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 are generalized EEG spikes (interictal spikes; IIS). IIS also are common in other mouse models and occur in AD patients. Im mouse models, hyperexcitability is also reflected by elevated expression of the transcription factor ∆FosB in the granule cells (GCs) of the dentate gyrus (DG), which are the principal cell type. Therefore we studied ΔFosB expression in GCs.

We also studied the the neuronal marker NeuN within hilar neurons of the DG because other studies have reduced NeuN protein expression is a sign of oxidative stress or other pathology. This is potentially important because hilar neurons regulate GC excitability.

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. Using the novel object location task, spatial memory improved. 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 protein expression. 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 (Bezzina et al. 2015; Kam et al. 2016; Lisgaras and Scharfman 2023), and BFCNs are likely to play a role in the hyperexcitabililty (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, Presenilin2$^{-/-}$, Lisgaras and Scharfman 2023; Amyloid precursor protein (APP) /Presenilin1 (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 reflected by high protein expression of ΔFosB, 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 longer-lasting epileptiform discharges, interictal epileptiform discharges (IEDs), are increased when cognitive dysfunction is impaired 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 were made every month shortly thereafter and ending at 6 months of age

The time span (1-6 months of age) is relevant because IIS are present at these ages (Bezzina et al., 2016; Kam et al., 2016; Lisgaras and Scharfman, 2023). The novel object location (NOL) task is impaired also (at 3-4 months of age in Tg2576 mice;(Duffy et al. 2015), so we evaluated NOL at 3 months. We also testeda 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 family with AD (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). WT mice received the intermediate diet during breeding, gestation, and after birth.

B. Behavior

1. *General information*

Starting at least 24 hrs prior to all experiments, mice were housed in the room where they would be tested behaviorally . Both NOL and NOR were preceded by 3 acclimation sessions (5 min each). Acclimations were conducted between 10:00 a.m. and 12:00 p.m.NOL and NOR were composed of a training and a testing session (5 min each; Figure 1B-D). ,Training and testing was conducted between 1:00 p.m. and 4:00 p.m. The interval between training and testing was 60 minutes and therefore the tasks 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). All equipment was cleaned using 70% ethanol before each use. In pilot studies, we confirmed that objects were explored equally by animals (see Results).. One object was composed of different 2 green LEGO pieces (approximately 5 cm x 7 cm x 7 cm); the other object was a pineapple (3 cm x 3 cm x 5 cm) and made of painted metal (Supplemental Figure 1; Botterill et al. 2021).

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. 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).

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

4. *Quantification*

The experimenter who conducted the analysis was blind to the genotypeand 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 the 2014 recommendations, we also analyzedthe number of approaches to an object. Time spent exploring each object was calculated as well, defined as the duration of time the nose was pointed at the object and the nose was located within 2 cm of the object. The time spent exploring also 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. Cognitive impairment was defined as significantly less than 50%.

290 **C. Anatomy**
291 **1.** *Perfusion-fixation and sectioning*
292 Mice were initially anesthetized by isoflurane inhalation (NDC# 07-893-1389,
293 Patterson Veterinary), followed by urethane (2.5 g/kg; i.p.; Cat# U2500, Sigma-
294 Aldrich). Under deep anesthesia, the abdominal and heart cavities were opened
295 with surgical scissors and a 23g needle inserted into the heart. The atria was
296 clipped, the needle was clamped in place with a hemostat, and the animal was
297 transcardially-perfused using 10 mL of room temperature (RT) saline (0.9%
298 sodium chloride in double distilled (dd) H_2O) using a peristaltic pump (Minipuls1;
299 Gilson), followed by 30 mL of 4°C 4% paraformaldehyde (PFA, Cat# 19210,
300 Electron Microscopy Sciences) in 0.1 M phosphate buffer (PB; pH 7.4). The
301 brains were immediately removed, hemisected, and post-fixed for at least 24 hrs
302 in a scintillation vial with 10 mL 4% PFA (at 4°C).
303 Following post-fixation, 50 µm-thick coronal sections were made using a
304 vibratome (VT 1000P, Leica Biosystems). Sections were collected serially and
305 stored at 4°C in 24-well tissue culture plates containing cryoprotectant solution
306 (30% glycerol, 30% ethylene glycol, 40% 0.1 M phosphate buffer, pH 6.7).
307 Throughout the hippocampus, every 6th section was processed with an antibody
308 to NeuN or ΔFosB. For analysis of anterior DG, 2-3 sections that were 300 µm
309 apart were selected from ~1.5-2.5 mm posterior to Bregma. For posterior DG, 2-3
310 sections that were 300 µm apart were selected starting at ~3.5 mm posterior to
311 Bregma. The values of the 2-3 sections were averaged so that there was one
312 measurement for anterior and one for posterior for each mouse.
313
314 **2.** *NeuN and ΔFosB immunohistochemistry*
315 Free floating sections were washed in 0.1 M Tris Buffer (TB; 3 x 5 min),
316 followed by a 3 min wash in 1% H_2O_2. Sections were then washed in 0.1 M TB (3
317 x 5 min) and incubated for 60 min in 5% horse serum for NeuN (Cat# S-2000,
318 Vector) or 5% goat serum for ΔFosB (Cat# S-1000, Vector), diluted in a solution
319 of 0.25% (volume/volume or v/v) Triton-X 100, and 1% (weight/volume or w/v)
320 bovine serum albumin in 0.1 M TB. Sections were then incubated overnight at
321 4°C in primary antibody to NeuN (mouse monoclonal, 1:5000; Cat# MAB377,
322 Chemicon International) or anti-ΔFosB (rabbit monoclonal, 1:1000; Cat# D3S8R,
323 Cell Signaling), diluted in a solution of 0.25% (v/v) Triton-X 100, and 1% (w/v)
324 bovine serum albumin in 0.1M TB. Both NeuN and ΔFosB have been well-
325 characterized as antigens (Mullen et al. 1992; Wolf et al. 1996; Chen et al. 1997;
326 Sarnat et al. 1998) and the antibodies we used have been commonly employed
327 in the past (Chen et al. 1997; Duffy et al. 2013; Corbett et al. 2017). On the
328 following day, sections were washed in 0.1 M TB (3 x 5 min) and then incubated
329 for 60 min in biotinylated horse anti-mouse secondary antibody to NeuN (1:500,
330 Cat# BA-2000, Vector) or biotinylated goat anti-rabbit secondary antibody to
331 ΔFosB (1:500, Cat# BA-1000), diluted in a solution of 0.25% (v/v) Triton-X 100,
332 and 1% (w/v) bovine serum albumin in 0.1 M TB. The sections were then washed
333 in 0.1 M TB (3 x 5 min) and incubated in avidin-biotin complex for 2 hrs (1:1000;
334 Cat# PK-6100, Vector). They were washed in 0.1 M TB (3 x 5 min) and then
335 reacted in a solution containing 0.5 mg/mL 3, 3'-diaminobenzidine (DAB; Cat#

336 D5905, Sigma-Aldrich), 40 µg/mL ammonium chloride (Cat# A4514, Sigma-
337 Aldrich), 25 mg/mL D(+)-glucose (Cat# G5767, Sigma-Aldrich), and 3 g/mL
338 glucose oxidase (Cat# G2133, Sigma-Aldrich) in 0.1 M TB. This method slowed
339 the reaction time so that the reaction could be stopped when the
340 immunoreactivity was robust but background was still low. The sections were
341 then washed in 0.1 M TB (3 x 5 min), mounted on gelatin-coated slides (1%
342 bovine gelatin, Cat# G9391, Sigma-Aldrich) and dried at RT overnight. The
343 following day they were dehydrated in increasing concentrations of ethanol (90%,
344 10 min; 95% 10 min; 100%, 10 min; 100% again, 10 min), washed in Xylene (10
345 min; Cat# 534056, Sigma-Aldrich) and cover-slipped with Permount (Cat# 17986-
346 01, Electron Microscopy Sciences).
347
348 **3.** *Analysis*
349 Photomicrographs were acquired using ImagePro Plus V7.0 (Media
350 Cybernetics) and a digital camera (Model RET 2000R-F-CLR-12, Q-Imaging).
351 NeuN and ΔFosB staining were quantified from micrographs using ImageJ
352 (V1.44, National Institutes of Health). All images were first converted to grayscale
353 and in each section, the hilus was traced, defined by zone 4 of Amaral (1978). A
354 threshold was then calculated to identify the NeuN-stained cell bodies but not
355 background. Then NeuN-stained cell bodies in the hilus were quantified
356 manually. Note that the threshold was defined in ImageJ using the distribution of
357 intensities in the micrograph. A threshold was then set using a slider in the
358 histogram provided by Image J (see Figure 6B,7B). The slider was pushed from
359 the low level of staining (similar to background) to the location where staining
360 intensity made a sharp rise, reflecting stained cells. Cells with labeling that was
361 above threshold were counted.
362 To quantify ΔFosB-stained cells, images were converted to grayscale and in
363 each section, the GCL was outlined and defined as a region of interest (ROI). A
364 threshold was then set to identify the well-stained cells but not the background,
365 analogous to the method to define threshold in the for NeuN. Two thresholds
366 were used because some cells were lightly stained and others much more robust
367 in their staining. The first threshold was most inclusive of immunoreactive cells.
368 Presumably this group reflected cells with less neuronal activity as well as those
369 with intense activity. Then a second analysis was done with the same sections
370 using a higher threshold. This group corresponded to cells with the highest
371 activity. ImageJ was used to calculate the area (in pixels) within the ROI that was
372 above threshold (see Figure 7B).
373
374 **D. Video-electroencephalographic (video-EEG) recordings**
375 **1.** *Stereotaxic surgery*
376 EEG electrodes (Cat# 8209, 0.10” stainless steel screws, Pinnacle
377 Technology) were implanted in Tg2576 animals at 4 weeks of age. WT
378 littermates were not implanted because previous work from our laboratory has
379 shown that WT mice have no IIS (Kam et al. 2016). The animals were
380 anesthetized by isoflurane inhalation (3% isoflurane. 2% oxygen for induction) in
381 a rectangular transparent plexiglas chamber (18 cm long x 10 cm wide x 8 cm

382 high) made in-house. For maintenance during surgery, isoflurane was ≤ 1.75%
383 and flow rate was 1.2 L/min. Mice were placed in a stereotaxic apparatus (David
384 Kopf Instruments). Prior to the implantation of the electrodes, animals were
385 injected with the analgesic Buprenex (0.2 mg/kg, s.c.; buprenorphine hydroxide,
386 NDC# 12496-0757-5, Reckitt Benckiser), which was diluted in saline (0.03
387 mg/mL in sterile 0.9% sodium chloride solution, Vedco Inc.). The skull was
388 exposed with a midline incision and 6 holes were drilled for the placement of
389 subdural screw electrodes.
390 The coordinates for the electrode placement were: right occipital cortex (AP -
391 3.5 mm, ML 2.0 mm), left frontal cortex (AP -0.5 mm, ML -1.5 mm), left
392 hippocampus (AP -2.5 mm, ML -2.0 mm), and right hippocampus (AP -2.5 mm,
393 ML 2.0 mm (Kam et al. 2016). An additional screw over the right olfactory bulb
394 was used as ground (AP 2.3 mm, ML 1.8 mm) and another screw was placed at
395 the midline over the cerebellum (relative to Lambda: AP -1.5 mm, ML -0.5 mm)
396 and used as reference. Screws were attached to an 8-pin connector (Cat#
397 ED85100-ND, Digi-Key Corporation), which was placed over the skull and
398 secured with dental cement (Cat# 4734FIB, Lang Dental Mfg. Co.).
399 After surgery, animals were placed on a heating blanket overnight and
400 injected with lactated Ringer's solution (50 mL/kg at 31°C; NDC# 099355000476,
401 Aspen Veterinary Resources Ltd). They were then transferred to the room where
402 the video-EEG was recorded.
403
404 **2.** *Video-EEG recording*
405 Video-EEG started 1 week after surgery, at 1.25 months (5 weeks) of age.
406 The animals were recorded at 5 weeks, and subsequently 2, 3, 4, 5 and 6
407 months of age. Each recording session lasted 24 hrs so that a long period of
408 sleep could be acquired, since IIS occur primarily in sleep (Kam et al. 2016).
409 Mice were placed into 21 cm x 19 cm transparent cages with food and water
410 provided *ad libitum* and corncob bedding. A pre-amplifier was inserted into the 8-
411 pin connector on the skull, which was connected to a multichannel commutator
412 (Cat# 8408, Pinnacle Technology). This arrangement allowed for free range of
413 movement throughout the recording. EEG signals were acquired at 2 kHz and
414 bandpass filtered at 0.5 – 200 Hz using Sirenia Acquisition (V2.0.4, Pinnacle
415 Technology). Simultaneous video recordings were captured using an infrared
416 camera (Cat# AP-DCS100W, Apex CCTV).
417
418 **3.** *Analysis*
419 EEG recordings were analyzed offline with Neuroscore V3.2.1 (Data Science
420 International). IIS were defined as large amplitude, 10-75 msec deflections
421 occurring synchronously in all 4 leads (Kam et al. 2016). They were quantified by
422 first setting an amplitude threshold (calculated separately for each recording) and
423 a duration criterion (10-75 msec). To determine the amplitude threshold, we
424 calculated the root mean square (RMS) amplitude for noise in each recording
425 during a 60 sec segment of baseline activity. A period of baseline activity was
426 selected that did not include any artifacts, which were defined as extremely large
427 deflections much greater than any other activity, and composed of a waveform

that was unlike any physiological activity. They typically were accompanied by a large shake in the animal, a cable hitting a part of the cage, or scratching the implant. 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 artifacts. The IIS in one of the hippocampal channels was used instead of another channel 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.

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 software (V 9.4.1, Graphpad).

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 5 weeks, 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.

474 **B. Behavior**
475 NOL and the NOR were selected because they show deficits in Tg2576 mice
476 at just 3 months of age (Duffy et al. 2015). This is before deficits have been
477 shown in other tasks (i.e., Morris Water Maze, Radial Arm Water Maze, Y-Maze)
478 which occur after 9 months of age (Yassine et al. 2013; Wolf et al. 2016). Figure
479 1B-D shows a schematic of the specific experimental procedures for NOL and
480 NOR.
481
482 **1.** *NOL*
483 The results of the NOL task are presented in Figures 2-4. In Figure 2, lines
484 connect the data for a given animal in training and testing. Data are the time
485 spent exploring the objects. Exploration of the object that is moved in the
486 training session (the novel oboject) is expressed as a percentage of the total
487 exploration time of both objects. When the slope of the line increases between
488 training and testing, the time spent exploring the moved object increased in
489 testing relative to training. This increase reflects a preference to explore the
490 moved object during the testing period, and suggests the mouse can recall the
491 old object locations and can recognize the new object location. Supplemental
492 Figure 2 shows the data with mean ± sem.
493 An important foundation for this task is equal preference for the objects
494 during training. The lack of preference reflects no inherent bias for one object vs.
495 the other. We confirmed the lack of bias for each genotype and each diet during
496 training. To conduct statistics we first conducted a two-way ANOVA based on the
497 training data with genotype or age (3 or 6 months) as factors. There were no
498 effects of genotype ($F(1,97)=0.40$, $p=0.530$) or age ($F(1,70)=1.94$, $p=0.167$) on
499 object exploration time. Then we conducted a two-way ANOVA to ask if diet
500 played a role. The main factors were diet and age and there also was no effect of
501 diet ($F(2,97)=0.06$, $p=0.941$) or age ($F(2,70)=2.94$, $p=0.058$) on object
502 exploration time.. Thus, novel object exploration during training approached 50%
503 in all treatment groups, independent of genotype and maternal diet, and at both
504 ages.
505 Next, we confirmed that Tg2576 mice were deficient in the test phase of NOL
506 when they were fed the standard mouse diet (the intermediate diet). First we
507 studied 3 months-old mice (Figure 2A). We used a two-way ANOVA with
508 genotype and task phase (training versus testing) as main factors. There was a
509 significant main effect of the task phase ($F(1,48)=8.51$, $p=0.005$) and a trend for
510 a genotype effect ($F(1,48)=3.80$, $p=0.057$). Consistent with previous studies
511 (Duffy et al. 2015), Tukey-Kramer post-hoc analyses revealed a significant
512 increase in the exploration of the novel object during testing in WT ($p=0.017$) but
513 not Tg2576 mice ($p=0.676$; Figure 2A). Thus, Tg2576 mice were impaired in NOL
514 when fed the intermediate diet.
515 In contrast to Tg2576 mice fed the intermediate diet, Tg2576 mice that
516 received the high choline diet showed memory for object location. Thus, a two-
517 way ANOVA revealed a main effect of the task phase ($F(1,72)=46.16$, $p<0.0001$)
518 but not genotype ($F(1,72)=1.74$, $p=0.191$). Tukey-Kramer post-hoc analyses
519 revealed a significant increase in the exploration of the novel object during

520 testing in both WT ($p=0.0003$) and Tg2576 mice ($p<0.0001$; Figure 2A3).
521 Therefore, high choline supplementation improved memory in Tg2576 mice.
522 In animals treated with the low choline diet, there was no effect of the phase
523 of the task ($F(1,74)=1.41$, $p=0.250$) or genotype ($F(1,74)=0.027$, $p=0.871$). These
524 data suggest that relatively low levels of choline during early life impaired spatial
525 memory in both WT and Tg2576 mice. Thus, low choline had a significant
526 adverse effect. Adverse effects are further supported by survival plots showing
527 that there was more mortality at earlier ages in offspring exposed to the low
528 choline diet (Supplemental Figure 1).
529 Figure 2B shows the results in 6 months-old WT and Tg2576 mice. In
530 animals that received the intermediate diet, a two-way ANOVA showed no effect
531 of genotype ($F(1,36)=0.01$, $p=0.907$) or task phase ($F(1,36)=2.36$, $p=0.133$),
532 revealing memory deficits in WT and Tg2576 mice (Figure 2B). In contrast, the
533 high choline group showed a main effect of task phase ($F(1,56)=22.18$,
534 $p<0.0001$) but not genotype ($F(1,56)=1.78$, $p=0.188$). Tukey-Kramer post-hoc
535 analyses showed a significant increase in the exploration of the novel object
536 during testing in both WT ($p<0.001$) and Tg2576 ($p=0.020$; Figure 2B). Thus, the
537 high choline-treated mice showed object location memory but the mice fed the
538 intermediate diet did not. The mice that received the relatively low choline diet
539 showed an effect of genotype ($F(1,50)=4.36$, $p=0.042$; Figure 2B) in that training
540 in WT mice differed from testing in Tg2576 mice ($p=0.031$). However, there was
541 no effect of task phase ($F(1,50)=3.75$, $p=0.058$). Thus, WT mice did not differ
542 between training and testing ($p=0.114$) and Tg2576 mice did not either
543 ($p=0.921$). Therefore, mice fed the low choline and intermediate diets were
544 impaired and the high choline-treated mice were not.
545 The discrimination indices are shown in Figure 3 and results led to the same
546 conclusions as the analyses in Figure 2. For the 3 months-old mice (Figure 3A),
547 the low choline group did not show the ability to perform the task for WT or
548 Tg2576 mice. Thus, a two-way ANOVA showed no effect of genotype
549 ($F(1,74)=0.027$, $p=0.870$) or task phase ($F(1,74)=1.41$, $p=0.239$). For the
550 intermediate diet-treated mice, there was no effect of genotype ($F(1,50)=0.3.52$,
551 $p=0.067$) but there was an effect of task phase ($F(1,50)=8.33$, $p=0.006$). WT mice
552 showed a greater discrimination index during testing relative to training ($p=0.019$)
553 but Tg2576 mice did not ($p=0.664$). Therefore, Tg2576 mice fed the intermediate
554 diet were impaired. In contrast, high choline-treated mice performed well. There
555 was a main effect of task phase ($F(1,68)=39.61$, $p=<0.001$) with WT ($p<0.0001$)
556 and Tg2576 mice ($p=0.0002$) showing preference for the moved object in the test
557 phase. Interestingly, there was a main effect of genotype ($F(1,68)=4.50$, $p=0.038$)
558 because the discrimination index for WT training was significantly different from
559 Tg2576 testing ($p<0.0001$) and Tg2576 training was significantly different from
560 WT testing ($p=0.0003$).
561 The discrimination indices of 6 months-old mice led to the same conclusions
562 as the results in Figure 2. There was no evidence of discrimination in low choline-
563 treated mice by two-way ANOVA (no effect of genotype, ($F(1,42)=3.25$, $p=0.079$;
564 no effect of task phase, $F(1,42)=0.278$, $p=0.601$). The same was true of mice fed
565 the intermediate diet (genotype, $F(1,12)=1.44$, $p=0.253$; task phase,

566 F(1,12)=2.64, p=0.130). However, both WT and Tg2576 mice performed well
567 after being fed the high choline diet (effect of task phase, (F(1,52)=58.75,
568 p=0.0001, but not genotype (F(1,52)=1.197, p=0.279). Tukey-Kramer post-hoc
569 tests showed that both WT (p<0.0001) and Tg2576 mice that had received the
570 high choline diet (p=0.0005) had elevated discrimination indices for the test
571 session.
572 Taken together, these results demonstrate the lasting beneficial effects of the
573 high choline diet and adverse effects of low choline on offspring in a spatial
574 memory task in Tg2576 as well as WT littermates.
575
576 **2.** *NOR*
577 We first confirmed that animals did not show preference for one object over
578 the other in training. Indeed, a two-way ANOVA revealed no main effects of
579 genotype at 3 months (F(1,71)=2.59, p=0.536) and no effect of diet
580 (F(2,71)=0.22, p=0.809). The same result was obtained at 6 months of age
581 (genotype, (F(1,66)=0.11, p=0.746); diet, (F(2,66)=0.98, p=0.376). Thus, novel
582 object exploration during training approached 50% in all treatment groups,
583 independent of genotype and maternal diet, and at both ages.
584 Figure 4 shows the results for the NOR task. Lines connect the performance
585 of a given animal in training and in testing. Supplemental Figure 3 shows the
586 data with mean ± sem. Figure 4A shows that there were no impairments at 3
587 months of age. In animals treated with the low choline diet, there was a main
588 effect of task phase (F(1,52)=19.81, p<0.0001), no effect of genotype
589 (F(1,52)=0.020, p=0.887), and more exploration of the novel object during the
590 test phase (WT, p=0.007; Tg2576, p<0.002). In animals that received the
591 intermediate diet, there also was a significant main effect of task phase
592 (F(1,38)=30.88, p<0.0001) and no effect of genotype (F(1,38)=0.97, p=0.330).
593 Animals explored the novel object significantly more during testing in WT
594 (p=0.014) and Tg2576 mice (p=0.002; Figure 3B). For the high choline group,
595 there also was an effect of task phase (F(1,26)=17.51, p=0.0003) and not
596 genotype (F(1,26)=3.53, p=0.072) and a significant increase in the exploration of
597 the novel object during testing in both WT (p=0.006) and Tg2576 (p=0.027;
598 Figure 3B).
599 Figure 4B shows the data for 6 months of age. Animals that received the low
600 choline diet performed the NOR task at 6 months of age (two-way ANOVA, task
601 phase (F(1,26)=32.66, p<0.0001; genotype (F(1,26)=0.048, p=0.821). Tukey-
602 Kramer post-hoc tests showed that there was a significant increase in the
603 exploration of the novel object in testing both in WT (p<0.001) and Tg2576 (p=
604 0.003). For mice that had been fed the intermediate choline diet, they also
605 performed the NOR task at 6 months of age (two-way ANOVA, task phase
606 (F(1,19)=13.65, p=0.002); genotype (F(1,19)=0.28, p=0.604). Tukey-Kramer
607 post-hoc analyses revealed a significant increase in the exploration of the novel
608 object during testing compared to training for both WT (p=0.0027) and Tg2576
609 (p=0.039; Figure 4B). Mice fed the high choline diet also showed no deficit in
610 NOR at 6 months of age. Thus, a two-way ANOVA revealed a main effect of task
611 phase (F(1,27)=16.26, p=0.0004) but not genotype (F(1,27)=0.24, p=0.625).

612 Tukey-Kramer post-hoc-hoc analyses revealed a significant increase in the
613 exploration of the novel object during testing in both WT (p=0.003) and Tg2576
614 (p=0.007; Figure 4B).
615 Thus, at both 3 and 6 months of age, WT and Tg2576 mice performed well in
616 the NOR task. These results suggest that young Tg2576 mice are less sensitive
617 to NOR than NOL. The greater sensitivity to NOL is consistent with past
618 demonstrations that the DG contributes to NOL (Sahay et al. 2011; Kesner et al.
619 2015; Spyrka and Hess 2018; Vandrey et al. 2020; Gulmez Karaca et al. 2021;
620 GoodSmith et al. 2022). Also, our implementation of NOL may have increased
621 the DG-dependence of the task by making the object locations relatively close
622 together, because in studies by Pofahl and colleagues, it was shown that
623 distances between objects like those we used made NOL DG-dependent (Pofahl
624 et al. 2021).
625
626 **3.** *Exploration time*
627 Total object exploration (TOE) was measured to address any effects of
628 genotype or diet on the total duration of exploration of objects (Figure 5). For
629 NOL TOE at 3 months of age, there were effects of genotype (F(1,67)=6.891,
630 p=0.01) but not diet (F(2,87)=0.67, p=0.63). Tukey-Kramer post-hoc tests
631 showed that Tg2576 mice fed the low choline diet showed more exploration than
632 WT mice fed the same diet but the effect was on the border of significance
633 (p=0.049; data not shown). Three months-old mice tested with NOR showed no
634 significant effect of genotype (F(1,76)=1.35, p=0.25) or diet (F(2,76)=0.30,
635 p=0.61). Since there were weak or no effects of genotype, all genotypes are
636 pooled for Figure 5A1 (NOL) and Figure 5B1 (NOR).
637 For NOL TOE at 6 months of age, there was no effect of genotype
638 (F(1,66)=0.33; p=0.57). For NOR TOE, the same was true (genotype:
639 F(1,72)=0.96, p=0.33; diet: F(2,72)=8.50, p=0.0005). Because genotype was not
640 a significant factor, we pooled genotypes (NOL, Figure 5C1; NOR, Figure 5D2).
641 With genotypes pooled we determined how diets differed. For NOL TOE,
642 mice treated with the low choline diet had less exploration than mice fed the high
643 choline diet (one-way ANOVA, F(2,77)=6.90; p=0.002; Tukey post-hoc test,
644 p=0.005; Figure 5C1) and the mice that were fed the intermediate diet also had
645 less exploration than mice fed the high choline diet (Tukey post-hoc test,
646 p=0.008; Figure 5C1). Results for NOR were similar: mice treated with the low
647 choline diet had less exploration than mice fed the high choline diet (one-way
648 ANOVA, F(2,74)=4.81; p=0.020; Tukey post-hoc test, p=0.016; Figure 5D1) and
649 the mice that were fed the intermediate diet also had less exploration than mice
650 fed the high choline diet (Tukey post-hoc test, p=0.03; Figure 5D1).
651 To gain insight into the potential reason for the effect of diet on exploration at
652 6 months of age, we measured total object approaches (TOA was defined as the
653 number of approaches to the familiar object + number of approaches to the novel
654 object (Figure 5). There was no effect of diet on TOA for NOL (F(2,75)=2.88;
655 p=0.092; Figure 5C2) or NOR (F(2,74)=1.81, p=0.171; Figure 5D2).
656 Taken together, the results indicate that, in 6 months-old mice, animals that
657 received the high choline diet spent more time with objects at each approach.

658 This could explain the increased object memory in high choline-treated mice
659 because there would bemore time for information processing during an
660 approach. Another possibility is that as Tg2576 mice age they show
661 compensatory changes that enhance memory. This has been suggested for
662 procedural learning (Middei et al. 2004) but to the best of our knowledge, it has
663 not been shown for memory of objects. A third possibility is that the high choline
664 diet reduces anxiety (Glenn et al. 2012; Langley et al. 2015; McCall et al. 2015).
665 Reduced anxiety may lessen fear of exploring objects, and as a result, animals
666 may spend more time with objects.
667
668 **C. Anatomy**
669 **1. NeuN**
670 To further analyze the effects of dietary choline early in life, we used an
671 antibody against a neuronal nuclear antigen, NeuN. Previous studies have found
672 that reduced expression of NeuN often occurs in neurons after insults or injury
673 (e.g., ischemia, toxicity and even aging; Lind et al. 2005; Portiansky et al. 2006;
674 Buckingham et al. 2008; Kadriu et al. 2009; Matsuda et al. 2009; Won et al.
675 2009; Duffy et al. 2011). For example, when NeuN is reduced after a
676 heterozygous deletion in ankyrin-rich membrane spanning kinase D-interacting
677 substrate of 220K, ARMS/Kidins, a protein critical to neurotrophin signaling,
678 neurons in the entorhinal cortex demonstrate loss of NeuN immunoreactivity and
679 the cellular morphology is abnormal (Duffy et al. 2011; Duffy et al. 2015). NeuN is
680 also reduced when Aβ levels are elevated (Wu et al. 2016) and NeuN is reduced
681 in AD patients (Camporez et al. 2021). Therefore, we initially examined NeuN-ir
682 in Tg2576 mice. We found it was reduced and then asked in the high choline diet
683 could restore it.
684 As shown in Figure 6A, relatively weak NeuN-ir was observed in Tg2576 that
685 received the low choline diet and the intermediate diet compared to the high
686 choline diet (Figure 6A). This observation is consistent with the vulnerability of
687 the hilus to insult and injury (Scharfman 1999). To examine the septotemporal
688 axis, quantification from 2-3 anterior coronal sections were averaged to provide a
689 value for the septal pole of the DG and 2-3 posterior sections were averaged to
690 assess the caudal DG. The most ventral, temporal levels were not sampled.
691 There was no effect of diet ($F(2,30)=2.11$, $p=0.137$) nor rostral-caudal level
692 ($F(1,30)=0.02$, $p=0.877$) but there was a significant interaction of diet and rostral-
693 caudal level ($F(2,30)=3.88$, $p=0.036$). A one-way ANOVA using anterior values
694 showed a significant effect of diet ($F(2,21)=7.58$, $p=0.003$) but this was not the
695 case for posterior values ($F(2,23)=0.13$; $p=0.876$). In the anterior DG, there was
696 less NeuN-ir in mice fed the low choline diet versus the high choline diet (Tukey-
697 Kramer post-hoc test, $p=0.029$) and less NeuN in mice fed the intermediate vs
698 high choline diet ($p=0.003$). The results suggest that hilar neurons of Tg2576
699 mice have reduced NeuN-ir and choline enrichment protected dorsal hilar
700 neurons from NeuN loss in Tg2576 mice.
701 To ask if the improvement in NeuN after the high choline diet in Tg256
702 restored NeuN to WT levels, we used WT mice. The WT mice were fed the
703 intermediate diet because it is the standard mouse chow, and this group was

704 intended to reflect normal mice. For the analysis, we used a one-way ANOVA
705 with 4 groups: Low choline Tg2576, Intermediate Tg2576, High choline Tg2576,
706 and Intermediate WT (Figure 5C). Tukey-Kramer multiple comparisons tests
707 were used as the post hoc tests. The results showed a significant group
708 difference for anterior DG ($F(3,25)=9.20$; $p=0.0003$; Figure 5C1) but not posterior
709 DG ($F(3,28)=0.867$; $p=0.450$; Figure 5C2). Regarding the anterior DG, there
710 were more NeuN-ir cells in high choline-treated mice than both low choline
711 ($p=0.046$) and intermediate choline-treated Tg2576 mice ($p=0.003$). WT mice
712 had more NeuN-ir cells than Tg2576 mice fed the low ($p=0.011$) or intermediate
713 diet ($p=0.003$). Tg2576 mice that were fed the high choline diet were not
714 significantly different from WT ($p=0.827$). Thus, we found reduced hilar NeuN in
715 Tg2576 mice and the high choline diet increased NeuN protein expression to WT
716 levels.
717
718 **2. ΔFosB**
719 To complement the information from the video-EEG recordings (see below)
720 we used a marker of elevated neuronal activity, ΔFosB (Figure 7). ΔFosB is a
721 truncated variant of the transcription factor FosB, which is increased by
722 enhanced neuronal activity; ΔFosB has a half-life of approximately 8 days (Ulery-
723 Reynolds et al., 2009), so when ΔFosB is elevated, it reflects increased neuronal
724 activity over the last 10-14 days (McClung et al. 2004). Previous studies have
725 shown that when the J20 mouse model of AD is examined with an antibody to
726 ΔFosB, the GC layer shows extremely high levels of ΔFosB expression (Corbett
727 et al. 2017), similar to a mouse with chronic spontaneous seizures (Morris et al.
728 2000). This is not surprising since the J20 mice have recurrent seizures (Palop et
729 al. 2007). Therefore, we asked if Tg2576 mice would have robust ΔFosB in the
730 GC layer, and choline supplementation would reduce it. We also included WT
731 mice fed the intermediate (standard) diet. These WT mice would allow us to
732 address the possibility that the high choline diet restored ΔFosB back to normal.
733 There was strong expression of ΔFosB in Tg2576 GCs in mice fed the low
734 choline diet (Figure 7A1). The high choline diet and intermediate diet appeared to
735 show less GCL ΔFosB-ir (Figure 7A2-3). A two-way ANOVA was conducted with
736 the experimental group (Tg2576 low choline diet, Tg2576 intermediate choline
737 diet, Tg2576 high choline diet, WT intermediate choline diet) and location
738 (anterior or posterior) as main factors. There was a significant effect of group
739 ($F(3,32)=13.80$, $p=<0.0001$) and location ($F(1,32)=8.69$, $p=0.006$). Tukey-Kramer
740 post-hoc tests showed that Tg2576 mice fed the low choline diet had significantly
741 greater ΔFosB-ir than Tg2576 mice fed the high choline diet ($p=0.0005$) and WT
742 mice ($p=0.0007$). Tg2576 mice fed the low and intermediate diets were not
743 significantly different ($p=0.275$). Tg2576 mice fed the high choline diet were not
744 significantly different from WT ($p>0.999$). There were no differences between
745 groups for the posterior DG (all $p>0.05$).
746 ΔFosB quantification was repeated with a lower threshold to define ΔFosB-ir
747 GCs (see Methods) and results were the same (Figure 7D). Two-way ANOVA
748 showed a significant effect of group ($F(3,32)=14.28$, $p< 0.0001$) and location
749 ($F(1,32)=7.07$, $p=0.0122$) for anterior DG but not posterior DG (Figure 7D). For

750 anterior sections, Tukey-Kramer post hoc tests showed that low choline mice had
751 greater ΔFosB-ir than high choline mice (p=0.0024) and WT mice (p=0.005) but
752 not Tg2576 mice fed the intermediate diet (p=0.275); Figure 7D1). Mice fed the
753 high choline diet were not significantly different from WT (p=0.993; Figure 7D1).
754 These data suggest that high choline in the diet early in life can reduce neuronal
755 activity of GCs in offspring later in life. In addition, low choline has an opposite
756 effect, suggesting low choline in early life has adverse effects.
757
758 **D. IIS**
759 Previous research has shown that Tg2576 mice exhibit IIS (Figure 8A)
760 starting at very young ages: 4 weeks (Kam et al. 2016) or 6 weeks (Bezzina et al.
761 2015). Therefore, we performed video-EEG using cortical (left frontal cortex, right
762 occipital cortex) and hippocampal (left and right) electrodes in WT and Tg2576
763 mice (Figure 8B-C). Animals were recorded for 24 hrs each session so that the
764 major behavioral states (exploration, awake rest, and sleep) were sampled well.
765 Consistent with previous studies (Bezzina et al. 2015; Kam et al. 2016) we
766 observed IIS in Tg2576 mice but not WT littermates. Therefore, analyses below
767 were only in Tg2576 mice.
768 As shown in Figure 8, animals that received the intermediate diet had a
769 significantly higher number of IIS in the 24 hrs-long recording periods compared
770 to animals that received the high choline and the low choline diets. A two-way
771 ANOVA (mixed model analysis) showed that there was a significant effect of age
772 ($F(2,37)=3.38$; $p=0.036$) and maternal diet ($F(2,36)=8.12$; $p=0.089$). At the 5
773 weeks-old recording, Tukey-Kramer post-hoc analyses showed that IIS
774 frequency in animals treated with the intermediate diet was higher compared to
775 animals treated with the low (p=0.027) or high choline diets (p=0.038). This also
776 was true for 2 months and 3 months (low choline, high choline, $p<0.05$). At 4
777 months, the low choline group had significantly reduced IIS frequency compared
778 to the mice that had received the intermediate diet (p=0.009) but this was not the
779 case for the high choline group compared to the intermediate group (p=0.976). At
780 5-6 months, IIS frequencies were not significantly different in the mice fed the
781 different diets (all $p>0.05$), probably because IIS frequency becomes increasingly
782 variable with age (Kam et al. 2016). One source of variability is seizures,
783 because there was a sharp increase in IIS during the day before and after a
784 seizure (Supplemental Figure 4). Another reason that the diets failed to show
785 differences was that the IIS frequency generally declined at 5-6 months. This can
786 be appreciated in Figure 8B and Supplemental Figure 6B. These data are
787 consistent with prior studies of Tg2576 mice where IIS increased from 1 to 3
788 months but then waxed and waned afterwards (Kam et al., 2016).
789
790 **E. Seizures and premature mortality in mice fed the low choline diet**
791 We found that mice fed the low choline diet had greater ΔFosB-ir in GCs and
792 the hilus showed very low NeuN-ir. Therefore, we asked whether low choline-
793 treated mice had more seizures than mice fed the other diets. We recorded 8
794 mice by video-EEG for 5 days each (at 6 months of age) to examine seizures
795 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 we think 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 during a severe seizure that leads to death , 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 and were tested behaviorally were not always tested with EEG.

For NOL, IIS frequency over 24 hrs was plotted against the preference for the novel object in the test phase (Figure 9A). 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 5 weeks, 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 differed from females ($p=0.002$) and males ($p<0.0001$) fed the intermediate diet, and males fed the high choline diet differed 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, and reduced 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-ir and most GCL ΔFosB protein 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 an early age in that hilar neurons are deficient in immunostaining with an anti-NeuN antibody, and this occurs before amyloid plaque develops (after 6 months of age; Hsaio et al., 1996). Tg2576 offspring that were fed 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 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 (Sarkisova et al. 2023) and audiogenic seizure severity (Poletaeva et al. 2014) in the offspring. 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 memory impairment in subsequent weeks; MCS reduced the impairment (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, where mutations found in AD are present in APP and PS1, it was shown that life-long choline supplementation, using the high choline diet, improved memory in aged mice compared to a low choline 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, where there are 3 familial AD mutations, 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 low 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. In contrast, 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 of the Tg2576 mice, 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. Mice also had high ΔFosB expression in the GCs. Another adverse effect was weak NeuN-ir in the hilus.. For both WT and Tg2576 mice, NOL was impaired. These data suggest that the offspring of mothers fed the low choline diet were unhealthy.. If mice were unhealthy, IIS might have been reduced due to impaired synchronization (despite high excitability in the DG). Another reason for reduced IIS is that the mice that had the low choline diet had seizures which interrupted REM 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 perspective 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.

979 **NeuN**
980 As mentioned above, NeuN is a neuronal nuclear antigen that can be
981 phosphorylated, and when that occurs the antibody to NeuN no longer binds to
982 NeuN. NeuN is phosphorylated in response to oxidative damage, brain injury,
983 and toxicity (Lind et al. 2005). Therefore, it was of interest when we saw that hilar
984 neurons of the dentate gyrus showed reduced NeuN in Tg2576 mice. Hilar
985 neurons are mainly glutamatergic mossy cells and somatostatin
986 (SOM)/neuropeptide Y (NPY)-expressing GABAergic neurons (HIPP cells;
987 (Houser 2007; Scharfman and Myers 2012; Scharfman 2016), and both neuronal
988 types are implicated in spatial memory functions of the GCs, as well as their
989 excitability (Myers and Scharfman 2009; 2011; Scharfman and Myers 2012;
990 Jinde et al. 2013; Scharfman 2016; Raza et al. 2017; Bui et al. 2018; GoodSmith
991 et al. 2019; Li et al. 2021). If damaged by the high intracellular APP and Aβ levels
992 in young Tg2576 mice, the hilar neurons would be expected to show NeuN loss.
993 This idea is consistent with deficits in SOM and NPY-stained cells in AD (Chan-
994 Palay 1987), indicating a vulnerability.
995 The reason for rescue of anterior hilar but not posterior hilar NeuN with the
996 high choline diet is unclear. The greater sensitivity of dorsal neurons may be
997 related to differences in gene expression patterns, since some of the genes that
998 are differentially expressed along the dorsal-ventral axis could affect vulnerability
999 to insult or injury (Cembrowski et al. 2016; Zhang et al. 2018). However, there
1000 was a similar trend for the data in both anterior and posterior regions. The lack of
1001 statistical significance in posterior DG may simply have been due to be greater
1002 variance in the posterior DG data.
1003
1004 **ΔFosB**
1005 Given that seizures are associated with elevated GC expression of ΔFosB
1006 (Chen et al. 1997; McClung et al. 2004; Corbett et al. 2017; You et al. 2017), the
1007 differences in ΔFosB protein expression levels observed here could be the result
1008 of increased seizures in animals that received the low choline diet. However, we
1009 did not detect a significant difference in seizures at 6 months of age, although
1010 there may have been a difference earlier in life or if we had recorded for a longer
1011 time. Interestingly, there may have been more seizures due to a reduced number
1012 of IIS. Thus, some evidence suggests that IIS can abort a seizure (Staley and
1013 Dudek 2006).
1014 ΔFosB is a transcription factor that is linked to cognition. In the J20 mouse
1015 model of AD, elevated ΔFosB in GCs led to reduced cognition, and when ΔFosB
1016 was selectively reduced the cognition improved (Corbett et al. 2017). Therefore,
1017 the reduction in ΔFosB by the high choline diet was important to show
1018 hyperexcitability was reduced and also important because it showed how high
1019 choline may benefit cognition.
1020
1021 **Choline and cholinergic neurons**
1022 There are many suggestions for the mechanisms that allow MCS to improve
1023 health of the offspring. One hypothesis that we are interested in is that MCS
1024 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 consolidation is a notable function of sleep (Graves et al. 2001; Poe et al. 2010). Indeed, in patients with AD, IIS and similar events, IEDs, are correlated with memory impairment (Vossel et al. 2016). Sleep disruption also has other negative consequences such as impairing normal clearance of Aβ (Nedergaard and Goldman 2020).

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 (Lisgaras and Scharfman, 2023). 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 diets would have been useful. Sample sizes were also not matched exactly, because several mice died.. 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. Recordings for 24 hrs started 1 week later (5 weeks-old; 1.2 months-old in the grahps).Additional recordings were made at, 2, 3, 4, 5, and 6 months of age. At 3 and 6 months of age behavior was tested, 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).

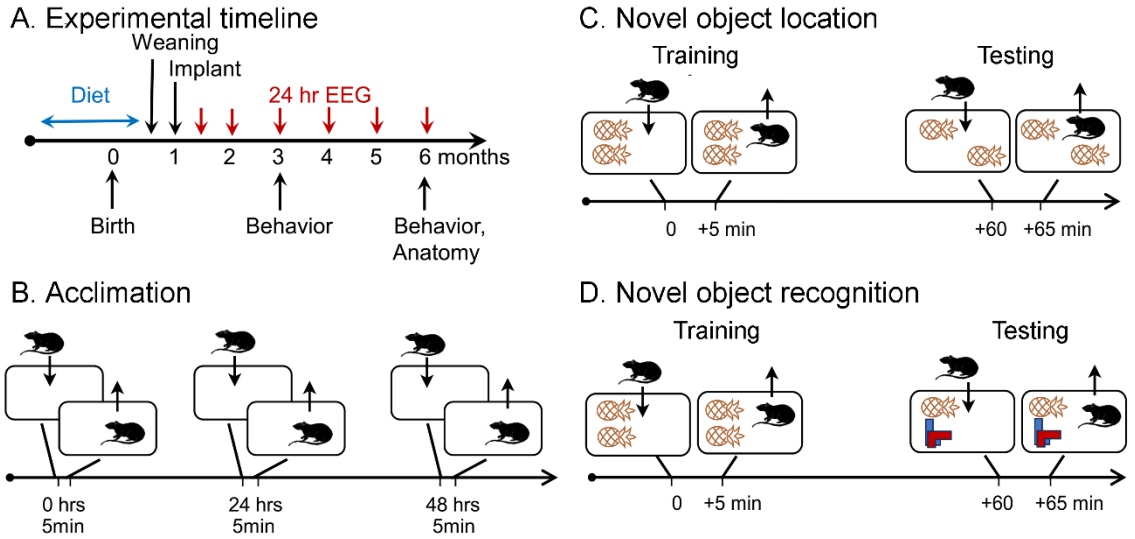


Figure 2. Choline enrichment reduced spatial memory deficits.

A. Three months-old mice. For A and B, breeders were fed the specified diet and offspring were tested.

1. Low choline diet. WT and Tg2576 offspring showed spatial memory deficits.
2. Intermediate diet. Tg2576 showed spatial memory deficits but not WT.
3. High choline diet. Spatial memory was improved..

B. Six months-old mice.

1. Low choline diet. WT and Tg2576 offspringshowed spatial memory deficits.
2. Intermediate diet. WT and Tg2576 mice showed spatial memory deficits.
3. High choline diet. WT and Tg2576 mice had improved spatial memory.

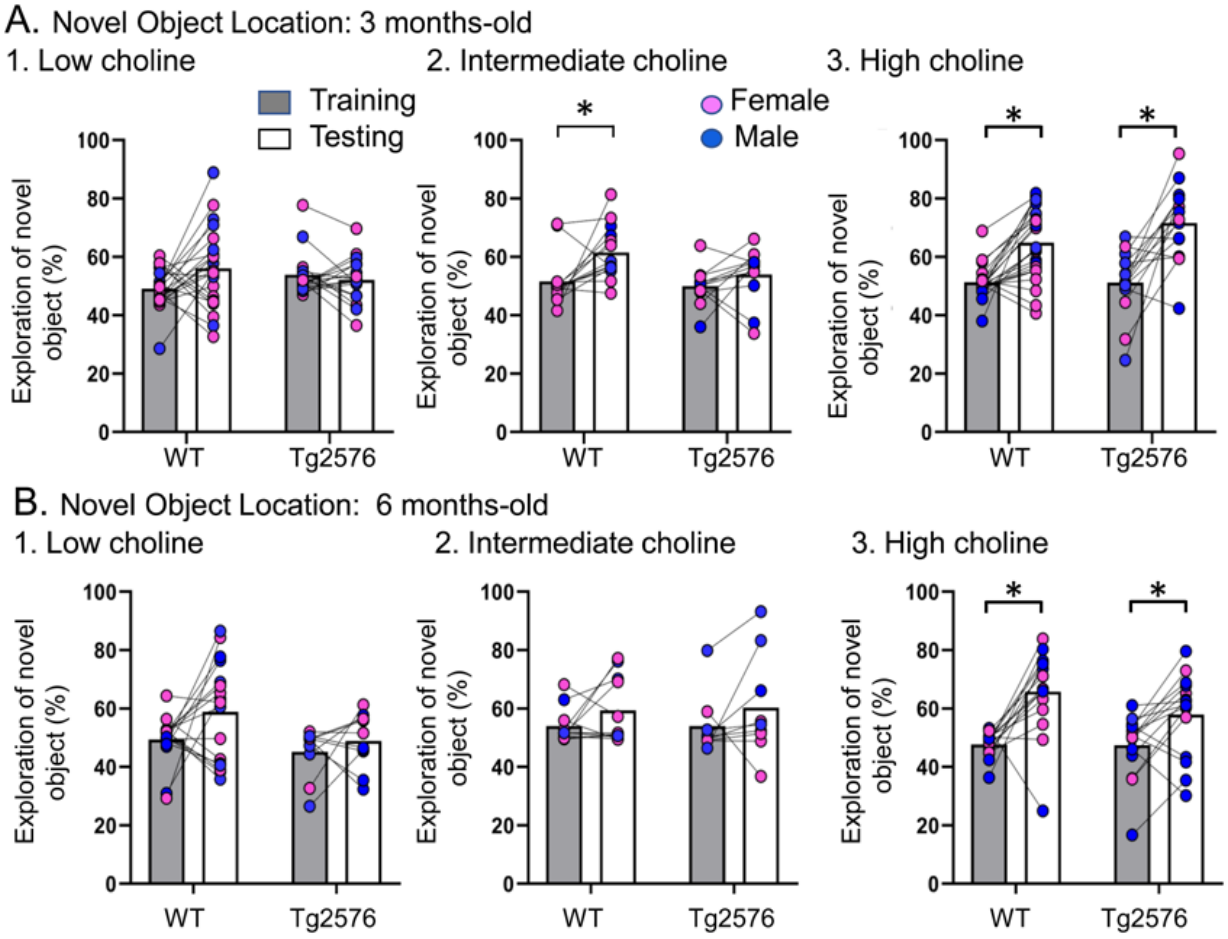


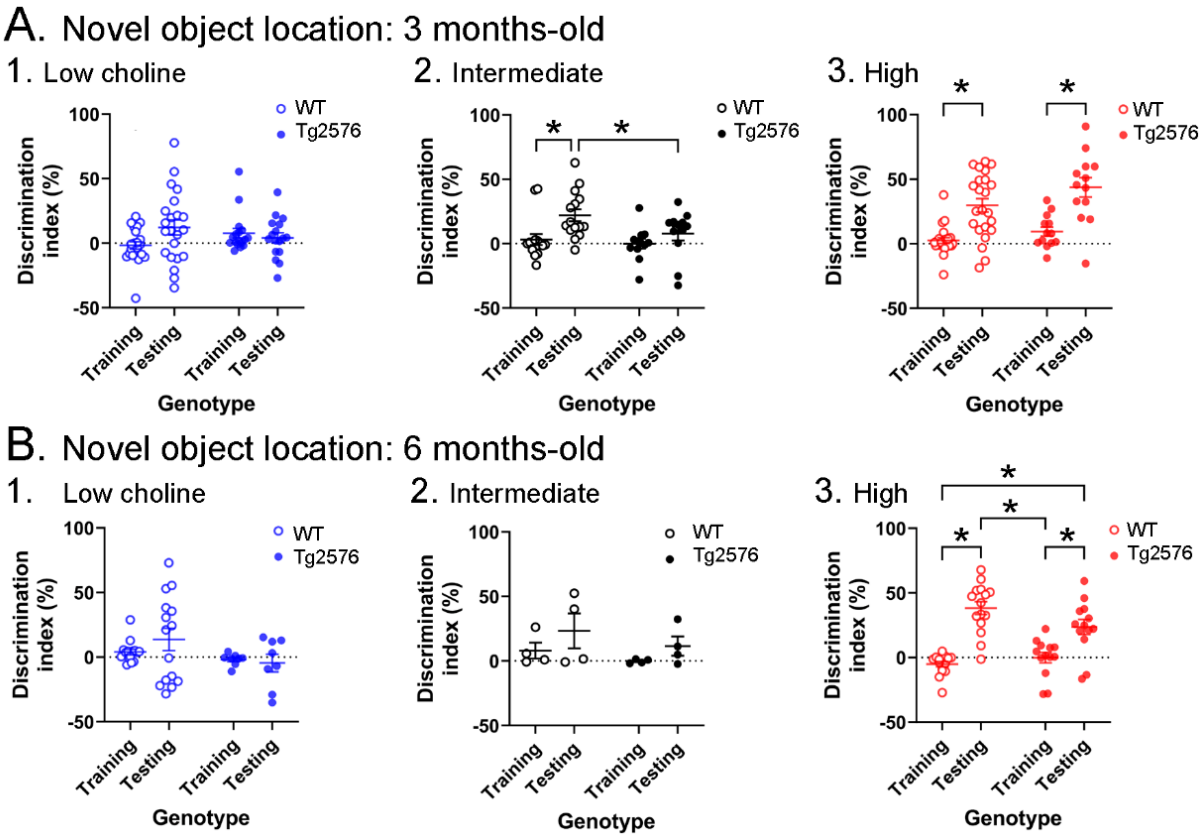
Figure 3. Novel object location results based on the discrimination index.
A. Results are shown for the 3 months-old WT and Tg2576 mice based on the discrimination index.

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.



1142 **Figure 4. WT and Tg2576 mice showed object recognition memory**
1143 **regardless of diet.**
1144 **A.** Three months-old mice.
1145 1-3. WT and Tg2576 mice performed the NOR task.
1146 **B.** Six months-old mice.
1147 1-3. WT and Tg2576 mice performed the NOR task.
1148

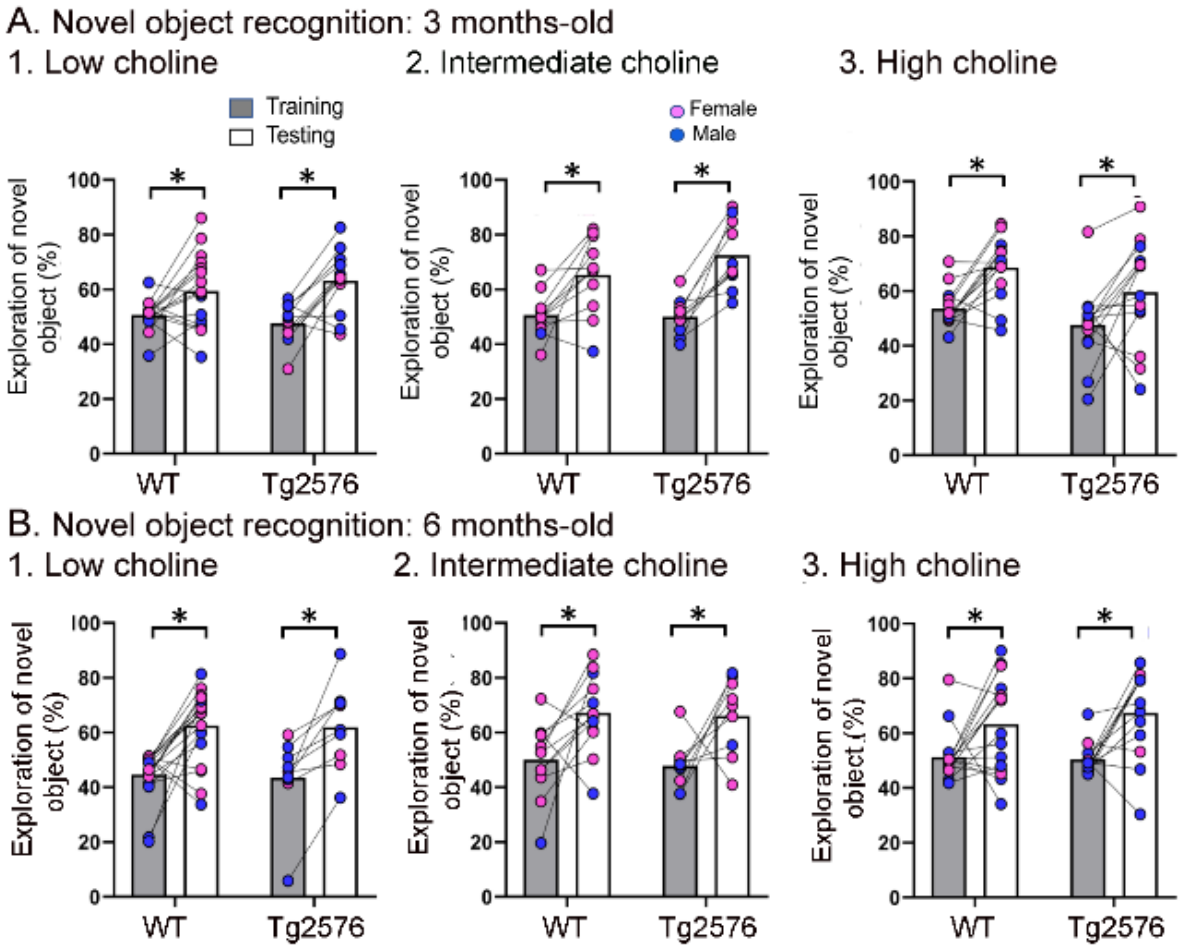


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Figure 5. Tg2576 mice treated with the high choline diet spent more time with the objects at each approach.

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.

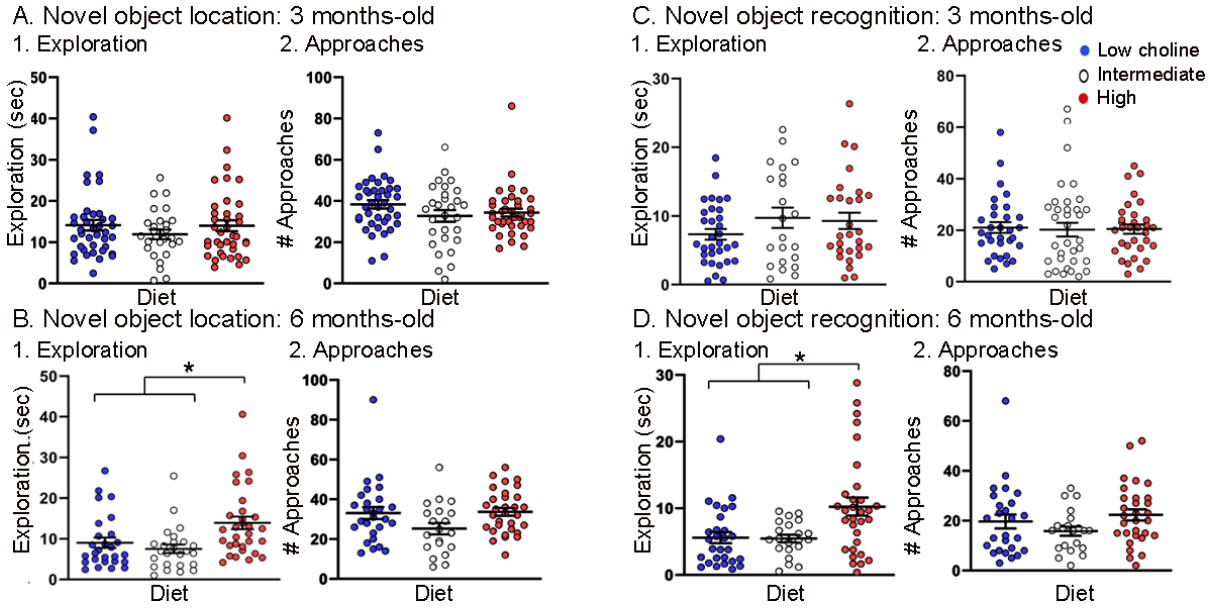


Figure 6. Choline supplementation improved NeuN immunoreactivity (ir) in hilar cells in Tg2576 animals

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

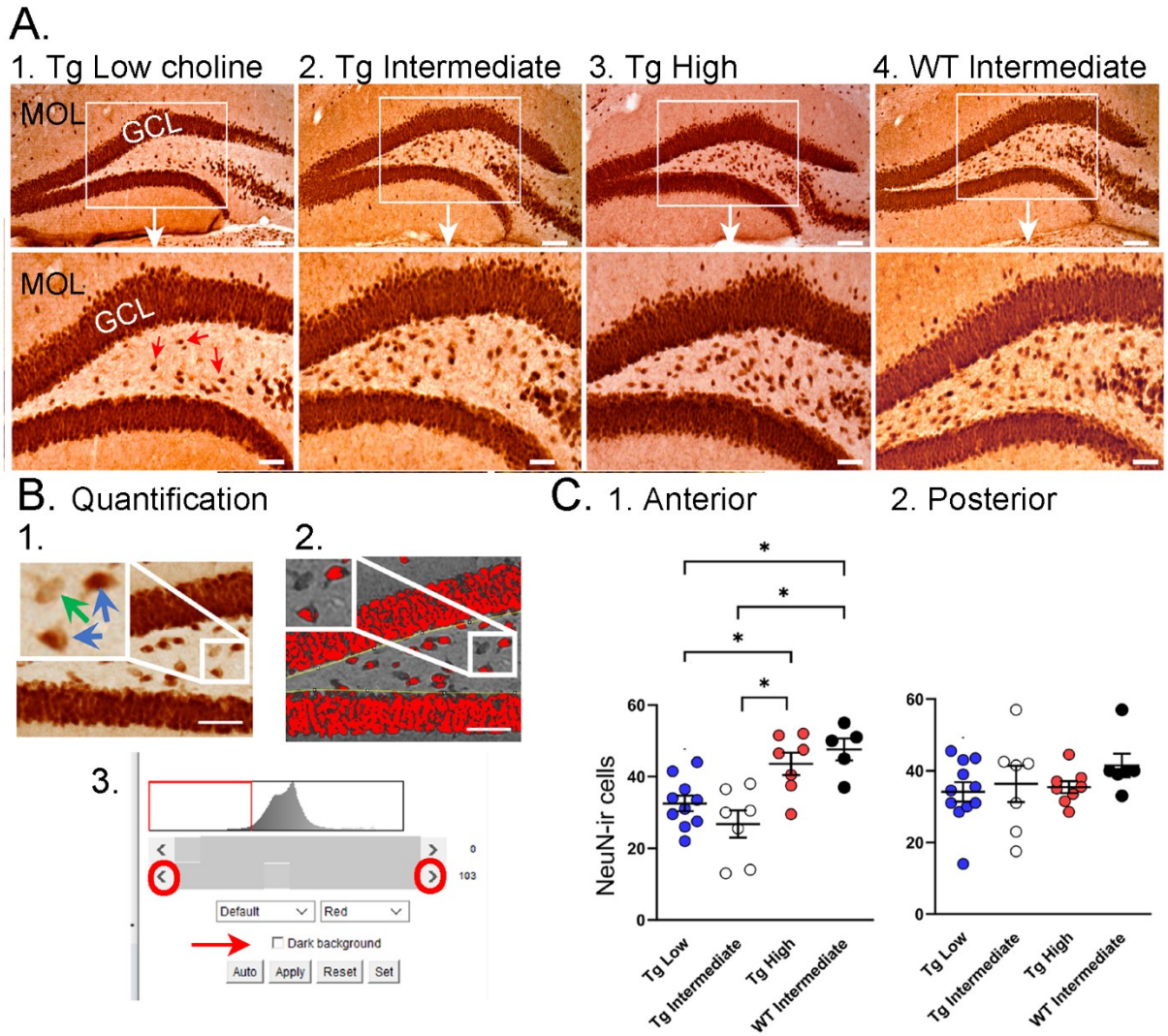
1. A section from a Tg2576 mouse fed the low choline diet. The area surrounded by a box is expanded below. Red arrows point to NeuN-ir hilar cells. Mol=molecular layer, GCL=granule cell layer. Calibration for the top row, 100 μm; for the bottom row, 50 μm.
2. A section from a Tg2576 mouse fed the intermediate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

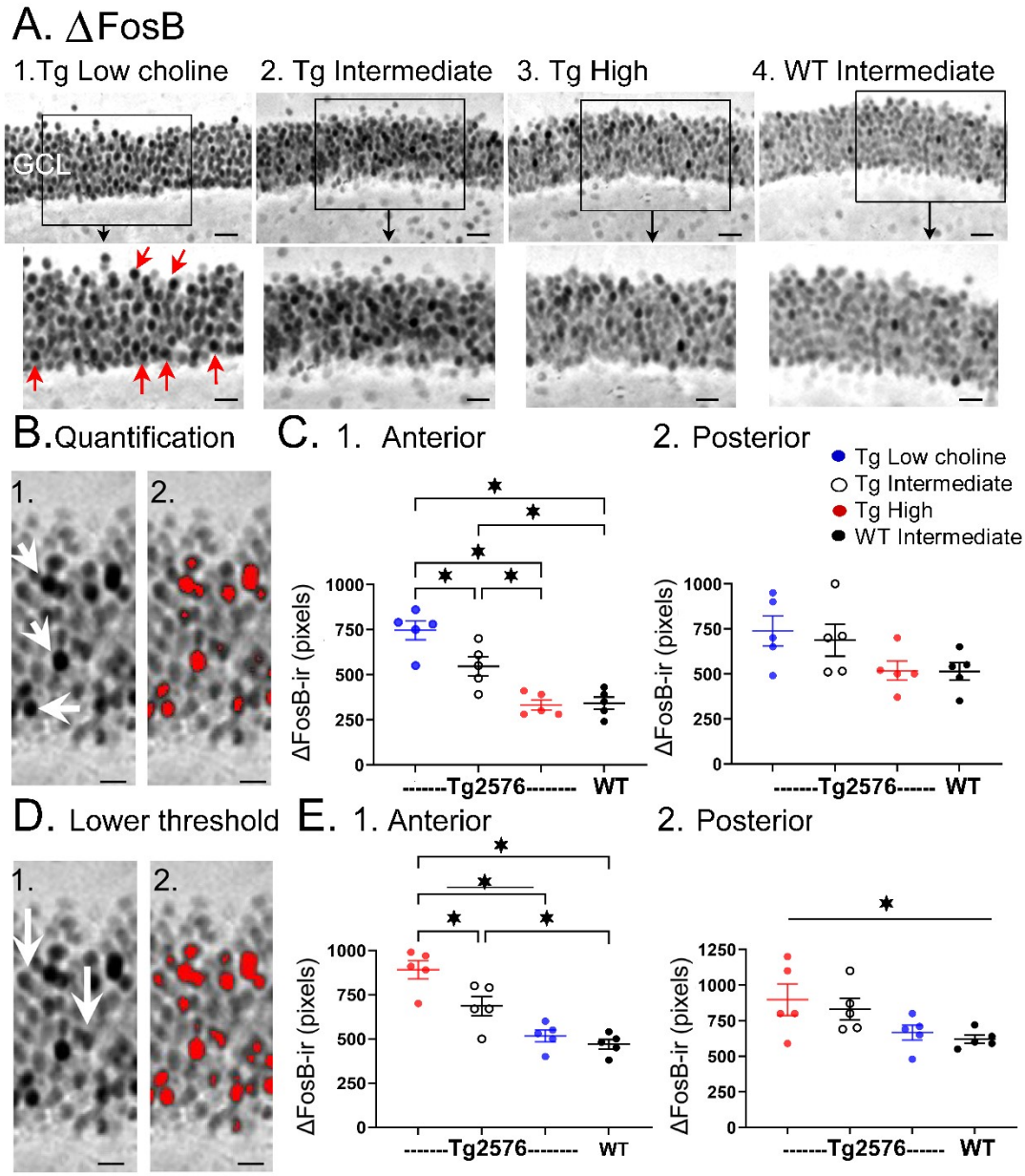
C. NeuN-ir in Tg2576 and WT mice. Tg2576 mice had either the low, intermediate, or high choline diet in early life. WT mice were fed the standard diet (intermediate choline)**.**

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.



1199
1200
1201

1202 **Figure 7. Choline supplementation reduced ΔFosB expression in dorsal**
1203 **GCs of Tg2576 mice.**
1204 **A.** Representative images of ΔFosB staining in GCL..
1205 1. A section from a low choline-treated Tg2576 mouse shows robust ΔFosB-ir in
1206 the GCL. The area outlined by the box is expanded below. Red arrows point to Δ
1207 **F**fosB-labeled cells. GCL=granule cell layer. Calibration for the top row, 100 μm;
1208 for the bottom row, 50 μm.2-3. Sections from intermediate (2) and high choline
1209 (3)-treated Tg2576 mice. Same calibrations as 1.
1210 4, A section from a WT mouse treated with the intermediate diet. Same
1211 calibrations as 1.
1212 **B.** Quantification methods**.** Representative images demonstrating the
1213 thresholding criteria established to quantify ΔFosB.
1214 1. A ΔFosB -stained section shows strongly-stained cells (white arrows).
1215 2. A strict thresholding criteria was used to make only the darkest stained cells
1216 red.
1217 **C.** Use of the strict threshold to quantify ΔFosB-ir.
1218 1. Anterior DG. Tg2576 mice treated with the choline supplemented diet had
1219 significantly less ΔFosB-ir compared to the Tg2576 mice fed the low or
1220 intermediate diets. Tg2576 mice fed the high choline diet were not significantly
1221 different from WT mice, suggesting a rescue of ΔFosB-ir.
1222 2. There were no significant differences in ΔFosB-ir in posterior sections.
1223 **D.** Methods are shown using a threshold that was less strict.
1224 1. Some of the stained cells that were included are not as dark as those used for
1225 the strict threshold (white arrows).
1226 2. All cells above the more permissive threshold are shown in red.
1227 **E.** Use of the less strict threshold to quantify ΔFosB-ir.
1228 1. Anterior DG. Tg2576 mice that were fed the high choline diet had less ΔFosB-
1229 ir pixels than the mice that were fed the other diets. There were no differences
1230 from WT mice, suggesting restoration of ΔFosB-ir by choline enrichment in early
1231 life.
1232 2. Posterior DG. There were no significant differences between Tg2576 mice fed
1233 the 3 diets or WT mice.


A. ΔFosB
1.Tg Low choline
2. Tg Intermediate
3. Tg High
4. WT Intermediate
GCL
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
E. 1. Anterior
2. Posterior
1250

1234
1235

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 (*, $p<0.05$).

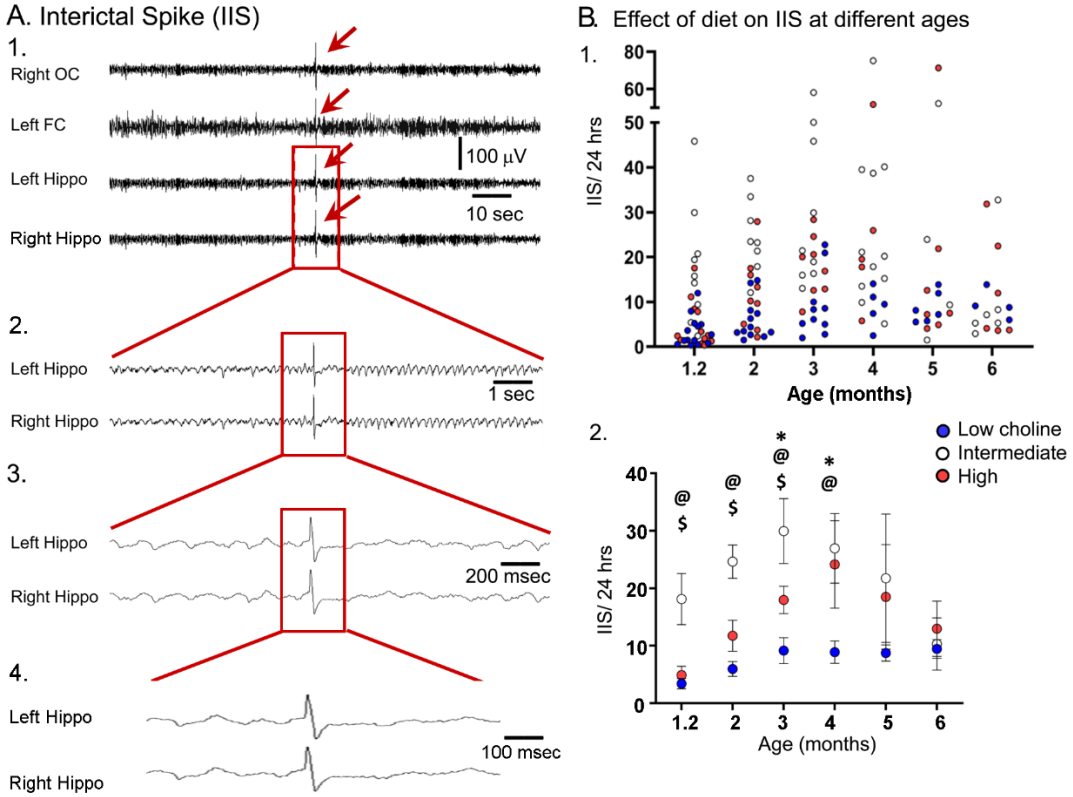


Figure 9. Correlations between IIS, Behavior, and hilar NeuN-ir.

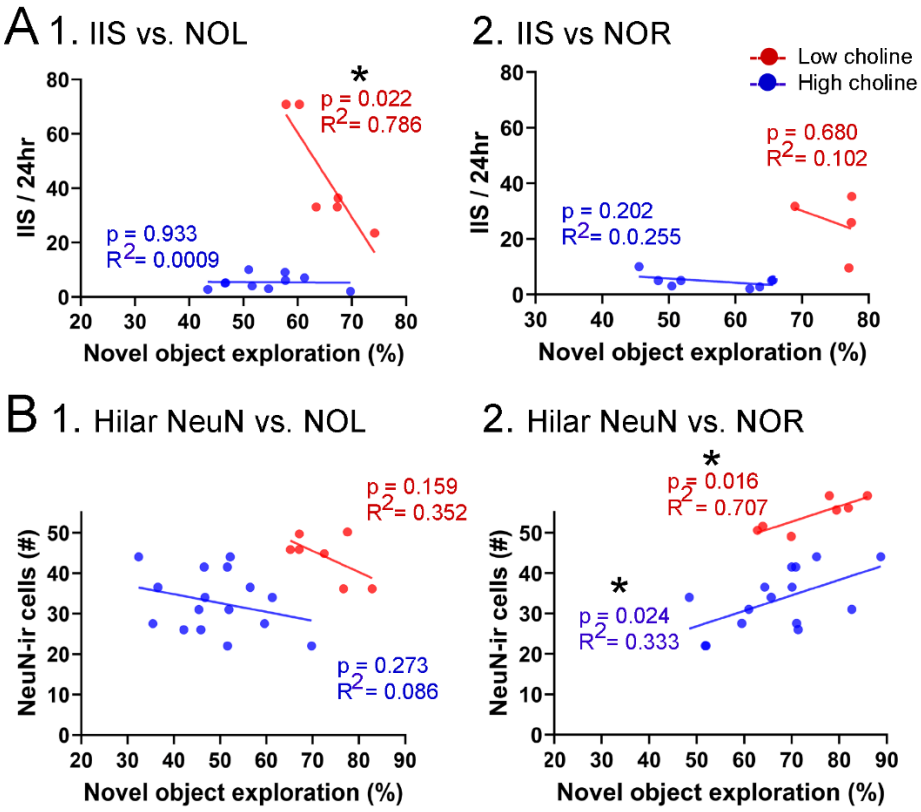
A. IIS frequency over 24 hrs is plotted against the preference for the novel object in the test phase of NOL. A greater preference is reflected by a greater percentage of time exploring the novel object.

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).

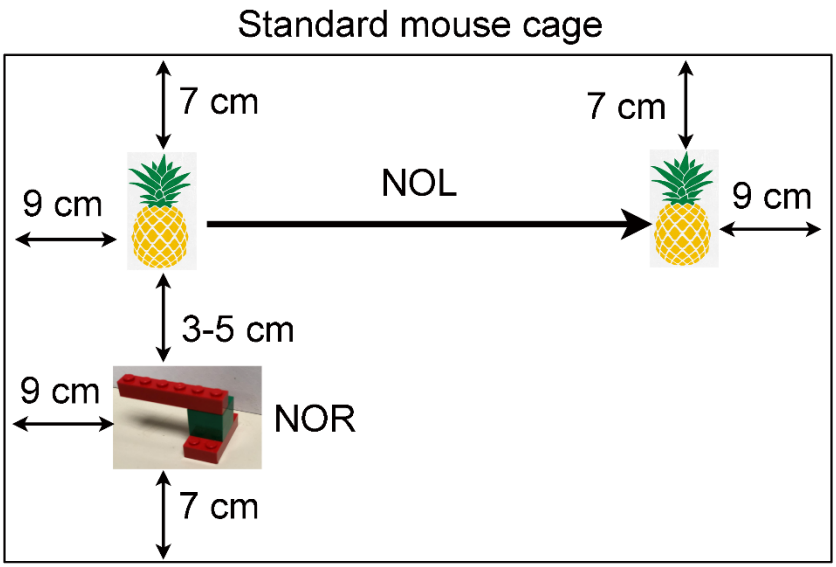


1272 **Supplemental Table 1. Comparison of the 3 diets used in this study.**
1273

	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

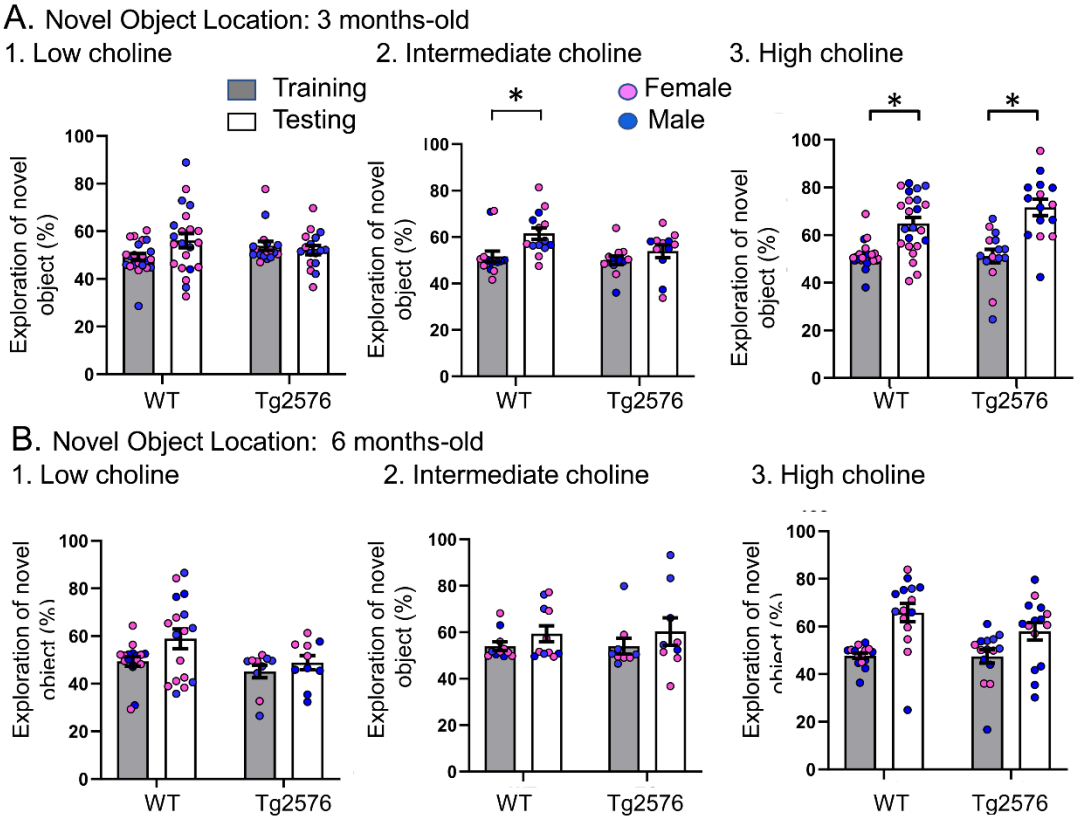
1274
1275 **Legend.**
1276 The major constituents of the diets are shown. Concentrations are g nutrient/kg
1277 chow. Food was provided *ad libitum* to breeders during mating, gestation and
1278 until weaning. After weaning all mice were fed the intermediate diet. WT mice
1279 were fed the intermediate diet during breeding, gestation, until weaning and after
1280 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.



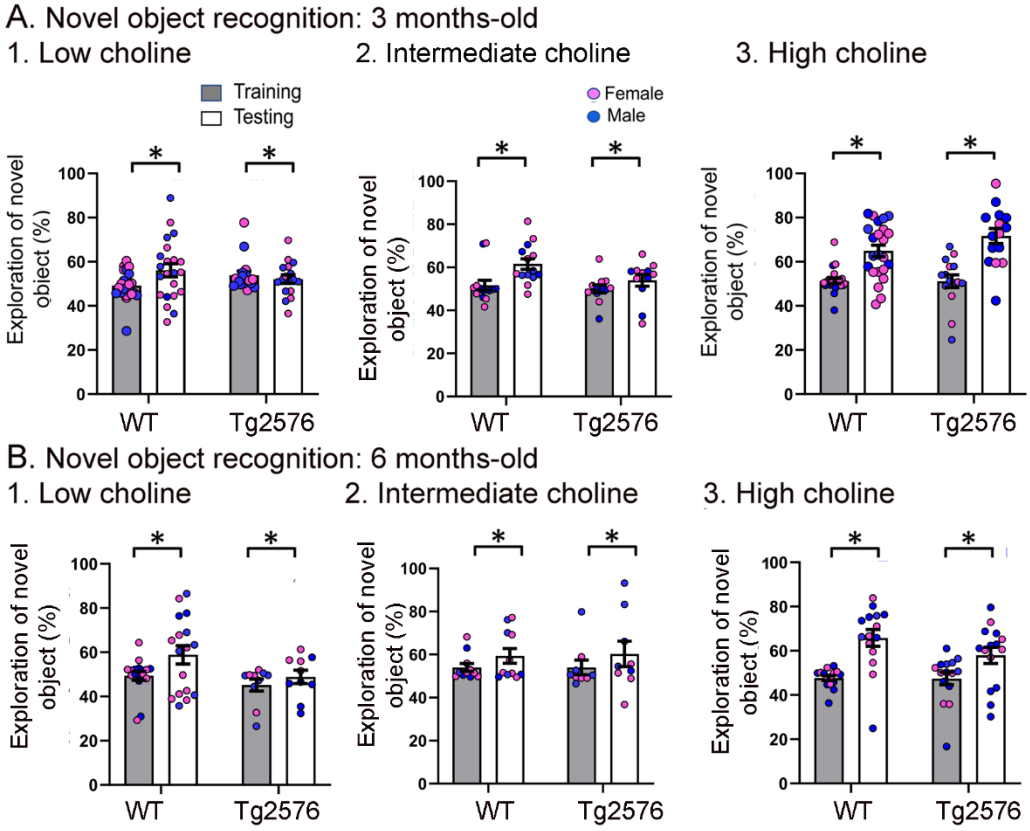
Supplemental Figure 2.

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.



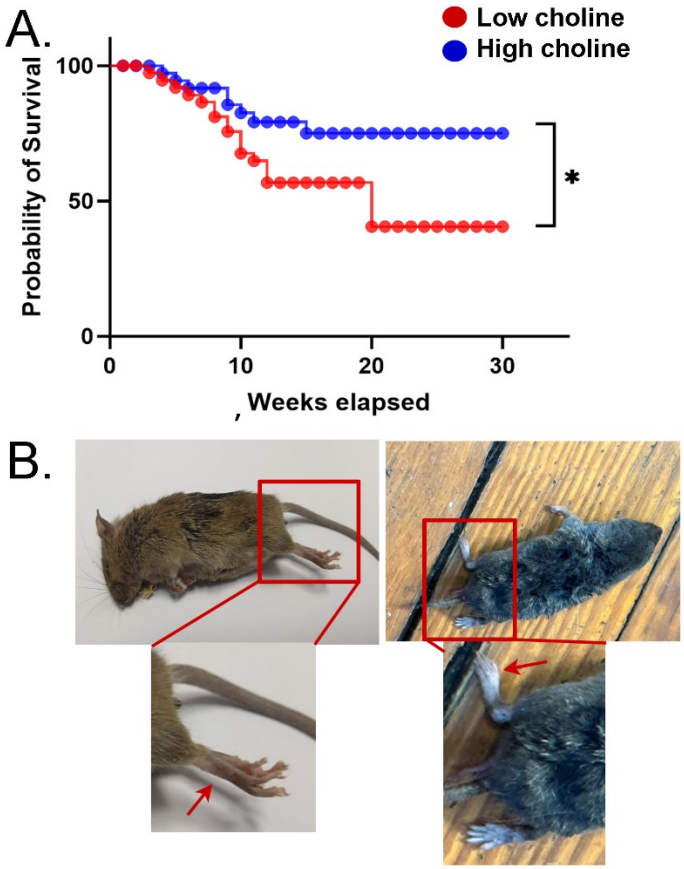
Supplemental Figure 4. Mortality was high in mice treated with the low choline diet.

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.

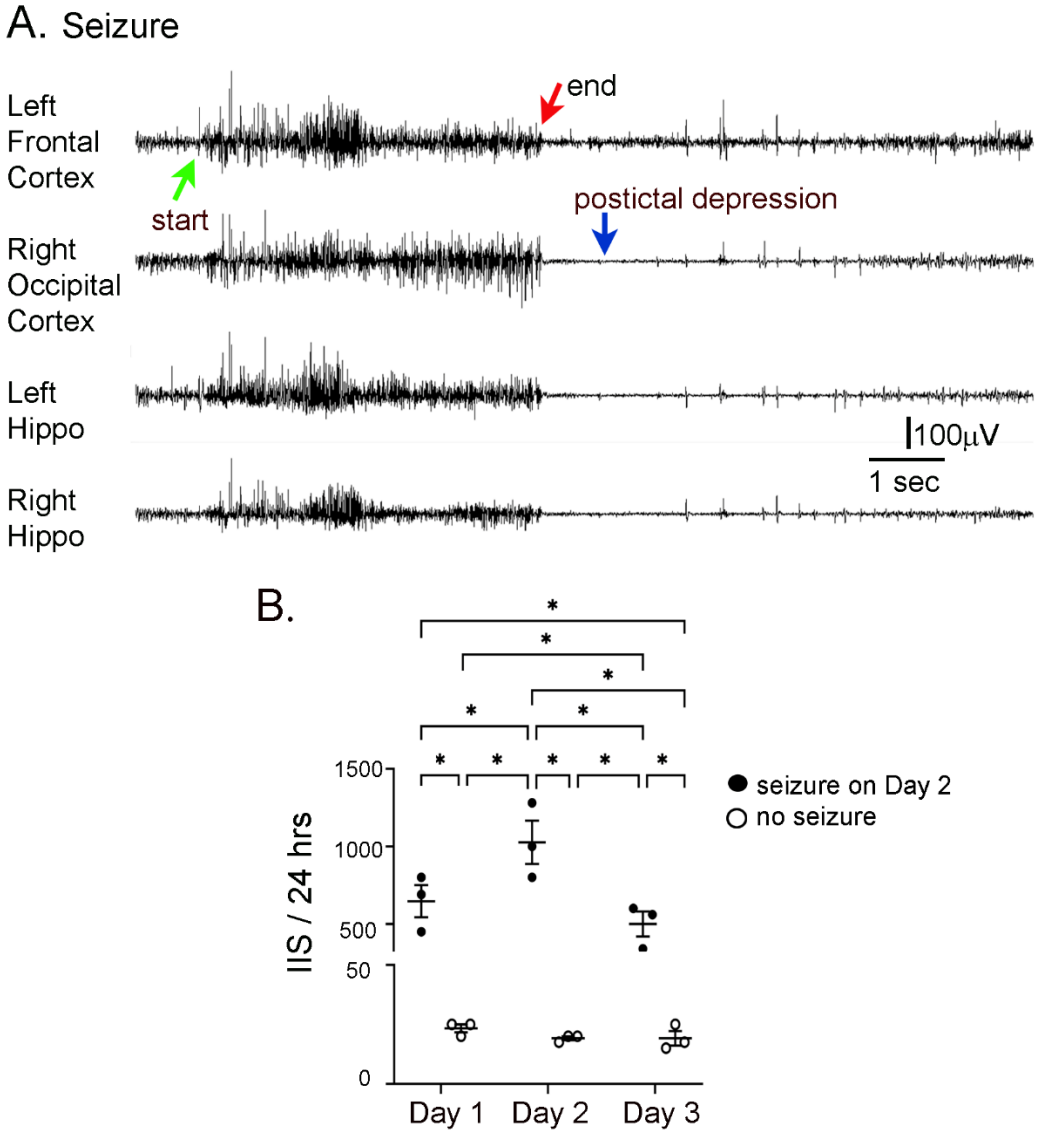
B.

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.

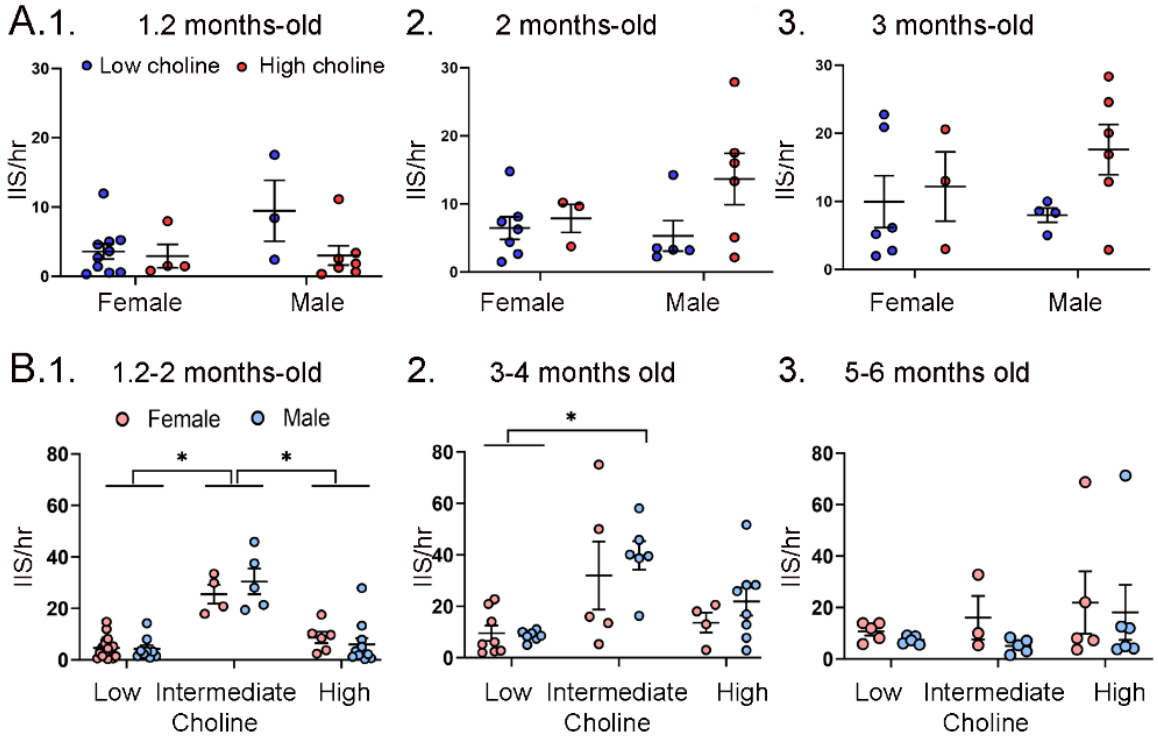


Supplemental Figure 5. IIS frequency before and after seizures.
A. Representative EEG traces recorded from electrodes implanted in the skull over the left frontal cortex, right occipital cortex, left hippocampus (Hippo) and right hippocampus during a spontaneous seizure in a 5 months-old Tg2576 mouse. 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
Right Occipital Cortex
Left Hippo
Right Hippo
end
start
postictal depression
100μV
1 sec
B.
IIS / 24 hrs
1500
1000
500
50
0
Day 1
Day 2
Day 3
seizure on Day 2
no seizure

1335

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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