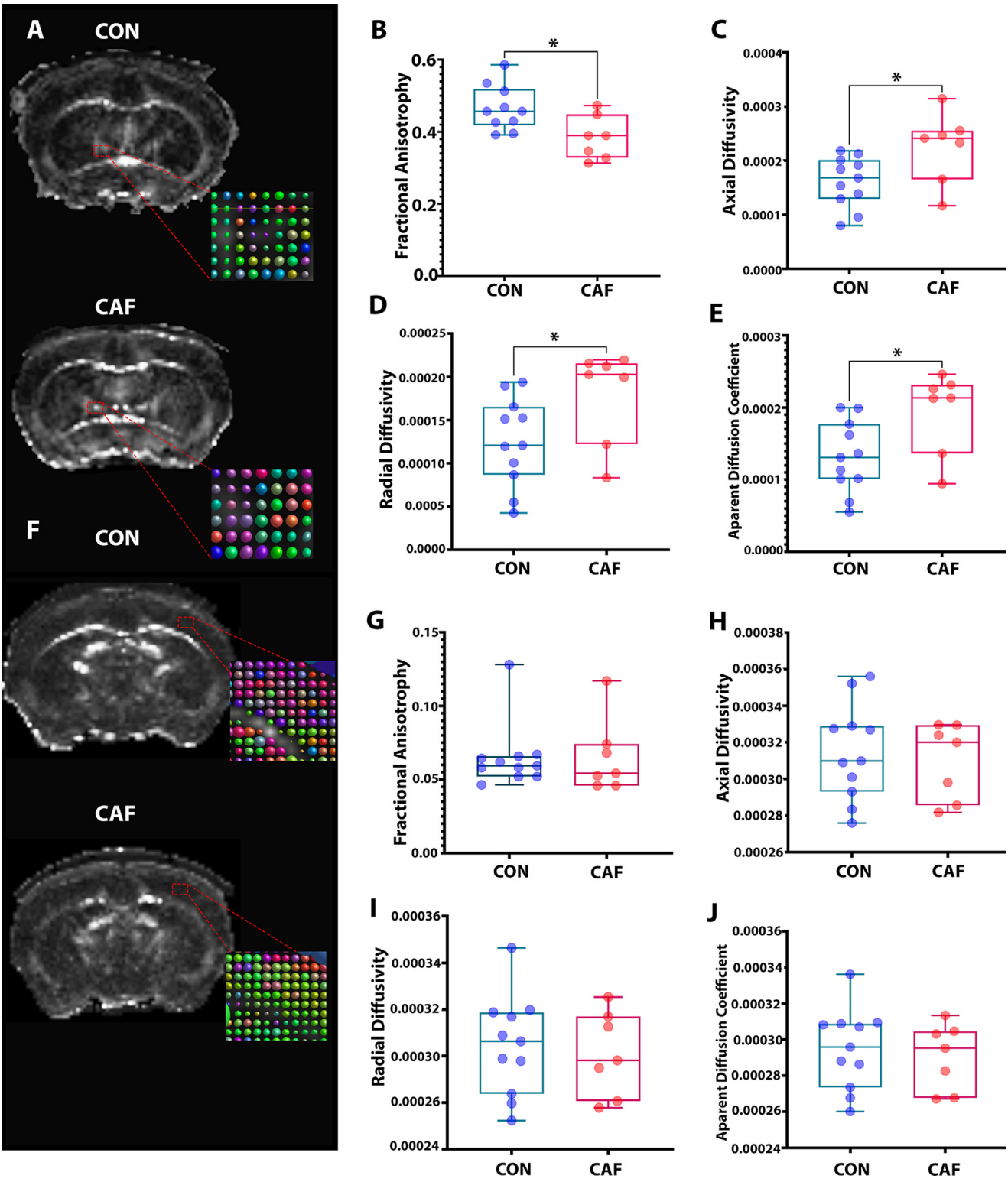

Fig. 4. Prenatal exposure to high-energy-diets promoted diffusion brain changes in the offspring. Female C57BL6 were fed with chow (CON) or CAF diets as described in Fig. 1, and offspring were analyzed using MRI analysis. **(A, F)** RGB-FA maps of CAF (bottom left) and Control offspring (top left). FA maps are based on the fiber direction (blue, caudal-rostral; red, left–right; and green, dorsal ventral). Image zoomed into the fimbria-fornix ROI shows the diffusion tensor ellipses. **(B–E)** Boxplots show the comparison of fractional anisotropy, radial diffusivity, apparent diffusion coefficient, apparent diffusion coefficient values in the fimbria-fornix. Data are expressed as mean ± SEM followed by *t*-test. **p* = 0.03, ***p* = 0.002, ****p* = 0.0002. CAF (*n* = 7) CON (*n* = 11). **(G–J)** Boxplots show the comparison of fractional anisotropy, axial diffusivity, radial diffusivity, apparent diffusion coefficient values in the primary somatosensory cortex. Data are expressed as mean ± SEM and statistical differences were depicted followed "*t*" test analysis. **p* = 0.03. Abbreviations: CAF: cafeteria; CON: Control; FA: fractional anisotropy; ROI: region of interest analyses; RGB-FA: Red green blue of fractional anisotropy.

Table 2. Diffusion tensor parameters of regions of interest

Structure	CON	CAF	*p* value
Corpus callosum			
FA	0.491	0.4603	0.5032
ADC	0.000106	0.000117	0.5484
AD	0.000157	0.0002	**0.0113***
RD	8.32E-05	9.24E-05	0.5608
Internal Capsule Right			
FA	0.4749	0.4735	0.9705
ADC	0.000136	0.00014	0.8296
AD	0.000197	0.00021	0.593
RD	0.000108	0.000107	0.9632
Internal Capsule Left			
FA	0.4276	0.4088	0.4901
ADC	0.000147	0.000151	0.8376
AD	0.00019	0.000212	0.4083
RD	0.000115	0.000122	0.6332
Fimbria Right			
FA	0.4658	0.3842	**0.0166***
ADC	0.000129	0.000144	0.1778
AD	0.000178	0.000198	0.1145
RD	0.000105	0.000118	0.2312
Fimbria Left			
FA	0.3969	0.4034	0.886
ADC	0.000155	0.00015	0.7221
AD	0.000215	0.000212	0.8649
RD	0.000131	0.000121	0.5036
Fornix body			
FA	0.219	0.2438	0.5323
ADC	0.000137	0.000154	0.2585
AD	0.000161	0.000182	0.2314
RD	0.000125	0.00014	0.3204

during development followed an exposure to caloric food formulas are not completely understood. In this study, we characterized diet-dependent brain volume and diffusion alterations using DBM and dMRI analysis, predicting cognitive behavior in the offspring at early stages of life. We newly identified that offspring exposed to CAF diet during the prenatal stage promoted a volume decrease in the fimbria-fornix region and in the fractional anisotropy, whereas it increased the axial diffusivity, radial diffusivity, and apparent diffusion coefficient values. Biological modeling identified that a decrease in volume in the fimbria-fornix and an increase in the apparent diffusion coefficient predicts time spent in the edges as an anxiety trait in the offspring exposed to CAF diet during prenatal programming. Our data proposes that prenatal exposure to high-energy diets primes selective volumetric changes in the fimbria-fornix circuit that integrate white matter alterations resembling an anxiety-like phenotype in the offspring.

Prenatal programming is a susceptible neurodevelopmental stage where external triggers might modulate refinement and establishment of brain circuits, coding for positive or negative outcomes after birth. We and others have reported that exposure to high-energy diets or ‘‘western diet formulas”, such as CAF diet, favors aberrant behaviors in the offspring including depression-like (de la Garza et al., 2019; Trujillo-Villarreal et al., 2021), addiction-like (Camacho, 2017; Cruz-Carrillo et al., 2020) and asocial-like behaviors (Maldonado-Ruiz et al., 2022). Here, we reported that prenatal exposure to CAF diet impaired spatial memory and learning performance during the long-term schedule (day 12) and no changes in performance of forced and spontaneous alternation or NORT in the offspring. Previous reports documented that prenatal exposure to CAF diet impaired learning and memory performance during puberty (30 days), which disappears during adulthood (Mucellini et al., 2019). However, two reports documented that prenatal exposure to 45% kcal fat diet from gestational day 14th to postnatal day 21st resulted in impairment in NORT by postnatal day 19–20 but not by 1–2 months of age in both sexes (Bengoetxea et al., 2017; Tsan et al., 2021). In fact, the detrimental effect of high-fat-diet exposure on memory acquisition was documented in murine models at 15–16 weeks old (Di Meco et al., 2021; Lin et al., 2021). Accordingly, our results agree with these findings confirming disruption in cognitive performance during the long-term schedule (day 12) in the offspring prenatally exposed to CAF diet, whereas they disagree with other studies. In fact, a recent report documented that a prenatal high-fat diet exposure to the 3xTg murine model of Alzheimer’s disease improved spatial learning and memory, and reverted synaptic dysfunction, Aβ, and tau neuropathology in the offspring (Di Meco et al., 2021). One potential explanation of this diversity of findings might be related to the behavioral test used by other researchers. For instance, to assess spatial memory performance the Morris water maze was used (Pistell et al., 2010; Gerstein et al., 2013; Lin et al., 2021; Page et al., 2014; Robb et al., 2017) whereas we performed our analysis using the Barnes maze. We conceive that in comparison to the Barnes maze, the Morris water maze exposes the subject to a major ‘‘stressful” scenario determined by water, where the subject must push itself for escape. A second potential variable of data discordance might be related to diet formula and time of exposure given that some authors used a 45% kcal fat diet (Bengoetxea et al., 2017; Tsan et al., 2021), or a high-fat-diet formula (Di Meco et al., 2021; Lin et al., 2021), compared with our CAF formula made of high-fat-high-sugar ingredients. Accordingly, diet formulas provide 60% or 42% calories from fat, in contrast to 49% calories from our own CAF diet formula (Di Meco et al., 2021; Lin et al., 2021). Finally, while still under investigation, authors also proposed that the timing of *in utero* exposure to high-fat-diets is critical for offspring cognitive performance later in adulthood.

One of the major contributions in the current study is that prenatal exposure to CAF diet is associated to lower volume in the fimbria-fornix and an increase in brain volume was detected in the primary somatosensory cortex of offspring. However, additional brain regions are structurally disrupted according to the jacobian values extracted from significant peaks in brain and we compared them between the two groups using a general lineal model and analyses were corrected for multiple comparisons using the false-discovery rate (FDR) at 10%, so these brain regions did not survive the correction. The fornix-fimbria modulates emotions linked to spatial and episodic long-term memory (Benear

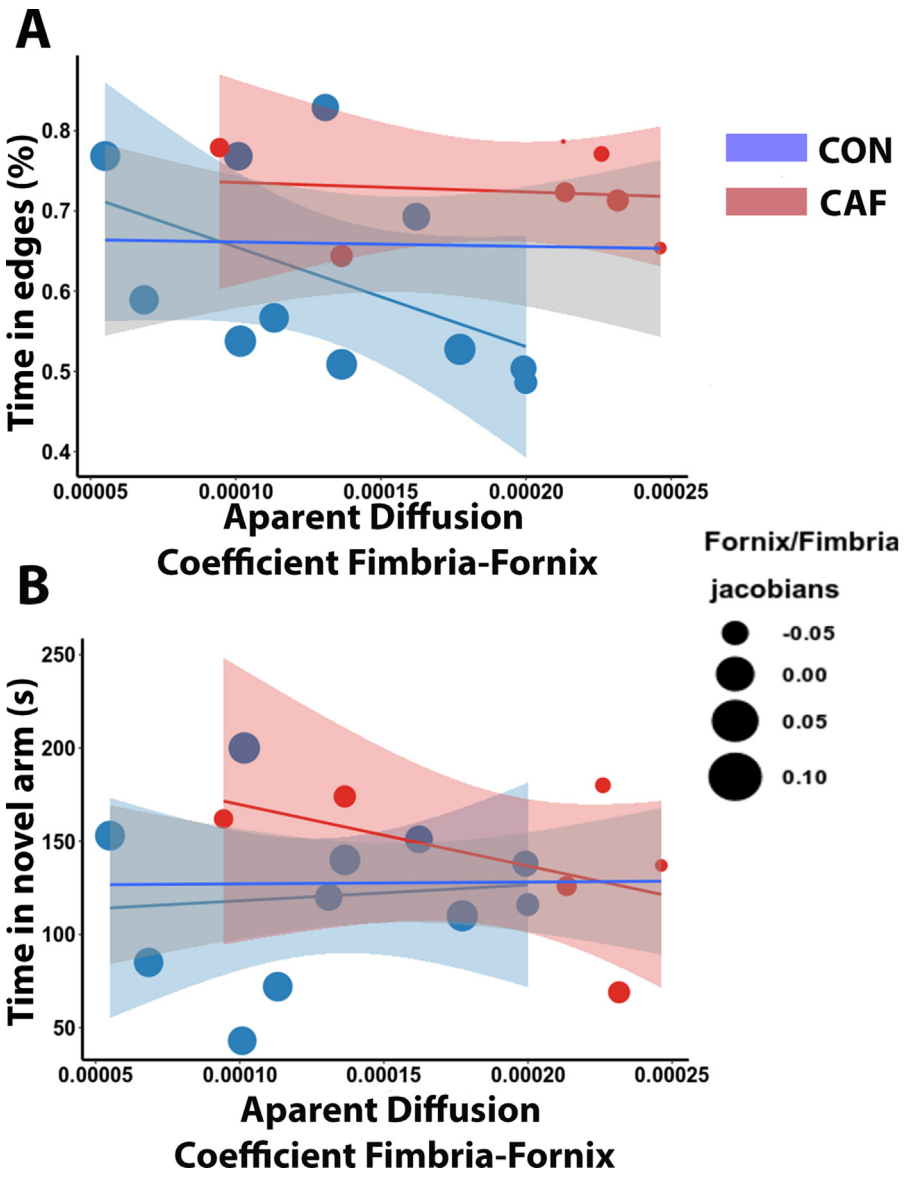

Fig. 5. Changes in diffusion metrics in the fimbria-fornix followed CAF diet exposure predict anxiety in the offspring. (A) Forced alternation time spent in the novel arm in the Y-maze of the CAF and control groups according to the volume decrease in fimbria-fornix and the apparent diffusion coefficient in the fimbria-fornix. **(B)** Time spent in edges expressed as anxiety in open field test of the CAF and control groups according to the volume decrease in fimbria-fornix and the apparent diffusion coefficient in the fimbria-fornix.

et al., 2020; Rootman et al., 2022). Structural defects in selective brain regions after exposure to high-energy diets are a hallmark of neuropsychiatric disorders (Kalyan-Masih et al., 2016). In fact, we previously reported that prenatal exposure to CAF diet promoted reduction in the volume of the nucleus accumbens, hippocampus, and prefrontal cortex (Trujillo-Villarreal et al., 2021). Also, volume decrease was documented in the medial amygdala and the basal forebrain of offspring exposed to high-fat diet during the prenatal stage, which are preserved even if the offspring is shifted to a low-fat diet at weaning (Fernandes et al., 2021), which agrees with our results. In fact, prenatal exposure to aberrant stimulus such as alcohol or infections promoted lower volumes in the hippocampus and defective memory function (Roediger et al., 2021; Guma et al., 2022) and a decrease in fornix volume was found in subjects with Alzheimer's disease (Benear et al., 2020). Accordingly, an increase in brain volume was detected in the primary somatosensory cortex of offspring subjects exposed to CAF diet during maternal programming. Primary somatosensory cortex receives sensory innervation from the hippocampus and amygdala, which by itself, facilitates higher-order processing and problem-solving by integrating previously stored experience, and respond to them by integrating other brain regions ("Medical Gallery of Blausen Medical 2014," 2014). An increased in gray matter volume in the primary somatosensory cortex has been found in Autism spectrum according to the disease severity (Wang et al., 2017). However, no extensive data has been documented regarding volumetric changes in the primary somatosensory cortex dictated by overnutrition during prenatal stages and its effects on behavioral alteration. Together, this evidence suggests that prenatal programming by high-energy diets primes volume brain changes in the fimbria-fornix and primary somatosensory cortex of offspring at an early stage of life.

While still under investigation, recent reports have documented potential traits of maternal programming on brain structure and behavior in the offspring after birth. Two recent studies documented the effect of maternal undernutrition (low protein diet) on brain structure and connectivity in the offspring of murine models (Barbeito-Andrés et al., 2018, 2019). Authors reported volume decrease in cerebellum, hippocampus and thalamus in the offspring exposed to low protein diet during prenatal stage (Barbeito-Andrés et al., 2019). Notably, the growth of central brain regions and long integrative myelinated tracts were relatively preserved, while the frequency of short tracts was relatively reduced in central brain regions such as the temporo-parietal cortex, confirming that maternal undernutrition produces long-term changes in brain structural connectivity in the offspring (Barbeito-Andrés et al., 2018). Also, behavioral alterations in the offspring have been reproduced by prenatal exposure to deleterious stimuli. For instance, a recent

report confirmed that perinatal fentanyl exposure in mice impaired the function of primary somatosensory cortex at adolescence (Alipio et al., 2021). Accordingly, maternal exposure to busulfan (an anticarcinogenic reagent), or poly I:C (a double-stranded RNA molecule activator of the Toll-like receptor 3), reduce the cell number in the somatosensory cortex leading to behavioral abnormalities (Shin Yim et al., 2017; Gouveia et al., 2020). While the authors did not describe volumetric brain changes in the somatosensory cortex followed maternal exposure to fentanyl, busulfan or poly I:C, it is tentative to speculate that morphological brain alterations reported by nutritional stress might resemble macrostructural alterations in the somatosensory cortex found in our model of maternal CAF diet exposure.

We enriched the analysis of the fornix-fimbria in the offspring prenatally exposed to CAF diet by using diffusion tensor magnetic resonance imaging (DTI). The DTI is an MRI-based analysis that quantifies the restriction of Brownian motion of water molecules in brain tissue, providing microstructural analysis of the white matter (Barry et al., 2021). The DTI provides an indirect measure of tissue characteristics according to their metrics; for instance, a DTI parallel diffusion is sensitive to axonal integrity, while a DTI perpendicular diffusion shows (but not specific) a myelin alteration and decreased axonal density. We found that CAF offspring had lower fractional anisotropy, and a higher axial diffusivity, radial diffusivity and apparent diffusion coefficient. Accordingly, quantitative fiber tracking revealed inverse correlations between fractional anisotropy and working memory and problem solving in healthy human subjects (Thomas et al., 2011), and major fimbria-fornix volume and fractional anisotropy value predicts better spatial memory (Dahmani et al., 2020) and adult memory performance (Benear et al., 2020), which both support our major findings. Notably, in a recent report using diffusion MRI and spherical deconvolution tractography, authors associated changes in fornix microstructure metrics to novelty seeking and curiosity qualities (Valji et al., 2019). This result supports our major finding, suggesting that offspring prenatally exposed to CAF diet displayed different behavior to novel places during the testing.

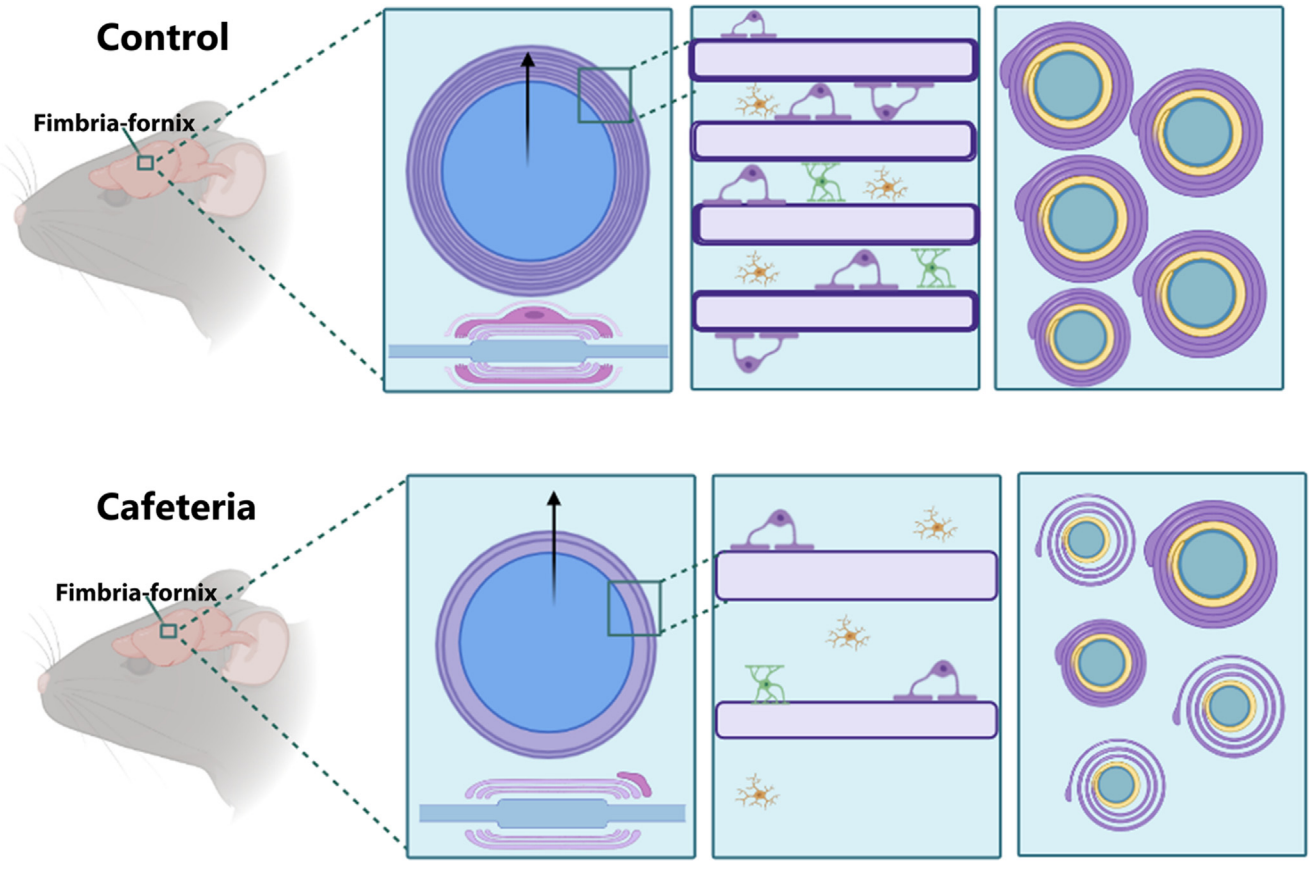

Fig. 6. Theoretical model summarizing current findings in the offspring prenatally exposed to CAF vs control diets. In control offspring, the axons are surrounded by myelin sheaths and tightly arranged, restricting the diffusion of water Conversely, we propose that according to higher metrics of axial diffusivity, radial diffusivity and apparent diffusion coefficient values resembling degradation of physiological barriers to the diffusion of water diffusion and an increase of diffusivity in all directions in the CAF offspring. These metrics might potentially suggest distortion of the axonal architecture and demyelination. Water diffusion along the perpendicular direction rises as myelin is disrupted, resulting in increased radial diffusivity. Axial diffusivity is thought to be increased by cellular infiltration, and changes in extracellular water. Finally, CAF offspring present also lower volume in fimbria-fornix could indicate less cellularity. As commented at discussion, some potentials limitations are still to be assessed to totally confirm our tentative model. For instance, a multi-compartment T2 or magnetization transfer must be integrated to precisely solve the DTI ambiguities and to directly assess myelin conformation, which would be of interest to a future line of research.

Finally, we performed a biological multiple regression model to predict the effect of prenatal diet exposure on changes in brain structure favoring cognitive failure in the offspring. Previous reports documented that offspring from dams fed CAF diet developed anxiety (Ramírez-López et al., 2016; Ribeiro et al., 2018). Our model predicts that a decrease in volume in the fimbria-fornix and an increase in its apparent diffusion coefficient associates with long time spent at the edges resembling an anxiety-like behavior in the offspring exposed to CAF diet during prenatal stage. The fimbria-fornix is the main axonal white matter tract that surrounds the hippocampus, and it is involved in the coordination of learning and memory response and emotions (Benear et al., 2020; Rootman et al., 2022) and volume changes in the fornix have been described in humans showing anxiety. A hippocampal atrophy it is expected to be associated to less hippocampal output in the fimbria, causing a smaller volume of it. For instance, a human DTI study found a positive correlation between trait anxiety scores and fractional anisotropy values in the fornix (Modi et al., 2013). Notably, anxiety-related traits were found in several brain pathologies such as anorexia nervosa or Huntington's disease which correlated with either a low fractional anisotropy values, a high mean diffusion, or decreased fornix volume in the fimbria-fornix (Kazlouski et al., 2011; Frank et al., 2013; Martin Monzon et al., 2016; Gabery et al., 2021). Notably, some reports also documented microstructural alterations related to high-fat diet (60% calories from fat) exposure, such as impaired neurogenesis in the hippocampus (Niculescu & Lupu, 2009) or reduced myelination in the medial cortex (Graf et al., 2016). Accordingly, this evidence suggests that prenatal exposure to high-energy diets promotes defective white matter integrity in the fornix predicting an anxiety-like behavior in the offspring.

We propose a tentative model where prenatal exposure to CAF diet primes selective changes in volume and diffusional parameters in the fimbria-fornix. Precisely, based on the dMRI data combining to lower volume values obtained by the DMB analysis, we suggest that the fimbria-fornix displays less axonal integrity, demyelination and poorer fiber organization along with less cellularity (Fig. 6). This demyelination and aberrant organization may lead to anxiety-like behavior in the offspring. Evidence identified an age-dependent decline in fimbria-fornix integrity by quantitative fiber tracking, where both increased axonal diffusion and radial diffusion coefficients were observed (Zahr et al., 2009; Thomas et al., 2011). Some potentials limitations are still to be assessed to totally confirm our tentative model. For instance, a multi-compartment T2 or magnetization transfer must be integrated to precisely solve the DTI ambiguities and to directly assess myelin conformation, which would be of interest to a future line of research.

In summary, prenatal exposure to CAF diet primes volume, structural and diffusional anomalies in the offspring, resulting in long time spent in the edges of the arena resembling an anxiety-like behavior phenotype. We propose that structural defects of the fimbria-fornix might be a major predictor to anxiety in subjects prenatally exposed to CAF diet. Our study highlights the fimbria-fornix circuitry as an underlying node affected by diet during the prenatal window, which might increase the risk for developing aberrant behaviors at early stages of life.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript and/or the supplementary files.

For more information or raw data access please contact at: acm590@hotmail.com or gabriela.cycr@gmail.com.

ETHICS STATEMENT

All the experiments were performed using two-month-old, wild-type male C57bl6 mice. Animals were handled according to the NIH guide for the care and use of laboratory animals (NIH Publications No. 80–23, revised in 1996). We followed the Basel Declaration to implement the ethical principles of Replacement, Reduction and Refinement of experimental animal models. Our study was approved by the local Animal Care Committee (BI20-00006) at the Universidad Autónoma de Nuevo León, México.

AUTHOR CONTRIBUTIONS

GCC, LATV, LC, EAGV and ACM conceived and designed the study. GCC, LATV, LC and EAGV the in vivo experiments. GCC, LATV, LC, EAGV and ACM discussed and wrote the paper. All authors read and approved the final manuscript. Dr. Alberto Camacho-Morales is the guarantor of this work.

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APPENDIX A. SUPPLEMENTARY MATERIAL

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