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). However, the effects of MCS on neuronal hyperexcitability in AD are unknown. Therefore, we investigated the effects of MCS in a well-established mouse model of AD, the Tg2576 mouse. Like many AD mouse models and patients, Tg2576 mice exhibit hyperexcitability, typically 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 that hilar neurons reduce expression of the neuronal marker NeuN in Tg2576 mice, which other studies have shown is a sign of oxidative stress or 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 of mice fed the relatively low choline diet had several adverse effects, such as increased mortality, suggesting poor health. 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 rodent and human populations 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 (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). 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 after 3 months of age (Kam et al., 2016). 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. We further evaluated 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). Therefore, we asked if MCS would restore NeuN expression.

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 studies of MCS in rodents (Meck et al., 1988; Loy et al., 1991; Holler et al., 1996; Meck & 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 hr-long recordings began after 1 week for recovery, followed by 24 hr-long recording each month. At 3 and 6 months, NOL and NOR were tested, and then we perfusion-fixed mice, sectioned the brains, and evaluated ΔFosB and NeuN protein expression (Figure 1A).

The results showed a remarkable effect of the high choline diet. IIS and spatial memory were improved, as was ΔFosB and NeuN expression. Interestingly, the low choline diet had mixed effects, reducing IIS frequency, but increasing mortality and having other adverse outcomes, suggesting IIS were probably reduced due to poor health. In summary, we make a strong argument for choline supplementation in early life to improve outcomes in an AD model, and potentially AD.

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) at the Nathan Kline Institute for Psychiatric Research. Tg2576 mice express a mutant form of human APP (isoform 695) with a mutation found in a Swedish cohort (Lys670Arg, Met671Leu). The expression is driven by the hamster prion protein promoter . Mice were bred in-house from heterozygous Tg2576 males and non-transgenic female mice (C57BL6/SJL F1 hybrid). WT mice were obtained from Jackson Laboratories (Stock# 100012). At weaning, genotypes were determined using a protocol to detect APP695.

Mice were housed in standard mouse cages with corn cob bedding and a 12-hr light-dark cycle. Food and water were provided *ad libitum*.

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

B. Behavior

1. *General information*

Mice were housed in the room where they would be tested behaviorally starting at least 24 hr prior to all experiments. Both NOL and NOR included three acclimations (2-3 min each) followed by a training and a testing session (5 min each; Figure 1B-D). The interval between training and testing was 60 minutes

and therefore tested short-term memory (Vogel-Ciernia & Wood, 2014). NOL and NOR tests were separated by 7 days to ensure effects of one task did not affect the next. The order was randomized. 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).

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. for all animals, while training and testing was conducted between 1:00 p.m. that were selected 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. As reported in the Results, animals showed no preference for either of the objects (i.e., LEGO object and pineapple, Supplemental Figure 1).

2. *NOL*

The task 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 ~5 cm x 5 cm x 5 cm. During the acclimations, animals were allowed to freely explore the cage. During the training session, mice were placed in the cage where they were 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). They 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 (e.g., right top; Supplemental Figure 1). The identical objects were identical pineapple-like objects made of hard material that was painted and approximately 3 x 3 x 5 cm (Supplemental Figure 1; Botterill et al., 2021).

3. *NOR*

The task 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 between the one of the cage walls (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 identical objects were 2 pineapple-like objects and the new object was made of 2 green LEGO pieces approximately 5 x 7 x 7 cm (Supplemental Figure 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 pointed at the

239 object and the nose was located within 2 cm of the object. In addition, exploration
240 time included the time animals spent on top of the object if they were looking
241 down and sniffing it. An approach was defined by a movement toward the object
242 and ending at least 2 cm from the edge of the object.
243 Animals that remembered the objects explored during the training phase
244 were expected to demonstrate an increased preference for novelty in the test
245 phase (Ennaceur & Delacour, 1988). In other words, exploration of the novel
246 object during testing was expected to be higher than 50% of the total time of
247 object exploration.
248
249 **C. Anatomy**
250 **1.** *Perfusion-fixation and sectioning*
251 Mice were initially anesthetized by isoflurane inhalation (NDC# 07-893-1389,
252 Patterson Veterinary), followed by urethane (2.5 g/kg; i.p.; Cat# U2500, Sigma-
253 Aldrich). Under deep anesthesia, the abdominal and heart cavities were opened
254 and the animal was perfused transcardially using 10 mL of room temperature
255 (RT) saline (0.9% sodi um chloride in double distilled (dd) H_2O) using a peristaltic
256 pump (Minipuls1; Gilson), followed by 30 mL of 4°C 4% paraformaldehyde (PFA,
257 Cat# 19210, Electron Microscopy Sciences) in 0.1 M phosphate buffer (PB; pH
258 7.4). The brains were immediately extracted, hemisected, and post-fixed for at
259 least 24 hr in 4% PFA (at 4°C).
260 Following post-fixation, the left hemisphere was sectioned in the coronal
261 plane and the right hemisphere was sectioned in the horizontal plane (50 µm-
262 thick sections) using a vibratome (VT 1000P, Leica Biosystems). Sections were
263 collected serially and stored at 4°C in 24-well tissue culture plates containing
264 cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% 0.1 M
265 phosphate buffer, pH 6.7). Throughout the hippocampus, every 6th section was
266 processed with an antibody to NeuN or ΔFosB.
267
268 **2.** *Immunohistochemistry- NeuN and ΔFosB*
269 Free floating sections were washed in 0.1 M Tris Buffer (TB; 3 x 5 min),
270 followed by a 3 min wash in 1% H_2O_2. They were then washed in 0.1 M TB (3 x 5
271 min) and incubated for 60 min in 5% horse serum for NeuN (Cat# S-2000,
272 Vector) or 5% goat serum for ΔFosB (Cat# S-1000, Vector), diluted in a solution
273 of 0.25% (volume/volume or v/v) Triton-X 100, and 1% (weight/volume or w/v)
274 bovine serum albumin in 0.1 M TB. They were then incubated overnight at 4°C in
275 primary antibody to NeuN (mouse monoclonal, 1:5000; Cat# MAB377, Chemicon
276 International) or anti-ΔFosB (rabbit monoclonal, 1:1000; Cat# D3S8R, Cell
277 Signaling), diluted in a solution of 0.25% (v/v) Triton-X 100, and 1% (w/v) bovine
278 serum albumin in 0.1M TB. Both NeuN and ΔFosB have been well-characterized
279 as antigens (Mullen et al., 1992; H. K. Wolf et al., 1996; Chen et al., 1997; Sarnat
280 et al., 1998) and the antibodies we used have been commonly employed in the
281 past (Chen et al., 1997; Duffy et al., 2013; Corbett et al., 2017).
282 On the following day, sections were washed in 0.1 M TB (3 x 5 min) and
283 then incubated for 60 min in biotinylated horse anti-mouse secondary antibody
284 for NeuN (1:500, Cat# BA-2000, Vector) or biotinylated goat anti-rabbit

285 secondary antibody for ΔFosB (1:500, Cat# BA-1000), diluted in a solution of
286 0.25% (v/v) Triton-X 100, and 1% (w/v) bovine serum albumin in 0.1M TB. The
287 sections were then washed in 0.1 M TB (3 x 5 min) and incubated in avidin-biotin
288 complex for 2 hr (1:1000; Cat# PK-6100, Vector). They were washed in 0.1 M TB
289 (3 x 5 min) and then reacted in a solution containing 0.5 mg/mL 3, 3'-
290 diaminobenzidine (DAB; Cat# D5905, Sigma-Aldrich), 40 µg/mL ammonium
291 chloride (Cat# A4514, Sigma-Aldrich), 25 mg/mL D(+)-glucose (Cat# G5767,
292 Sigma-Aldrich), and 3 g/mL glucose oxidase (Cat# G2133, Sigma-Aldrich) in 0.1
293 M TB. The purpose of this method was to slow the reaction time so that the
294 reaction could be temporally controlled, meaning that the reaction could be
295 stopped when the immunoreactivity was robust but background was still low. The
296 sections were then washed in 0.1 M TB (3 x 5 min), mounted on gelatin-coated
297 slides (1% bovine gelatin, Cat# G9391, Sigma-Aldrich) and dried at RT overnight.
298 The following day they were dehydrated in increasing concentrations of ethanol
299 (90%, 10 min; 95% 10 min; 100%, 10 min; 100% again, 10 min), washed in
300 Xylene (10 min; Cat# 534056, Sigma-Aldrich) and cover-slipped with Permount
301 (Cat# 17986-01, Electron Microscopy Sciences).
302
303 **2.** *Analysis*
304 Photomicrographs were acquired using ImagePro Plus V7.0 (Media
305 Cybernetics) and a digital camera (Model RET 2000R-F-CLR-12, Q-Imaging).
306 NeuN and ΔFosB staining were quantified from micrographs using ImageJ V1.44
307 (National Institutes of Health). All images were first converted to grayscale and in
308 each section, the hilus was traced, defined by zone 4 of Amaral (1978). A
309 threshold was then calculated to identify the NeuN-stained cell bodies but not
310 background. Then NeuN-stained cell bodies in the hilus were quantified
311 manually. Note that the threshold was defined in Image J using the distribution of
312 intensities in the micrograph. A slider was pushed from the low level of staining to
313 the location where staining intensity makes a sharp rise. Below that was
314 considered background and above that was defined as above threshold.
315 To count ΔFosB-stained cells, images were converted to grayscale and in
316 each section, the dentate gyrus granule cell layer was outlined and defined as a
317 region of interest (ROI). A threshold was then set to identify the dark cells but not
318 the background, analogous to the was threshold was defined in the preceding
319 paragraph. Two thresholds were used. The first threshold was most inclusive of
320 immunoreactive cells even if staining was not too dark. Presumably this group
321 reflected cells with less neuronal activity. Then counts were made a second time
322 with a higher threshold. This group probably corresponded to cells with the
323 highest activity. ImageJ was used to calculate the area (in pixels) within the ROI
324 that was above threshold.
325
326 **D. Video-electroencephalographic (video-EEG) recordings**
327 **1.** *Stereotaxic surgery*
328 EEG electrodes were implanted in Tg2576 animals at 4 weeks of age. WT
329 littermates were not implanted because previous work from our laboratory has
330 shown that WT mice have no IIS (Kam et al., 2016). The animals were

anesthetized by isoflurane inhalation (3% isoflurane. 2% oxygen for induction). The Isoflurane for maintenance during surgery was ≤ 1.75%, The 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); 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 near 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°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 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. IIS occur primarily in sleep so only sleep was analyzed.

Mice were placed into 21 cm x 19 cm transparent cages (similar to standard laboratory 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 basal 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 (Graphpad).

401 **III. RESULTS**
402
403 **A. Behavior**
404 Tg2576 mice develop impairments in synaptic plasticity and behavior very
405 early in life, prior to Aβ deposition (Duffy et al., 2015) (Ohno et al., 2004;
406 Jacobsen et al., 2006). In this study, we examined short-term spatial memory
407 and recognition memory using the NOL and the NOR tasks respectively. These
408 tasks, unlike others previously used to evaluate cognitive deficits in >9 months-
409 old Tg2576 mouse model (i.e., Morris Water Maze, Radial Arm Water Maze, Y-
410 Maze; (Yassine et al., 2013; A. Wolf et al., 2016)), provided an opportunity to
411 capture cognitive deficits in animals as young as three months-old (Duffy et al.,
412 2015).
413 Figure 1B-C shows a schematic of the specific experimental procedures
414 followed for NOL and NOR.
415
416 **1.** *NOL*
417 The results of the NOL task are presented in Figure 2. Exploration of the
418 novel object is expressed as a percentage of the total exploration of both objects.
419 During the training session, animals did not show preference for one object over
420 the other and this result was not dependent on genotype. Thus, a two-way
421 ANOVA revealed no main effects of genotype on novel object exploration during
422 training at three ($F(1,97) = 0.40$, $p = 0.53$) or six months of age ($F(1,70) = 1.94$, p
423 $= 0.17$). There also was no effect of maternal diet on training at three ($F(2,97) =$
424 0.06, $p = 0.94$) or six months of age ($F(2,70) = 2.94$, $p = 0.06$).
425 Next, we asked it Tg2576 mice were deficient in the test phase of NOL. We
426 used a two-way ANOVA with genotype and task phase (training versus testing)
427 as main factors. In animals that received the intermediate diet, a two-way
428 ANOVA revealed a significant main effect of the phase of the task (training or
429 testing; $F(1,24) = 7.48$, $p = 0.01$). Consistent with previous studies (Duffy et al.,
430 2015), post-hoc analyses revealed a significant increase in the exploration of the
431 novel object during testing in WT ($p = 0.02$) but not Tg2576 ($p = 0.50$; Figure 2A).
432 Thus, Tg2576 mice were impaired in NOL at 3 and 6 months of age when fed the
433 intermediate diet.
434 In animals that received the high choline diet, a two-way ANOVA revealed a
435 main effect of the task phase ($F(1,36) = 48.23$, $p < 0.001$). Post-hoc analyses
436 revealed a significant increase in the exploration of the novel object during
437 testing in both WT ($p < 0.001$) and Tg2576 ($p < 0.001$). Therefore, high choline
438 supplementation improved memory in Tg2576 mice.
439 In animals treated with the low choline diet, there was no effect of phase of
440 the task ($F(1,37) = 1.39$, $p = 0.25$). These data suggesting that low levels of
441 choline during early life impaired spatial memory in both WT and Tg2576 mice
442 (Figure 2A). Thus, low choline had a significant adverse effect on NOL. Adverse
443 effects are supported by survival plots showing that there was more mortality at
444 earlier ages in low choline-treated mice (Supplemental Figure 1).
445 Figure 2B shows the results in 6 months-old WT and Tg2576 mice. In
446 animals that received the intermediate diet, a two-way ANOVA showed no main

447 effect of genotype ($F(1,18) = 0.01$, $p = 0.92$) or task phase ($F(1,18) = 3.40$, $p =$
448 0.08), revealing age-related memory deficits even in WT mice at six months of
449 age (Figure 2B). In contrast, the high choline group showed a main effect of task
450 phase ($F(1,29) = 28.32$, $p < 0.001$). Post-hoc analyses showed a significant
451 increase in the exploration of the novel object during testing in both WT ($p <$
452 0.001) and Tg2576 ($p = 0.02$). In animals that received the low choline diet, there
453 was no effect of task phase ($F(1,25) = 3.00$, $p = 0.10$; Figure 2B). Thus, low
454 choline had adverse effects on NOL in both WT and Tg2576 mice.
455 Taken together, these results demonstrate the lasting beneficial effects of the
456 high choline diet and adverse effects of low choline on a spatial memory task in
457 Tg2576 as well as WT littermates.
458
459 **2.** *NOR*
460 The results of the NOR task are presented in Figure 3. During the training
461 session animals did not show preference for one object over the other, exploring
462 both objects equally. A two-way ANOVA revealed no main effects of genotype at
463 three months ($F(1,71) = 2.59$, $p = 0.54$) and no effect of diet ($F(2,71) = 0.22$, $p =$
464 0.81). The same result was obtained at six months of age (genotype: ($F(1,66) =$
465 0.11, $p = 0.75$); diet: ($F(2,66) = 0.98$, $p = 0.38$). Thus, novel object exploration
466 during training approached 50% in all treatment groups, independent of genotype
467 and maternal diet, and at both ages.
468 Figure 3A shows the results of the testing phase of the NOR task in 3
469 months-old WT and Tg2576 mice. In animals that received the intermediate
470 choline diet, a two-way ANOVA revealed a significant main effect of task phase
471 ($F(1,19) = 40.53$, $p < 0.01$) and no effect of genotype ($F(1,19) = 0.78$, $p = 0.39$).
472 Post-hoc analyses revealed a significant increase in the exploration of the novel
473 object during testing compared to training for both WT ($p < 0.01$) and Tg2576 (p
474 < 0.01; Figure 3A). These results suggest that Tg2576 did not have a deficit in
475 NOR at 3 months of age.
476 Mice fed the high choline diet also showed no deficit in NOR at 3 months of
477 age. Thus, a two-way ANOVA revealed a main effect of task phase ($F(1,26) =$
478 17.51, $p < 0.01$) but not genotype ($F(1,26) = 3.53$, $p = 0.07$). Post-hoc analyses
479 revealed a significant increase in the exploration of the novel object during
480 testing in both WT ($p < 0.01$) and Tg2576 ($p = 0.03$; Figure 3A).
481 In animals treated with low choline, there was no impairment in NOR at 3
482 months of age either (task phase ($F(1,26) = 22.61$, $p < 0.01$; genotype ($F(1,26) =$
483 0.02, $p = 0.89$). There was a significant increase in the exploration of the novel
484 object in testing both in WT ($p = 0.03$) and Tg2576 ($p < 0.01$).
485 Figure 3B shows that there were no impairments at 6 months of age. In
486 animals that received the intermediate diet, a two-way ANOVA revealed a
487 significant main effect of task phase ($F(1,19) = 13.65$, $p < 0.01$) but no main
488 effect of genotype ($F(1,19) = 0.28$, $p = 0.60$). Animals explored the novel object
489 significantly more during testing in WT ($p = 0.03$) and Tg2576 mice ($p = 0.04$;
490 Figure 3B). In animals that received the other diets, results were the same. For
491 the high choline group, there was an effect of task phase ($F(1,27) = 16.26$, $p <$
492 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 (Jacobsen et al., 2006; 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 exploration, independent of the object (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 ($p = 0.049$). Three month-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 and asked to determine which diets differed. For NOL, mice treated with the low choline diet had less exploration than mice fed the high choline diet (one-way ANOVA, $F(2,77) = 6.90$; $p = 0.002$; Tukey post-hoc test, $p = 0.005$) 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, $p = 0.02$) and the

538 mice that were fed the intermediate diet also had less exploration than mice fed
539 the high choline diet (Tukey post-hoc test, $p = 0.03$; Figure 4D).
540
541 *2) Approaches*
542 To gain insight into the differences in exploration, we measured total object
543 approaches (TOA; Figure 4A-D). TOA was defined as the number of approaches
544 to the familiar object + number of approaches to the novel object. There were no
545 differences by genotype (two-way ANOVA, $F(1,73) = 2.930$, $p = 0.09$) or maternal
546 diet ($F(2,73) = 2.36$, $p = 0.10$; Figure 4A-D).
547 Taken together, the results indicate that, in 6 months-old mice, animals that
548 received the high choline diet spent more time with objects at each approach.
549 This may underlie increased object location memory in high choline-treated mice
550 by increasing time for information processing during an approach. It may also
551 help in the NOR task with object recognition memory.
552 Another possibility is that as the Tg2576 mice age they show compensatory
553 changes that enhance memory. This has been suggested for procedural learning
554 (Middei et al., 2004) but not for memory of objects.
555 Interestingly, it has been suggested that the high choline diet reduces anxiety
556 (Glenn et al., 2012; Langley et al., 2015; McCall et al., 2015). Reduced anxiety
557 may reduce fear of exploring objects, and as a result, animals may spend more
558 time with objects.
559 For 3 months-old mice there were no effects of maternal diet on exploration,
560 suggesting that increased time with objects for a given approach is a change that
561 develops with age. To our knowledge, this age-related change has not been
562 previously reported. It could be a result of impairments in Tg2576 mice with age,
563 or a strategy to cope with impairments that are developing as AD pathology
564 increases.
565
566 **B. Anatomy**
567 **1. NeuN**
568 To further analyze the effects of dietary choline early in life, an antibody
569 against a neuronal nuclear antigen, NeuN. Previous studies have found that
570 reduced expression of NeuN often occurs in neurons after insults or injury (e.g.,
571 ischemia, toxicity and even aging (Lind et al., 2005; Portiansky et al., 2006;
572 Buckingham et al., 2008; Kadriu et al., 2009; Matsuda et al., 2009; Won et al.,
573 2009; Duffy et al., 2011). When NeuN is reduced after a reduction in
574 ARMS/Kiddins, a protein critical to neurotrophin signaling, neurons in the
575 entorhinal cortex demonstrate additional signs of toxicity such as pkynosis (Duffy
576 et al., 2011; Duffy et al., 2015). Since NeuN is reduced when Aβ levels are
577 elevated (Wu et al., 2016) and NeuN is reduced in AD patients (Camporez et al.,
578 2021), we used NeuN expression to ask if the high choline diet could rescue the
579 loss of NeuN-ir.
580 As shown in Figure 5, relatively weak NeuN-ir was observed in Tg2576
581 animals in the hilus, an area known to be highly vulnerable to insult and injury
582 (Scharfman, 1999). The most NeuN loss appeared to be in mice that received
583 the low choline diet and the intermediate diet compared to the high choline diet

584 (Figure 5B). Therefore, we conducted a two-way ANOVA with diet and
585 septotemporal levels as main factors. To examine the septotemporal axis,
586 quantification from 2-3 dorsal coronal sections were averaged to provide a value
587 for dorsal DG and 2-3 ventral horizontal sections were averaged for a ventral
588 value. There was no effect of diet ($F(2,30) = 2.11$, $p = 0.14$) nor septotemporal
589 level ($F(1,30) = 0.02$, $p = 0.88$) but there was a significant interaction of diet and
590 septotemporal level ($F(2,30) = 3.88$, $p = 0.04$). A one-way ANOVA using dorsal
591 values showed a significant effect of diet ($F(2,21)= 7.58$, $p = 0.003$) but this was
592 not the case for ventral values ($F(2,23) = 0.13$; $p = 0.88$; Figure 5D). In the dorsal
593 DG, there was less NeuN-ir in mice fed the low choline diet vs the high choline
594 diet (Tukey-Kramer post hoc test, $p = 0.03$) and less NeuN in mice fed the
595 intermediate vs high choline diet ($p = 0.003$; Figure 5D). The results suggest that
596 choline enrichment protected hilar neurons from NeuN loss in Tg2576 mice.
597
598 **2. ΔFosB**
599 To complement the video-EEG recordings we used a marker of elevated
600 neuronal activity, ΔFosB (Figure 6). ΔFosB is a truncated variant of the
601 transcription factor FosB, which is increased by neuronal activity; ΔFosB has a
602 half-life of approximately 8 days (Ulery-Reynolds et al., 2009), so when ΔFosB is
603 elevated, it reflects increased neuronal activity over the last 10-14 days. Previous
604 studies have shown that when the J20 mouse model of AD is examined with an
605 antibody to ΔFosB, the GC layer shows extremely high levels of ΔFosB
606 expression, similar to a mouse with chronic spontaneous seizures (epilepsy;
607 REF). This is not surprising since the J20 mice have recurrent seizures (REF).
608 Therefore, we asked if Tg2576 mice would have robust ΔFosB in the GC layer,
609 and choline supplementation would reduce it.
610 There was strong expression of ΔFosB in Tg2576 GCs especially in mice fed
611 the low choline diet (Figure 6). Two-way ANOVA showed a main effect of
612 maternal diet ($F(2,18) = 3.76$, $p = 0.04$) but there was not a main effect of
613 septotemporal level ($F(1,18) = 1.3$, $p = 0.26$).
614 To clarify the effect of maternal diet, a one-way ANOVA was conducted. For
615 dorsal sections, there was an effect of diet ($F(2,9) = 10.74$, $p = 0.004$) with post-
616 hoc tests showing significantly less GC ΔFosB expression in high choline-treated
617 mice compared to low choline ($p = 0.003$). Low-choline-treated mice were not
618 significantly different from mice that were fed the intermediate diet ($p = 0.08$). For
619 the ventral DG, there was no significant effect of diet ($F2,9) = 0.79$, $p = 0.52$;
620 Figure 6).
621 ΔFosB quantification was repeated with a lower threshold to define a ΔFosB-
622 ir GCs (see Methods) and results were the same (Figure 6). One-way ANOVA
623 showed a significant effect of diet ($F(2,41) = 14.66$, $p < 0.0001$), and low choline
624 mice had greater ΔFosB-ir than high choline mice ($p =0.003$). Mice fed the
625 intermediate diet had higher expression than mice fed the high choline diet ($p =$
626 0.0001). These data suggest that high choline in the diet early in life can reduce
627 hyperexcitability of GCs in offspring later in life. In addition, low choline has an
628 opposite effect, again suggesting this maternal diet has adverse effects.
629

630 **C. IIS**

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

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

652 As shown previously (Kam et al., 2016), and in Figure 7, at 4-6 months of
653 age there was high variability in IIS frequency between animals. It is possible that
654 IIS frequency becomes more variable because we found that seizures occur in
655 the Tg2576 mice after 2 months of age (Kam et al., 2016), and there was a sharp
656 increase in IIS during the day before and after a seizure (Supplemental Figure
657 20.

658

659 **D. Seizures and premature mortality in mice that had been fed the low**
660 **choline diet**

661 We found that mice that had been fed the low choline diet had greater
662 ΔFosB-ir in GCs and the hilus showed very low NeuN-ir. Therefore, we asked
663 whether low choline-treated mice had more seizures than mice fed the other
664 diets. We recorded 8 mice by video-EEG for 5 days each (at 6 months of age) to
665 examine seizures and found 2 mice from the low choline group had seizures (11
666 seizures over 2 of the 5 days in one mouse, 1 seizure in the other mouse)
667 whereas none of the mice in the intermediate or high choline group had seizures
668 (n=0/4, 2 intermediate and 2 high choline).

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

673 The reason that low choline-treated mice appeared to die in a seizure was
674 that they were found in a specific posture in their cage which occurs when a
675 severe seizure leads to death (Supplemental Figure 2). They were typically found

676 in a prone posture with extended, rigid limbs (Supplemental Figure 2).
677 Regardless of how the mice died, there was greater mortality in the low choline
678 group compared to mice that had been fed the high choline diet (Supplemental
679 Figure 2).
680
681 **E. Sex differences**
682 As shown in Figures 2-3, there appeared to be no sex differences in NOL or
683 NOR. To confirm, we studied NOL further, since that was the task that showed
684 effects of diet. A three-way ANOVA with sex, genotype and task phase as factors
685 showed no significant effect of sex at 3 months of age (low choline diet, $F(1,35) =$
686 0.0001; $p = 0.99$). Results were the same for the 3 months-old mice fed the
687 intermediate diet ($F(1,22) = 0.062$; $p = 0.81$) and 3 months-old mice that were fed
688 the high choline diet ($F(1,34) = 0.43$; $p = 0.52$). At 6 months of age there also
689 were no effects of sex (low choline diet, $F(1,23) = 0.34$; $p = 0.57$; intermediate
690 diet, $F(1,16) = 2.49$; $p = 0.13$; high choline diet, $F(1,27) = 0.29$; $p = 0.87$).
691 For IIS, we did not find sex differences (Supplemental Figure 4). A two-way
692 ANOVA with sex and diet as factors showed no effect of sex ($F(1,20) =2.48$; $p =$
693 0.13) or diet ($F(1,20) = 3.56$; $p = 0.07$). (Note that for this comparison we used
694 the low choline and high choline groups but not the intermediate group because
695 sex information was unavailable for these mice.) The results for months 2-3 also
696 showed no effect of sex (2 months: $F(1,17) = 0.64$; $p = 0.43$; 3 months: $F(1,15) =$
697 0.19; $p = 0.67$).
698
699 **IV. DISCUSSION**
700
701 **Summary**
702 This study showed that early life choline supplementation had several
703 beneficial effects in Tg2576 mice. The high choline diet led toz reduced IIS
704 frequency, improved behavior in the NOL task, and improved expression of
705 ΔFosB.
706 We showed for the first time that Tg2576 mice have pathology in the hilus at
707 6 months of age in that hilar neurons are deficient in immunostaining with an anti-
708 NeuN antibody. Tg2576 offspring on the high choline diet showed a restoration of
709 hilar NeuN staining. Because hilar neurons play a role in NOL (Bui et al., 2018), it
710 is possible that rescue of hilar neurons by the high choline diet led to improved
711 spatial memory. Hilar neurons have been suggested to contribute to excitability
712 of GCs; Scharfman, 1999, 2016), so phenotypic rescue of hilar neurons may also
713 have contributed to the ability of the high choline diet to reduce hyperexcitability.
714 We also found surprising effects of treating mice with relatively low choline in
715 the maternal diet. On the one hand, IIS frequency was reduced. However, these
716 mice showed premature mortality. Furthermore, in WT mice where no
717 impairments in NOL normally occur, WT mice fed the low choline diet were
718 impaired. In Tg2576 mice, the group that had the low maternal choline diet had
719 the highest ΔFosB expression and the lowest NeuN expression of any
720 experimental group. Reasons for these surprising effects are discussed below.
721

722 **Benefits of high choline**

723 The results of this study are consistent with previous reports that prenatal or
724 postnatal choline supplementation improves object memory. One study of iron
725 deficiency showed that choline supplementation improved NOR (Kennedy et al.,
726 2014). Wistar rats treated with a diet high in choline had improved NOR relative
727 to rats that had been fed a diet low in choline (Moreno et al., 2013). In rats that
728 were aged to 24 months, animals that had been fed a high choline diet showed
729 improved NOR, but only in females (Glenn et al., 2008).

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

737 To our knowledge our study is the first to show that MCS can exert effects of
738 IIS and ΔFosB. However, prior studies have shown that MCS in rats improved
739 memory after severe seizures. Using a convulsant to induce several hours of
740 severe seizures (status epilepticus, SE), there is usually extensive hippocampal
741 neuronal loss and when tested in the subsequent days and weeks, memory
742 impairment. MCS reduced the impairment in the Morris water maze (Yang et al.,
743 2000; Holmes et al., 2002; Wong-Goodrich et al., 2011).

744 Our results are consistent with previous studies of mouse models of AD. In
745 the APP/PS1 mouse model, it was shown that life-long choline supplementation,
746 to the level we used (5.0 g/kg) improved memory in aged mice compared to the
747 1.1 g/kg diet (Velazquez et al., 2019). Postnatal choline supplementation also
748 improved memory in APP/PS1 mice (Wang et al., 2019). Other improvements
749 were also shown in the APP/PS1 mice, such as improved expression of choline
750 acetyl-transferase (Mellott et al., 2017; Velazquez et al., 2019).

751

752 **Adverse effects of relatively low choline**

753 The effects of the relatively low choline diet were surprising because several
754 adverse effects were found. This is surprising because 1.1 g/kg choline has not
755 been considered low by others who study rats. The past studies suggest that
756 there could be a species difference. Also, some of the past studies used the diet
757 between day 11 and 17 of gestation, which is shorter than what we used, and
758 therefore could have led to fewer adverse effects.

759 One adverse effect was weak NeuN expression in hilar neurons of mice fed
760 the low choline diet. Also, when WT mice were tested, NOL was impaired in the
761 low choline group but not in the mice exposed to diets with higher levels of
762 choline.

763 One of the surprising findings was a high mortality and appeared to die from
764 seizures. The surviving mice had very high ΔFosB expression in the GCs,
765 supporting the idea they had more seizures. Indeed, the survivors did have more
766 seizures than mice fed the other diets. These data suggest that the offspring of

767 mothers fed the low choline diet were unhealthy, possibly leading to or because
768 of seizures.
769 On the other hand, IIS were reduced in frequency in the same mice. There
770 are several possible explanations for this apparent paradox. The most
771 parsimonious explanation is the mice were unhealthy. If principal cells or
772 synapses degenerate, IIS would be reduced. Another explanation is seizures
773 disrupted the behavioral states when IIS occur, such as REM sleep. If REM
774 would be consistently disrupted, one would expect that IIS would be reduced.
775 Also, seizures in the Tg2576 mice were followed by a depression of the EEG that
776 would reduce IIS (postictal depression; Supplemental Figure 3).
777 A more radical explanation is that the intermediate diet promoted IIS rather
778 than low choline reducing IIS. The idea is attractive because it would potentially
779 explain why low and high choline groups had low IIS frequency compared to the
780 intermediate group. Thus, choline may not affect IIS very much but something
781 else in the diet that was very different in the intermediate group led to a
782 facilitation of IIS. This idea is also consistent with the data showing that the low
783 IIS in months 1-3 rose in months 4-6 as time when they were fed the
784 intermediate diet increased and there was a longer time since exposure to the
785 low/high choline diets.
786
787 **NeuN**
788 As mentioned above, NeuN is a neuronal nuclear antigen that can be
789 phosphorylated, and when that occurs the antibody to NeuN no longer binds to
790 NeuN. NeuN is phosphorylated in response to oxidative damage, brain injury,
791 and oxidative stress (Lind et al., 2005). Therefore, it was of interest when we saw
792 that hilar neurons of the dentate gyrus showed reduced NeuN in Tg2576 mice.
793 Hilar neurons are mainly glutamatergic mossy cells and somatostatin
794 (SOM)/neuropeptide Y (NPY)-expressing GABAergic neurons (HIPP cells;
795 (Houser, 2007; Scharfman & Myers, 2012; Scharfman, 2016), and both neuronal
796 types are implicated in spatial memory functions of the GCs, as well as their
797 excitability (Myers & Scharfman, 2009, 2011; Scharfman & Myers, 2012; Jinde et
798 al., 2013; Scharfman, 2016; Raza et al., 2017; Bui et al., 2018; GoodSmith et al.,
799 2019; Li et al., 2021). If damaged by the high intracellular APP/Aβ levels in young
800 Tg2576 mice, the hilar neurons would be expected to show NeuN loss. This
801 idea is consistent with prior studies of the HIPP cells in AD, stained using NPY.
802 The prior studies showed deficits in SOM and NPY-stained cells (Chan-Palay,
803 1987), indicating a vulnerability.
804 The rescue of dorsal hilar but not ventral hilar NeuN with the high choline diet
805 is unclear. However, there was a trend for the high choline mice to have greater
806 NeuN in ventral tissue sections. Therefore, the dorsal neurons appear to have
807 shown a more robust change. The greater sensitivity of dorsal neurons may be
808 related to differences in dorsal and ventral dentate gyrus gene expression
809 patterns, since some of the genes that are differentially expressed could affect
810 vulnerability to insult or injury (Cembrowski et al., 2016; Zhang et al., 2018). It is
811 possible that dorsal hilar neurons are inherently more resilient to insults,
812 especially in the context of AD. Resilience of vulnerable neuronal populations,

especially in the hippocampus, is a high interest topic across the AD spectrum, and in relevant AD models, including the Tg2576 mouse.

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. 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). The intermediate diet was useful however, because numerous studies in AD mouse models employ this standard diet.

There was high variability in the IIS data in the mice fed the intermediate diet after 3 months of age, and this may have led to an underestimation of effects. This variability was observed before and it may be due to the emergence of aspects of AD pathophysiology that emerge after 3 months of age (Kam et al.., 2016).

Because IIS occur primarily in sleep, it is possible that the effects of choline on IIS were indirectly due to their effects on sleep. For example, if the high choline diet led to reduced sleep, it would reduce IIS. However, there is no evidence that MCS influences sleep in humans or laboratory animals. The one study that looked at the sleep/wake cycle in rats did not find effects (Cheng et al., 2008).

Although we failed to detect sex differences, there may actually be 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 on diestrus have low excitability in hippocampal slices. At other cycle stages hyperexcitability occurs.

Limitations of the Tg2576 model should be considered because the mice do not recapitulate all characteristics of AD, such as tau pathology. Also, the Swedish mutation does not represent the majority of AD, which 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 (Strupp, 2016; Powers 2017, 2021; Alldred 2021, 2023).

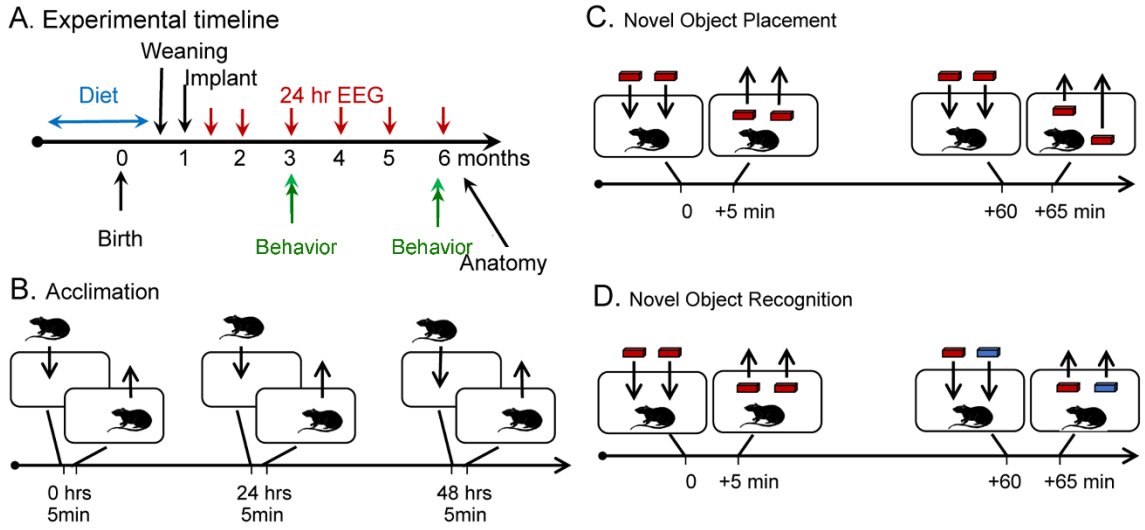
Figure 1. Schematic of the behavioral procedures for the NOL and NOR tasks.

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 24hr 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).



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

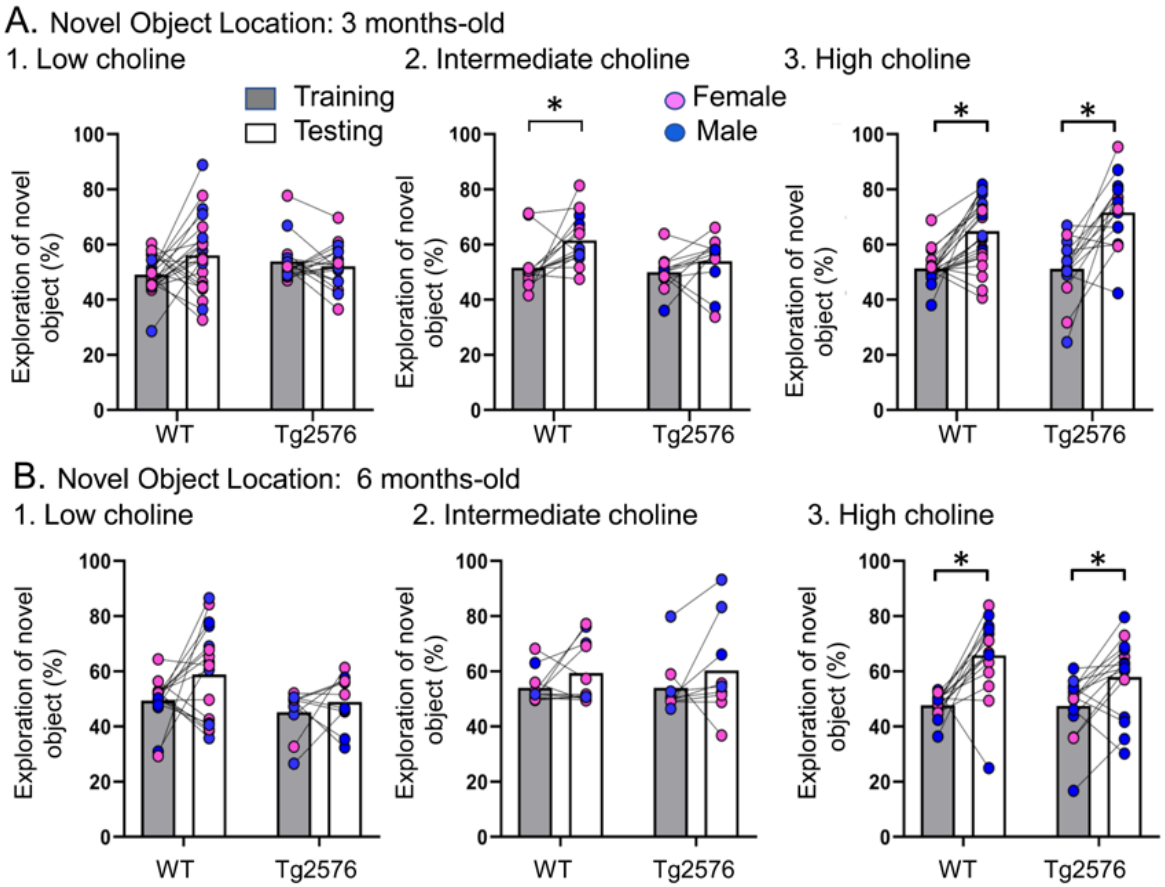

A. Novel Object Location: 3 months-old
1. Low choline
2. Intermediate choline
3. High choline
Training
Testing
Female
Male
Exploration of novel object (%)
WT
Tg2576
B. Novel Object Location: 6 months-old
1. Low choline
2. Intermediate choline
3. High choline

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.

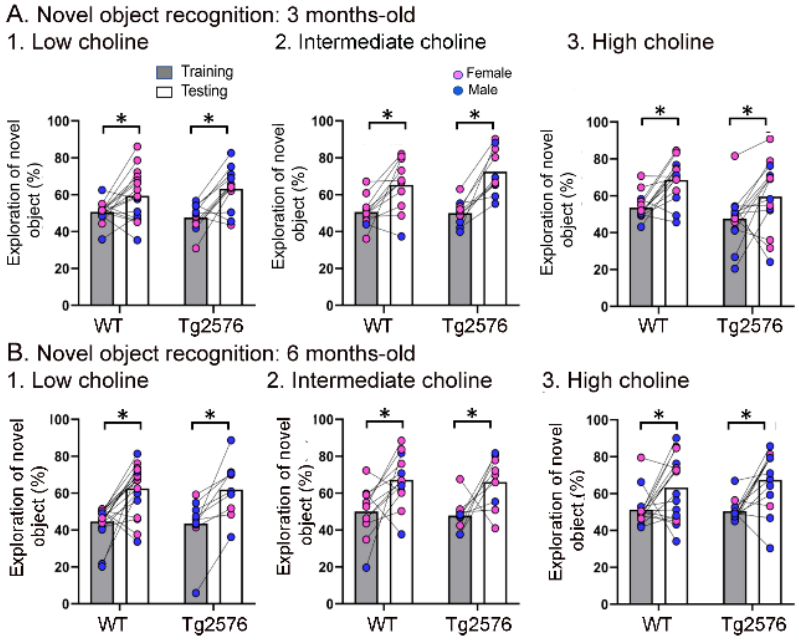


Figure 4. Tg2576 mice treated with the choline supplemented diet spent more time with the objects at each approach.

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.

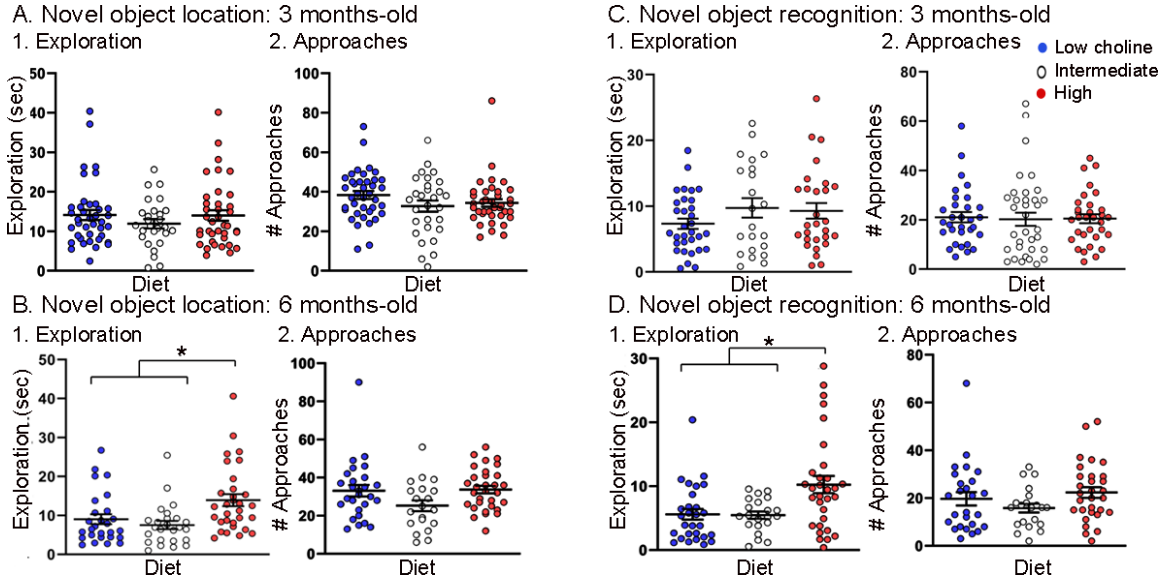


Figure 5. Choline supplementation improves NeuN immunoreactivity (ir) in hilar cells in Tg2576 animals

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 choline supplemented 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).

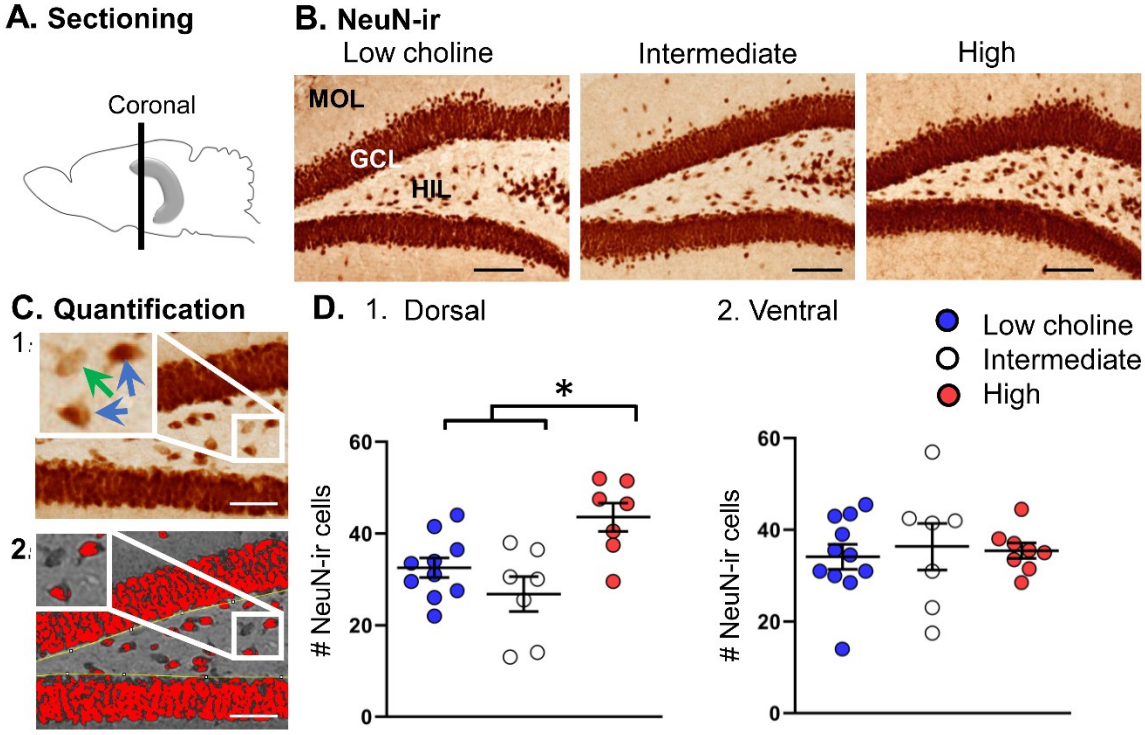


Figure 6. Choline supplementation reduced ΔFosB expression in dorsal GCs.

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.

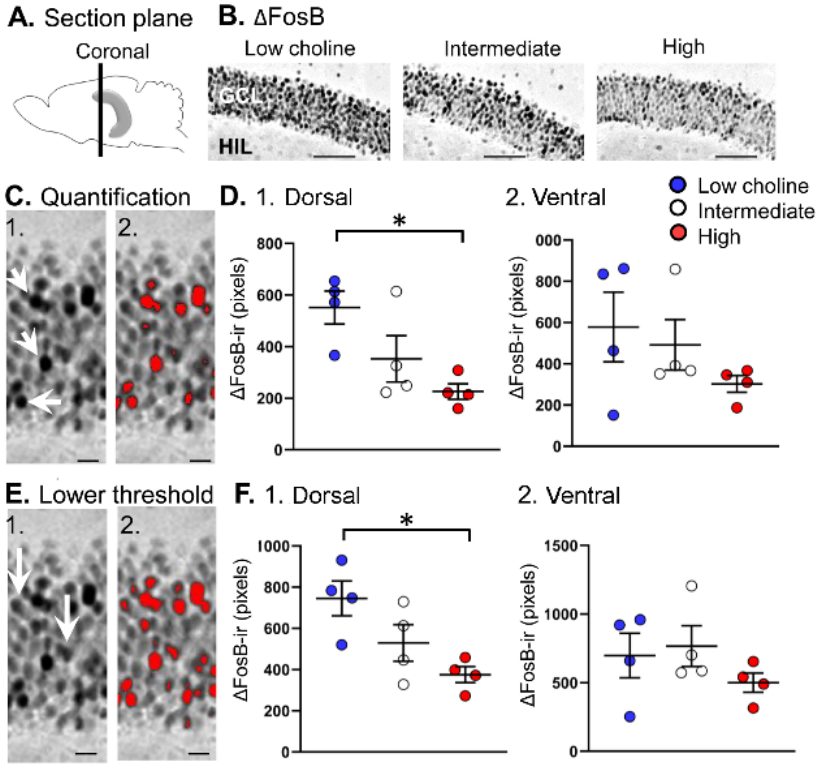
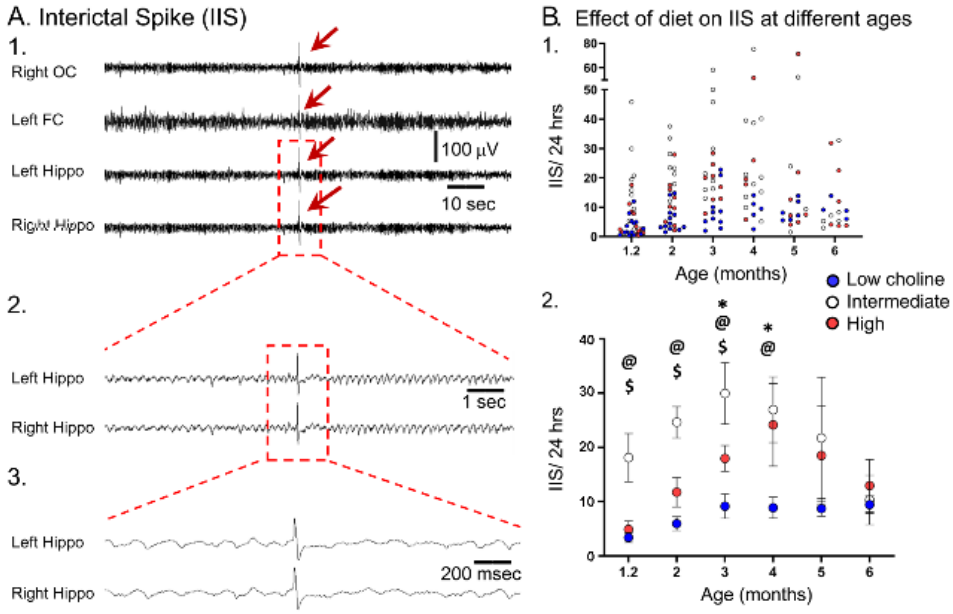


Figure 7. The high choline diet reduced IIS frequency in Tg2576 animals.
A. 1. Representative example of an IIS (red arrow). IIS were defined as occurring in all four channels (generalized) to distinguish them from focal spikes that can often be artifact.
2-3. The IIS shown in A1 is expanded.
B. 1. Scatter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

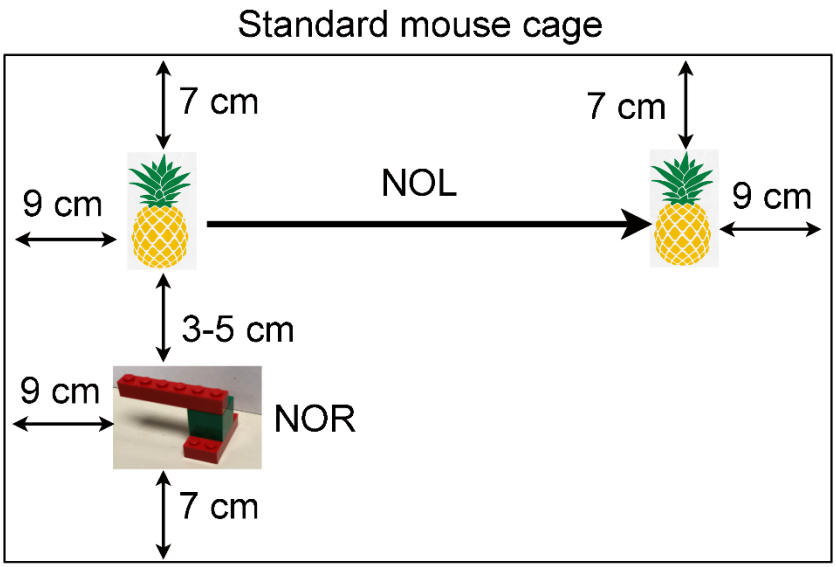


976 **Supplemental Table 1. Comparison of the three diets used in this study.**
977

	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

978
979

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.

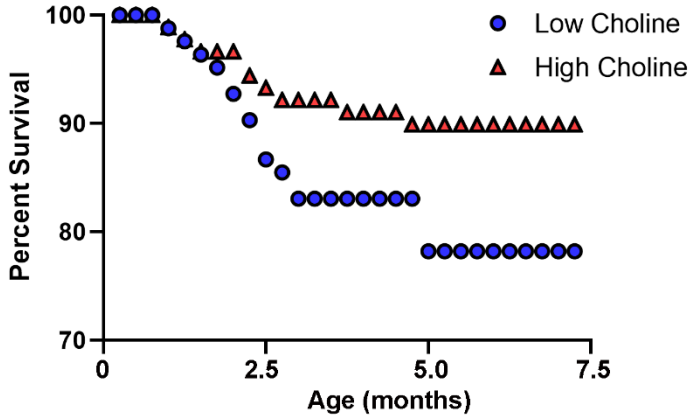


Supplemental Figure 2. Mortality was high in ice treated with the low choline diet.

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

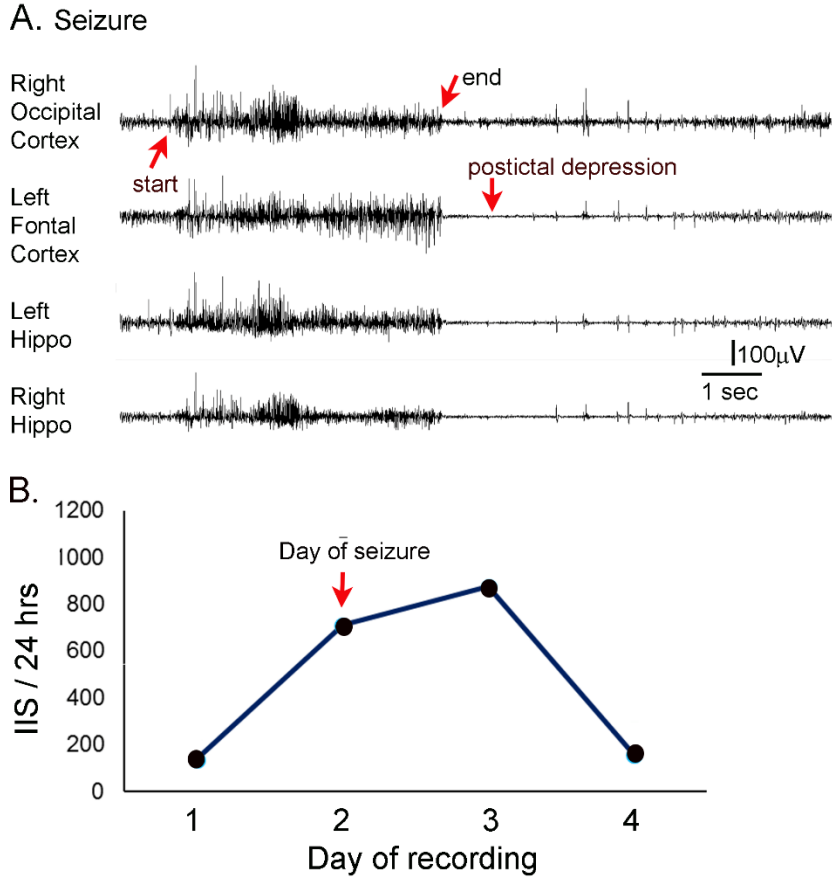
B. A photo of a mouse after sudden unexplained death. The mouse was found in a posture consistent with death during a convulsive seizure.

A.

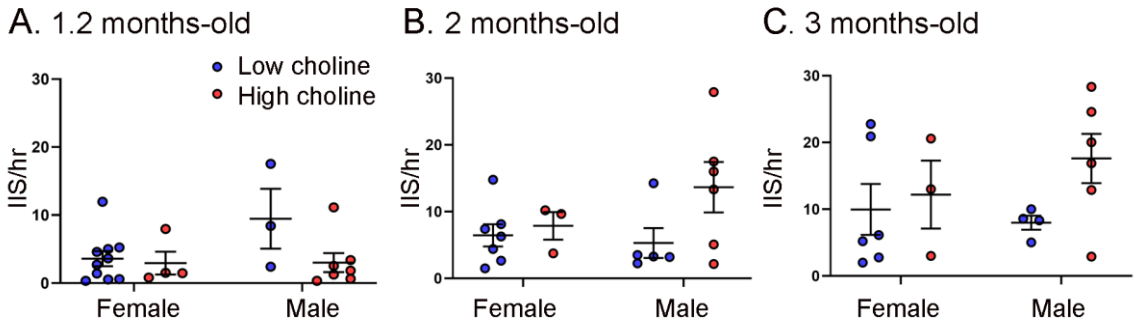


B.

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 hr, 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.



1005 **Supplemental Figure 4. IIS frequency was similar for each sex.**
1006 **A.** At 1.2 months-old, female and male Tg2576 mice were not significantly
1007 different in IIS frequency for the low and high choline diets.
1008 **B-C.** There were no significant sex differences at 2 (B) and 3 (C) months of age.
1009



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