# 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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**Key words:** dementia, maternal choline supplementation, hippocampus, dentate gyrus, hilus, interictal spike, novel object location, novel object recognition

**Word count:**
Abstract: 250
Introduction: 726
Discussion: 1705

**Number of figures:** 7
Number of supplemental figures: 4

**Acknowledgements:**
Supported by NIH R01 AG055328 (HES, SDG), MH109305 (HES) and AG077103 (SDG) and the New York State Office of Mental Health (HES, JJL). We thank Dr. Christos Lisgaras for comments on the manuscript and Dr. Korey Kam for contributions at an early stage of this project.

**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 principal cells of the dentate gyrus (DG), granule cells (GCs). 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 restored. Spatial memory improved. In contrast, offspring exposed to the relatively low choline diet had several adverse effects, such as increased mortality. 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 negative consequences. This is the first study showing that dietary choline can regulate hyperexcitability, hilar neurons, 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 & 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 (for review see Meck & 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). The recommendation to use MCS is based primarily on data from the Ts65Dn mouse model of Down syndrome (DS) and AD, where 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).

Based on the studies of Ts65Dn mice, we asked if MCS would improve another impairment that has been found in AD patients and mouse models of AD, hyperexcitability. We chose to study Tg2576 mice because hyperexcitability is robust, and BFCNs are likely to play a role (Kam et al. 2016, Lisgaras & 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. 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 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 & Lenck-Santini 2006, Kleen et al. 2010, Kleen et al. 2013, Gelinas et al. 2016) . Also, IIS in the Tg2576 mouse start early in life (by 1 month of age), before memory impairment, which occurs at 3-4 months of age (Duffy et al. 2015). Therefore, IIS might disrupt activity sufficiently to promote behavioral impairments. 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, and a related task, novel object recognition (NOR; Vogel-Ciernia & Wood 2014). We also studied expression of NeuN, a neuronal marker that is reduced in numerous disease conditions (Buckingham et al. 2008, Kadriu et al. 2009, Matsuda et al. 2009, Duffy et al. 2011, Duffy et al. 2015). Here we report 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 & Myers 2012).

We fed dams one of three diets, which were relatively low, intermediate or high in choline. The high choline diet provided levels of choline similar to other

141 studies of MCS in rodents (Meck et al. 1988, Loy et al. 1991, Holler et al. 1996,
142 Meck & Williams 1999, Sandstrom et al. 2002, Mellott et al. 2004, Glenn et al.
143 2012, Kelley et al. 2019). After weaning the intermediate diet was used. Offspring
144 were implanted with electrodes for EEG at 1 month of age, and 24 hr-long
145 recordings began 1/month starting at 1 week after surgery. NOL and NOR were
146 tested at 3 months and again at 6 months, and then we perfusion-fixed mice,
147 sectioned the brains, and evaluated ΔFosB and NeuN protein expression (Figure
148 1A).
149 The results showed a remarkable effect of the high choline diet. IIS and
150 spatial memory were improved, as was ΔFosB and NeuN expression.
151 Interestingly, the relatively low choline diet had mixed effects, reducing IIS
152 frequency, but making memory, ΔFosB, and NeuN worse. The mice also died
153 prematurely. In summary, we make a strong argument for choline
154 supplementation in early life to improve outcomes in an AD model, and
155 potentially AD.
156
157 **II. METHODS**
158
159 **A. Animals**
160 All experimental procedures followed the guidelines set by the National
161 Institute of Health (NIH) and were approved by the Institutional Animal Care and
162 Use Committee (IACUC) of the Nathan Kline Institute for Psychiatric Research.
163 Mice were housed in standard mouse cages with corn cob bedding and a 12-hr
164 light-dark cycle. Food and water were provided *ad libitum*.
165 Tg2576 mice express a mutant form of human APP (isoform 695) with a
166 mutation found in a Swedish cohort (Lys670Arg, Met671Leu). The expression is
167 driven by the hamster prion protein promoter. Mice were bred in-house from
168 heterozygous Tg2576 males and non-transgenic female mice (C57BL6/SJL F1
169 hybrid). Wildtype (WT) mice were obtained from Jackson Laboratories (Stock#
170 100012). At weaning, genotypes were determined using a protocol to detect
171 APP695.
172 Breeding pairs were randomly assigned to receive one of three diets with
173 different concentrations of choline chloride: 1.1 g/kg (AIN-76A, Dyets), 2.0 g/kg
174 (Purina 5008, W.F. Fisher and Son Inc.) and 5.0 g/kg (AIN-76A, Dyets;
175 Supplemental Table 1). These diets (relatively “low choline” diet, “intermediate
176 choline” diet and “high choline” diet, respectively) were used until weaning (25-30
177 days of age). After weaning, all mice were fed the intermediate diet. Mice were
178 housed with others of the same sex (1-4 per cage).
179
180 **B. Behavior**
181 **1.** *General information.*
182 Mice were housed in the room where they would be tested behaviorally
183 starting at least 24 hr prior to all experiments. Both NOL and NOR included three
184 acclimations (5 min each) followed by a training and a testing session (5 min
185 each; Figure 1B-D). The interval between training and testing was 60 minutes
186 and therefore tested short-term memory (Vogel-Ciernia & 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 a standard rat cage as described above. 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 offline. Exploration was quantified based on the guidelines of Vogel-Ciernia & Wood (2014), but in addition to time spent exploring, the number of approaches to an object was also quantified. Time spent exploring each object was defined as the duration of time the nose was

233 pointed at the object and the nose was located within 2 cm of the object. In
234 addition, exploration time included the time animals spent on top of the object if
235 they were looking down and sniffing it. An approach was defined by a movement
236 toward the object and ending at least 2 cm from the edge of the object.
237 Animals that remembered the objects explored during the training phase
238 were expected to demonstrate an increased preference for novelty in the test
239 phase (Ennaceur & Delacour 1988). In other words, exploration of the novel
240 object during testing was expected to be higher than 50% of the total time of
241 object exploration.
242
243 **C. Anatomy**
244 **1.** *Perfusion-fixation and sectioning.*
245 Mice were initially anesthetized by isoflurane inhalation (NDC# 07-893-1389,
246 Patterson Veterinary), followed by urethane (2.5 g/kg; i.p.; Cat# U2500, Sigma-
247 Aldrich). Under deep anesthesia, the abdominal and heart cavities were opened
248 and the animal was transcardially-perfused using 10 mL of room temperature
249 (RT) saline (0.9% sodium chloride in double distilled (dd) $H_2O$) using a peristaltic
250 pump (Minipuls1; Gilson), followed by 30 mL of 4°C 4% paraformaldehyde (PFA,
251 Cat# 19210, Electron Microscopy Sciences) in 0.1 M phosphate buffer (PB; pH
252 7.4). The brains were immediately extracted, hemisected, and post-fixed for at
253 least 24 hr in 4% PFA (at 4°C).
254 Following post-fixation, the left hemisphere was sectioned in the coronal
255 plane and the right hemisphere was sectioned in the horizontal plane (50 µm-
256 thick sections) using a vibratome (VT 1000P, Leica Biosystems). Sections were
257 collected serially and stored at 4°C in 24-well tissue culture plates containing
258 cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% 0.1 M
259 phosphate buffer, pH 6.7). Throughout the hippocampus, every 6th section was
260 processed with an antibody to NeuN or ΔFosB.
261
262 **2.** *NeuN and ΔFosB immunohistochemistry*
263 Free floating sections were washed in 0.1 M Tris Buffer (TB; 3 x 5 min),
264 followed by a 3 min wash in 1% $H_2O_2$. Sections were then washed in 0.1 M TB (3
265 x 5 min) and incubated for 60 min in 5% horse serum for NeuN (Cat# S-2000,
266 Vector) or 5% goat serum for ΔFosB (Cat# S-1000, Vector), diluted in a solution
267 of 0.25% (volume/volume or v/v) Triton-X 100, and 1% (weight/volume or w/v)
268 bovine serum albumin in 0.1 M TB. Sections were then incubated overnight at
269 4°C in primary antibody to NeuN (mouse monoclonal, 1:5000; Cat# MAB377,
270 Chemicon International) or anti-ΔFosB (rabbit monoclonal, 1:1000; Cat# D3S8R,
271 Cell Signaling), diluted in a solution of 0.25% (v/v) Triton-X 100, and 1% (w/v)
272 bovine serum albumin in 0.1M TB. Both NeuN and ΔFosB have been well-
273 characterized as antigens (Mullen et al. 1992, Wolf et al. 1996, Chen et al. 1997,
274 Sarnat et al. 1998) and the antibodies we used have been commonly employed
275 in the past (Chen et al. 1997, Duffy et al. 2013, Corbett et al. 2017).
276 On the following day, sections were washed in 0.1 M TB (3 x 5 min) and
277 then incubated for 60 min in biotinylated horse anti-mouse secondary antibody
278 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).

**2.** *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 to identify the dark cells but not the background slide using A slider was pushed from the low level of staining corresponding to background to the location where staining intensity makes a sharp rise, reflecting strongly stained cells. Below that was considered background and above that was defined as above threshold.

To count ΔFosB-stained cells, images were converted to grayscale and in each section, the dentate gyrus GCL was outlined and defined as a region of interest (ROI). A threshold was then set to identify the dark cells but not the background, analogous to the method to define threshold in the preceding paragraph. Two thresholds were used. The first threshold was most inclusive of immunoreactive cells. Presumably this group reflected cells with less neuronal activity. Then counts were made a second time with a higher threshold. This group probably 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). 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 six 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 eight-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$^{o}$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 hr 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 eight-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 four 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). 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 four 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). P-value was set to <0.05 prior to all experiments. 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 unless they were significant.

For the analysis of IIS frequency, a repeated measures ANOVA was not possible because animal numbers were not the same at all ages. This was due to mortality in low choline-treated mice or other unidentified reasons. Therefore, a mixed effects analysis was conducted.

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. Tests were conducted using Prism (V 9.4.1, Graphpad Software).

398 **III. RESULTS**
399
400 **A. Behavior**
401 NOL and the NOR, unlike other tasks previously used to evaluate cognitive
402 deficits in >9 months-old Tg2576 mouse model (i.e., Morris Water Maze, Radial
403 Arm Water Maze, Y-Maze; Yassine et al. 2013, Wolf et al. 2016), provided an
404 opportunity to capture cognitive deficits in animals as young as three months-old
405 (Duffy et al. 2015). Figure 1B-D shows a schematic of the specific experimental
406 procedures for NOL and NOR.
407
408 **1.** *NOL*
409 The results of the NOL task are presented in Figure 2. Exploration of the
410 novel object is expressed as a percentage of the total exploration of both objects.
411 During the training session, animals did not show preference for one object over
412 the other and this result was independent of genotype. Thus, a two-way ANOVA
413 revealed no main effects of genotype on novel object exploration during training
414 at three ($F(1,97) = 0.40$, $p = 0.53$) or six months of age ($F(1,70) = 1.94$, $p = 0.17$).
415 There also was no effect of maternal diet on training at three ($F(2,97) = 0.06$, $p =$
416 $0.94$) or six months of age ($F(2,70) = 2.94$, $p = 0.06$).
417 Next, we asked whether Tg2576 mice were deficient in the test phase of
418 NOL. First we studied the 3 months-old mice. We used a two-way ANOVA with
419 genotype and task phase (training versus testing) as main factors. In animals that
420 received the intermediate diet, a two-way ANOVA revealed a significant main
421 effect of the phase of the task (training or testing; $F(1,24) = 7.48$, $p = 0.01$).
422 Consistent with previous studies (Duffy et al. 2015), post-hoc analyses revealed
423 a significant increase in the exploration of the novel object during testing in WT ($p$
424 $= 0.02$) but not Tg2576 mice ($p = 0.50$; Figure 2A2). Thus, Tg2576 mice were
425 impaired in NOL when fed the intermediate diet.
426 In animals that received the high choline diet, a two-way ANOVA revealed a
427 main effect of the task phase ($F(1,36) = 48.23$, $p < 0.001$). Post-hoc analyses
428 revealed a significant increase in the exploration of the novel object during
429 testing in both WT ($p < 0.001$) and Tg2576 mice ($p < 0.001$; Figure 2A3).
430 Therefore, high choline supplementation improved memory in Tg2576 mice.
431 In animals treated with the low choline diet, there was no effect of phase of
432 the task ($F(1,37) = 1.39$, $p = 0.25$). These data suggesting that relatively low
433 levels of choline during early life impaired spatial memory in both WT and
434 Tg2576 mice (Figure 2A). Thus, low choline had a significant adverse effect.
435 Adverse effects are further supported by survival plots showing that there was
436 more mortality at earlier ages in offspring exposed to the low choline diet
437 (Supplemental Figure 1).
438 Figure 2B shows the results in 6 months-old WT and Tg2576 mice. In
439 animals that received the intermediate diet, a two-way ANOVA showed no main
440 effect of genotype ($F(1,18) = 0.01$, $p = 0.92$) or task phase ($F(1,18) = 3.40$, $p =$
441 $0.08$), revealing age-related memory deficits even in WT mice at six months of
442 age (Figure 2B). In contrast, the high choline group showed a main effect of task
443 phase ($F(1,29) = 28.32$, $p < 0.001$). Post-hoc analyses showed a significant

increase in the exploration of the novel object during testing in both WT ($p < 0.001$) and Tg2576 ($p = 0.02$; Figure 2B). In animals that received the relatively low choline diet, there was no effect of task phase ($F(1,25) = 3.00$, $p = 0.10$; Figure 2B). Thus, low choline had adverse effects on NOL in both WT and Tg2576 mice.

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. A two-way ANOVA revealed no main effects of genotype at three months ($F(1,71) = 2.59$, $p = 0.54$) and no effect of diet ($F(2,71) = 0.22$, $p = 0.81$). The same result was obtained at six months of age (genotype: ($F(1,66) = 0.11$, $p = 0.75$); diet: ($F(2,66) = 0.98$, $p = 0.38$). Thus, novel object exploration during training approached 50% in all treatment groups, independent of genotype and maternal diet, and at both ages.

Figure 3A shows the results of the testing phase of the NOR task in 3 months-old WT and Tg2576 mice. In animals that received the intermediate choline diet, a two-way ANOVA revealed a significant main effect of task phase ($F(1,19) = 40.53$, $p < 0.01$) and no effect of genotype ($F(1,19) = 0.78$, $p = 0.39$). Post-hoc analyses revealed a significant increase in the exploration of the novel object during testing compared to training for both WT ($p < 0.01$) and Tg2576 ($p < 0.01$; Figure 3A). These results suggest that Tg2576 did not have a deficit in NOR at 3 months of age.

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 ($F(1,26) = 17.51$, $p < 0.01$) but not genotype ($F(1,26) = 3.53$, $p = 0.07$). Post-hoc analyses revealed a significant increase in the exploration of the novel object during testing in both WT ($p < 0.01$) and Tg2576 ($p = 0.03$; Figure 3A).

In animals treated with the low choline diet in early life, there was no impairment in NOR at 3 months of age (task phase ($F(1,26) = 22.61$, $p < 0.01$; genotype ($F(1,26) = 0.02$, $p = 0.89$). There was a significant increase in the exploration of the novel object in testing both in WT ($p = 0.03$) and Tg2576 ($p < 0.01$).

Figure 3B shows that there were no impairments at 6 months of age. In animals that received the intermediate diet, a two-way ANOVA revealed a significant main effect of task phase ($F(1,19) = 13.65$, $p < 0.01$) but no main effect of genotype ($F(1,19) = 0.28$, $p = 0.60$). Animals explored the novel object significantly more during testing in WT ($p = 0.03$) and Tg2576 mice ($p = 0.04$; Figure 3B). In animals that received the other diets, results were the same. For the high choline group, there was an effect of task phase ($F(1,27) = 16.26$, $p < 0.01$) but not genotype ($F(1,27) = 0.24$, $p = 0.63$) and a significant increase in the exploration of the novel object during testing in both WT ($p = 0.04$) and Tg2576 ($p < 0.01$; Figure 3B). Similarly, in animals treated with the low choline diet, there was a main effect of task phase ($F(1,26) = 32.66$, $p < 0.01$), no effect of genotype

(F(1, 6) = 0.05, p = 0.82), and more exploration of the novel object during the test phase (WT, p < 0.01; Tg2576, p < 0.01).

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 dentate gyrus contributes to NOL (Sahay et al. 2011, Kesner et al. 2015, Spyrka & Hess 2018, Vandrey et al. 2020, Gulmez Karaca et al. 2021, GoodSmith et al. 2022), and early signs of adverse effects in the dentate gyrus in mouse models of AD (impaired LTP; Jacobsen et al. 2006, altered gene expression; Palop et al. 2011). Our implementation of NOL may have increased the dentate gyrus-dependence of the task by making the object locations relatively close together, because the dentate gyrus is critical to distinguishing small differences in locations. In studies by Pofahl and colleagues, it was shown that distances between objects like those we used made NOL dentate gyrus-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 4).

a. 3 months of age

For NOL 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; Figure 4A). 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; Figure 4C).

b. 6 months of age

*1) Exploration*

NOL and NOR showed significant effects of diet on exploration at 6 months of age (Figure 4B,D). For NOL, there was no effect of genotype (F(1,66) = 0.33; p = 0.57) but there was an effect of maternal diet (F(2,66) = 8.82; p = 0.0004). For NOR, the same was true (genotype: F(1,72) = 0.96, p = 0.33; diet: F(2,72) = 8.50, p = 0.0005). We then pooled genotypes to determine which diets differed. For NOL, mice treated with the relatively 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 4B) 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 4B). Results for NOR were the same: 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 = 0.02; Tukey post-hoc test, p = 0.02; Figure 4D) 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 4D).

536
537 *2) Approaches*
538 To gain insight into the potential reason for differences in exploration, we
539 measured total object approaches (TOA; Figure 4A-D). TOA was defined as the
540 number of approaches to the familiar object + number of approaches to the novel
541 object. There were no differences by genotype (two-way ANOVA, $F(1,73) =$
542 $2.930$, $p = 0.09$) or maternal diet ($F(2,73) = 2.36$, $p = 0.10$; Figure 4A-D).
543 Taken together, the results indicate that, in 6 months-old mice, animals that
544 received the high choline diet spent more time with objects at each approach.
545 This may underlie increased object location memory in high choline-treated mice
546 by increasing time for information processing during an approach. It may also
547 help in the NOR task with object recognition memory, although it did not seem
548 impaired in mice fed other diets. Another possibility is that as Tg2576 mice age
549 they show compensatory changes that enhance memory. This has been
550 suggested for procedural learning (Middei et al. 2004) but not for memory of
551 objects. A third possibility is that the high choline diet reduces anxiety (Glenn et
552 al. 2012, Langley et al. 2015, McCall et al. 2015). Reduced anxiety may lessen
553 fear of exploring objects, and as a result, animals may spend more time with
554 objects.
555 For 3 months-old mice there were no effects of maternal diet on exploration,
556 suggesting that increased time with objects for a given approach is a change that
557 develops with age. To our knowledge, this age-related change has not been
558 previously reported. It could be a strategy to cope with impairments that are
559 developing as AD pathology manifests.
560
561 **B Anatomy**
562 **1. NeuN**
563 To further analyze the effects of dietary choline early in life, we used an
564 antibody against a neuronal nuclear antigen, NeuN. Previous studies have found
565 that reduced expression of NeuN often occurs in neurons after insults or injury
566 (e.g., ischemia, toxicity and even aging; Lind et al. 2005, Portiansky et al. 2006,
567 Buckingham et al. 2008, Kadriu et al. 2009, Matsuda et al. 2009, Won et al.
568 2009, Duffy et al. 2011). For example, when NeuN is reduced after a reduction in
569 ankyrin-rich membrane spanning kinase D-interacting substrate of 220K,
570 ARMS/Kidins, a protein critical to neurotrophin signaling, neurons in the
571 entorhinal cortex demonstrate signs of toxicity such as pkynosis (Duffy et al.
572 2011, Duffy et al. 2015). Since NeuN is reduced when A$\beta$ levels are elevated
573 (Wu et al. 2016) and NeuN is reduced in AD patients (Camporez et al. 2021), we
574 used NeuN expression to ask if the high choline diet could rescue the loss of
575 NeuN-ir.
576 As shown in Figure 5, relatively weak NeuN-ir was observed in Tg2576
577 animals in the hilus, an area known to be highly vulnerable to insult and injury
578 (Scharfman 1999). The most NeuN loss appeared to be in mice that received the
579 low choline diet and the intermediate diet compared to the high choline diet
580 (Figure 5B). Therefore, we conducted a two-way ANOVA with diet and
581 septotemporal levels as main factors. To examine the septotemporal axis,

quantification from 2-3 dorsal coronal sections were averaged to provide a value for dorsal DG and 2-3 ventral horizontal sections were averaged for a ventral value. There was no effect of diet ($F(2,30) = 2.11$, $p = 0.14$) nor septotemporal level ($F(1,30) = 0.02$, $p = 0.88$) but there was a significant interaction of diet and septotemporal level ($F(2,30) = 3.88$, $p = 0.04$). A one-way ANOVA using dorsal values showed a significant effect of diet ($F(2,21)= 7.58$, $p = 0.003$) but this was not the case for ventral values ($F(2,23) = 0.13$; $p = 0.88$; Figure 5D). In the dorsal DG, there was less NeuN-ir in mice fed the low choline diet versus the high choline diet (Tukey-Kramer post hoc test, $p = 0.03$) and less NeuN in mice fed the intermediate vs high choline diet ($p = 0.003$; Figure 5D). The results suggest that choline enrichment protected hilar neurons from NeuN loss in Tg2576 mice.

**2. ΔFosB**

To complement the information from the video-EEG recordings we used a marker of elevated neuronal activity, ΔFosB (Figure 6). 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 (epilepsy; 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.

There was strong expression of ΔFosB in Tg2576 GCs especially in mice fed the low choline diet (Figure 6). Two-way ANOVA showed a main effect of maternal diet ($F(2,18) = 3.76$, $p = 0.04$) but there was not a main effect of septotemporal level ($F(1,18) = 1.3$, $p = 0.26$).

To clarify the effect of maternal diet, a one-way ANOVA was conducted. There was an effect of diet ($F(2,9) = 10.74$, $p = 0.004$) with post-hoc tests showing significantly less GC ΔFosB expression in high choline-treated mice compared to low choline ($p = 0.003$). Low choline-treated mice were not significantly different from mice that were fed the intermediate diet ($p = 0.08$). These data used dorsal sections so we repeated the analysis for the ventral DG, and there also was no significant effect of diet ($F2,9) = 0.79$, $p = 0.52$; Figure 6).

ΔFosB quantification was repeated with a lower threshold to define ΔFosB-ir GCs (see Methods) and results were the same (Figure 6). One-way ANOVA showed a significant effect of diet ($F(2,41) = 14.66$, $p < 0.0001$), and low choline mice had greater ΔFosB-ir than high choline mice ($p =0.003$). Mice fed the intermediate diet had higher expression than mice fed the high choline diet ($p = 0.0001$). These data suggest that high choline in the diet early in life can reduce hyperexcitability of GCs in offspring later in life. In addition, low choline has an opposite effect, again suggesting this maternal diet has adverse effects.

628 **C. IIS**

629 Previous research has shown that Tg2576 mice exhibit IIS (Figure 7A)
630 starting at very young ages: 4 weeks (Kam et al. 2016) or 6 weeks (Bezzina et al.
631 2015). Therefore, we performed video-EEG using cortical (left frontal cortex, right
632 occipital cortex) and hippocampal (left and right) electrodes in WT and Tg2576
633 mice (Figure 7B-C). Animals were recorded for 24 hr each session so that there
634 was sufficient time to capture major behavioral states (exploration, awake rest,
635 and sleep). Consistent with previous studies (Bezzina et al. 2015, Kam et al.
636 2016) we observed IIS in Tg2576 mice but not WT littermates.

637 As shown in Figure 7, animals that received the intermediate diet had a
638 significantly higher number of IIS in the 24 hr recording periods compared to
639 animals that received the high choline and the low choline diets. A mixed model
640 analysis showed that there was a significant effect of age ($F(2,37) = 3.38$; $p =$
641 $0.04$) and maternal diet ($F(2,36) = 8.12$; $p = 0.01$). At the 1.25-month recording,
642 Tukey post-hoc analyses showed that IIS frequency in animals treated with the
643 intermediate diet was higher compared to animals treated with the low ($p = 0.03$)
644 or high choline diets ($p = 0.04$). This also was true for 2 months and 3 months
645 (low choline, high choline, $p < 0.05$). At 4 months, the low choline groups had
646 significantly reduced IIS frequency compared to the intermediate diet ($p = 0.009$)
647 but not the high choline group ($p = 0.98$). At 5-6 months, IIS frequencies were not
648 significantly different in the mice fed the maternal diets (all $p > 0.05$).

649 As shown previously (Kam et al. 2016), and in Figure 7, at 4-6 months of age
650 there was high variability in IIS frequency between animals. It is possible that IIS
651 frequency becomes more variable because we found that seizures occur in the
652 Tg2576 mice after 2 months of age (Kam et al. 2016), and there was a sharp
653 increase in IIS during the day before and after a seizure (Supplemental Figure 3).
654

655 **D. Seizures and premature mortality in mice fed the low choline diet**

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

664 These values are probably an underestimate for the low choline group
665 because many mice in this group appeared to die in a severe seizure prior to 6
666 months of age (Supplemental Figure 2). Therefore, the survivors at 6 months
667 probably were the subset with few seizures.

668 The reason that low choline-treated mice appeared to die in a seizure was
669 that they were found in a specific posture in their cage which occurs when a
670 severe seizure leads to death (Supplemental Figure 2). They were found in a
671 prone posture with extended, rigid limbs (Supplemental Figure 2). Regardless of
672 how the mice died, there was greater mortality in the low choline group compared
673 to mice that had been fed the high choline diet (Supplemental Figure 2).

674 **E. Sex differences**
675 As shown in Figures 2-3, there appeared to be no sex differences in NOL or
676 NOR. For confirmation, we studied NOL further, since that was the task that
677 showed effects of diet. A three-way ANOVA with sex, genotype and task phase
678 as factors showed no significant effect of sex at three months of age (low choline
679 diet, $F(1,35) = 0.0001$; $p = 0.99$). Results were the same for the three months-old
680 mice fed the intermediate diet ($F(1,22) = 0.062$; $p = 0.81$) and three months-old
681 mice that were fed the high choline diet ($F(1,34) = 0.43$; $p = 0.52$). At six months
682 of age there also were no significant effects of sex (low choline diet, $F(1,23) =$
683 $0.34$; $p = 0.57$; intermediate diet, $F(1,16) = 2.49$; $p = 0.13$; high choline diet,
684 $F(1,27) = 0.29$; $p = 0.87$).
685 Regarding IIS, we did not find sex differences (Supplemental Figure 4). A
686 two-way ANOVA with sex and diet as factors showed no effect of sex ($F(1,20)$
687 $=2.48$; $p = 0.13$) or diet ($F(1,20) = 3.56$; $p = 0.07$). Note that for this comparison
688 we used the low choline and high choline groups only because sex information
689 was only available for these mice. The results for months 2-3 also showed no
690 effect of sex (2 months: $F(1,17) = 0.64$; $p = 0.43$; 3 months: $F(1,15) = 0.19$; $p =$
691 $0.67$).
692
693 **IV. DISCUSSION**
694
695 **Summary**
696 This study showed that early life choline supplementation had several
697 beneficial effects in Tg2576 mice. The high choline diet led to reduced IIS
698 frequency, improved behavior in the NOL task, and improved expression of
699 ΔFosB.
700 We showed for the first time that Tg2576 mice have pathology in the hilus at
701 six months of age in that hilar neurons are deficient in immunostaining with an
702 anti-NeuN antibody. Tg2576 offspring on the high choline diet showed a
703 restoration of hilar NeuN staining. Because hilar neurons play a role in NOL (Bui
704 et al. 2018), it is possible that rescue of hilar neurons by the high choline diet led
705 to improved spatial memory. Hilar neurons have been suggested to contribute to
706 excitability of GCs (Sloviter 1994, Scharfman 1999, Scharfman & Myers 2012,
707 Jinde et al. 2013, Bui et al. 2018, Botterill et al. 2019), so phenotypic rescue of
708 hilar neurons may also have contributed to the ability of the high choline diet to
709 reduce hyperexcitability.
710 We also found surprising effects of treating mice with relatively low choline in
711 the maternal diet. On the one hand, IIS frequency was reduced. However, these
712 mice showed premature mortality. Furthermore, in WT mice where no
713 impairments in NOL normally occur, WT mice fed the low choline diet were
714 impaired. In Tg2576 mice, the group that had the low maternal choline diet had
715 the highest ΔFosB expression and the lowest NeuN expression of any
716 experimental group. Potential reasons for these surprising effects are discussed
717 below.
718
719

720 **Benefits of high choline**
721 The results of this study are consistent with previous reports that prenatal or
722 postnatal choline supplementation improves object memory. One study of iron
723 deficiency showed that choline supplementation improved NOR (Kennedy et al.
724 2014). Wistar rats treated with a diet high in choline had improved NOR relative
725 to rats that had been fed a diet low in choline (Moreno et al. 2013). In rats that
726 were aged to 24 months, animals that had been fed a high choline diet showed
727 improved NOR, but only in females (Glenn et al. 2008).
728 Although few studies have examined the effects of MCS on hyperexcitability,
729 our results showing the benefits of high choline are consistent with reports that a
730 methyl-enriched diet (including choline but also betaine, folic acid, vitamin B12,
731 L-methionine and zinc) reduced spike wave discharges in the offspring
732 (Sarkisova et al. 2023) and audiogenic seizure severity (Poletaeva et al. 2014).
733 Perinatal ethanol treatment that increased excitability was mitigated by choline
734 chloride injection from postnatal day 10-30 (Grafe et al. 2022).
735 To our knowledge our study is the first to show that MCS can exert effects on
736 IIS and ΔFosB. However, prior studies have shown that MCS in rats improved
737 memory after severe seizures. Using a convulsant to induce several hours of
738 severe seizures (status epilepticus, SE), there is usually extensive hippocampal
739 neuronal loss and when tested in the subsequent days and weeks, memory
740 impairment. MCS reduced this impairment following SE in the Morris water maze
741 (Yang et al. 2000, Holmes et al. 2002, Wong-Goodrich et al. 2011).
742 Our results are consistent with previous studies of mouse models of AD. In
743 the APP/PS1 mouse model, it was shown that life-long choline supplementation,
744 to the level we used (5.0 g/kg) improved memory in aged mice compared to the
745 1.1 g/kg diet (Velazquez et al. 2019). Postnatal choline supplementation also
746 improved memory in APP/PS1 mice (Wang et al. 2019). Other improvements
747 were also shown in the APP/PS1 mice, such as improved cholinergic expression
748 via upregulation of choline acetyltransferase (Mellott et al. 2017, Velazquez et al.
749 2019).
750
751 **Adverse effects of low choline**
752 The effects of the relatively low choline diet were surprising because several
753 adverse effects were found. This is unexpected because 1.1 g/kg choline is not
754 considered very low in several studies of the past, and some publications
755 consider it normal (e.g., Lamoureux et al. 2008). The majority of past studies
756 used rats (Meck et al. 1988, Meck & Williams 1999, 2003, Meck et al. 2007,
757 Lamoureux et al. 2008), however, so there could be a species difference. Also,
758 some of the past studies used the diet for a shorter timespan than we did (e.g.,
759 Lamoureux et al. 2008) because of evidence that perinatal choline
760 supplementation exerts its maximal effects during critical periods (Meck &
761 Williams 2003, Meck et al. 2007).
762 It was also surprising to find high mortality in mice exposed to the low choline
763 diet in early life. Also, the offspring of mothers fed the low choline diet had very
764 high ΔFosB expression in the GCs, suggesting they had chronic seizures. The
765 observation that mice died from seizures is consistent with the ΔFosB data.

766 There also was weak NeuN in the hilus. When WT mice were tested, NOL was
767 impaired in the low choline group. These data suggest that the offspring of
768 mothers fed the low choline diet were unhealthy, possibly leading to or because
769 of seizures.
770 On the other hand, IIS were reduced in frequency in the same mice. There
771 are several possible explanations for this apparent paradox. The most
772 parsimonious explanation is the mice were unhealthy. If there was disruption of
773 sleep, IIS might be reduced since IIS occur in sleep primarily. However, there is
774 no evidence that MCS influences sleep in humans or laboratory animals. The
775 one study that looked at the sleep/wake cycle in rats did not find effects (Cheng
776 et al. 2008).
777 A more radical explanation is that the intermediate diet promoted IIS rather
778 than low choline reducing IIS. The idea here is that choline is not critical to IIS
779 because both low and high choline groups had low IIS frequency compared to
780 the intermediate group, at least for 3 months of life. Instead of choline, a
781 constituent of the intermediate diet may have promoted IIS. The reason why the
782 effect of diet waned over time could have been due to the fact that after weaning
783 all animals had the intermediate diet.
784 More seizures could explain premature mortality in the low choline group.
785 However, why it would decrease IIS is not clear. One reason seizures could
786 cause IIS to be reduced is that seizures in the Tg2576 mice typically started
787 during sleep and woke mice from sleep. The arousal from sleep would interrupt
788 the behavioral state when IIS usually occur as mentioned above. Also, seizures
789 in the Tg2576 mice were followed by a depression of the EEG that would reduce
790 IIS (postictal depression; Supplemental Figure 3).
791
792 **NeuN**
793 As mentioned above, NeuN is a neuronal nuclear antigen that can be
794 phosphorylated, and when that occurs the antibody to NeuN no longer binds to
795 NeuN. NeuN is phosphorylated in response to oxidative damage, brain injury,
796 and toxicity (Lind et al. 2005). Therefore, it was of interest when we saw that hilar
797 neurons of the dentate gyrus showed reduced NeuN in Tg2576 mice. Hilar
798 neurons are mainly glutamatergic mossy cells and somatostatin
799 (SOM)/neuropeptide Y (NPY)-expressing GABAergic neurons (HIPP cells;
800 (Houser 2007, Scharfman & Myers 2012, Scharfman 2016), and both neuronal
801 types are implicated in spatial memory functions of the GCs, as well as their
802 excitability (Myers & Scharfman 2009, 2011, Scharfman & Myers 2012, Jinde et
803 al. 2013, Scharfman 2016, Raza et al. 2017, Bui et al. 2018, GoodSmith et al.
804 2019, Li et al. 2021). If damaged by the high intracellular APP/Aβ levels in young
805 Tg2576 mice, the hilar neurons would be expected to show NeuN loss. This idea
806 is consistent with deficits in SOM and NPY-stained cells (Chan-Palay 1987),
807 indicating a vulnerability.
808 The reason for rescue of dorsal hilar but not ventral hilar NeuN with the high
809 choline diet is unclear. However, there was a trend for the high choline mice to
810 have greater NeuN in ventral tissue sections. The greater sensitivity of dorsal
811 neurons may be related to differences in dorsal and ventral dentate gyrus gene

expression patterns, since some of the genes that are differentially expressed could affect vulnerability to insult or injury (Cembrowski et al. 2016, Zhang et al. 2018). Resilience of vulnerable neuronal populations, especially in the hippocampus, is a topic of high interest in AD research.

**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.

**Additional considerations**

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. Moreover, it may have promoted IIS as discussed above.

Although we failed to detect sex differences, there may actually have been differences if females had been analyzed according to their stage of the estrous cycle at death. This possibility is raised by prior data showing that rats and mice during diestrus have low excitability in hippocampal slices; at other cycle stages hyperexcitability occurs (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 of cerebral amyloid overexpression lacks tau pathology and recapitulates familial AD, whereas the majority of AD is sporadic. However, MCS has now demonstrated structural/functional benefits in several AD-relevant models, namely Tg2576 (this report), APP-PS1 (Mellott et al. 2017), and Ts65Dn (Powers et al. 2016, Strupp et al. 2016, Powers et al. 2017, Alldred et al. 2021, Alldred et al. 2023).

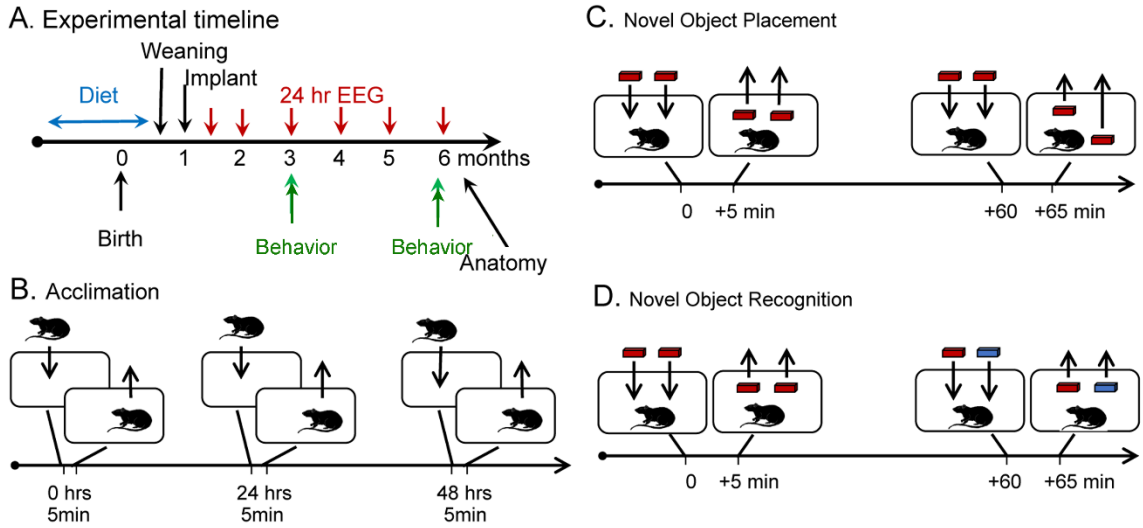
**Figure 1. Schematic of the behavioral procedures for the NOL and NOR tasks.**

**A.** Experimental timeline. Dams were fed one of three 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 hr 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 three acclimation sessions separated by 24hrs 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).



875 **Figure 2. Choline enrichment reduced spatial memory deficits.**
876 **A.** Three months-old mice.
877 **1.** WT and Tg2576 mice exposed to the low choline diet showed spatial memory
878 deficits.
879 **2.** Tg2576 exposed to the intermediate diet showed spatial memory deficits but
880 not WT.
881 **3.** Spatial memory was improved in animals exposed to the high choline diet.
882 **B.** Six months-old mice.
883 **1.** WT and Tg2576 mice exposed to the low choline diet showed spatial memory
884 deficits.
885 **2.** WT and Tg2576 mice exposed to the intermediate diet showed spatial
886 memory deficits.
887 **3.** WT and Tg2576 mice exposed to the high choline diet had improved spatial
888 memory.

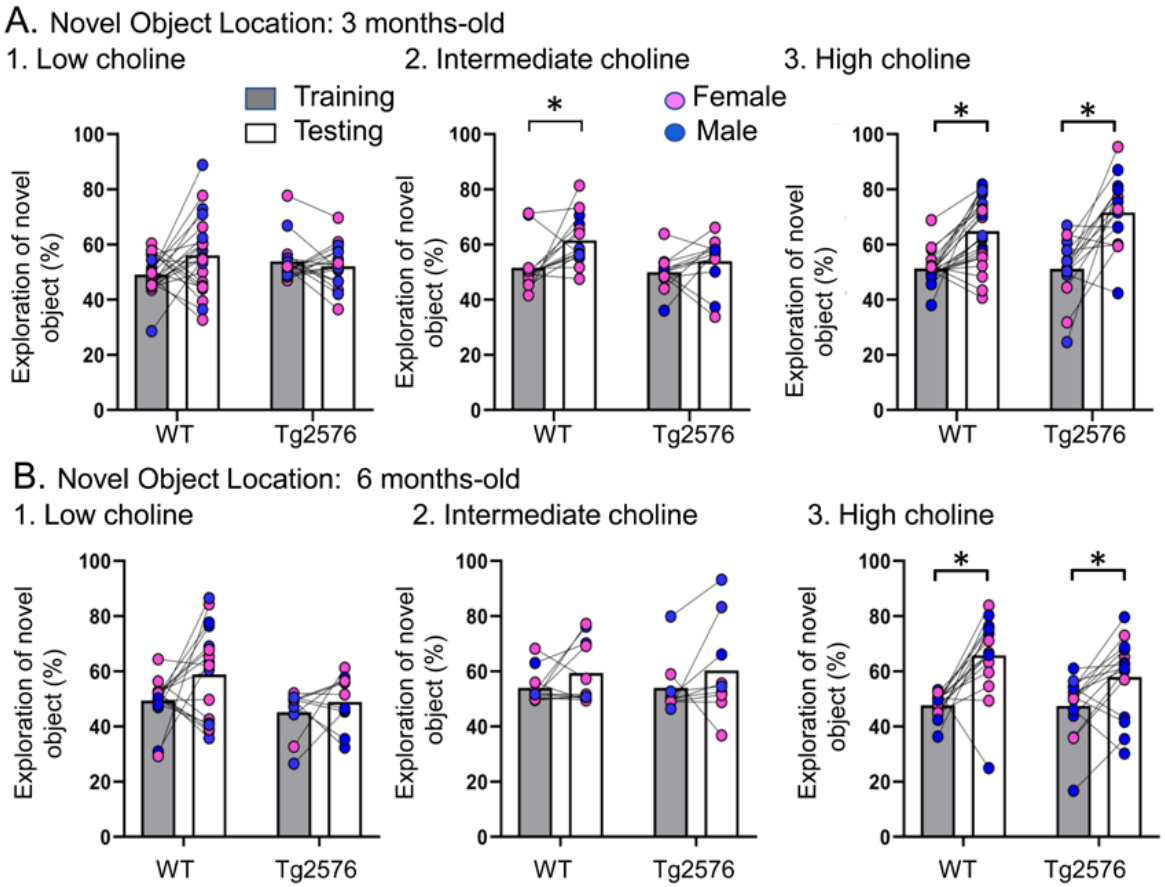

A. Novel Object Location: 3 months-old
1. Low choline
2. Intermediate choline
3. High choline
Training
Testing
Female
Male
Exploration of novel object (%)
WT
Tg2576
B. Novel Object Location: 6 months-old
1. Low choline
2. Intermediate choline
3. High choline

**Figure 3. WT and Tg2576 mice showed 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.

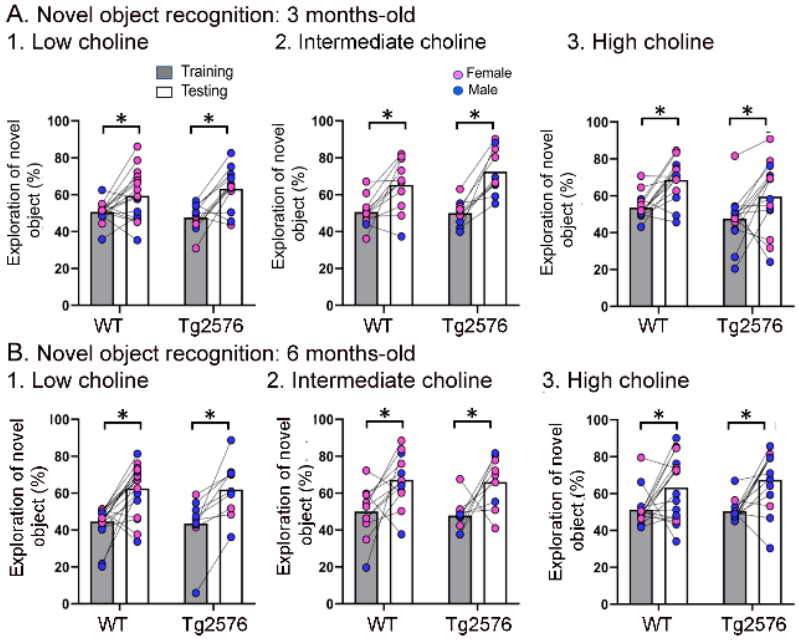


**Figure 4. Tg2576 mice treated with the choline supplemented diet spent more time with the objects at each approach.**

**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.

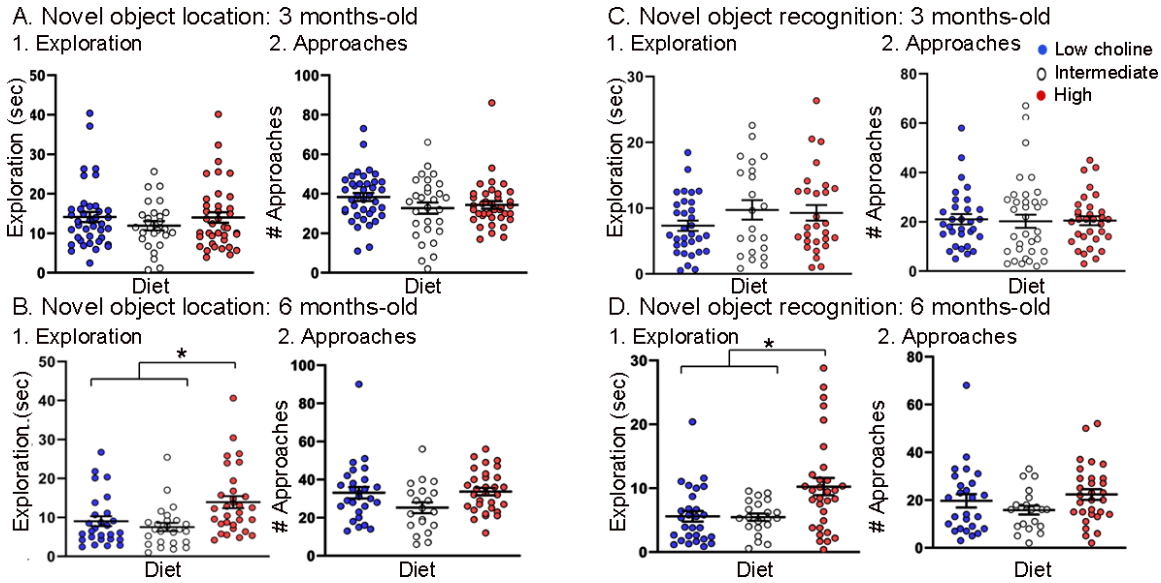


**Figure 5. Choline supplementation improves NeuN immunoreactivity (ir) in hilar cells in Tg2576 animals**

**A.** A sagittal view of the brain illustrating the coronal sectioning plane.

**B.** Representative images of NeuN-ir staining in the dorsal dentate gyrus of Tg2576 animals from each treatment group.

**C.** Quantification methods. Representative images demonstrating the thresholding criteria established to quantify NeuN-ir.

1. A NeuN-stained section shows an inset (arrow) expanded at the top left to show 3 hilar cells. NeuN-ir cells are marked by blue arrows. NeuN-ir that was too close to background staining to be considered positive is marked by a green arrow.

2. After converting the image to grayscale, a threshold was set so that all cells stained well with NeuN antibody were red. The inset shows that the two cells that were darkest met the threshold while the weakly stained cell did not.

**D.** Six months-old Tg2576 mice treated with the high choline diet had significantly more NeuN-ir cells in the hilus in the dorsal dentate gyrus (1) compared to other treatment groups but not ventral dentate gyrus (2).

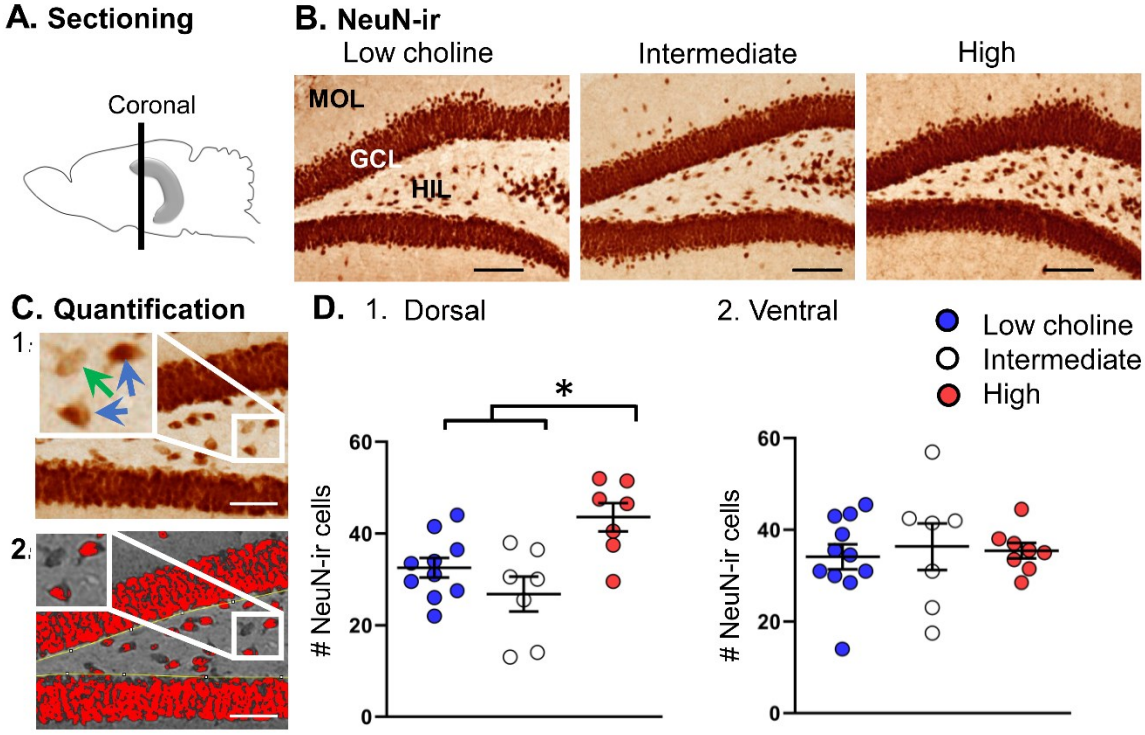


**Figure 6. Choline supplementation reduced ΔFosB expression in dorsal GCs of Tg2576 mice.**

**A.** A sagittal view of the brain illustrating the coronal sectioning plane.

**B.** Representative images of ΔFosB staining in the anterior dentate gyrus of Tg2576 animals from each treatment group.

**C.** 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.

**D.** Six months-old Tg2576 mice treated with the choline supplemented diet had significantly more ΔFosB -ir pixels above threshold in the dorsal dentate gyrus (1) compared to other treatment groups but not ventral dentate gyrus (2).

**E.** 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.

**F.** With the less conservative threshold, the results were the same as D. Mice that were fed the high choline diet had less ΔFosB-ir pixels than the mice that were fed the other diets. However, this was true for dorsal (1) but not ventral (2) sections.

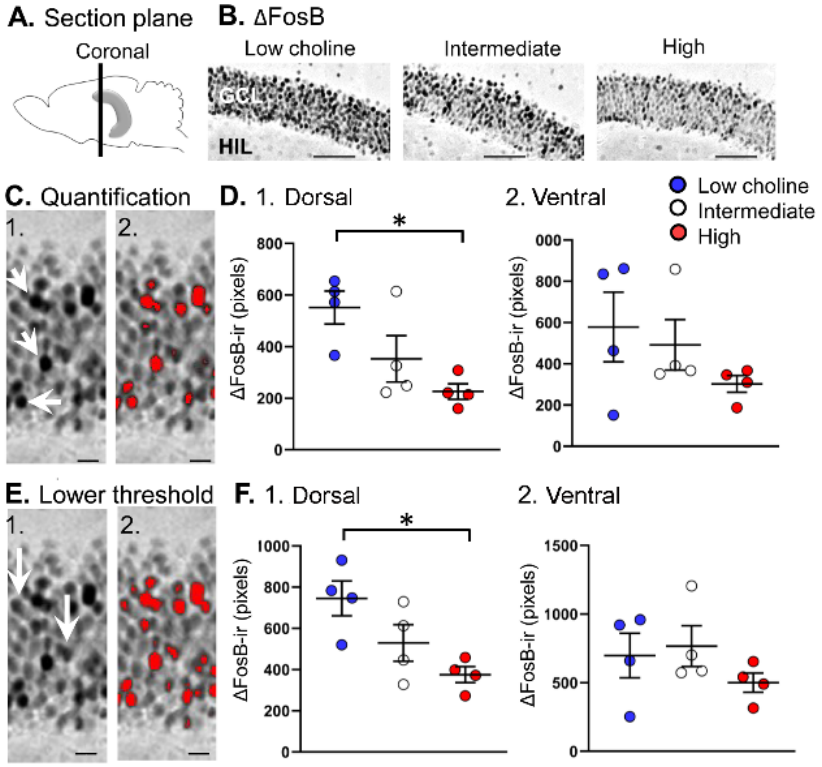
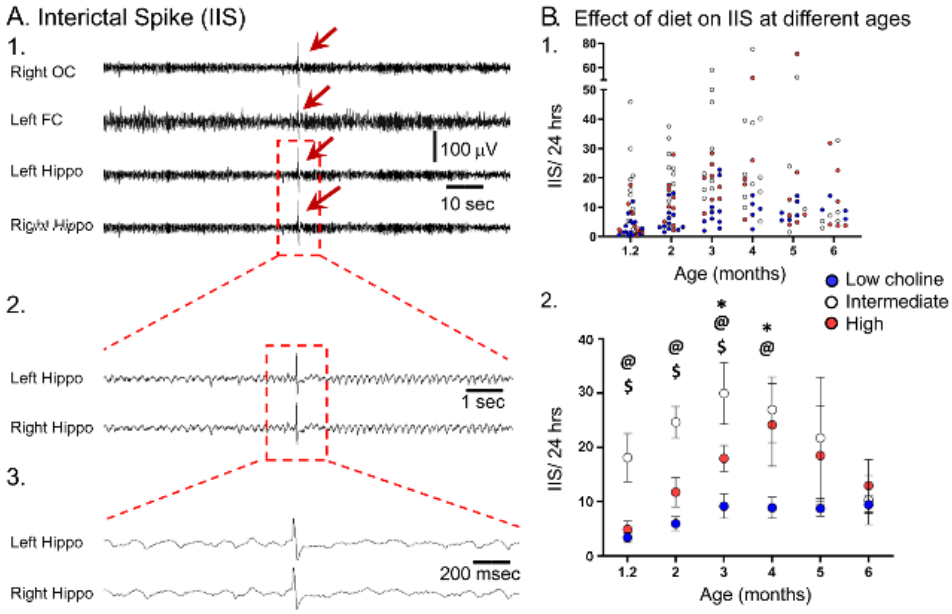


**Figure 7. The high choline diet reduced IIS frequency in Tg2576 animals.**
**A. 1.** Representative example of an IIS (red arrow). IIS were defined as occurring in all four channels (generalized) to distinguish them from focal spikes that can often be artifact.
2-3. The IIS shown in A1 is expanded.
**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 (*, $p<0.05$).



970 **Supplemental Table 1. Comparison of the three diets used in this study.**
971

| | 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 |

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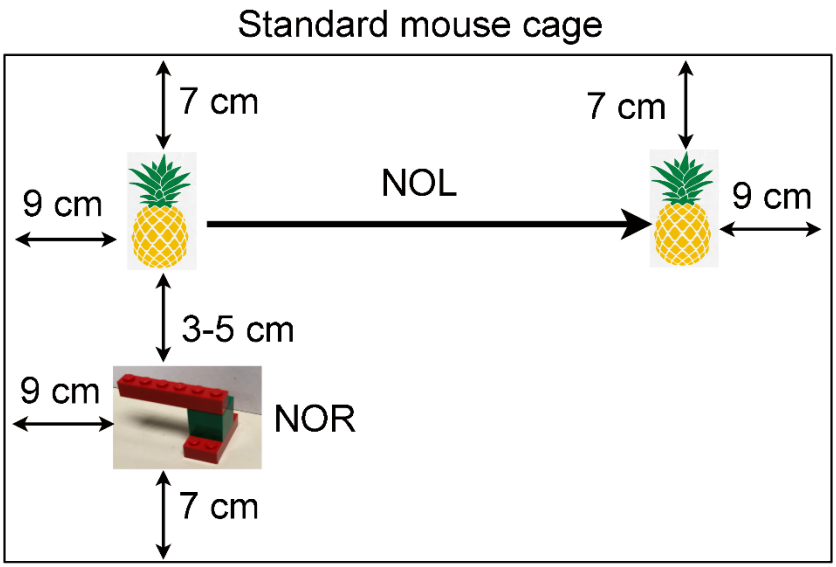
**Supplemental Figure 1. Details of behavioral tasks.**
A standard mouse cage is diagrammed with the locations of objects inside it. For NOL, there were two pineapple-like objects and one was moved across the cage for the test session. For the NOR test session, one pineapple-like object was removed and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

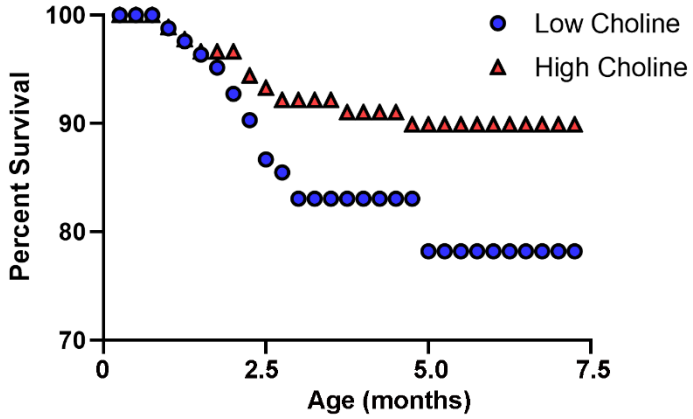


**Supplemental Figure 2. Mortality was high in mice treated with the low choline diet.**

**A.** Survival curves are shown for mice fed the low choline diet and mice fed the high choline diet.

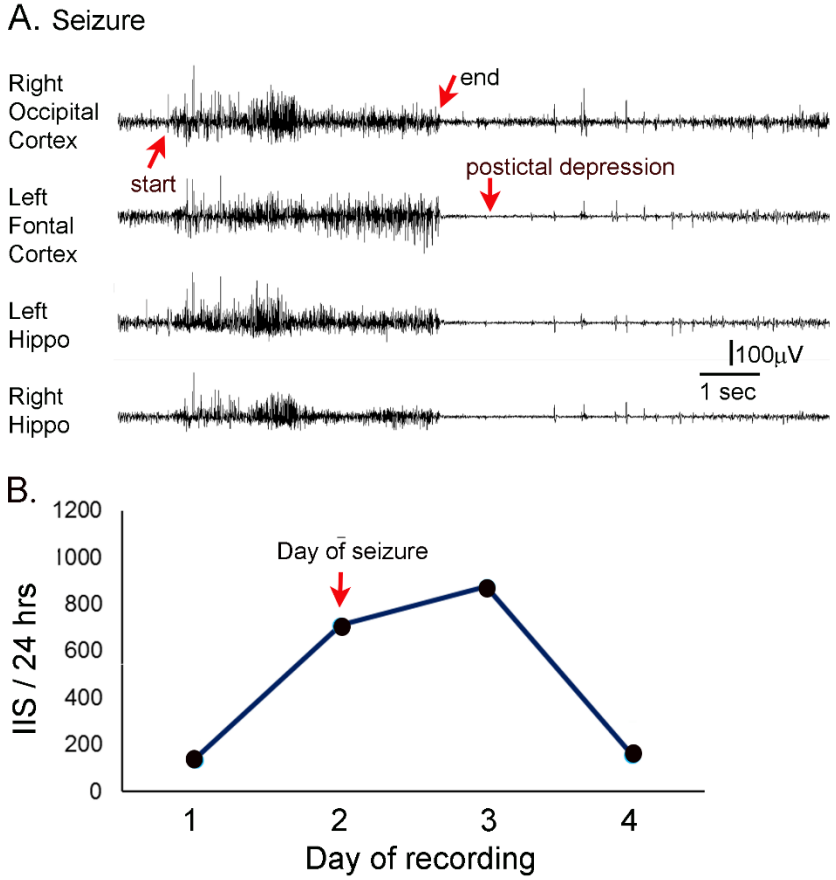
**B.** A photo of a mouse after sudden unexplained death. The mouse was found in a posture consistent with death during a convulsive seizure.

A.

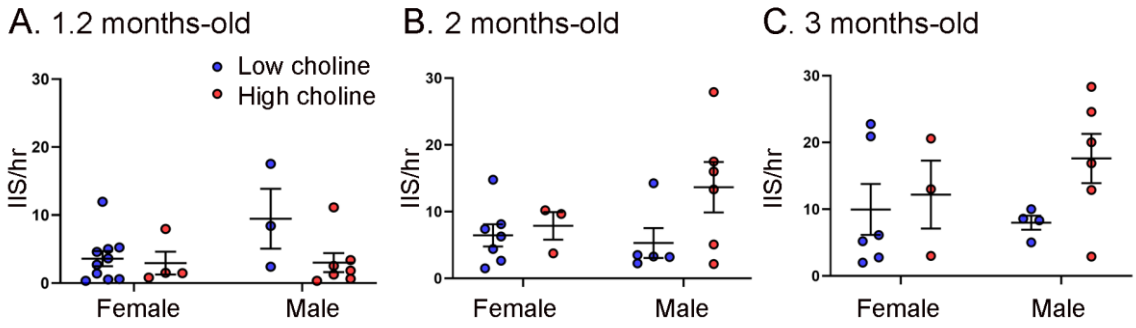



B.

**Supplemental Figure 3. IIS frequency before and after seizures.**
**A.** Representative EEG trace of a seizure in a 5 months-old Tg2576 mouse.
**B.** IIS frequency was quantified from continuous video-EEG for 4 days. During the first 24 hrs, IIS frequency was low. The next day a seizure occurred (arrow), and IIS frequency increased approximately 5 fold, and for the next 24 hr period. On day 4, IIS frequency returned to lower levels.



**Supplemental Figure 4. IIS frequency was similar for each sex.**
**A.** At 1.2 months-old, female and male Tg2576 mice were not significantly different in IIS frequency for the low and high choline diets.
**B-C.** There were no significant sex differences at 2 (B) and 3 (C) months of age.



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