Choline supplementation in early life improves and low levels of choline can impair outcomes in a mouse model of Alzheimer's disease.

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ABSTRACT

Maternal choline supplementation (MCS) improves cognition in Alzheimer's disease (AD) models. However, effects of MCS on neuronal hyperexcitability in AD are unknown. We investigated effects of MCS in a well-established mouse model of AD with hyperexcitability, the Tg2576 mouse. The most common type of hyperexcitability in Tg2576 mice, and many other mouse models and AD patients, are generalized EEG spikes (interictal spikes; IIS). Hyperexcitability is also reflected by elevated expression of the transcription factor ΔFosB in the granule cells (GCs) of the dentate gyrus (DG), which are the principal cell type. We also studied the hilus of the DG because hilar neurons regulate GC

excitability. We found reduced expression of the neuronal marker NeuN within hilar neurons in Tg2576 mice, which other studies have shown is a sign of oxidative stress or other pathology.

Tg2576 breeding pairs received a diet with a relatively low, intermediate or high concentration of choline. After weaning, all mice received the intermediate diet. In offspring of mice fed the high choline diet, IIS frequency declined, GC ΔFosB expression was reduced, and NeuN expression was restored. Spatial memory improved using the novel object location task. In contrast, offspring exposed to the relatively low choline diet had several adverse effects, such as increased mortality. They had the weakest hilar NeuN immunoreactivity and greatest GC ΔFosB. However, their IIS frequency was low, which was surprising. The results provide new evidence that a diet high in choline in early life can improve outcomes in a mouse model of AD, and relatively low choline can have mixed effects. This is the first study showing that dietary choline can regulate hyperexcitability, hilar neurons, ΔFosB and spatial memory in an animal model of AD.

I. INTRODUCTION

Diet has been suggested to influence several aspects of brain health. One dietary intervention that has been studied extensively in rodents and humans is supplementation of the maternal diet with the nutrient choline (maternal choline supplementation; MCS). MCS improves many aspects of brain health in humans (Zeisel and da Costa 2009; Jiang et al. 2014). In normal rats, MCS also is beneficial, with numerous studies showing improved behavior in the adult offspring (for review see Meck and Williams 2003).

In Alzheimer's disease (AD), changes to the diet have been recommended (Bourre 2006; Power et al. 2019; Mao et al. 2021; Lobo et al. 2022), including MCS (Strupp et al. 2016; Velazquez et al. 2020; Dave et al. 2023; Judd et al. 2023a; b). One reason for the recommendation is that serum levels of choline are low in individuals with AD (Dave et al. 2023; Judd et al. 2023b). In addition, using mouse models of AD, increased choline improved many characteristics of the disease, ranging from inflammation to glucose metabolism and the hallmark amyloid and tau pathology (Dave et al. 2023; Judd et al. 2023a; b). In the Ts65Dn mouse model of Down syndrome (DS) and AD, MCS led to improved memory and attention in Ts65Dn offspring (Strupp et al. 2016; Powers et al. 2017; Powers et al. 2021). In addition, degeneration of basal forebrain cholinergic neurons (BFCNs) in Ts65Dn mice, a hallmark of DS and AD, was reduced (Kelley et al. 2016; Powers et al. 2017; Alldred et al. 2023; Gautier et al. 2023).

Here we asked if MCS would improve Tg2576 mice, a model of familial AD where a mutation in the precursor to amyloid β (Aβ), amyloid precursor protein (APP) is expressed by the hamster prion protein promoter (Hsiao et al. 1996). This is a commonly used mouse model that simulates aspects of AD.

One of the reasons to use Tg2576 mice was to ask if MCS would improve the hyperexcitability found in the mice. The Tg2576 mouse is ideal because hyperexcitability is robust, and BFCNs are likely to play a role in the hyperexcitabililty (Bezzina et al. 2015; Kam et al. 2016; Lisgaras and Scharfman 2023). In our past work, the primary type of hyperexcitability was interictal spikes (IIS), which are named because they occur in between seizures (ictal events) in epilepsy. They are studied by EEG. Importantly, IIS are found in numerous mouse models of AD (J20, Brown et al. 2018; Ts65Dn, PS2$^{-/-}$, Lisgaras and Scharfman 2023; APP/PS1, Shoob et al. 2023), as well as patients (Sanchez et al. 2012; Vossel et al. 2016; Beagle et al. 2017; Vossel et al. 2017; Vossel and Karageorgiou 2021; Vossel 2023).

We also studied hyperexcitability using ΔFosB expression, a transcription factor that is increased when neurons have been highly active over the prior 10-14 days (McClung et al. 2004). We studied ΔFosB in granule cells (GCs) of the dentate gyrus (DG), because numerous ΔFosB-expressing GCs occur when there are IIS (You et al. 2017; You et al. 2018). We also focused on GCs because we found that the cell layer of GCs (GCL) is where IIS are largest relative to area CA1 and overlying neocortex (Lisgaras and Scharfman 2023). Moreover, closed loop optogenetic silencing of GCs reduced IIS (Lisgaras and Scharfman 2023).

We also asked if behavior would improve if MCS reduced IIS frequency in Tg2576 mice. The basis for this question is in studies of epilepsy, where IIS disrupt cognition (Rausch et al. 1978; Aarts et al. 1984; Holmes and Lenck-Santini 2006; Kleen et al. 2010; Kleen et al. 2013; Gelinas et al. 2016) . Also, both IIS and an epileptiform discharge that is usually longer lasting, interictal epileptiform discharges (IEDs), are correlated with cognitive dysfunction in AD patients (Vossel et al. 2016).

We fed dams one of 3 diets, which were relatively low, intermediate or high in choline (Figure 1A; Supplemental Table 1). The high choline diet provided levels of choline similar to other studies of MCS in rodents (Meck et al. 1988; Loy et al. 1991; Holler et al. 1996; Meck and Williams 1999; Sandstrom et al. 2002; Mellott et al. 2004; Glenn et al. 2012; Kelley et al. 2019). After weaning the intermediate diet was used. Offspring were implanted with electrodes for EEG at 1 month of age, and 24 hrs-long recordings began 1/month starting at 1 week after surgery. After 6 months the recordings ended. The time span (1-6 months of age) was selected because this is when cognitive impairment and IIS first develop. Therefore we could determine if these characteristics were delayed.

We previously found that the novel object location (NOL) task is impaired at 3-4 months of age in Tg2576 mice (Duffy et al. 2015), so we evaluated NOL at 3 months, and a related task, novel object recognition (NOR; Vogel-Ciernia and Wood 2014). To understand the persistence of any effects of the diets, we repeated NOL and NOR at 6 months. The interval between testing was sufficiently long that it is unlikely that testing at 3 months affected testing at 6 months, but we can not exclude the possibility. At 6 months of age we perfusion-fixed mice, sectioned the brains, and evaluated NeuN and ΔFosB protein expression. NeuN was studied because it is a neuronal marker that is reduced in numerous pathological conditions (Buckingham et al. 2008; Kadriu et al. 2009; Matsuda et al. 2009; Duffy et al. 2011; Duffy et al. 2015). We also assayed ΔFosB because it is a method complementary to EEG recordings of hyperexcitabililty. It also can be used to assess hyperexcitability of GCs.

The results showed a remarkable effect of the high choline diet. IIS and spatial memory were improved, as was NeuN and ΔFosB expression. Interestingly, the relatively low choline diet had mixed effects, reducing IIS frequency, but making NOL, ΔFosB, and NeuN worse. The mice also died prematurely relative to mice that were fed the high choline diet. We also report for the first time that there is loss of NeuN-ir in the DG hilus of Tg2576 mice, which is important because impaired hilar neurons could cause GC hyperexcitability (Sperk et al. 2007; Scharfman and Myers 2012). In summary, we make a strong argument for choline supplementation in early life to improve outcomes in an AD model, especially the DG.

II. METHODS

A. Animals

All experimental procedures followed the guidelines set by the National Institute of Health (NIH) and were approved by the Institutional Animal Care and

Use Committee (IACUC) of the Nathan Kline Institute for Psychiatric Research. Mice were housed in standard mouse cages (26 cm wide x 40 cm long x 20 cm high) with corn cob bedding and a 12 hrs-long light-dark cycle. Food and water were provided *ad libitum*.

Tg2576 mice express a mutant form of human APP (isoform 695) with a mutation found in a Swedish cohort (Lys670Arg, Met671Leu), driven by the hamster prion protein promoter (Hsiao et al. 1996). Mice were bred in-house from heterozygous Tg2576 males and non-transgenic female mice (C57BL6/SJL F1 hybrid). Wildtype (WT) mice were obtained from Jackson Laboratories (Stock# 100012). Genotypes were determined by the New York University Mouse Genotyping Core facility using a protocol to detect APP695.

Breeding pairs were randomly assigned to receive one of 3 diets with different concentrations of choline chloride: 1.1 g/kg (AIN-76A, Dyets Inc.), 2.0 g/kg (Purina 5008, W.F. Fisher and Son Inc.) and 5.0 g/kg (AIN-76A, Dyets Inc.; Supplemental Table 1). These diets ("low choline" diet, "intermediate choline" diet and "high choline" diet, respectively) were used until weaning (25-30 days of age). After weaning, all mice were fed the intermediate diet. Mice were housed with others of the same sex (1-4 per cage).

B. Behavior

1. *General information*

Mice were housed in the room where they would be tested behaviorally starting at least 24 hrs prior to all experiments. Both NOL and NOR included 3 acclimations (5 min each) followed by a training and a testing session (5 min each; Figure 1B-D). The interval between training and testing was 60 minutes and therefore tested short-term memory (Vogel-Ciernia and Wood 2014). NOL and NOR tests were separated by 7 days to ensure effects of one task did not affect the next. Prior studies have failed to find an effect on one task on the next when a 7 day-long interval is used (Botterill et al. 2021). The order of testing (NOL before NOR or NOR before NOL) was randomized.

Video recordings of all training and testing sessions were captured using a USB camera (Logitech HD Pro C920, Logitech). Acclimations were conducted between 10:00 a.m. and 12:00 p.m., while training and testing was conducted between 1:00 p.m. and 4:00 p.m. All equipment was cleaned using 70% ethanol before each use. In pilot studies, we chose objects that would be explored equally by animals. Indeed, animals showed no preference for either of the objects (i.e., LEGO object and pineapple, Supplemental Figure 1) during training, as reported in the Results.

2. *NOL*

NOL was conducted in a standard rat cage (26 cm wide x 40 cm long x 20 cm high) with different pictures on 3 sides of the cage to provide a consistent context and therefore foster acclimation. Pictures included several shapes and colors. The dimensions of the pictures were 1) 10 cm x 21 cm, 2) 16 cm x 18 cm, and 3) 17 cm x 20 cm. During the acclimations, animals were allowed to freely explore the cage. During the training session, mice were placed in the cage

where they had been acclimated, with two identical objects, 1 in a corner of the cage (e.g., left top) and the second in the adjacent corner (e.g., left bottom, Supplemental Figure 1). The mice were then removed and placed in their home cage for 1 hr. During the test session, one of the objects was moved to the opposite end of the cage (left top to right top; Supplemental Figure 1). The two objects were identical pineapple-like objects made of metal that were painted and approximately 3 cm x 3 cm x 5 cm (Supplemental Figure 1; Botterill et al. 2021).

3. *NOR*

NOR was conducted in the cage described above for NOL. As for NOL, during the acclimations animals were allowed to freely explore the cage. During the training session, mice were placed in the cage used for acclimation with two identical objects centered along the shortest cage wall (Supplemental Figure 1). The mouse was then removed and placed in their home cage for 1 hr. During the testing session, one of the objects was replaced with a different one. The objects that were identical were 2 pineapple-like objects, and the new object was made of 2 green LEGO pieces approximately 5 cm x 7 cm x 7 cm (Supplemental Fig. 1; Botterill et al. 2021).

4. *Quantification*

The experimenter who conducted the analysis was blind to the genotype of the offspring and which object was novel or familiar. Videos of the training and testing sessions were analyzed manually. A subset of data was analyzed by two independent blinded investigators and they were in agreement. Exploration was quantified based on the guidelines of Vogel-Ciernia and Wood (2014). However, in addition to time spent exploring, the number of approaches to an object was quantified. Time spent exploring each object was defined as the duration of time the nose was pointed at the object and the nose was located within 2 cm of the object. In addition, exploration time included the time animals spent on top of the object if they were looking down and sniffing it. An approach was defined by a movement toward the object and ending at least 2 cm from the edge of the object.

Animals that remembered the objects that were explored during the training phase were expected to demonstrate an increased preference for novelty in the test phase (Ennaceur and Delacour 1988). In other words, exploration of the novel object during testing was expected to be higher than 50% of the total time of object exploration.

C. Anatomy

1. *Perfusion-fixation and sectioning*

Mice were initially anesthetized by isoflurane inhalation (NDC# 07-893-1389, Patterson Veterinary), followed by urethane (2.5 g/kg; i.p.; Cat# U2500, Sigma-Aldrich). Under deep anesthesia, the abdominal and heart cavities were opened with surgical scissors and a 23g needle inserted into the heart. The atria was clipped, the needle was clamped in place with a hemostat, and the animal was transcardially-perfused using 10 mL of room temperature (RT) saline (0.9%

sodium chloride in double distilled (dd) H_2O) using a peristaltic pump (Minipuls1; Gilson), followed by 30 mL of 4°C 4% paraformaldehyde (PFA, Cat# 19210, Electron Microscopy Sciences) in 0.1 M phosphate buffer (PB; pH 7.4). The brains were immediately extracted, hemisected, and post-fixed for at least 24 hrs in 4% PFA (at 4°C).

Following post-fixation, 50 µm-thick coronal sections were made using a vibratome (VT 1000P, Leica Biosystems). Sections were collected serially and stored at 4°C in 24-well tissue culture plates containing cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% 0.1 M phosphate buffer, pH 6.7). Throughout the hippocampus, every 6th section was processed with an antibody to NeuN or ΔFosB. For analysis of anterior DG, 2-3 sections that were 300 µm apart were selected from ~1.5-2.5 mm posterior to Bregma. For posterior DG, 2-3 sections that were 300 µm apart were selected starting at ~3.5 mm posterior to Bregma. The values of the 2-3 sections were averaged so that there was one measurement for anterior and one for posterior for each mouse.

2. *NeuN and ΔFosB immunohistochemistry*

Free floating sections were washed in 0.1 M Tris Buffer (TB; 3 x 5 min), followed by a 3 min wash in 1% H_2O_2. Sections were then washed in 0.1 M TB (3 x 5 min) and incubated for 60 min in 5% horse serum for NeuN (Cat# S-2000, Vector) or 5% goat serum for ΔFosB (Cat# S-1000, Vector), diluted in a solution of 0.25% (volume/volume or v/v) Triton-X 100, and 1% (weight/volume or w/v) bovine serum albumin in 0.1 M TB. Sections were then incubated overnight at 4°C in primary antibody to NeuN (mouse monoclonal, 1:5000; Cat# MAB377, Chemicon International) or anti-ΔFosB (rabbit monoclonal, 1:1000; Cat# D3S8R, Cell Signaling), diluted in a solution of 0.25% (v/v) Triton-X 100, and 1% (w/v) bovine serum albumin in 0.1M TB. Both NeuN and ΔFosB have been well-characterized as antigens (Mullen et al. 1992; Wolf et al. 1996; Chen et al. 1997; Sarnat et al. 1998) and the antibodies we used have been commonly employed in the past (Chen et al. 1997; Duffy et al. 2013; Corbett et al. 2017). On the following day, sections were washed in 0.1 M TB (3 x 5 min) and then incubated for 60 min in biotinylated horse anti-mouse secondary antibody for NeuN (1:500, Cat# BA-2000, Vector) or biotinylated goat anti-rabbit secondary antibody for ΔFosB (1:500, Cat# BA-1000), diluted in a solution of 0.25% (v/v) Triton-X 100, and 1% (w/v) bovine serum albumin in 0.1 M TB. The sections were then washed in 0.1 M TB (3 x 5 min) and incubated in avidin-biotin complex for 2 hrs (1:1000; Cat# PK-6100, Vector). They were washed in 0.1 M TB (3 x 5 min) and then reacted in a solution containing 0.5 mg/mL 3, 3'-diaminobenzidine (DAB; Cat# D5905, Sigma-Aldrich), 40 µg/mL ammonium chloride (Cat# A4514, Sigma-Aldrich), 25 mg/mL D(+)-glucose (Cat# G5767, Sigma-Aldrich), and 3 g/mL glucose oxidase (Cat# G2133, Sigma-Aldrich) in 0.1 M TB. This method slowed the reaction time so that the reaction could be temporally controlled, meaning that the reaction could be stopped when the immunoreactivity was robust but background was still low. The sections were then washed in 0.1 M TB (3 x 5 min), mounted on gelatin-coated slides (1% bovine gelatin, Cat# G9391, Sigma-Aldrich) and dried at RT overnight. The following day they were dehydrated in

increasing concentrations of ethanol (90%, 10 min; 95% 10 min; 100%, 10 min; 100% again, 10 min), washed in Xylene (10 min; Cat# 534056, Sigma-Aldrich) and cover-slipped with Permount (Cat# 17986-01, Electron Microscopy Sciences).

3. *Analysis*

Photomicrographs were acquired using ImagePro Plus V7.0 (Media Cybernetics) and a digital camera (Model RET 2000R-F-CLR-12, Q-Imaging). NeuN and ΔFosB staining were quantified from micrographs using ImageJ (V1.44, National Institutes of Health). All images were first converted to grayscale and in each section, the hilus was traced, defined by zone 4 of Amaral (1978). A threshold was then calculated to identify the NeuN-stained cell bodies but not background. Then NeuN-stained cell bodies in the hilus were quantified manually. Note that the threshold was defined in ImageJ using the distribution of intensities in the micrograph. A threshold was then set using a slider in the histogram provided by Image J. The slider was pushed from the low level of staining (similar to background) to the location where staining intensity made a sharp rise, reflecting stained cells. Cells with labeling that was above threshold were counted.

To quantify ΔFosB-stained cells, images were converted to grayscale and in each section, the GCL was outlined and defined as a region of interest (ROI). A threshold was then set to identify the well-stained cells but not the background, analogous to the method to define threshold in the for NeuN. Two thresholds were used because some cells were lightly stained and others much more robust in their staining. The first threshold was most inclusive of immunoreactive cells. Presumably this group reflected cells with less neuronal activity as well as those with intense activity. Then a second analysis was done with the same sections using a higher threshold. This group corresponded to cells with the highest activity. ImageJ was used to calculate the area (in pixels) within the ROI that was above threshold.

D. Video-electroencephalographic (video-EEG) recordings

1. *Stereotaxic surgery*

EEG electrodes (stainless steel jeweler's screws) were implanted in Tg2576 animals at 4 weeks of age. WT littermates were not implanted because previous work from our laboratory has shown that WT mice have no IIS (Kam et al. 2016). The animals were anesthetized by isoflurane inhalation (3% isoflurane. 2% oxygen for induction) in a rectangular transparent plexiglas chamber (18 cm long x 10 cm wide x 8 cm high) made in-house. For maintenance during surgery, isoflurane was $\leq$ 1.75% and flow rate was 1.2 L/min. Mice were placed in a stereotaxic apparatus (David Kopf Instruments). Prior to the implantation of the electrodes, animals were injected with the analgesic Buprenex (0.2 mg/kg, s.c.; buprenorphine hydroxide, NDC# 12496-0757-5, Reckitt Benckiser), which was diluted in saline (0.03 mg/mL in sterile 0.9% sodium chloride solution, Vedco Inc.). The skull was exposed with a midline incision and 6 holes were drilled for

the placement of subdural screw electrodes (Cat# 8209, 0.10” stainless steel screws, Pinnacle Technology).

The coordinates for the electrode placement were: right occipital cortex (AP -3.5 mm, ML 2.0 mm), left frontal cortex (AP -0.5 mm, ML -1.5 mm), left hippocampus (AP -2.5 mm, ML -2.0 mm), and right hippocampus (AP -2.5 mm, ML 2.0 mm (Kam et al. 2016). Two additional screws were placed, one over the right olfactory bulb as ground (AP 2.3 mm, ML 1.8 mm) and another at the midline over the cerebellum as a reference (relative to Lambda: AP -1.5 mm, ML -0.5 mm). Screws were attached to an 8-pin connector (Cat# ED85100-ND, Digi-Key Corporation), which was placed over the skull and secured with dental cement (Cat# 4734FIB, Lang Dental Mfg. Co., Inc.).

After surgery, animals were placed on a heating blanket overnight and injected with lactated Ringer’s solution (50 mL/kg at 31°C; NDC# 099355000476, Aspen Veterinary Resources Ltd). They were then transferred to the room where the video-EEG is recorded. Implanted animals were allowed to recover for one week following the surgery.

2. *Video-EEG recording*

Video-EEG was recorded stating at 1.25 months (5 weeks) of age. The animals were then recorded at 2, 3, 4, 5 and 6 months of age. Each recording session lasted 24 hrs so that a long period of sleep could be acquired, since IIS occur primarily in sleep (Kam et al. 2016).

Mice were placed into 21 cm x 19 cm transparent cages with food and water provided *ad libitum* and corncob bedding. A pre-amplifier was inserted into the 8-pin connector on the skull, which was connected to a multichannel commutator (Cat# 8408, Pinnacle Technology). This arrangement allowed for free range of movement throughout the recording. EEG signals were acquired at 2000 Hz and bandpass filtered at 0.5 – 200 Hz using Sirenia Acquisition)V2.0.4, Pinnacle Technology). Simultaneous video recordings were captured using an infrared camera (Cat# AP-DCS100W, Apex CCTV).

3. *Analysis*

EEG recordings were analyzed offline with Neuroscore V3.2.1 (Data Science International). IIS were defined as large amplitude, 10-75 msec deflections occurring synchronously in all 4 leads (Kam et al. 2016). They were quantified by first setting an amplitude threshold (calculated separately for each recording) and a duration criterion (10-75 msec). To determine the amplitude threshold, we calculated the root mean square (RMS) amplitude for noise in each recording during a 60 sec segment of baseline activity that did not include any artifacts. The threshold for IIS was set at 9 standard deviations above the RMS amplitude. This threshold was selected because it identified IIS extremely well and excluded movement or other artifacts. The IIS in one of the hippocampal channels was used because of data suggesting IIS begin in the hippocampus (Kam et al. 2016; Lisgaras and Scharfman 2023). Following the automatic analysis of the IIS, the software’s detection accuracy was verified manually for each recording to ensure

that all spikes appeared in all 4 channels and that no artifacts were included in the final IIS count.

E. Statistical comparisons

Data are expressed as mean ± standard error of the mean (SEM). The significant was set to <0.05 prior to all experiments. Tests were conducted using Prism (V 9.4.1, Graphpad Software).

Parametric data comparing two groups used unpaired two-tailed t-tests. For >2 groups, one-way ANOVA was used followed by Tukey-Kramer post-hoc tests that corrected for multiple comparisons. For data with two main factors, two-way ANOVA was followed by Tukey-Kramer post-hoc tests. Interactions are not reported in the Results unless they were significant. For the analysis of IIS frequency from 1.2 months of age up to 6 months-of age, a repeated measures ANOVA (RMANOVA) was used.

Tests for normality (Shapiro-Wilk) and homogeneity of variance (Bartlett's test) were used to determine if parametric statistics could be used. When data were not normal, non-parametric data were used. When there was significant heteroscedasticity of variance, data were log transformed. If log transformation did not resolve the heteroscedasticity, non-parametric statistics were used. For non-parametric data, Mann-Whitney *U* tests were used to compare two groups and Kruskal-Wallis for >2 groups. The post-hoc tests were Dunn's test for multiple comparisons.

For correlations, Pearson's r was calculated. To compare survival curves, a Log rank (Mantel-Cox) test was performed.

III. RESULTS

A. Approach

As shown in Figure 1A, animals were implanted with electrodes at 1 month of age and recorded for a continuous 24 hrs-long period at 1.2, 2, 3, 4, 5,and 6 months of age. At 3 and 6 months of age, mice were tested for NOL and NOR. Afterwards they were perfused and immunocytochemistry was conducted for NeuN and ΔFosB. Sections were made in the coronal plane and relatively anterior and posterior levels were compared to sample different parts of hippocampus. Some mice were not possible to include in all assays either because they died before reaching 6 months or for other reasons.

B. Behavior

NOL and the NOR were selected because they show deficits in Tg2576 mice at just 3 months of age (Duffy et al. 2015). This is before deficits have been shown in other tasks in >9 months-old Tg2576 mice (i.e., Morris Water Maze, Radial Arm Water Maze, Y-Maze; Yassine et al. 2013; Wolf et al. 2016) . Figure 1B-D shows a schematic of the specific experimental procedures for NOL and NOR.

1. *NOL*

The results of the NOL task are presented in Figures 2-4. In Figure 2 the diets, age, sex, and individual animals are presented. Lines connect the behavior of a given animal in training with the behavior during testing. Behavior is measured as the time spent exploring the object that is moved during the testing period. Exploration of the novel object is expressed as a percentage of the total exploration of both objects. When the slope of the line increases between training and testing, the time spent exploring the moved object increased in testing relative to training. This increase reflects a preference to explore the moved object during the testing period, and suggests the mouse can recall the old object locations and can recognize the new object location. Supplemental Figure 2 shows the data with mean ± sem.

An important foundation for this task is equal preference for the objects during training. The lack of preference reflects no inherent bias for one object vs. the other. We confirmed the lack of bias for each genotype and each diet. Thus, a two-way ANOVA with genotype or age (3 or 6 months) revealed no effect of genotype on object exploration during training ($F(1,97)=0.40$, $p=0.530$).There also was no effect of age ($F(1,70)=1.94$, $p=0.167$). When the factors were diet and age there also was no effect of diet ($F(2,97)=0.06$, $p=0.941$) or age ($F(2,70)=2.94$, $p=0.058$) on object exploration during training.

Next, we asked whether Tg2576 mice were deficient in the test phase of NOL when they were fed the standard mouse diet (the intermediate diet). First we studied 3 months-old mice (Figure 2A). We used a two-way ANOVA with genotype and task phase (training versus testing) as main factors. In animals that received the intermediate diet, a two-way ANOVA revealed a significant main effect of the task phase ($F(1,48)=8.51$, $p=0.005$) and a trend for a genotype effect ($F(1,48)=3.80$, $p=0.057$). Consistent with previous studies (Duffy et al. 2015), Tukey-Kramer post-hoc analyses revealed a significant increase in the exploration of the novel object during testing in WT ($p=0.017$) but not Tg2576 mice ($p=0.676$; Figure 2A). Thus, Tg2576 mice were impaired in NOL when fed the intermediate diet.

In contrast to Tg2576 mice fed the intermediate diet, Tg2576 mice that received the high choline diet showed memory for object location. Thus, a two-way ANOVA revealed a main effect of the task phase ($F(1,72)=46.16$, $p<0.0001$) but not genotype ($F(1,72)=1.74$, $p=0.191$). Tukey-Kramer post-hoc analyses revealed a significant increase in the exploration of the novel object during testing in both WT ($p=0.0003$) and Tg2576 mice ($p<0.0001$; Figure 2A3). Therefore, high choline supplementation improved memory in Tg2576 mice.

In animals treated with the low choline diet, there was no effect of the phase of the task ($F(1,74)=1.41$, $p=0.250$) or genotype ($F(1,74)=0.027$, $p=0.871$). These data suggest that relatively low levels of choline during early life impaired spatial memory in both WT and Tg2576 mice. Thus, low choline had a significant adverse effect. Adverse effects are further supported by survival plots showing that there was more mortality at earlier ages in offspring exposed to the low choline diet (Supplemental Figure 1).

Figure 2B shows the results in 6 months-old WT and Tg2576 mice. In animals that received the intermediate diet, a two-way ANOVA showed no effect of genotype ($F(1,36)=0.01$, $p=0.907$) or task phase ($F(1,36)=2.36$, $p=0.133$), revealing memory deficits in WT and Tg2576 mice (Figure 2B). In contrast, the high choline group showed a main effect of task phase ($F(1,56)=22.18$, $p<0.0001$) but not genotype ($F(1,56)=1.78$, $p=0.188$). Tukey-Kramer post-hoc analyses showed a significant increase in the exploration of the novel object during testing in both WT ($p<0.001$) and Tg2576 ($p=0.020$; Figure 2B). Thus, the high choline-treated mice showed object location memory but the mice fed the intermediate diet did not. The mice that received the relatively low choline diet showed an effect of genotype ($F(1,50)=4.36$, $p=0.042$; Figure 2B) in that training in WT mice differed from testing in Tg2576 mice ($p=0.031$). However, there was no main effect of task phase ($F(1,50)=3.75$, $p=0.058$). WT mice did not differ between training and testing ($p=0.114$) and Tg2576 mice did not either ($p=0.921$). Therefore, mice fed the low choline and intermediate diets were impaired and the high choline-treated mice were not.

The discrimination indices are shown in Figure 3 and results led to the same conclusions as the analyses in Figure 2. For the 3 months-old mice (Figure 3A), the low choline group did not show the ability to perform the task for WT or Tg2576 mice. Thus, a two-way ANOVA showed no effect of genotype ($F(1,74)=0.027$, $p=0.870$) or task phase ($F(1,74)=1.41$, $p=0.239$). For the intermediate diet-treated mice, there was no effect of genotype ($F(1,50)=0.3.52$, $p=0.067$) but there was an effect of task phase ($F(1,50)=8.33$, $p=0.006$). WT mice showed a greater discrimination index during testing relative to training ($p=0.019$) but Tg2576 mice did not ($p=0.664$). Therefore, Tg2576 mice fed the intermediate diet were impaired. In contrast, high choline-treated mice performed well. There was a main effect of task phase ($F(1,68)=39.61$, $p=<0.001$) with WT ($p<0.0001$) and Tg2576 mice ($p=0.0002$) showing preference for the moved object in the test phase. Interestingly, there was a main effect of genotype ($F(1,68)=4.50$, $p=0.038$) because the discrimination index for WT training was significantly different from Tg2576 testing ($p<0.0001$) and Tg2576 training was significantly different from WT testing ($p=0.0003$).

The discrimination indices of 6 months-old mice led to the same conclusions as the results in Figure 2. There was no evidence of discrimination in low choline-treated mice by two-way ANOVA (no effect of genotype, ($F(1,42)=3.25$, $p=0.079$; no effect of task phase, $F(1,42)=0.278$, $p=0.601$). The same was true of mice fed the intermediate diet (genotype, $F(1,12)=1.44$, $p=0.253$; task phase, $F(1,12)=2.64$, $p=0.130$). However, both WT and Tg2576 mice performed well after being fed the high choline diet (effect of task phase, ($F(1,52)=58.75$, $p=0.0001$, but not genotype ($F(1,52)=1.197$, $p=0.279$). Tukey-Kramer post-hoc tests showed that both WT ($p<0.0001$) and Tg2576 mice that had received the high choline diet ($p=0.0005$) had elevated discrimination indices for the test session.

Taken together, these results demonstrate the lasting beneficial effects of the high choline diet and adverse effects of low choline on offspring in a spatial memory task in Tg2576 as well as WT littermates.

2. *NOR*

We first confirmed that animals did not show preference for one object over the other in training. Indeed, a two-way ANOVA revealed no main effects of genotype at 3 months ($F(1,71)=2.59$, $p=0.536$) and no effect of diet ($F(2,71)=0.22$, $p=0.809$). The same result was obtained at 6 months of age (genotype, ($F(1,66)=0.11$, $p=0.746$); diet, ($F(2,66)=0.98$, $p=0.376$). Thus, novel object exploration during training approached 50% in all treatment groups, independent of genotype and maternal diet, and at both ages.

Figure 4 shows the results for the NOR task. In this figure the diets, age, sex, and individual animals are presented. Lines connect the performance of a given animal in training and in testing. Supplemental Figure 3 shows the data with mean ± sem. Figure 4A shows that there were no impairments at 3 months of age. In animals treated with the low choline diet, there was a main effect of task phase ($F(1,52)=19.81$, $p<0.0001$), no effect of genotype ($F(1,52)=0.020$, $p=0.887$), and more exploration of the novel object during the test phase (WT, $p=0.007$; Tg2576, $p<0.002$). In animals that received the intermediate diet, there also was a significant main effect of task phase ($F(1,38)=30.88$, $p<0.0001$) and no effect of genotype ($F(1,38)=0.97$, $p=0.330$). Animals explored the novel object significantly more during testing in WT ($p=0.014$) and Tg2576 mice ($p=0.002$; Figure 3B). For the high choline group, there also was an effect of task phase ($F(1,26)=17.51$, $p=0.0003$) and not genotype ($F(1,26)=3.53$, $p=0.072$) and a significant increase in the exploration of the novel object during testing in both WT ($p=0.006$) and Tg2576 ($p=0.027$; Figure 3B).

Figure 4B shows the data for 6 months of age. Animals that received the low choline diet performed the NOR task at 3 months of age (two-way ANOVA, task phase ($F(1,26)=32.66$, $p<0.0001$; genotype ($F(1,26)=0.048$, $p=0.821$). Tukey-Kramer post-hoc tests showed that there was a significant increase in the exploration of the novel object in testing both in WT ($p<0.001$) and Tg2576 ($p=0.003$). For mice that had been fed the intermediate choline diet, they also performed the NOR task at 3 months of age (two-way ANOVA, task phase ($F(1,19)=13.65$, $p=0.002$); genotype ($F(1,19)=0.28$, $p=0.604$). Tukey-Kramer post-hoc analyses revealed a significant increase in the exploration of the novel object during testing compared to training for both WT ($p=0.0027$) and Tg2576 ($p=0.039$; Figure 4B). Mice fed the high choline diet also showed no deficit in NOR at 3 months of age. Thus, a two-way ANOVA revealed a main effect of task phase ($F(1,27)=16.26$, $p=0.0004$) but not genotype ($F(1,27)=0.24$, $p=0.625$). Tukey-Kramer post-hoc-hoc analyses revealed a significant increase in the exploration of the novel object during testing in both WT ($p=0.003$) and Tg2576 ($p=0.007$; Figure 4B).

Thus, at both 3 and 6 months of age, WT and Tg2576 mice performed well in the NOR task. These results suggest that young Tg2576 mice are less sensitive to NOR than NOL. The greater sensitivity to NOL is consistent with past demonstrations that the DG contributes to NOL (Sahay et al. 2011; Kesner et al. 2015; Spyrka and Hess 2018; Vandrey et al. 2020; Gulmez Karaca et al. 2021; GoodSmith et al. 2022). Also, our implementation of NOL may have increased the DG-dependence of the task by making the object locations relatively close

together, because in studies by Pofahl and colleagues, it was shown that distances between objects like those we used made NOL DG-dependent (Pofahl et al. 2021).

3. *Exploration time*

Total object exploration (TOE) was measured to address any effects of genotype or diet on the total duration of exploration of objects (Figure 5).

For NOL TOE at 3 months of age, there were effects of genotype ($F(1,67)=6.891$, $p=0.01$) but not maternal diet ($F(2,87)=0.67$, $p=0.63$). Tukey-Kramer post-hoc tests showed that Tg2576 mice fed the low choline diet showed more exploration than WT mice fed the same diet but the effect was on the border of significance ($p=0.049$; data not shown). Three months-old mice tested with NOR showed no significant effect of genotype ($F(1,76)=1.35$, $p=0.25$) or diet ($F(2,76)=0.30$, $p=0.61$). Since there were weak or no effects of genotype, all genotypes are pooled for Figure 5A1 (NOL) and Figure 5B1 (NOR).

For NOL TOE at 6 months of age, there was no effect of genotype ($F(1,66)=0.33$; $p=0.57$). For NOR TOE, the same was true (genotype: $F(1,72)=0.96$, $p=0.33$; diet: $F(2,72)=8.50$, $p=0.0005$). Because genotype was not a significant factor, we pooled genotypes (NOL, Figure 5C1; NOR, Figure 5D2).

With genotypes pooled we determined how diets differed. For NOL TOE, mice treated with the low choline diet had less exploration than mice fed the high choline diet (one-way ANOVA, $F(2,77)=6.90$; $p=0.002$; Tukey post-hoc test, $p=0.005$; Figure 5C1) and the mice that were fed the intermediate diet also had less exploration than mice fed the high choline diet (Tukey post-hoc test, $p=0.008$; Figure 5C1). Results for NOR were similar: mice treated with the low choline diet had less exploration than mice fed the high choline diet (one-way ANOVA, $F(2,74)=4.81$; $p=0.020$; Tukey post-hoc test, $p=0.016$; Figure 5D1) and the mice that were fed the intermediate diet also had less exploration than mice fed the high choline diet (Tukey post-hoc test, $p=0.03$; Figure 5D1).

To gain insight into the potential reason for the effect of diet onexploration at 6 months of age, we measured total object approaches (TOATOA was defined as the number of approaches to the familiar object + number of approaches to the novel object (Figure 5). There was no effect of diet on TOA for NOL ($F(2,75)=2.88$; $p=0.092$; Figure 5C2) or NOR ($F(2,74)=1.81$, $p=0.171$; Figure 5D2).

Taken together, the results indicate that, in 6 months-old mice, animals that received the high choline diet spent more time with objects at each approach. This may underlie increased object memory in high choline-treated mice by increasing time for information processing during an approach. Another possibility is that as Tg2576 mice age they show compensatory changes that enhance memory. This has been suggested for procedural learning (Middei et al. 2004) but to the best of our knowledge, it has not been shown for memory of objects. A third possibility is that the high choline diet reduces anxiety (Glenn et al. 2012; Langley et al. 2015; McCall et al. 2015). Reduced anxiety may lessen fear of exploring objects, and as a result, animals may spend more time with objects.

C. Anatomy

1. NeuN

To further analyze the effects of dietary choline early in life, we used an antibody against a neuronal nuclear antigen, NeuN. Previous studies have found that reduced expression of NeuN often occurs in neurons after insults or injury (e.g., ischemia, toxicity and even aging; Lind et al. 2005; Portiansky et al. 2006; Buckingham et al. 2008; Kadriu et al. 2009; Matsuda et al. 2009; Won et al. 2009; Duffy et al. 2011). For example, when NeuN is reduced after a reduction in ankyrin-rich membrane spanning kinase D-interacting substrate of 220K, ARMS/Kidins, a protein critical to neurotrophin signaling, neurons in the entorhinal cortex demonstrate signs of toxicity such as pyknosis (Duffy et al. 2011; Duffy et al. 2015). Since NeuN is reduced when Aβ levels are elevated (Wu et al. 2016) and NeuN is reduced in AD patients (Camporez et al. 2021), we used NeuN expression to ask if the high choline diet could rescue the loss of NeuN-ir.

As shown in Figure 6A, relatively weak NeuN-ir was observed in Tg2576 that received the low choline diet and the intermediate diet compared to the high choline diet (Figure 6A). This observation is consistent with the vulnerability of the hilus to insult and injury (Scharfman 1999). Therefore, we conducted a two-way ANOVA with diet and septotemporal levels as main factors. To examine the septotemporal axis, quantification from 2-3 anterior coronal sections were averaged to provide a value for the septal pole of the DG and 2-3 posterior sections were averaged to assess the caudal DG. The most ventral, temporal levels were not sampled. There was no effect of diet ($F(2,30)=2.11$, $p=0.137$) nor rostral-caudal level ($F(1,30)=0.02$, $p=0.877$) but there was a significant interaction of diet and rostral-caudal level ($F(2,30)=3.88$, $p=0.036$). A one-way ANOVA using anterior values showed a significant effect of diet ($F(2,21)=7.58$, $p=0.003$) but this was not the case for posterior values ($F(2,23)=0.13$; $p=0.876$). In the anterior DG, there was less NeuN-ir in mice fed the low choline diet versus the high choline diet (Tukey-Kramer post-hoc test, $p=0.029$) and less NeuN in mice fed the intermediate vs high choline diet ($p=0.003$). The results suggest that choline enrichment protected dorsal hilar neurons from NeuN loss in Tg2576 mice.

To ask if the improvement in NeuN after MCS in Tg256 restored NeuN to WT levels we used WT mice. For this analysis we used a one-way ANOVA with 4 groups: Low choline Tg2576, Intermediate Tg2576, High choline Tg2576, and Intermediate WT (Figure 5C). Tukey-Kramer multiple comparisons tests were used as the post hoc tests. The WT mice were fed the intermediate diet because it is the standard mouse chow, and this group was intended to reflect normal mice. The results showed a significant group difference for anterior DG ($F(3,25)=9.20$; $p=0.0003$; Figure 5C1) but not posterior DG ($F(3,28)=0.867$; $p=0.450$; Figure 5C2). Regarding the anterior DG, there were more NeuN-ir cells in high choline-treated mice than both low choline ($p=0.046$) and intermediate choline-treated Tg2576 mice ($p=0.003$). WT mice had more NeuN-ir cells than Tg2576 mice fed the low ($p=0.011$) or intermediate diet ($p=0.003$). Tg2576 mice

that were fed the high choline diet were not significantly different from WT (p=0.827).

2. ΔFosB

To complement the information from the video-EEG recordings (see below) we used a marker of elevated neuronal activity, ΔFosB (Figure 7). ΔFosB is a truncated variant of the transcription factor FosB, which is increased by enhanced neuronal activity; ΔFosB has a half-life of approximately 8 days (Ulery-Reynolds et al., 2009), so when ΔFosB is elevated, it reflects increased neuronal activity over the last 10-14 days (McClung et al. 2004). Previous studies have shown that when the J20 mouse model of AD is examined with an antibody to ΔFosB, the GC layer shows extremely high levels of ΔFosB expression (Corbett et al. 2017), similar to a mouse with chronic spontaneous seizures (Morris et al. 2000). This is not surprising since the J20 mice have recurrent seizures (Palop et al. 2007). Therefore, we asked if Tg2576 mice would have robust ΔFosB in the GC layer, and choline supplementation would reduce it. We also included WT mice. These WT mice would allow us to address the possibility that the high choline diet restored ΔFosB back to normal. As for the NeuN immunocytochemistry, the WT mice were fed the intermediate diet.

There was strong expression of ΔFosB in Tg2576 GCs in mice fed the low choline diet (Figure 7A1). The high choline diet and intermediate diet appeared to show less GCL ΔFosB-ir (Figure 7A2-3). A two-way ANOVA was conducted with the experimental group (Tg2576 low choline diet, Tg2576 intermediate choline diet, Tg2576 high choline diet, WT intermediate choline diet) and location (anterior or posterior) as main factors. There was a significant effect of group ($F(3,32)=13.80$, $p=<0.0001$) and location ($F(1,32)=8.69$, $p=0.006$). Tukey-Kramer post-hoc tests showed that Tg2576 mice fed the low choline diet had significantly greater ΔFosB-ir than Tg2576 mice fed the high choline diet ($p=0.0005$) and WT mice ($p=0.0007$). Tg2576 mice fed the low and intermediate diets were not significantly different ($p=0.275$). Tg2576 mice fed the high choline diet were not significantly different from WT ($p>0.999$). There were no differences between groups for the posterior DG (all $p>0.05$).

ΔFosB quantification was repeated with a lower threshold to define ΔFosB-ir GCs (see Methods) and results were the same (Figure 7D). Two-way ANOVA showed a significant effect of group ($F(3,32)=14.28$, $p< 0.0001$) and location ($F(1,32)=7.07$, $p=0.0122$) for anterior DG but not posterior DG (Figure 7D). For anterior sections, Tukey-Kramer post hoc tests showed that low choline mice had greater ΔFosB-ir than high choline mice ($p=0.0024$) and WT mice ($p=0.005$) but not Tg2576 mice fed the intermediate diet ($p=0.275$); Figure 7D1). Mice fed the high choline diet were not significantly different from WT ($p=0.993$; Figure 7D1). These data suggest that high choline in the diet early in life can reduce neuronal activity of GCs in offspring later in life. In addition, low choline has an opposite effect, suggesting low choline in early life has adverse effects.

D. IIS

Previous research has shown that Tg2576 mice exhibit IIS (Figure 8A) starting at very young ages: 4 weeks (Kam et al. 2016) or 6 weeks (Bezzina et al. 2015). Therefore, we performed video-EEG using cortical (left frontal cortex, right occipital cortex) and hippocampal (left and right) electrodes in WT and Tg2576 mice (Figure 8B-C). Animals were recorded for 24 hrs each session so that the major behavioral states (exploration, awake rest, and sleep) were sampled well. Consistent with previous studies (Bezzina et al. 2015; Kam et al. 2016) we observed IIS in Tg2576 mice but not WT littermates. Therefore, analyses below were only in Tg2576 mice.

As shown in Figure 8, animals that received the intermediate diet had a significantly higher number of IIS in the 24 hrs-long recording periods compared to animals that received the high choline and the low choline diets. A two-way ANOVA (mixed model analysis) showed that there was a significant effect of age ($F(2,37)=3.38$; $p=0.036$) and maternal diet ($F(2,36)=8.12$; $p=0.089$). At the 1.25-month recording, Tukey-Kramer post-hoc analyses showed that IIS frequency in animals treated with the intermediate diet was higher compared to animals treated with the low ($p=0.027$) or high choline diets ($p=0.038$). This also was true for 2 months and 3 months (low choline, high choline, $p<0.05$). At 4 months, the low choline group had significantly reduced IIS frequency compared to the mice that had received the intermediate diet ($p=0.009$) but this was not the case for the high choline group compared to the intermediate group ($p=0.976$). At 5-6 months, IIS frequencies were not significantly different in the mice fed the different diets (all $p>0.05$), probably because IIS frequency becomes increasingly variable with age (Kam et al. 2016). One source of variability is seizures, because there was a sharp increase in IIS during the day before and after a seizure (Supplemental Figure 4). Another reason that the diets failed to show differences was that the IIS frequency generally declined at 5-6 months. This can be appreciated in Figure 8B and Supplemental Figure 6B. These data are consistent with prior studies of Tg2576 mice where IIS increased from 1 to 3 months but then waxed and waned afterwards (Kam et al., 2016).

E. Seizures and premature mortality in mice fed the low choline diet

We found mice fed the low choline diet had greater ΔFosB-ir in GCs and the hilus showed very low NeuN-ir. Therefore, we asked whether low choline-treated mice had more seizures than mice fed the other diets. We recorded 8 mice by video-EEG for 5 days each (at 6 months of age) to examine seizures and found 2 mice from the low choline group had seizures (11 seizures over 2 of the 5 days in one mouse, 1 seizure in the other mouse) whereas none of the other mice had seizures (n=0/4, 2 intermediate and 2 high choline, data not shown).

These values are probably an underestimate for the low choline group because many mice in this group appeared to die in a severe seizure prior to 6 months of age (Supplemental Figure 5). Therefore, the survivors at 6 months probably were the subset with few seizures.

The reason that low choline-treated mice appeared to die in a seizure was that they were found in a specific posture in their cage which occurs when a

severe seizure leads to death (Supplemental Figure 5). They were found in a prone posture with extended, rigid limbs (Supplemental Figure 5). Regardless of how the mice died, there was greater mortality in the low choline group compared to mice that had been fed the high choline diet (Log-rank (Mantel-Cox) test, Chi square 5.36, df 1, p=0.021; Supplemental Figure 5A).

F. Correlations between IIS and other measurements

As shown in Figure 9A, IIS were correlated to behavioral performance in some conditions. For these correlations, only mice that were fed the low and high choline diets were included because mice that were fed the intermediate diet did not have sufficient EEG recordings in the same mouse where behavior was studied. IIS frequency over 24 hrs was plotted against the preference for the novel object in the test phase (Figure 9A). For NOL, IIS were significantly less frequent when behavior was the best, but only for the high choline-treated mice (Pearson's r, p=0.022). In the low choline group, behavioral performance was poor regardless of IIS frequency (Pearson's r, p=0.933; Figure 9A1). For NOR, there were no significant correlations (low choliNe, p=0.202; high choline, p=0.680) but few mice were tested in the high choline-treated mice (Figure 9B2).

We also tested whether there were correlations between dorsal hilar NeuN-ir cell numbers and IIS frequency. In Figure 9B, IIS frequency over 24 hrs was plotted against the number of dorsal hilar cells expressing NeuN. The dorsal hilus was used because there was no effect of diet on the posterior hilus. For NOL, there was no significant correlation (low choline, p=0.273; high choline, p=0.159; Figure 9B1). However, for NOR, there were more NeuN-ir hilar cells when the behavioral performance was strongest (low choline, p=0.024; high choline, p=0.016; Figure 9B2). These data support prior studies showing that hilar cells, especially mossy cells (the majority of hilar neurons), contribute to object recognition (Botterill et al. 2021; GoodSmith et al. 2022).

G. Sex differences

As shown in Figures 2-3, there appeared to be no sex differences in NOL or NOR. For confirmation, we studied NOL further, since that was the task that showed effects of diet. A 3-way ANOVA with sex, genotype and task phase as factors showed no significant effect of sex at 3 months of age (low choline diet, F(1,35)=0.0001; p=0.99). Results were the same for the 3 months-old mice fed the intermediate diet (F(1,22)=0.062; p=0.810) and 3 months-old mice that were fed the high choline diet (F(1,34)=0.432; p=0.522). At 6 months of age there also were no significant effects of sex (low choline diet, F(1,23)=0.343; p=0.571; intermediate diet, F(1,16)=2.49; p=0.130; high choline diet, F(1,27)=0.29; p=0.873).

Regarding IIS, we did not find sex differences (Supplemental Figure 6). In Supplemental Figure 6A, comparisons are made of males and females fed the low or high choline diet at 1.2, 2, and 3 months-of age. A two-way ANOVA with sex and diet as factors showed no effect of sex (F(1,20) =2.48; p=0.132) or diet (F(1,20)=3.56; p=0.067). Note that for this comparison we used the low choline and high choline groups only because there were few mice for each sex at all

ages. The results for months 2-3 also showed no effect of sex (2 months: $F(1,17)=0.637$; $p=0.429$; 3 months: $F(1,15)=0.178$; $p=0.668$).

In Supplemental Figure 6B we grouped mice at 1-2 months, 3-4 months and 5-6 months so that there were sufficient females and males to compare each diet. A two-way ANOVA with diet and sex as factors showed a significant effect of diet ($F(2,47)=46.21$; $p<0.0001$) at 1-2 months of age, but not sex ($F1,47)=0.11$, $p=0.758$). Post-hoc comparisons showed that the low choline group had fewer IIS than the intermediate group, and the same was true for the high choline-treated mice. Thus, female mice fed the low choline diet differed from the females ($p<0.0001$) and males ($p<0.0001$) fed the intermediate diet. Male mice that had received the low choline diet different from females ($p<0.0001$) and males ($p<0.0001$) fed the intermediate diet. Female mice fed the high choline diet different from females ($p=0.002$) and males ($p<0.0001$) fed the intermediate diet, and males fed the high choline diet difference from females ($p<0.0001$) and males ($p<0.0001$) fed the intermediate diet.

For the 3-4 months-old mice there was also a significant effect of diet ($F(2,32)=10.82$, $p=0.0003$) but not sex ($F(1,32)=1.05$, $p=0.313$). Post-hoc tests showed that low choline females were different from males fed the intermediate diet ($p=0.007$), and low choline males were also significantly different from males that had received the intermediate diet ($p=0.006$). There were no significant effects of diet ($F(2,23)=1.21$, $p=0.317$) or sex ($F(1,23)=0.84$, $p=0.368$) at 5-6 months of age.

IV. DISCUSSION

Summary

This study showed that choline supplementation in early life had several beneficial effects in Tg2576 mice. The high choline diet led to improved behavior in the NOL task, improved expression of ΔFosB, rand educed IIS frequency.

We also found surprising effects of treating mice with relatively low choline in the maternal diet. The low choline-treated Tg2576 mice had the least hilar NeuN and most GCL ΔFosB expression. There was an impairment in the NOL task not only in low choline-treated Tg2576 mice but also WT mice. In addition, the low choline-treated Tg2576 mice showed premature mortality compared to Tg2576 mice fed the high choline diet. These results were surprising because the relatively low levels of choline have been considered by others to be a control (Moreno et al. 2013; Mellott et al. 2017; Velazquez et al. 2019). Our data suggest the low choline diet led to several adverse effects. Yet the IIS frequency was low in the low choline-treated Tg2576 mice, suggesting a beneficial effect. It is possible the chronic overexpression of GC ΔFosB led to reduced IIS by altering GC gene expression, since ΔFosB regulates the GC transcriptome (Corbett et al. 2017; You et al. 2018; Stephens et al. 2020). Alterations in GC transcription could impair IIS generation because the GCs appear to be a site where IIS are generated (Lisgaras and Scharfman 2023). Altered GC gene expression might also explain the reduced performance in the NOL task of low choline-treated

mice, because the DG plays an important role in this detecting novel object locations normally (Lee et al. 2005).

We showed for the first time that Tg2576 mice have pathology in the hilus at 6 months of age in that hilar neurons are deficient in immunostaining with an anti-NeuN antibody. Tg2576 offspring on the high choline diet showed a restoration of hilar NeuN staining. Because hilar neurons play a role in NOL (Bui et al. 2018), it is possible that rescue of hilar neurons by the high choline diet led to improved spatial memory. However, there was no significant correlation between the number of NeuN-ir hilar cells and testing for the NOL task. There was a significant correlation for the NOR task, with both low and high choline groups performing better when hilar NeuN-ir cell numbers were greater. The significant correlation with NOR test performance is consistent with a role of somatostatin-expressing hilar GABAergic neurons (Nagarajan et al. 2024) and hilar glutamatergic mossy cells in NOR and object-related activity (Botterill et al. 2021; GoodSmith et al. 2022). Somatostatin-expressing hilar neurons (Tallent 2007; Savanthrapadian et al. 2014; Hofmann et al. 2016) and mossy cells (Sloviter 1994; Scharfman 1999; Scharfman and Myers 2012; Jinde et al. 2013; Bui et al. 2018; Botterill et al. 2019) have been suggested to contribute to excitability of GCs so phenotypic rescue of mossy cells may also have contributed to the ability of the high choline diet to reduce hyperexcitability.

Benefits of high choline

The results of this study are consistent with previous reports that prenatal or postnatal choline supplementation improves object memory. One study of iron deficiency showed that choline supplementation improved NOR (Kennedy et al. 2014). Wistar rats treated with a diet high (5.0 g/kg) in choline had improved NOR relative to rats that had been fed a diet lower (1.1 g/kg) in choline (Moreno et al. 2013). In rats that were aged to 24 months, animals that had been fed a high choline diet showed improved NOR, but only in females (Glenn et al. 2008).

Although few studies have examined the effects of MCS on hyperexcitability, our results showing the benefits of high choline are consistent with reports that a methyl-enriched diet (including choline but also betaine, folic acid, vitamin B12, L-methionine and zinc) reduced spike wave discharges in the offspring (Sarkisova et al. 2023) and audiogenic seizure severity (Poletaeva et al. 2014). Perinatal ethanol treatment that increased excitability was mitigated by choline chloride injection from postnatal day 10-30 (Grafe et al. 2022).

To our knowledge our study is the first to show that MCS can exert effects on IIS and ΔFosB. However, prior studies have shown that MCS in rats improved memory after severe seizures. Using a convulsant to induce several hours of severe seizures (status epilepticus, SE), there is usually extensive hippocampal neuronal loss and when tested in the subsequent days and weeks, memory impairment. MCS reduced this impairment following SE in the Morris water maze (Yang et al. 2000; Holmes et al. 2002; Wong-Goodrich et al. 2011).

Our results are consistent with previous studies of mouse models of AD. In the APP/PS1 mouse model, it was shown that life-long choline supplementation, to the level we used (5.0 g/kg) improved memory in aged mice compared to the

1.1 g/kg diet (Velazquez et al. 2019). Postnatal choline supplementation also improved memory in APP/PS1 mice (Wang et al. 2019). Other improvements were also shown in the APP/PS1 mice, such as increased choline acetyltransferase, the major enyzme for acetylcholine synthesis (Mellott et al. 2017; Velazquez et al. 2019). In 3xFAD mice, a diet without choline had numerous deleterious consequences, including increased Aβ and tau phosporylation (Dave et al. 2023)

Adverse effects of the diet with relatively low choline

The relatively low choline diet had several adverse effects, which was surprising because the low choline levels are not considered very low in some prior studies (Moreno et al. 2013; Mellott et al. 2017; Velazquez et al. 2019). However, the past studies using 1.1 g/kg choline did not test Tg2576 mice. Past studies sometimes used the diet for only part of gestation rather than all of gestation and stopped after birth instead of continuing until weaning. We fed the diet throughout gestation and until weaning. Nevertheless, it is surprising. One possible explanation is an interaction of the low choline diet with the strain, SJL (Swiss James Lambert). To our knowledge this strain has not been tested with different diets before. SJL mice are descended from Swiss Webster, and are prone to reticulum cell sarcoma (Haran-Ghera et al. 1967). This strain is also characterized by vulnerability to infection causing a multiple sclerosis-like syndrome (Linzey et al. 2023).

One of the adverse effects was high mortality. Mice appeared to die in seizures. Consistent with chronic hyperexcitability, mice that had received the low choline diet had high ΔFosB expression in the GCs. Another adverse effect was weak NeuN in the hilus in mice that had received the low choline diet. For both WT and Tg2576 mice, NOL was impaired. These data suggest that the offspring of mothers fed the low choline diet were unhealthy, possibly leading to or because of seizures. If mice were unhealthy, IIS might have been reduced due to impaired excitatory synaptic function. Another reason for reduced IIS is that the mice that had the low choline diet had seizures which interrupted REM sleep. Thus, seizures in Tg2576 mice typically started in sleep. Less REM sleep would reduce IIS because IIS occur primarily in REM. Also, seizures in the Tg2576 mice were followed by a depression of the EEG (postictal depression; Supplemental Figure 3) that would transiently reduce IIS. A different, radical explanation is that the intermediate diet promoted IIS rather than low choline reducing IIS. Instead of choline, a constituent of the intermediate diet may have promoted IIS.

NeuN

As mentioned above, NeuN is a neuronal nuclear antigen that can be phosphorylated, and when that occurs the antibody to NeuN no longer binds to NeuN. NeuN is phosphorylated in response to oxidative damage, brain injury, and toxicity (Lind et al. 2005). Therefore, it was of interest when we saw that hilar neurons of the dentate gyrus showed reduced NeuN in Tg2576 mice. Hilar neurons are mainly glutamatergic mossy cells and somatostatin

(SOM)/neuropeptide Y (NPY)-expressing GABAergic neurons (HIPP cells; (Houser 2007; Scharfman and Myers 2012; Scharfman 2016), and both neuronal types are implicated in spatial memory functions of the GCs, as well as their excitability (Myers and Scharfman 2009; 2011; Scharfman and Myers 2012; Jinde et al. 2013; Scharfman 2016; Raza et al. 2017; Bui et al. 2018; GoodSmith et al. 2019; Li et al. 2021). If damaged by the high intracellular APP/Aβ levels in young Tg2576 mice, the hilar neurons would be expected to show NeuN loss. This idea is consistent with deficits in SOM and NPY-stained cells (Chan-Palay 1987), indicating a vulnerability.

The reason for rescue of anterior hilar but not posterior hilar NeuN with the high choline diet is unclear. The greater sensitivity of dorsal neurons may be related to differences in gene expression patterns, since some of the genes that are differentially expressed along the dorsal-ventral axis could affect vulnerability to insult or injury (Cembrowski et al. 2016; Zhang et al. 2018). However, there was a similar pattern to the data for both anterior and posterior. The lack of statistical significance appeared to be greater variance in the posterior data.

ΔFosB

Given that seizures are associated with elevated GC expression of ΔFosB (Chen et al. 1997; McClung et al. 2004; Corbett et al. 2017; You et al. 2017), the differences in ΔFosB expression levels observed here could be the result of increased seizures in animals that received the low choline diet. Therefore, we suggest that the high ΔFosB in Tg2576 mice fed the low choline diet reflects more seizures than in the other two diets.

ΔFosB is a transcription factor that is linked to cognition. In the J20 mouse model of AD, elevated ΔFosB in GCs led to reduced cognition, and when ΔFosB was selectively reduced the cognition improved (Corbett et al. 2017). Therefore, the reduction in ΔFosB by the high choline diet was important to show hyperexcitability was reduced and also important because it showed how high choline may benefit cognition.

Choline and cholinergic neurons

There are many suggestions for the mechanisms that allow MCS to improve health of the offspring. One hypothesis that we are interested in is that MCS improves outcomes by reducing IIS. Reducing IIS would potentially reduce hyperactivity, which is significant because neuronal activity can increase release of Aβ and tau (Cirrito et al. 2005; Cirrito et al. 2008; Bero et al. 2011; Yamada et al. 2014; Yamamoto et al. 2015; Hettinger et al. 2018). IIS would also be likely to disrupt sleep since it represents aberrant synchronous activity over widespread brain regions. The disruption to sleep could impair memory consolidation since it is a notable function of sleep (Graves et al. 2001; Poe et al. 2010). Sleep disruption also has other negative consequences such as impairing normal clearance of Aβ (Nedergaard and Goldman 2020). Indeed, in patients with AD, IIS and similar events, IEDs, are correlated with memory impairment (Vossel et al. 2016).

How would choline supplementation in early life reduce IIS of the offspring? It may do so by making BFCNs more resilient. That is significant because BFCN abnormalities appear to cause IIS. Thus, selective silencing of BFCNs reduced IIS. The cholinergic antagonist atropine also reduces IIS when injected systemically (Kam et al., 2016), and it reduced elevated synaptic activity of GCs in young Tg2576 mice in vitro (Alcantara-Gonzalez et al. 2021). These studies are consistent with the idea that early in AD there is elevated cholinergic activity (DeKosky et al. 2002; Ikonomovic et al. 2003; Kelley et al. 2014; Mufson et al. 2015; Kelley et al. 2016), while later in life there is cholinergic degeneration. Indeed, the overactivity of cholinergic neurons of the first months of life could cause the degeneration at older ages.

Why would MCS make BFCNs resilient? There are several possibilities that have been explored, based on genes upregulated by MCS. One attractive hypothesis is that neurotrophic support for BFCNs is retained after MCS but in aging and AD it declines (Gautier et al. 2023). The neurotrophins, notably nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) have been known for a long time to support the health of BFCNs (Mufson et al. 2003; Niewiadomska et al. 2011).

Limitations

One of the limitations of the study was that the maternal diets were not matched exactly. Although the low and high choline diets only had differences in choline, the intermediate diet had different amounts of choline as well as other constituents (Supplementary Table 1). Therefore, the only diet comparison with a difference restricted to choline is the low versus high choline diets (Supplemental Table 1). The intermediate diet was useful however, because numerous studies in AD mouse models employ this standard diet.

In addition, groups were not exactly matched. Although WT mice do not have IIS, a WT group for each of the Tg2576 groups would have been useful. Instead, we included WT mice for the behavioral tasks and some of the anatomical assays. Related to this point is that several mice died during the long-term EEG monitoring of IIS. However, this is unlikely to have had a major effect because IIS were low in frequency in all groups over the last months of the study.

Regarding sex differences, there may have been differences if females had been separated by stage of the estrous cycle at death. This possibility is raised by prior data showing that rats and mice during proestrous and estrous mornings have hyperexcitability but not at other cycle stages (Scharfman et al. 2003).

The Tg2576 mouse model is one of many murine models of AD, and as with all models there are inherent limitations. The Tg2576 model recapitulates familial AD, whereas the majority of AD is sporadic. Tg2576 mice also lack tau pathology. However, MCS has now demonstrated structural/functional benefits in several AD-relevant models, namely Tg2576 (this report), APP/PS1 (Alldred et al. 2021; Dave et al. 2023), Ts65Dn (Velazquez et al. 2013; Powers et al. 2016; Strupp et al. 2016; Powers et al. 2017; Alldred et al. 2021; Alldred et al. 2023) and 3xTg mice (Dave et al. 2023). Moreover, increasing evidence suggests that

humans with AD have low serum choline and are improved by dietary choline (Dave et al. 2023; Judd et al. 2023a; b).

Conclusions

There is now a substantial body of evidence that MCS promotes learning and memory in the offspring of normal rats and improves behavior and other abnormalities in mice that simulate AD. There also is evidence that serum levels of choline are low in AD. Therefore, it is exciting to think that dietary choline might improve AD. Given the past work also shows benefits to mice that simulate DS, the benefits of choline supplementation appear to extend to DS. The present study adds to the growing consensus that MCS is restorative by showing that hyperexcitability and pathology in the DG of Tg2576 mice are improved by MCS. They also suggest that there may be some adverse effects of a relatively low level of choline in the diet.

Figure 1. Schematic of the behavioral procedures for the NOL and NOR tasks.

A. Experimental timeline. Dams were fed one of 3 diets for mating, gestation, and offspring consumed the diet until weaning at 25-30 days of age. At 1 month-old, offspring were implanted with electrodes for EEG and recorded for a 24 hrs-long period at 1.2, 2, 3, 4, 5, and 6 months of age. At 3 and 6 months of age behavior was tested. At 6 months of age, after behavioral testing, mice were perfused, brains were sectioned, and sections were processed with antibodies against NeuN and ΔFosB.

B. Prior to NOL or NOR, animals were acclimated to the testing arena. There were 3 acclimation sessions separated by 24 hrs during which animals were allowed to freely explore for 5 min.

C. In the NOL task, animals were placed in a cage with two identical objects and allowed to freely explore for 5 min (Training). After one hour they were brought back to the cage, where one object was displaced, and allowed to freely explore for 5 min (Testing).

D. In the NOR task. animals were placed in a cage with two identical objects and allowed to freely explore for 5 min (Training). After one hour they were brought back to the cage, where one object was replaced, and allowed to freely explore for 5 min (Testing).

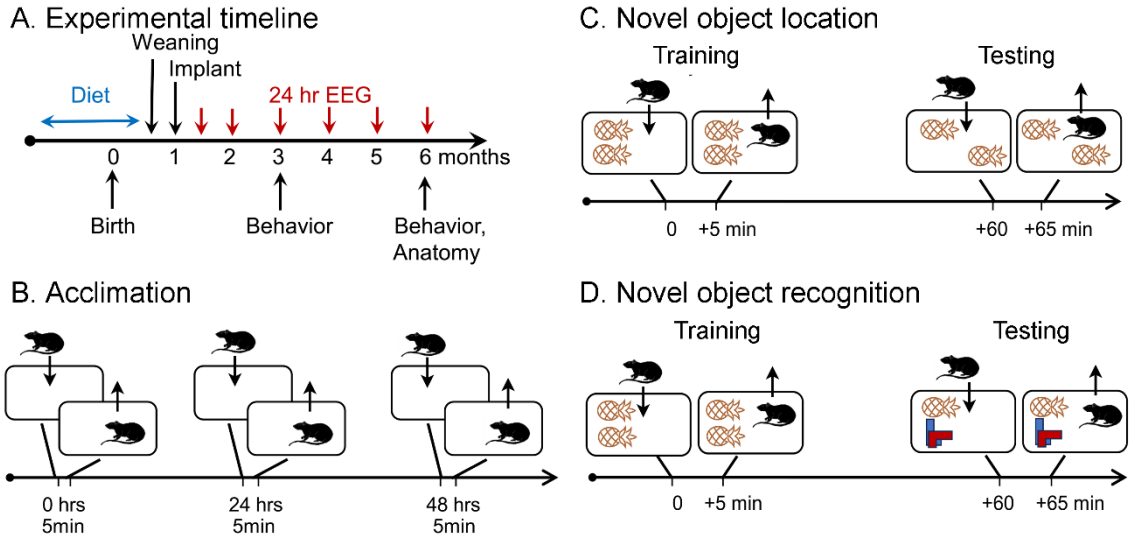


Figure 2. Choline enrichment reduced spatial memory deficits.

A. Three months-old mice.

1. WT and Tg2576 mice exposed to the low choline diet showed spatial memory deficits.
2. Tg2576 exposed to the intermediate diet showed spatial memory deficits but not WT.
3. Spatial memory was improved in animals exposed to the high choline diet.

B. Six months-old mice.

1. WT and Tg2576 mice exposed to the low choline diet showed spatial memory deficits.
2. WT and Tg2576 mice exposed to the intermediate diet showed spatial memory deficits.
3. WT and Tg2576 mice exposed to the high choline diet had improved spatial memory.

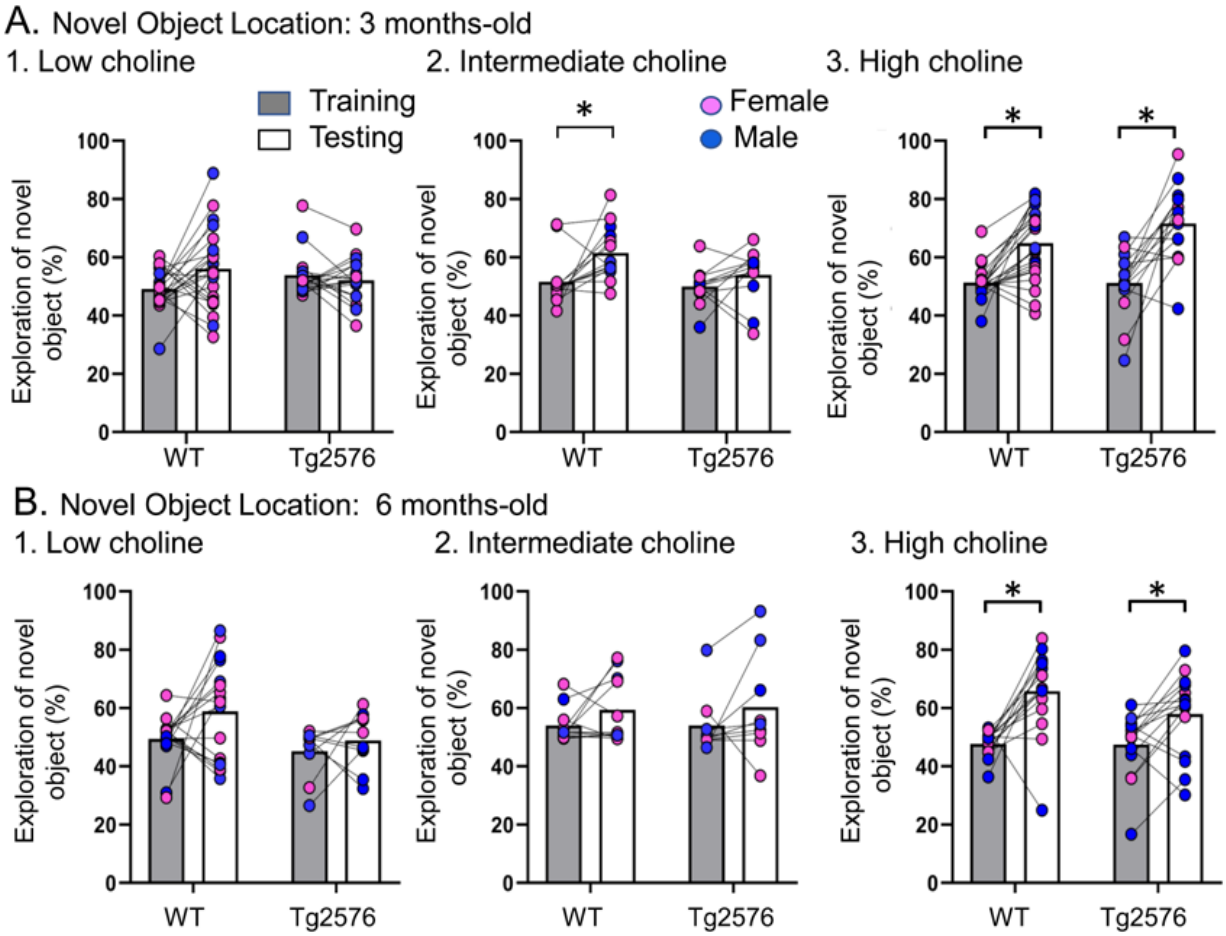


Figure 3. Novel object location results based on the discrimination index.
A. Results are shown for the 3 months-old WT and Tg2576 mice based on the discrimination index.

1. Mice fed the low choline diet showed object location memory only in WT.
2. Mice fed the intermediate diet showed object location memory only in WT.
3. Mice fed the high choline diet showed memory both for WT and Tg2576 mice. Therefore, the high choline diet improved memory in Tg2576 mice.

B. The results for the 6 months-old mice are shown.

1-2. There was no significant memory demonstrated by mice that were fed either the low or intermediate choline diet.

3. Mice fed a diet enriched in choline showed memory whether they were WT or Tg2576 mice. Therefore, choline enrichment improved memory in all mice.

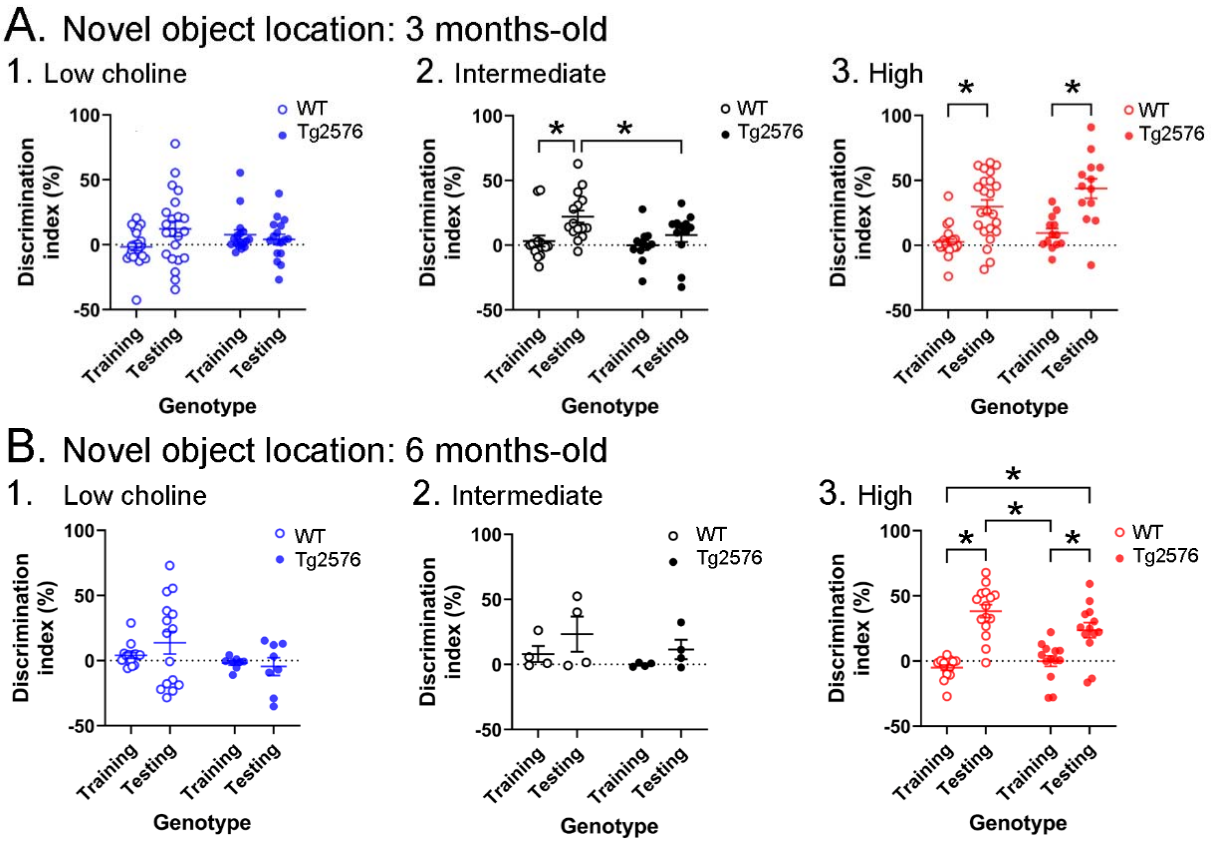


Figure 4. WT and Tg2576 mice showed object recognition memory regardless of diet.

A. Three months-old mice.

1-3. WT and Tg2576 mice treated performed the NOR task.

B. Six months-old mice.

1-3. WT and Tg2576 mice performed the NOR task.

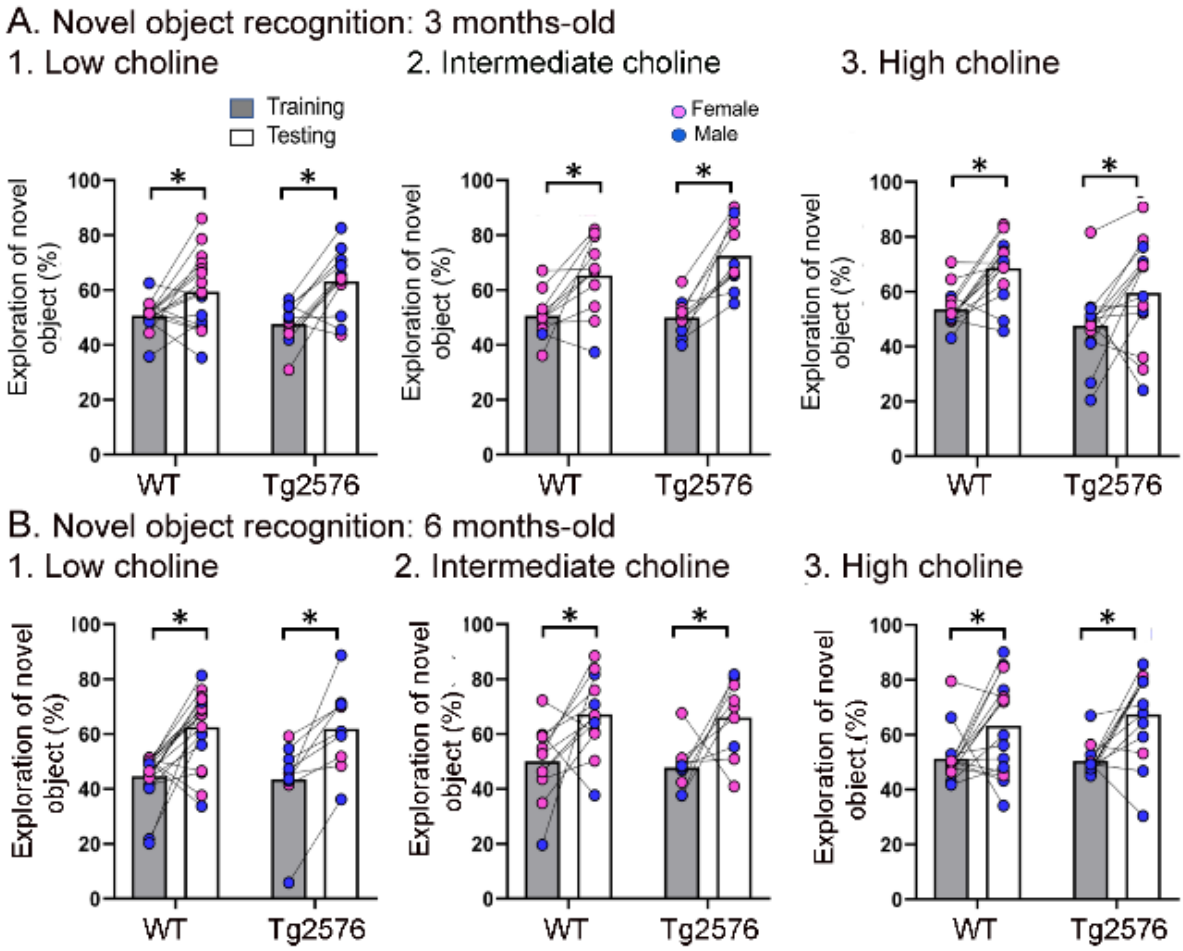


Figure 5. Tg2576 mice treated with the choline supplemented diet spent more time with the objects at each approach.

A-B. NOL.

There was no effect of diet on time exploring or approaches to the novel object in the NOL task at 3 months of age (A) but there were significant differences at 6 months of age (B). Mice exposed to the high choline diet spent more time exploring than mice that had been fed the low choline or the intermediate diet.

C-D. NOR.

There was no effect of diet on time exploring or approaches to the novel object in the NOR task at 3 months of age (C) but there were significant differences at 6 months of age (D). Mice that had been fed the high choline diet spent more time exploring than mice that had been fed the low choline or the intermediate diet.

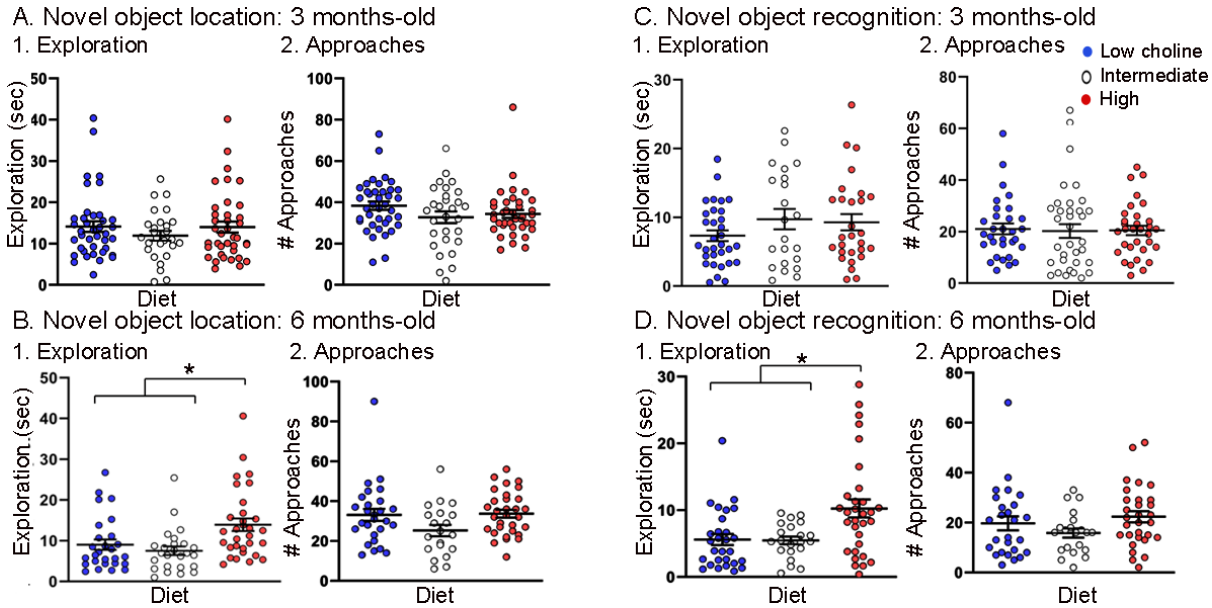


Figure 6. Choline supplementation improved NeuN immunoreactivity (ir) in hilar cells in Tg2576 animals

A. Representative images of NeuN-ir staining in the anterior DG of Tg2576 animals.

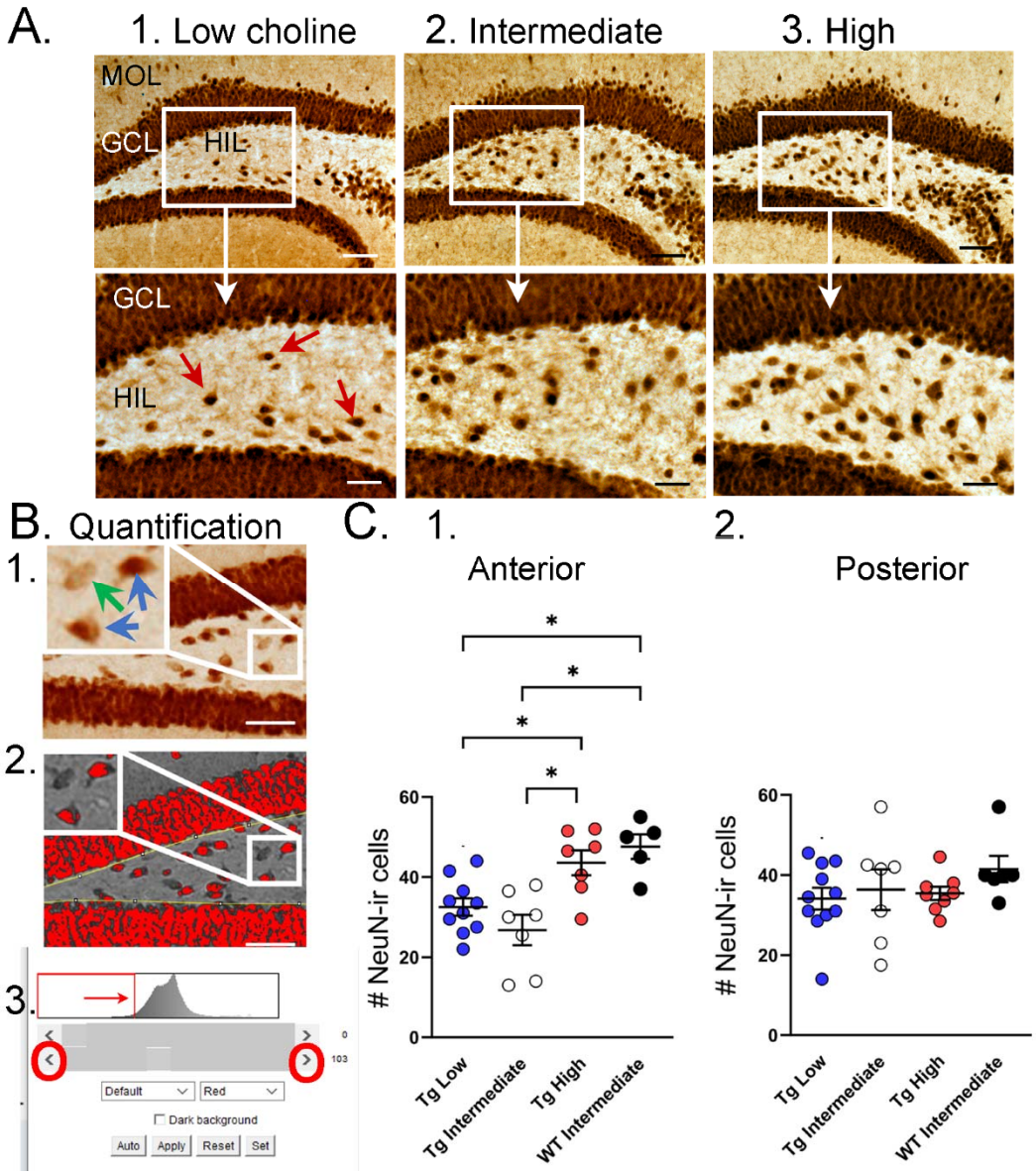
1. A section from a Tg2576 mouse fed the low choline diet. The area surrounded by a box is expanded below. Red arrows point to NeuN-ir hilar cells. Mol=molecular layer, GCL=granule cell layer, HIL=hilus. Calibration for the top row, 100 µm; for the bottom row, 50 µm.
2. A section from a Tg2576 mouse fed the intermediate diet. Same calibrations as for 1.
3. A section from a Tg2576 mouse fed the high choline diet. Same calibrations as for 1.

B. Quantification methods. Representative images demonstrate the thresholding criteria used to quantify NeuN-ir.

1. A NeuN-stained section. The area surrounded by the white box is expanded in the inset (arrow) to show 3 hilar cells. The 2 NeuN-ir cells above threshold are marked by blue arrows. The 1 NeuN-ir cell below threshold is marked by a green arrow.
2. After converting the image to grayscale, the cells above threshold were designated as red. The inset shows that the two cells that were marked by blue arrows are red while the cell below threshold is not.
3. An example of the threshold menu from ImageJ showing the way the threshold was set. Sliders (red circles) were used to move the threshold to the left or right of the histogram of intensity values. The final position of the slider (red arrow) was positioned at the onset of the steep rise of the histogram.

C. NeuN-ir in Tg2576 and WT mice. Tg2576 mice had either the low, intermediate, or high choline diet in early life. WT mice were fed the standard diet (intermediate choline)**.**

1. Tg2576 mice treated with the high choline diet had significantly more hilar NeuN-ir cells in the anterior DG compared to Tg2576 mice that had been fed the low choline or intermediate diet. The values for Tg2576 mice that received the high choline diet were not significantly different from WT mice, suggesting that the high choline diet restored NeuN-ir.
2. There was no effect of diet or genotype in the posterior DG, probably because the low choline and intermediate diet did not appear to lower hilar NeuN-ir.



1194
1195
1196

Figure 7. Choline supplementation reduced ΔFosB expression in dorsal GCs of Tg2576 mice.

A. Representative images of ΔFosB staining in GCL of Tg2576 animals from each treatment group.

1. A section from a low choline-treated mouse shows robust ΔFosB-ir in the GCL. Calibration, 100 µm.

2-3. Sections from intermediate (2) and high choline (3)-treated mice. Same calibration as 1.

B. Quantification methods**.** Representative images demonstrating the thresholding criteria established to quantify ΔFosB.

1. A ΔFosB -stained section shows strongly-stained cells (white arrows).

2. A strict thresholding criteria was used to make only the darkest stained cells red.

C. Use of the strict threshold to quantify ΔFosB-ir.

1. Anterior DG. Tg2576 mice treated with the choline supplemented diet had significantly less ΔFosB-ir compared to the Tg2576 mice fed the low or intermediate diets. Tg2576 mice fed the high choline diet were not significantly different from WT mice, suggesting a rescue of ΔFosB-ir.

2. There were no significant differences in ΔFosB-ir in posterior sections.

D. Methods are shown using a threshold that was less strict.

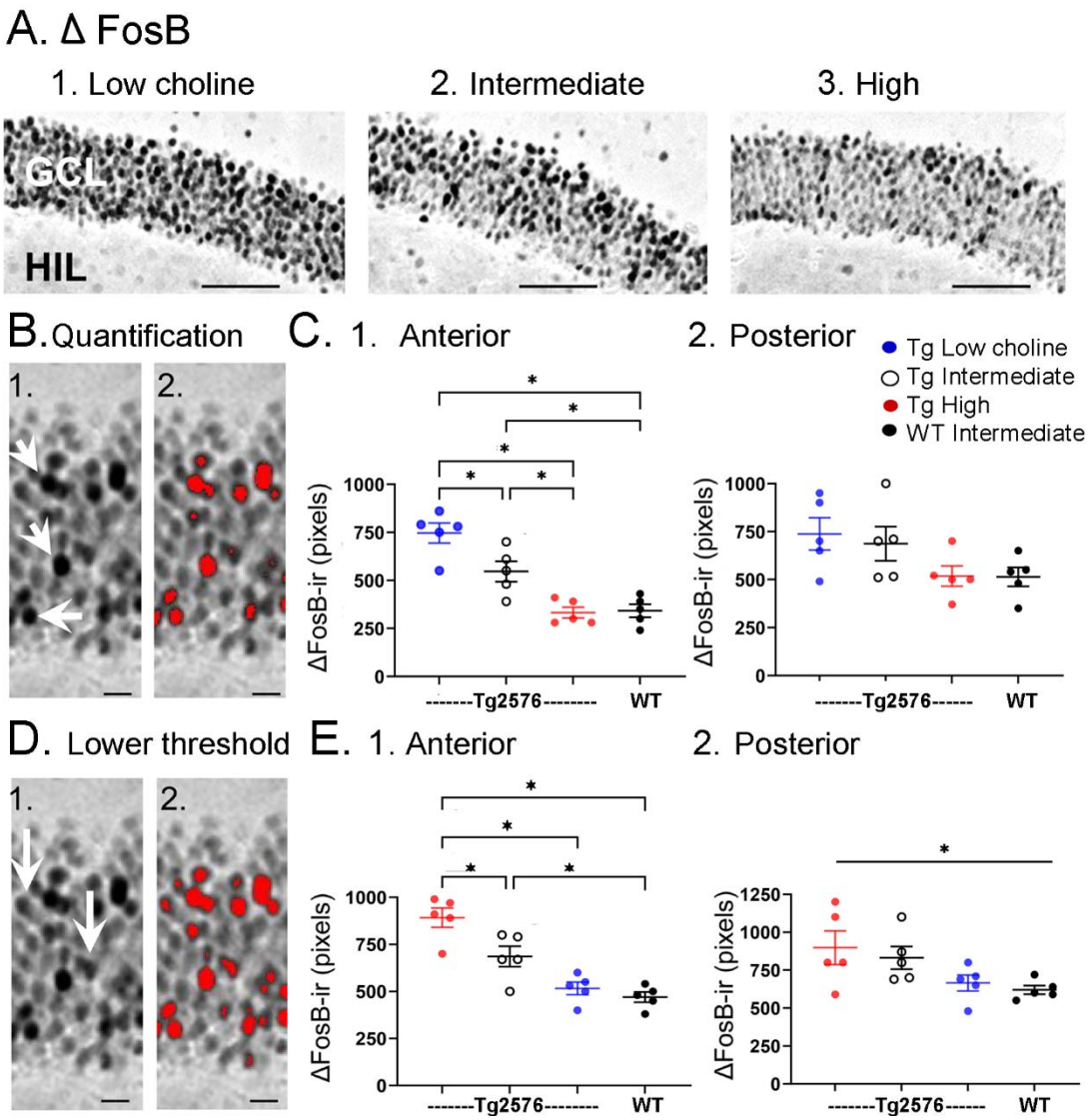
1. Some of the stained cells that were included are not as dark as those used for the strict threshold (white arrows).

2. All cells above the less conservative threshold are shown in red.

E. Use of the less strict threshold to quantify ΔFosB-ir.

1. Anterior DG. Tg2576 mice that were fed the high choline diet had less ΔFosB-ir pixels than the mice that were fed the other diets. There were no differences from WT mice, suggesting restoration of ΔFosB-ir by choline enrichment in early life.

2. Posterior DG. There were no significant differences between Tg2576 mice fed the 3 diets or WT mice.


A. Δ FosB
1. Low choline
2. Intermediate
3. High
GCL
HIL
B. Quantification
1.
2.
C. 1. Anterior
2. Posterior
Tg Low choline
Tg Intermediate
Tg High
WT Intermediate
ΔFosB-ir (pixels)
1000
750
500
250
0
-------Tg2576--------
WT
D. Lower threshold
1.
2.
E. 1. Anterior
2. Posterior
1250
-------Tg2576------

1227
1228

Figure 8. The high choline diet reduced IIS frequency in Tg2576 animals.
A. 1. Representative example of an IIS (red arrow). IIS were defined as occurring in all 4 channels (generalized) to distinguish them from focal spikes that can often be artifact.
2-4. The IIS shown in A1 is expanded.
B. 1. Scatter plot of IIS frequency at each age of recording.
2. Means and sem are plotted. The high choline diet group had fewer IIS than the intermediate diet group at ages 1-3 months ($, $p<0.05$) and the low choline group had less IIS than the intermediate diet at ages 1-4 months (@, $p<0.05$). The high and low choline diet groups were significantly different at ages 3 and 4 months (*, $p<0.05$).

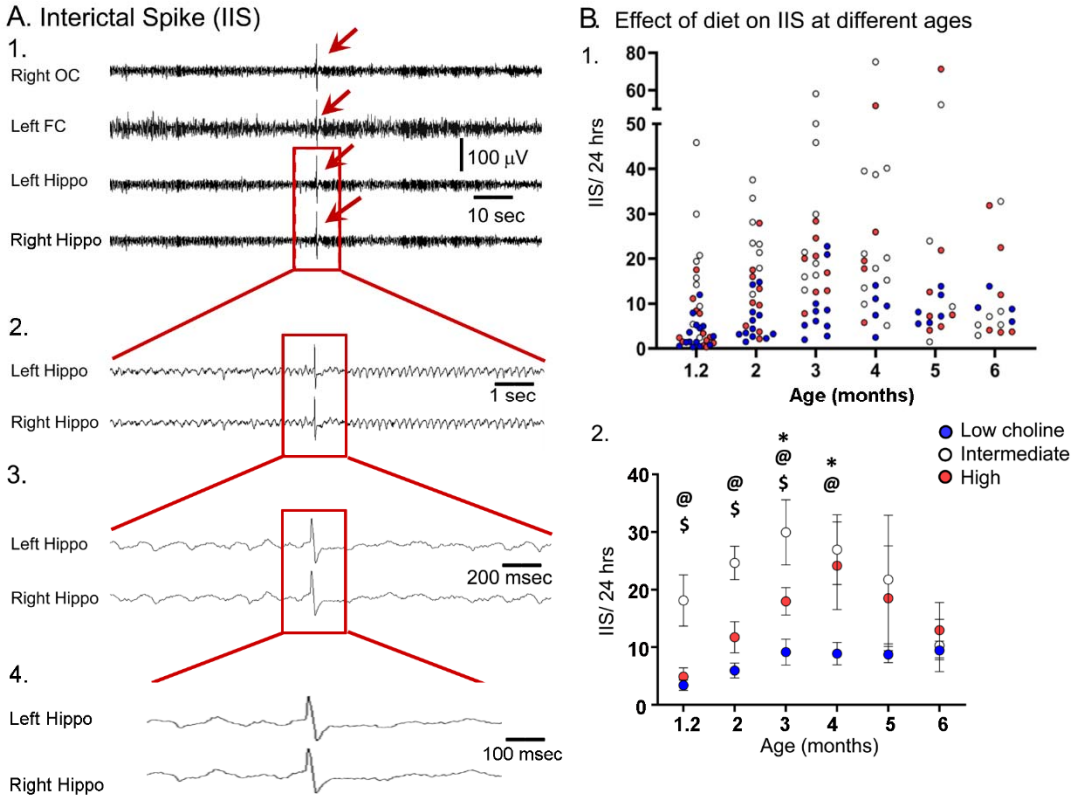


Figure 9. Correlations between IIS, Behavior, and hilar NeuN-ir.

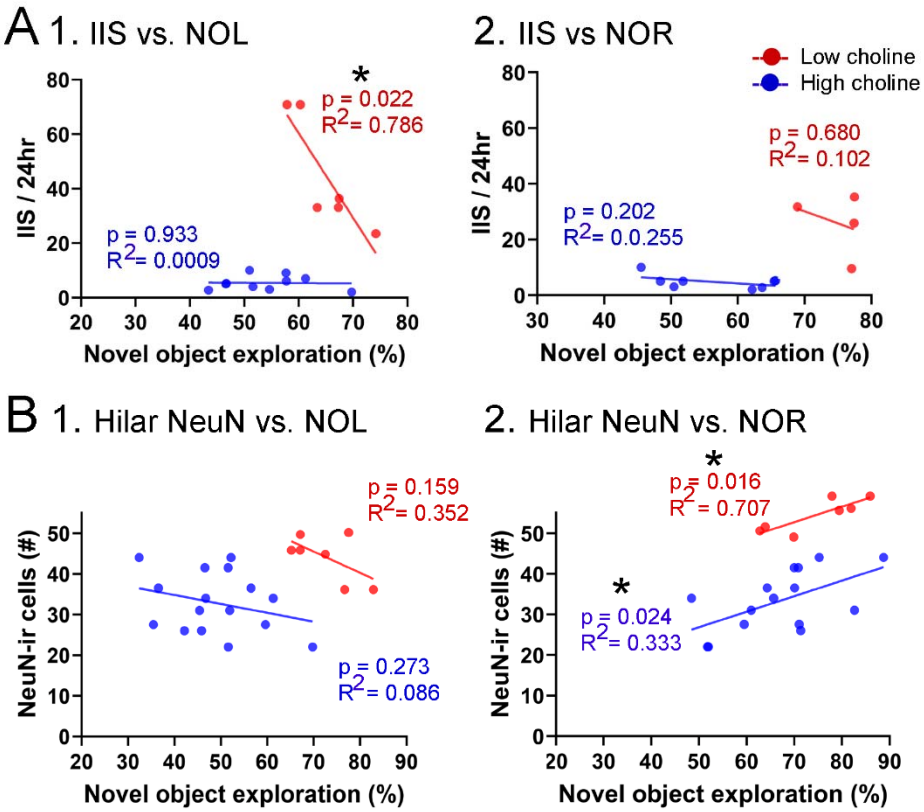
A. IIS frequency over 24 hrs is plotted against the preference for the novel object in the test phase of NOL. A greater preference is reflected by a greater percentage of time exploring the novel object.

1. The mice fed the high choline diet (red) showed greater preference for the novel object when IIS were low. These data suggest IIS impaired object location memory in the high choline-treated mice. The low choline-treated mice had very weak preference and very few IIS, potentially explaining the lack of correlation in these mice.
2. There were no significant correlations for IIS and NOR. However, there were only 4 mice for the high choline group, which is a limitation.

B. IIS frequency over 24 hrs is plotted against the number of dorsal hilar cells expressing NeuN. The dorsal hilus was used because there was no effect of diet on the posterior hilus..

1. Hilar NeuN-ir is plotted against the preference for the novel object in the test phase of NOL. There were no significant correlations.

2. Hilar NeuN-ir was greater for mice that had better performance in NOR, both for the low choline (blue) and high choline (red) groups. These data support the idea that hilar cells contribute to object recognition (Kesner et al. 2015; Botterill et al. 2021; GoodSmith et al. 2022).



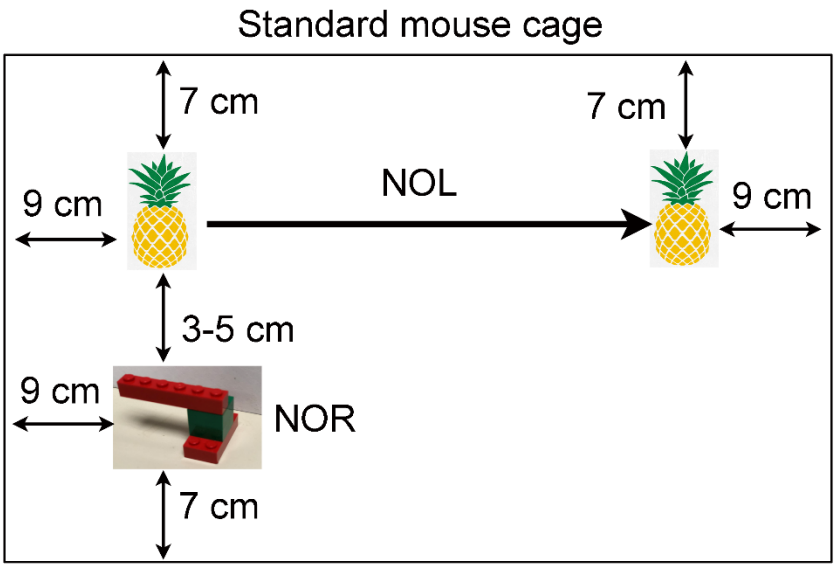
Supplemental Table 1. Comparison of the 3 diets used in this study.

	Low choline	Intermediate choline	High choline
Choline	1.1g/kg	2.0g/kg	5.0g/kg
Carbohydrate	690	570	690
Protein	177	236	177
Fat	120	170	120
Thiamin	0.020	0.015	0.020
Riboflavin	0.600	0.005	0.600
Pyridoxine	0.700	0.006	0.700
Niacin	3.000	0.780	3.000
Folic Acid	0.200	0.003	0.200
Biotin	0.020	0.002	0.020

Legend.

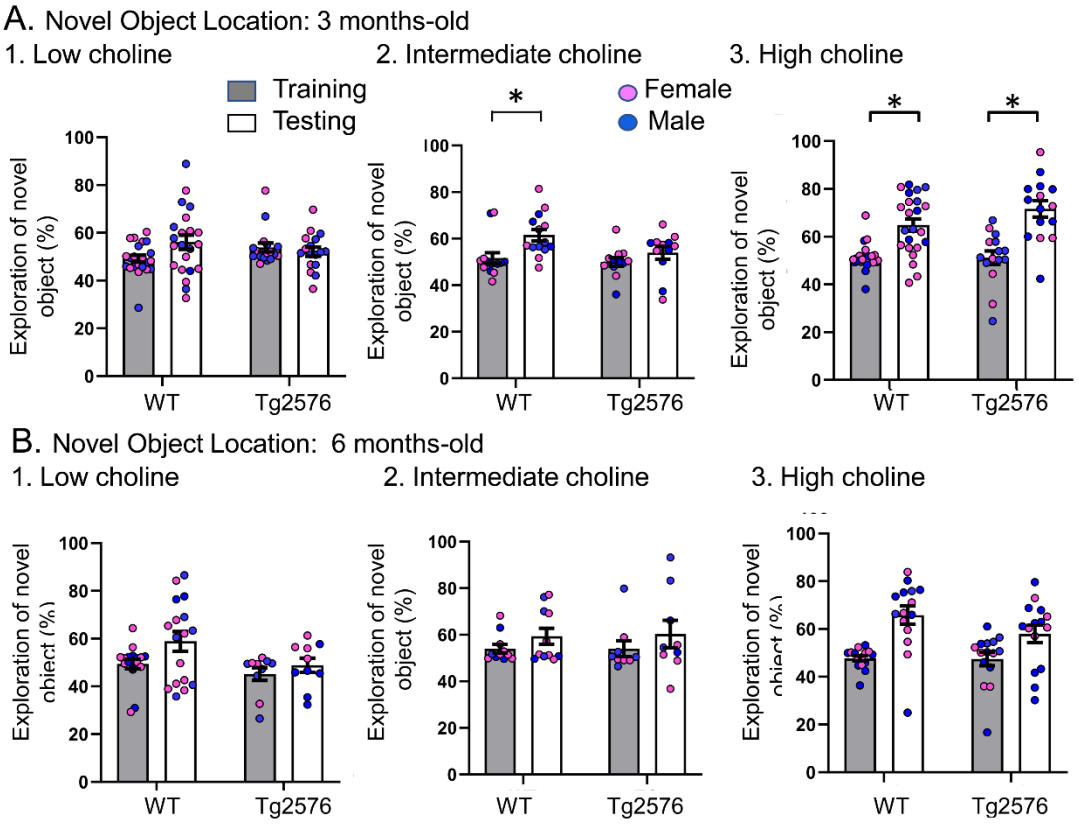
The major constituents of the diets are shown. Concentrations are g nutrient/kg chow. Food was provided *ad libitum* to breeders during mating, gestation and until weaning. After weaning all mice were fed the intermediate diet. WT mice were fed the intermediate diet during breeding, gestation, until weaning and after weaning.

Supplemental Figure 1. Details of behavioral tasks.
A standard mouse cage is diagrammed with the locations of objects inside it. For NOL, there were two pineapple-like objects and one was moved across the cage for the test session. For the NOR test session, one pineapple-like object was removed and an object made of LEGO was placed in the location where the original pineapple-like object had been.



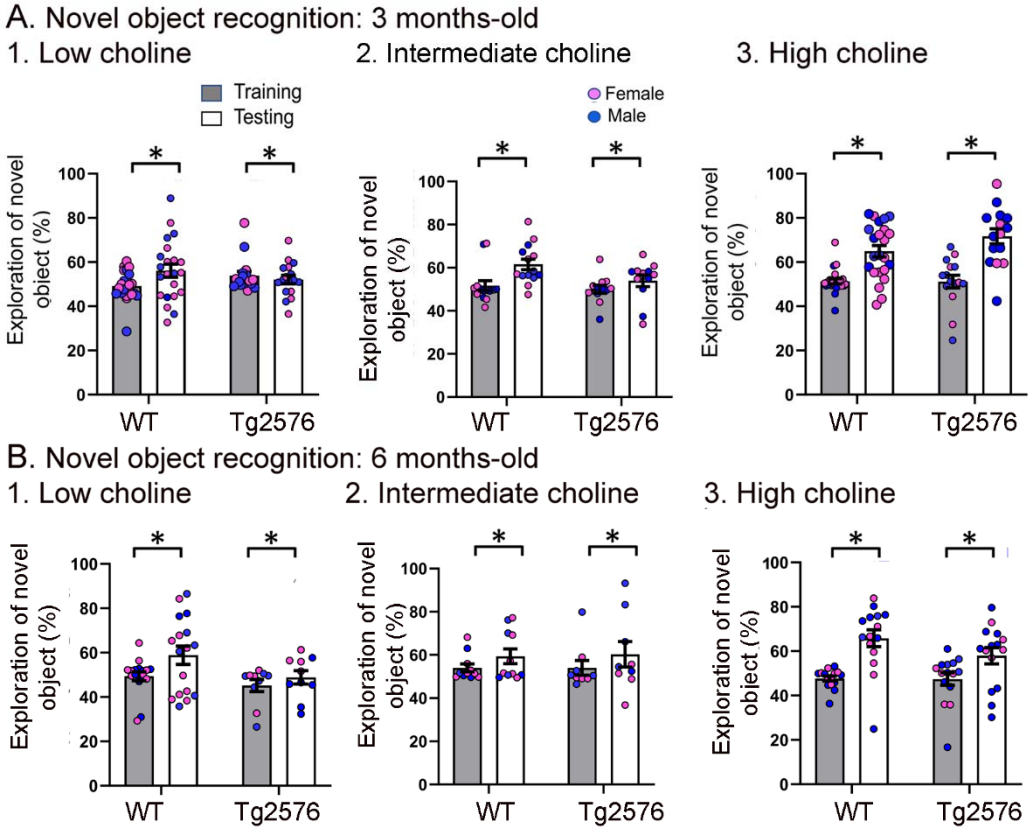
Supplemental Figure 2.

The data shown in Figure 2 are plotted with means ± sem.



Supplemental Figure 3.

The data shown in Figure 3 are plotted with means ± sem.



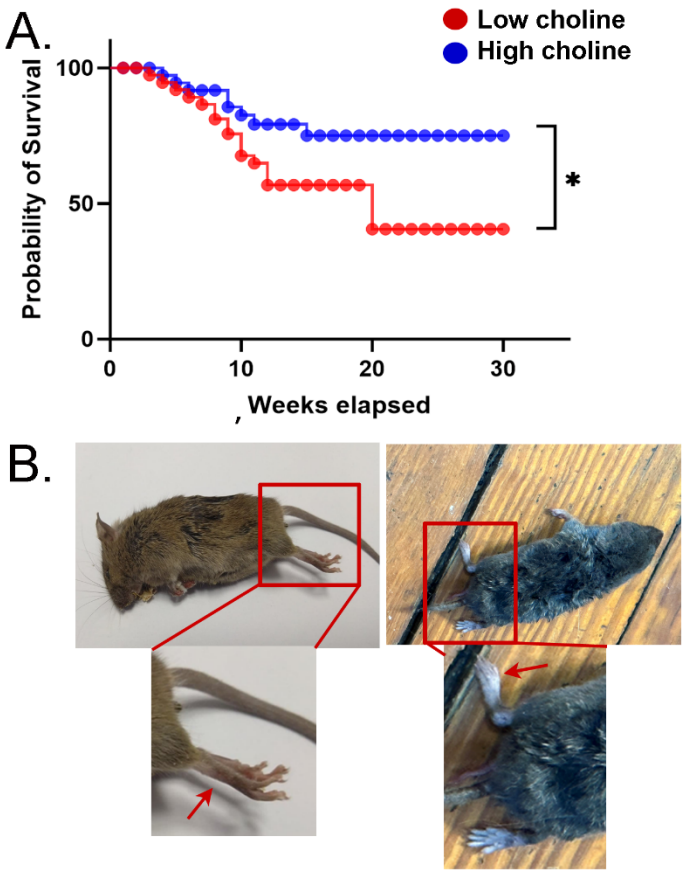
Supplemental Figure 4. Mortality was high in mice treated with the low choline diet.

A. Survival curves are shown for mice fed the low choline diet and mice fed the high choline diet. The mice fed the high choline diet had a significantly less severe survival curve.

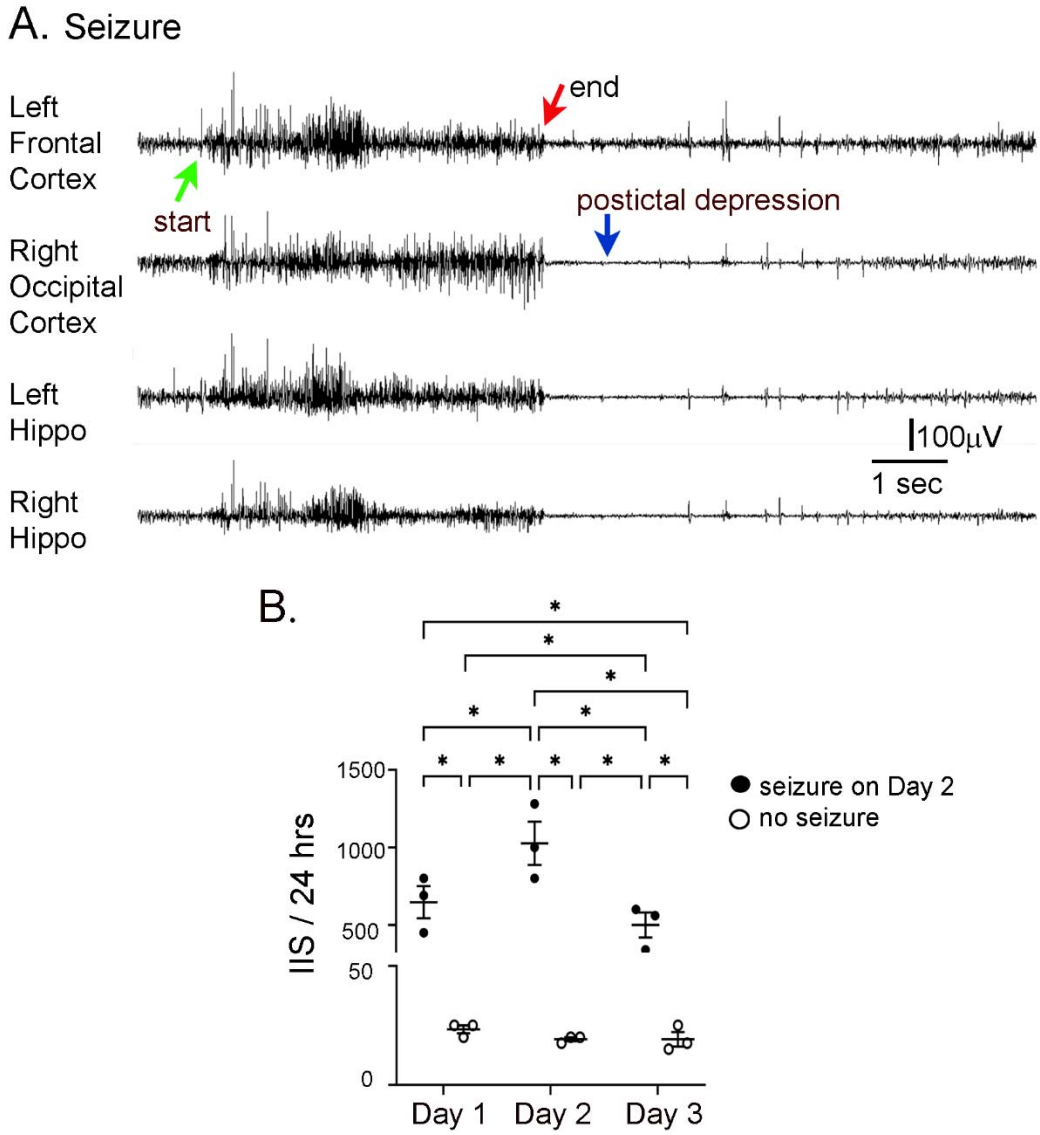
B.

Left: A photo of a mouse after sudden unexplained death. The mouse was found in a posture consistent with death during a convulsive seizure. The area surrounded by the red box is expanded below to show the outstretched hindlimb (red arrow).

Right: A photo of a mouse that did not die suddenly. The area surrounded by the box is expanded below to show that the hindlimb is not outstretched.

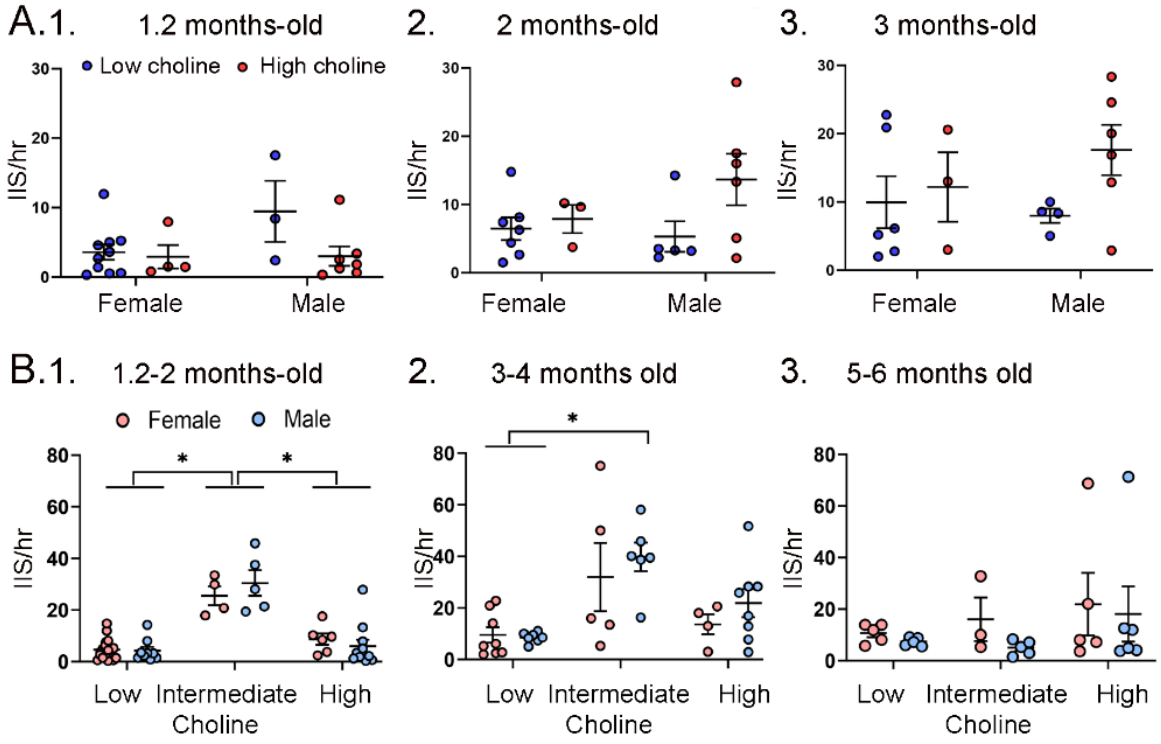


Supplemental Figure 5. IIS frequency before and after seizures.
A. Representative EEG traces recorded from electrodes implanted in the skull over the left frontal cortex, right occipital cortex, left hippocampus (Hippo) and right hippocampus during a spontaneous seizure in a 5 months-old Tg2576 mouse. Arrows point to the start (green arrow) and end of the seizure (red arrow), and postictal depression (blue arrow).
B. IIS frequency was quantified from continuous video-EEG for mice that had a spontaneous seizure during the recording period and mice that did not. IIS frequency is plotted for 3 consecutive days, starting with the day before the seizure (designated as day 1), and ending with the day after the seizure (day 3). A two-way RMANOVA was conducted with the day and group (mice with or without a seizure) as main factors. There was a significant effect of day ($F(2,4)=46.95$, $p=0.002$) and group (seizure vs no seizure; $F(1,2)=46.01$, $p=0.021$) and an interaction of factors ($F(2,4)=46.68$, $p=0.002$)..Tukey-Kramer post-hoc tests showed that mice with a seizure had significantly greater IIS frequencies than mice without a seizure for every day (day 1, $p=0.0005$; day 2, $p=0.0001$; day 3, $p=0.0014$). For mice with a seizure, IIS frequency was higher on the day of the seizure than the day before ($p=0.037$) or after ($p=0.010$). For mice without a seizure, there were no significant differences in IIS frequency for day 1, 2, or 3. These data are similar to prior work showing that from one day to the next mice without seizures have similar IIS frequencies (Kam et al. 2016).


A. Seizure
Left Frontal Cortex
end
start
postictal depression
Right Occipital Cortex
Left Hippo
100μV
1 sec
Right Hippo
B.
IIS / 24 hrs
1500
1000
500
50
0
Day 1
Day 2
Day 3
seizure on Day 2
no seizure

1328

Supplemental Figure 6. IIS frequency was similar for each sex.
A. IIS frequency was compared for females and males at 1.2 months (1), 2 months (2), and 3 months (3). Two-way ANOVA was used to analyze the effects of sex and diet. Female and male Tg2576 mice were not significantly different.
B. Mice were pooled at 1.2 and 2 months (1), 3 and 4 months (2) and 5 and 6 months (3). Two-way ANOVA analyzed the effects of sex and diet. There were significant effects of diet for (1) and (2) but not (3). There were no effects of sex at any age.
1. There were significant effects of diet ($F(2,47)=46.21$, $p<0.0001$) but not sex ($F(1,47)=0.106$, $p=0.746$). Female and male mice fed the low choline diet or high choline diet were significantly different from female and male mice fed the intermediate diet (all $p<0.05$, asterisk).
2. There were significant effects of diet ($F(2,32)=10.82$, $p=0.0003$) but not sex ($F(1,32)=1.05$, $p=0.313$). Both female and male mice of the low choline group were significantly different from male mice fed the intermediate diet (both $p<0.05$, asterisk) but no other pairwise comparisons were significant.
3. There were no significant differences (diet, $F(2,23)=1.21$, $p=0.317$); sex, $F(1,23)=0.844$, $p=0.368$).



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