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Paternal preconception donepezil exposure enhances learning in offspring

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Abstract

Background Recent research has indicated that parental use of central nervous system-targeting medications during periconceptional periods may affect offspring across various developmental and behavioral domains. The present study sought to investigate the potential influence of paternal use of donepezil, a specific reversible central acetylcholinesterase inhibitor that activates the cholinergic system to promote cognition, on offspring.

Results In this study, male rats were bred after 21 days of oral donepezil administration at a dose of 4 mg/kg to generate F1 offspring. Both male and female F_1 offspring displayed enhanced performance in learning and short-term memory tests, including novel object recognition, Y maze, and operant learning. Transcriptomic analysis revealed notable alterations in genes associated with the extracellular matrix in the hippocampal tissue of the F1 generation. Integration with genes related to intelligence identified potential core genes that may be involved in the observed behavioral enhancements.

Conclusions These findings indicate that prolonged paternal exposure to donepezil may enhance the learning and memory abilities of offspring, possibly by targeting nonneural, extracellular regions. Further research is required to fully elucidate any potential transgenerational effects.

Keywords Donepezil, Cross-generational inheritance, Short-term memory

Background

In recent years, there has been a growing interest in studying the intergenerational effects of prolonged medication use, with a particular focus on central nervous system (CNS) drugs and their epigenetic outcomes [1]. This research encompasses a wide array of substances. For example, maternal periconceptional use of selective serotonin reuptake inhibitors (SSRIs) has been associated with lower birth weight, increased risk of neonatal adaptation syndrome, increased susceptibility to autism spectrum disorder (ASD) [2], and attention deficiency [3] in offspring. Additionally, the use of psychostimulants such as methylphenidate during pregnancy has been linked to birth defects and limb abnormalities in offspring [4]. There has also been considerable evidence that addictive substances such as opioids [5], alcohol [6–8], cannabis

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[9], psychostimulants [10–12], and nicotine [13, 14], acting on the mesolimbic dopamine reward pathway, affecting behaviors such as anxiety, learning, memory, and cravings in offspring [15]. In addition to maternal effects, recent literature has also highlighted epigenetic features inherited from fathers [16–19]. This highlights the necessity of investigating the transgenerational effects of CNS-targeted treatments.

The cholinergic system in the CNS plays a critical role in maintaining consciousness and significantly contributes to learning and memory processes [20, 21]. Donepezil, a reversible inhibitor of acetylcholinesterase (AChE), slows the breakdown of acetylcholine, thus enhancing synaptic availability and promoting cognitive improvements [22]. Preclinical studies have demonstrated diverse pharmacological effects of donepezil, including enhancements of neuroplasticity, anti-inflammatory properties, reductions in oxidative stress, prevention of excitotoxic cell damage, and improvements in cerebral blood flow [23, 24]. These properties have led to the widespread use of donepezil in the management of mild to moderate Alzheimer's disease [25–28], cognitive impairment in patients with vascular dementia [29], and post breast cancer chemotherapy survivors [30] and its investigational application in the treatment of addiction [31, 32], post-COVID-19 memory impairment [33], depression and ASD [34, 35].

Despite the well-established cognitive benefits of donepezil, there remains a gap in understanding its intergenerational impacts. To address this issue, we established a rat model of chronic donepezil administration and evaluated the learning, memory, and other cognitive markers of offspring to investigate any potential effects on the next generation.

Methods

Animals and husbandry

The wild-type Sprague–Dawley (SD) rats used in this study were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. F1 and F2 rats were bred in the lab under clean animal breeding environment (CL). Adult rats were unisexually housed in cages, 4–5 each, under a reversed light/dark cycle (darkness from 6:30 PM to 6:30 AM) at a temperature of 22±2 °C and a humidity of 50±20%. The rats had free access to food and water. The animal treatments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Shanghai Medical College of Fudan University.

Intragastric donepezil administration

Eight-week-old male SD rats were randomly assigned to receive either donepezil (Done) or saline (Veh) treatment. The Done group received consecutive intragastric administrations of 4 mg/kg donepezil daily for 21 days, which was delivered as a 4 mg/mL saline solution. The Veh group received an equivalent volume of saline as a control.

Mating and breeding

One day after the last dose was delivered, each male rat was cohoused with two naïve female rats for mating. Male rats were separated from pregnant rats once a vaginal plug was observed in the female or after 3 days. Subsequently, the males were subjected to the open field test, the elevated plus maze test, and the operant conditioning tests in the following week, each separated by a three-day interval. After birth, the total number of pups and the sex ratio of the F1 rats were recorded. After weaning, they were housed unisexually in cages of 4–5 rats each. A portion of the F1 generation had access to food and drink ad libitum, and their weight was recorded weekly. Another portion of the F1 generation was used for behavioral testing, and individuals were not mixed for different purposes. Randomly selected naïve male rats from the Done-F1 and Veh-F1 groups were housed with naïve 8-week-old normal female rats to obtain the F2 generation. The pedigree of rats used for weighting, mating, behavioral tests, sampling were noted in Supplementary Fig. 1.

Behavioral tests

Three days before the test, rats were transferred to the test room for 30 min to acclimate to the environment and to the experimenter. The test environment was maintained at a noise level of less than 20 dB, with uniform illumination in the test box and a light intensity of 20–25 lx. To minimize the effects of earlier behavioral tests, we scheduled the experiments to follow a low-stress to high-stress paradigm. The sequence of testing was as follows: open field test, elevated plus maze, novel object recognition, Y maze (for F1 and F2 progeny), sucrose preference test (for F1 and F2 progeny), and operant conditioning. Rats were allowed to rest for 3 days between each test, except for the sucrose preference test and operant conditioning, which occurred 7 days apart.

Open field test

The open field test was used to evaluate the locomotion and anxiety-like behavior. The open-field test box, measuring 60 cm × 60 cm × 60 cm, was constructed of all-black acrylic with an antireflective black sticker on the bottom plate. During the experiment, the test animals were removed from their home cage and placed in the box facing one of the corners. The free movement of the test rats in the box was recorded for 15 min. The video of the open field experiment was processed using Tracking

Master V4.0 analysis software to determine the movement distance, time spent in the central area, and number of shuttles made by the experimental rats.

Elevated plus maze test

To assess the anxiety level of rats, elevated plus maze test was used. The experimental platform was elevated 50 cm above the ground, with both open and closed arms measuring 10 cm × 50 cm and intersecting at ninety degrees, with a central zone measuring 10 cm × 10 cm, and closed arms with 30 cm walls. The bottom was covered with an antireflective black sticker. On the day of the experiment, each animal was placed in the central area of the maze facing the open arms, and their movements in the maze were recorded for 5 min. Tracking Master V4.0 analysis software was used to analyze the time spent and distance traveled in the open and closed arms.

Y maze test

The Y maze test was used to evaluate short-term special memory of rats. A light-gray polyvinyl chloride Y-maze with three arms of equal length (30 cm × 10 cm × 30 cm, L × W × H) was used. During the training period, one arm was occluded. The animals were placed facing the center and allowed to freely explore the maze for 10 min. One hour after the training, the trained rats were placed in the maze again, with the unexplored arm open. Exploration within 5 min was recorded and analyzed with Tracking Master V4.0 to determine the number and duration of entries into the novel arm.

Sucrose preference test

The sucrose preference test was used to test if the rats have developed depression-like behavior. The experiment consisted of training and testing phases. During the 48-hour training period, individually housed rats were given two bottles of 2% sucrose solution for the first 24 h, one bottle of 2% sucrose solution and one bottle of plain water for the next 24 h. The positions of the two water bottles were changed every 12 h. The test period began after a 15-hour period of food and water deprivation. The rats were given one bottle of 2% sucrose solution and one bottle of plain water, and water consumption was measured over 12 h. The sucrose preference was calculated based on the amount of sucrose consumed relative to the total fluid intake:

$$Sucrose\ preference\ (100\%) = \frac{2\%\ Sucrose\ consumption\ (g)}{Total\ water\ consumption\ (g)} \times 100\%$$

Novel object recognition test

The novel object recognition test was used to assess novelty-seeking behavior and short-term memory. The experimental box (60 cm × 60 cm × 60 cm) was made of black acrylic material, with the floor covered with antiglare black stickers. The test consisted of two periods. During the training period, two 10 cm × 10 cm identical objects were fixed in the arena at equal distances to the wall. The animals were placed facing the corner and allowed to explore the objects for 10 min. Then, the rat was returned to the home cage at one-hour intervals. During the test period, one of two identical objects was replaced with a novel object of a different shape and color. The rats were again placed in the testing box facing the corner, and their free movements and exploration of the objects were recorded for 5 min. Tracking Master V4.0 was used to determine the time spent exploring the novel and familiar objects during the test phase. Discrimination index was calculated as:

$$Discrimination\ index\ = \frac{Time_{novel}\ (s)}{Time_{familiar}\ (s)}$$

Operant conditioning

Operant conditioning was carried out to test the learning capacity of rats. After a 12-hour food deprivation period, the rats were placed in a Skinner box equipped with two levers (a correct lever, leading to food delivery, and an incorrect lever, leading to lever retraction and no food), a buzzer, a yellow chamber light (at the rear end of the chamber), a blue signal light (at the front of the chamber), and a white signal light (above the correct lever). Pressing the correct lever led to extinguishment of the blue light and illumination of the white light for 4 s, paired with a 4-second tone stimulus and a food pellet reward (45 mg/pellet, BioServ). Pressing the incorrect lever lead to retraction of the levers for 4 s. Each training session lasted for 4 h per day. Training continued until the rat accumulated 100 food pellets by pressing the lever, or was terminated when the total training time exceeded 30 h. The time elapsed to obtain 100 pellets, and the incorrect lever press were recorded. The learning rate for the operant conditioning behavior is calculated using the following formula:

$$Learning\ Rate = \frac{Samples\ reaching\ 100\ pellets}{Total\ samples} \times 100\%$$

Sample preparation for transcriptomic sequencing

Rats were euthanized and then perfused with ice-cold PBS to remove blood. Hippocampal tissue was harvested and immediately subjected to Dounce homogenization in RNA Isolater Total RNA Extraction Reagent (Vazyme International LLC). Total RNA isolation was carried out by chloroform back-extraction and propanol

precipitation following the manufacturer's instructions. The RNA concentration was measured using a Qubit 3000 Fluorometer (Thermo Fisher Scientific, Inc.). A total RNA library was prepared using 200 ng of RNA. Ribosome depletion from total RNA was performed through probe hybridization and RNAse H digestion using the Ribo-off rRNA Depletion Kit (H/M/R, Vazyme International LLC) according to the manufacturer's protocol. A strand-specific total RNA library was then constructed using the VAHTS Universal V8 RNA-seq Library Prep Kit for Illumina (Vazyme International LLC). Specifically, ribosomal RNA-depleted RNA was fragmented to 200–300 bp using Mg^{2+}-based methods, followed by double-stranded cDNA synthesis, adapter ligation, size selection, and 15 cycles of PCR amplification. The purified libraries were quantified using PCR-based methods for multiplexing. An Illumina NovaSeq 6000 was used to acquire twenty million reads per library, conducted by Genewiz LLC.

Gene expression analyses of the hippocampus

Raw paired-end reads were subjected to the following processes: [1] quality filtering using Trimmomatic [36] by filtering reads shorter than 50 bp and truncating the bases with quality scores < 30; [2] paired-end read assembly using HISAT2 [37] with genome sequences from Ensembl (rn6); [3] read count calculation with featureCounts from the subread aligner package [38] with the annotation Rnor_6.0 V2015-07-24-10-09-53; and [4] DeSeq2 for differential analysis.

Gene ontology analysis and pathway overrepresentation were carried out using ClusterProfiler V4.6.0 [39]. Disease ontology annotation and GSEA were performed using DOSE V3.34.2 [40]. Cell type markers for each cell type were downloaded from CellMarker 2.0 [41], and enrichment analysis was carried out with Fisher's exact test. The functional grouping of pathways was carried out using ClueGO [42].

Quantitive real-time PCR

HIppocampal tissues were dissected directly into Trizol reagent and homogenized. Trizol-lysed samples were then used for RNA extraction using the Direct-zol® RNA Microprep Kit (Zymo Research) according to the manufacturer's instructions. Reverse transcription was performed using the HiScript II 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme Biotech Co., Ltd., China). Briefly, 13 μL reaction mix containing 900 ng total RNA, gDNA wiper, 100nM Oligo$(dT)_{23}$VN and 100 ng random hexamer primers was mixed, and heated at 42 °C for 2 min, and 1 μL HiScript II Enzyme Mix with 2 μL 10 × RT buffer were added. Then the reverse transcription was carried out in a PCR machine for 15 min at 50 °C, 2 min at 85 °C, and held at 4 °C.

Real-time quantitative PCR was with 10 μL reaction mix containing 0.4 μL RT product, 100 nM of each primer (see Supplementary Table 2), 5 μL 2 × ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., China). PCR was carried out on CFX Opus 384 (Bio-Rad Inc., USA), 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, and then 60 °C for 20 s. All reactions were run in triplicate. Relative expression level was calculated using DDCt, with *Hprt1* as internal control.

Statistical analyses

Statistical analyses were carried out using Graphpad Prism 9 or R. Differences between two groups were assessed using an unpaired Student's *t*-test (two-tailed). Learning curves of operant behavior were compared using the log-rank test. For data obtained from F1 and F2, which potentially involve litter effects, intraclass correlation (ICC) was used to estimate litter effect, and linear mixed-effects model fit by REML was carried out to interpret group effects, sex differences and potential interactions, with litter included as a random effect. Sample sizes were reported as the number of animals used in the experiments, and were estimated based on previous experience and are similar to those commonly employed in the field. Two to three animals from the same litter were used for each experiment, and litter size is reported in Supplementary Table 2. The R code used for linear mixed-effects model was included in Supplementary file 1. $P<0.05$ was considered statistically significant. Data and error bars are presented as the mean ± s.e.m.

Results

Establishment of a paternal preconception donepezil exposure model in rats

To investigate the potential effects of chronic donepezil exposure on offspring, we randomly assigned wild-type male SD rats to receive either donepezil (Done, 4 mg/kg) or saline (Veh) treatment for 21 days. One day after the last dose was delivered, the rats were housed with wild-type naïve female rats for mating (Fig. 1a). There was no significant difference in the body weight gain in the two groups, as indicated by the body weight on day 0 or day 21 (Fig. 1b, $F_{Group \times Time}(7, 133)=0.8628$, $P=0.5378$; F_{Group} $(1, 19)=0.4431$, $P=0.5136$). Donepezil has been reported to enhance short-term memory and cognition. Therefore, multiple behavioral tests were used to assess its effects after mating. In the open field test, both Done and Veh rats traveled comparable total distances in the arena (Fig. 1d, t $(19)=1.419$, $P=0.172$). There were no significant differences between the two groups in terms of the distance traveled in the center (Fig. 1e, t $(19)=1.212$, $P=0.241$) or time spent exploring the central area (Fig. 1f, t $(19)=1.294$, $P=0.211$). Similarly, Done and Veh rats spent comparable amounts of time exploring

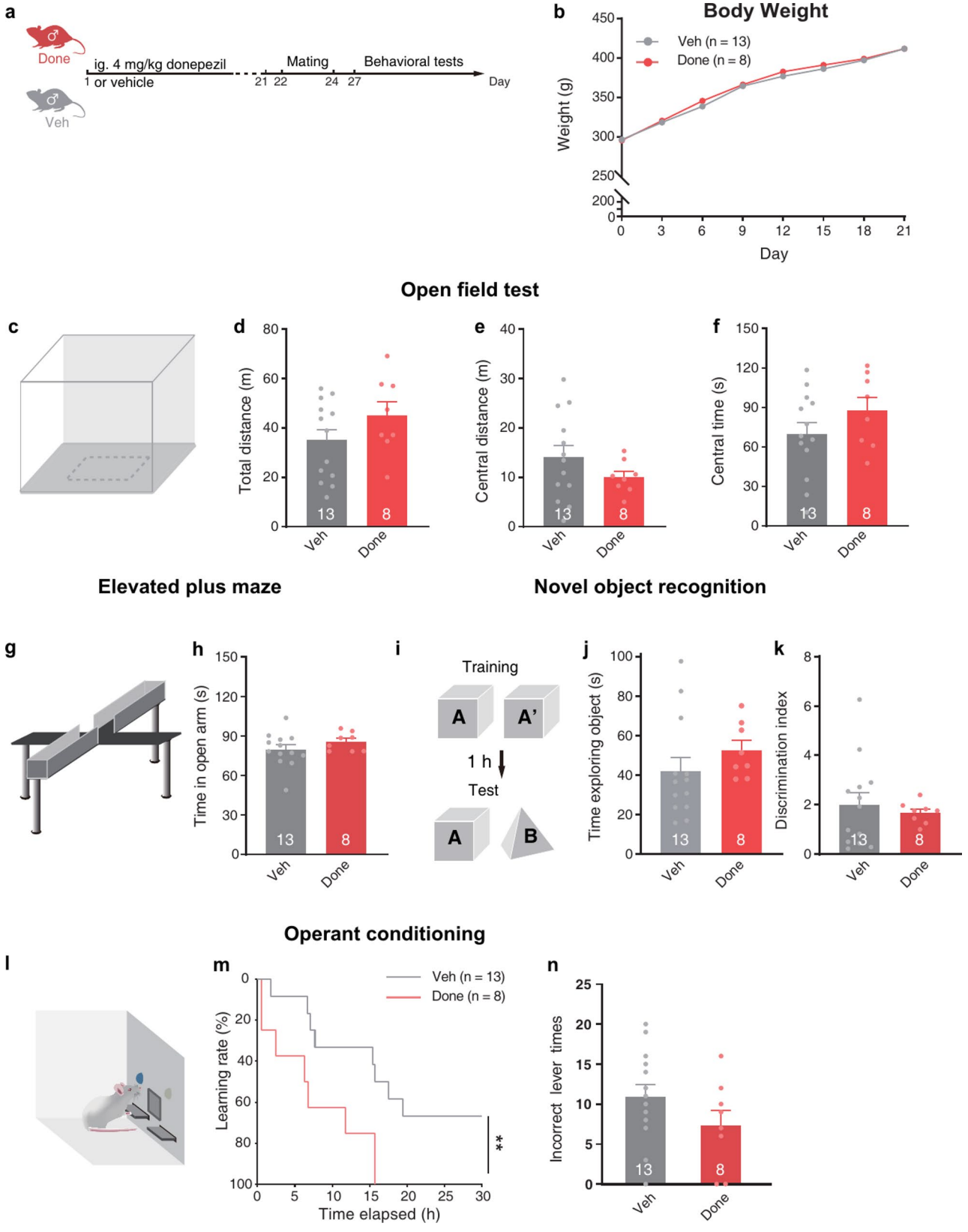

Fig. 1 (See legend on next page.)

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Fig. 1 Chronic donepezil exposure improved learning and memory in adult male rats. **(a)** Schematic for donepezil administration, tests, and mating. **(b)** Body weights of Veh- and Done-treated male rats between day 0 and day 21. **(c-f)** Schematics **(c)** and results of open field test. The total distance traveled **(d)**, distance traveled in the central area **(e)**, time spent in the central area **(f)** of male rats exposed to chronic donepezil treatment are shown. **(g-h)** Schematics **(g)** and results of elevated plus maze. Time spent exploring the open arm **(h)** of male rats exposed to chronic donepezil treatment are shown. **(i-k)** Schematics **(i)** and results of novel object recognition. The total time spent exploring objects during the test phase **(j)**, discrimination index **(k)** of male rats exposed to chronic donepezil treatment are shown. **(l-n)** Schematics **(l)** and results of operant conditioning in a Skinner box. Learning rate of operant behavior **(m)**, incorrect lever times **(n)** of male rats exposed to chronic donepezil treatment are shown. The data are shown as the mean ± s.e.m. The numbers within each column represent the number of animals tested

the open arm of the elevated plus arm maze (Fig. 1h, t (19) = 1.204, P = 0.243). In the test phase of novel object recognition, rats from the two groups spent comparable time exploring novel and familiar objects (Fig. 1j, t (19) = 1.081, P = 0.293), and were able to distinguish the novel object from the familiar object (Fig. 1k, t (19) = 0.515, P = 0.613). We then used operant-conditioned learning and novel object recognition tests to assess the learning capacity and short-term memory of both Done and Veh rats. The group of rats treated with donepezil achieved the goal of obtaining 100 food pellets in a Skinner box via lever pressing significantly faster than did the control group treated with Veh (Fig. 1m, log-rank test, c^2 = 8.354, P = 0.004), while the number of incorrect lever press was no different (Fig. 1n, t (19) = 1.386, P = 0.182). These data indicate enhanced learning induced by 21-day donepezil treatment in male adult rats. **Paternal donepezil exposure does not disrupt birth and weight gain in F1 or F2 progeny**.

F1 progeny were generated by mating with naïve female animals, as mentioned above, while the F2 generation was obtained by crossing naïve male Done-F1 and Veh-F1 with naïve female rats (Fig. 2a). The numbers of F1 offspring from vehicle-treated fathers (Veh) and those from donepezil-treated fathers (Done) were comparable per litter (Fig. 2b, F_{Group} (1, 10) = 0.502, P = 0.495). The body weights of male and female F1 offspring were measured between postnatal week 2 and week 8 and were found to be comparable (Fig. 2c, F_{Group} (1, 10) = 0.036, P = 0.853). Similarly, there was no difference in litter size (Fig. 2d, F_{Group} (1, 10) = 0.307, P = 0.592) or body weight between these two F2 groups (Fig. 2e, F_{Group} (1, 8) = 0.038, P = 0.849). These data indicate that paternal exposure to donepezil does not disrupt birth or weight gain in F1 or F2 progeny.

Paternal donepezil exposure enhanced learning and short-term memory in F1 progeny

Behavioral tests were performed on eight-week-old F1 rats. Similar to the results obtained for the F0 generation, the male and female F1 offspring sired by both Done and Veh rats traveled comparable total distances in the arena during the open field test (Fig. 3a, F_{Group} (1, 8) = 0.146, P = 0.712). Additionally, there was no significant difference in the distance traveled (Fig. 3b, F_{Group} (1, 8) = 0.801, P = 0.397) or time spent exploring the central area (Fig. 3c, F_{Group} (1, 8) = 0.391, P = 0.549) between the two groups. Furthermore, both Done and Veh F1 rats spent a comparable amount of time exploring the open arm of the elevated plus arm maze (Fig. 3d, F_{Group}(1, 8])=0.883, P = 0.375). In two-bottle choice tests, Done F1 consumed comparable 2% sucrose as compared to Veh F1, indicating a normal hedonic response (Fig. 3f, F_{Group} (1, 8) = 4.089, P = 0.078).

Additionally, while F1 progeny from the two groups were comparable in time spent exploring the objects during novel object recognition test (Fig. 3g, F_{Group} (1, 8) = 0.543, P = 0.482), Done-F1 outperformed Veh-F1 in discriminating novel objects (Fig. 3h, F_{Group} (1, 8) = 4.923, P = 0.058). Similarly in Y maze tests, they spent more time (Fig. 3j, F_{Group} (1, 8) = 14.32, P = 0.0054) and entries exploring the novel arm (Fig. 3k, F_{Group} (1, 8]) = 10.89, P = 0.0108). They were also significantly faster than Veh-F1 in achieving the goal of obtaining 100 food pellets in a Skinner box (Fig. 3l, Log-rank test, c^2 = 10.56, P = 0.0012), while the number of incorrect lever pressed were not significantly different (Fig. 3m, F_{Group} (1, 8) = 0.492, P = 0.503). Importantly, no differences were observed between males and females in any of the behavioral assessments performed. These behavioral assessments suggest that paternal exposure to donepezil enhances learning and short-term memory in F1 progeny.

Donepezil-sired F2 offspring show normalized learning compared to saline-sired controls

We conducted the same behavioral tests on the F2 offspring as on the F1 offspring. The results showed comparable performance of open field tests (Fig. 4a-c), and no statistical difference was observed in total distance traveled (F_{Group} (1, 6) = 0.150, P = 0.711), distance travelled in the central area (F_{Group} (1, 6) = 0.163, P = 0.701), or in time spent in the central area (F_{Group} (1, 6) = 0.0507, P = 0.829). In the elevated plus maze test, Done-F2 and Veh-F2 were not statistically different in time exploring the open arm (Fig. 4d, F_{Group} (1, 6) = 1.532, P = 0.262). Furthermore, the two groups of rats exhibited comparable sucrose preference in two-bottle choice tests (Fig. 4e, F_{Group} (1, 6) = 0.685, P = 0.439). They were also indifferent in performance of novel object recognition tests (Fig. 4f-g, time exploring, F_{Group} (1, 6) = 0.562, P = 0.482; discrimination index, F_{Group} (1, 6) = 0.667, P = 0.445), Y maze tests (Fig. 4h-i, time, F_{Group} (1, 6) = 0.0634, P = 0.810;

entries, F_{Group} (1, 6) = 0.278, P = 0.617), or operant learning (Fig. 4f-g, learning rate, Log-rank test, c^2 = 0.329, P = 0.566; incorrect lever press, F_{Group} (1, 6) = 1.769, P = 0.232). These results suggest intergenerational but not transgenerational effects of learning and memory enhancement due to paternal preconception donepezil exposure on offspring.

Transcriptomic changes in the hippocampus of donepezil-treated offspring

We sampled hippocampal tissues from the Done-F1 and Veh-F1 groups for transcriptome sequencing and differential expression analysis. A total of 726 differentially expressed genes (DEGs; 418 upregulated and 308 downregulated) were identified under P value ≤ 0.05 (Fig. 5a). First, functional overrepresentation was performed by comparing DEGs with genes expressed in the hippocampus as a background. Gene Ontology was used to analyze the function and distribution of these DEGs. The biological functions of DEGs were overrepresented in cell adhesion, morphogenesis, cell migration, and extracellular matrix composition, whereas the cellular component analysis revealed their distribution in the cell periphery, extracellular matrix, receptor complex, cell junction and vesicles [43]. Because the brain is heterogeneous, we used a single-cell sequencing-based cell marker database, CellMarker 2.0 [41], as a reference and predicted the cellular distribution of these DEGs. The results showed that the DEGs were primarily distributed in mesenchymal cells and microvascular pericytes (Fig. 5c). Interestingly, in contrast with the overrepresentation of pathways in nonneuronal cells of these DEGs, background genes were closely related to synapse function, with significant enrichment in synaptic long-term potentiation, the MAPK signaling pathway, and axon morphogenesis (Fig. 5b, left, yellow), and were primarily located in synapses, chromosomes, and the cytoplasm (Fig. 5b, right, yellow). These results suggest that paternal donepezil exposure might alter intercellular connections in the neurovascular units of offspring.

We also conducted a gene set enrichment analysis (GSEA) to identify potential disease associations of these genes. Comparison with the DisGeNET database revealed significant associations with autoimmune disease, cerebral ischemia, Alzheimer's disease, Parkinson's disease, and schizophrenia (Fig. 5d).

As donepezil-treated offspring show improvements in short-term memory and learning, it would be of potential interest to explore whether certain intelligence-related genes are altered. We retrieved a list of candidate genes related to intelligence and cognitive performance from two GWAS analyses of UK BioSample Bank samples [44] and [45]compared our DEGs with those candidates. Interestingly, out of the 726 DEGs, 130 collapsed (Fig. 5e). Based on the network analysis of the collapsed genes, the Wnt, VEGF, and TNF pathways were identified as significant (Fig. 5f). We proceeded to validate the expression of highly connected genes using qPCR and observed consistent and significant down-regulation of small GTPases *RhoA* (F_{Group} (1, 6) = 11.392, P = 0.014), and *Rac1* (F_{Group} (1, 6) = 11.392, P = 0.014), alongside the upregulation of *Micb* (F_{Group} (1, 6) = 6.9823, P = 0.039) and *Cdh20* (F_{Group} (1, 6) = 9.2607, P = 0.023) (Fig. 5g). Taken together, our data suggest epigenetic predisposition to learning and memory enhancement by genes involved in nonneural, extracellular regulation. Although our behavioral assessments of the F1 generation indicated improved learning and short-term memory in adulthood, there is concern about the potential for neurological disorders in middle and old age, which requires further behavioral and mechanistic investigations.

Discussions

There is growing evidence that parental exposure to adverse environmental factors can lead to pathological traits or increased susceptibility to diseases in offspring, a phenomenon known as intergenerational or transgenerational epigenetic inheritance [46]. These two terms are distinguished by the persistence of phenotypic changes across generations [47]. Historically, most studies have focused on maternal lineage, largely due to the presence of cellular components in oocytes that can transmit environmental signals to the developing embryo [48]. However, recent research has revealed that paternal exposure to environmental factors can also significantly impact offspring through modifications in the sperm epigenome [49]. A notable example is the Dutch famine of 1944, where epidemiological studies demonstrated that the offspring of those affected are more likely to develop coronary heart disease, diabetes, and exhibit an increased risk of obesity and cognitive aging phenotypes [50–54]. Additionally, exposure to various chemicals, including drugs and environmental toxins, has been reported to cause transgenerational effects. For instance, maternal exposure to vinclozolin during pregnancy can lead to stress sensitization that persists for up to three generations in female offspring [55, 56]. Similarly, exposure to alcohol during the fetal period has been shown to result in stress sensitization in subsequent generations, affecting two to three generations of offspring in adulthood [57, 58]. These findings underscore the intricate interplay between environmental exposures and epigenetic inheritance in shaping cognitive development across generations.

In line with the broader context of environmental exposure to neurotoxic substances, studies have shown potential transgenerational effects of other peripheral acetylcholinesterase inhibitors, such as organophosphorus (OP) and methylcarbamate (MC) insecticides [22,

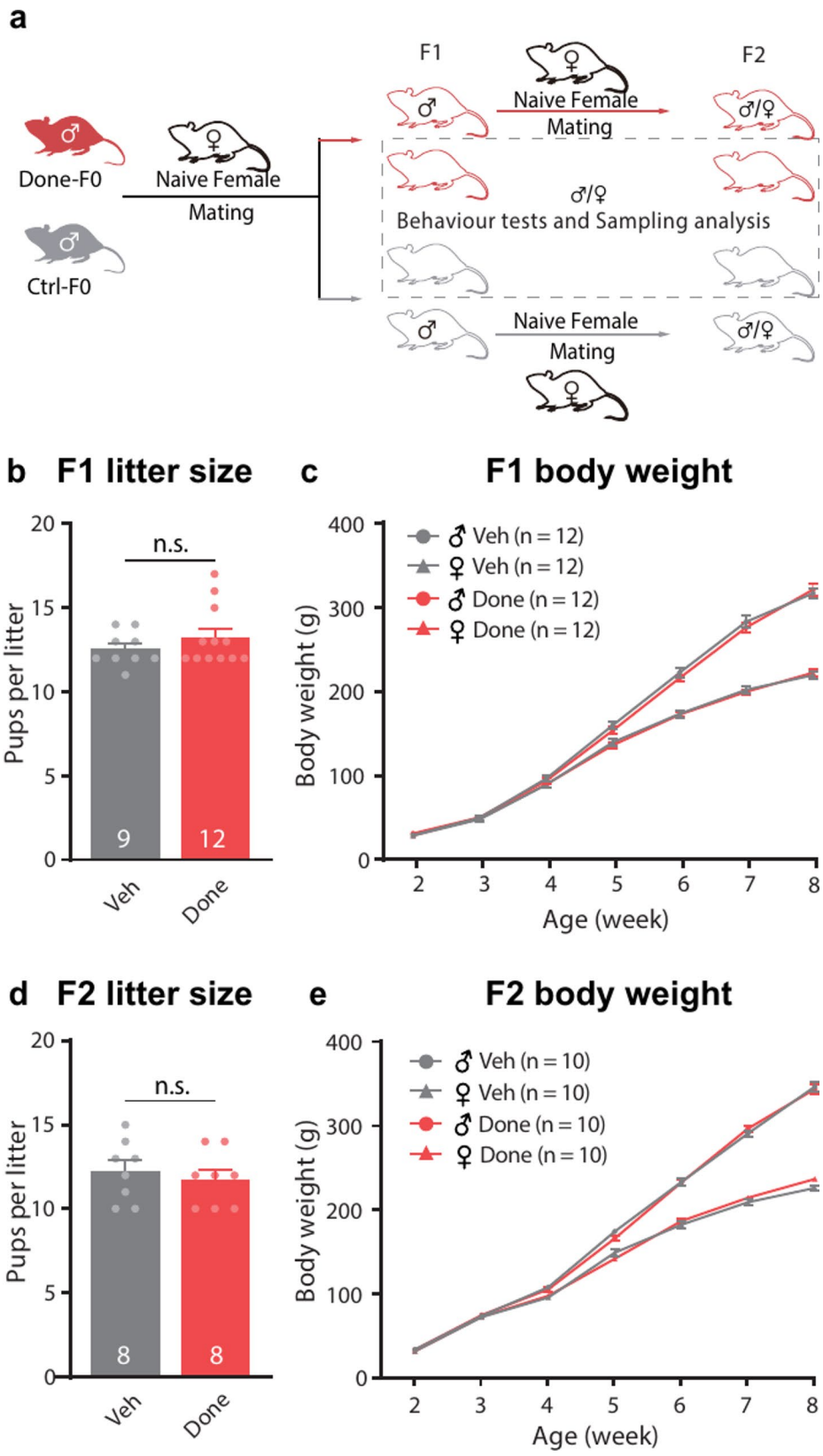

Fig. 2 Litter size and body weight of F1 and F2 offspring. **(a)** Schematic of the generation of F1 and F2 offspring. **(b)** Number of pups per litter sired by Veh and Done F0. **(c)** Body weights of Veh-F1 and Done-F1 offspring between 2 and 8 weeks. **(d)** Number of pups per litter sired by Veh and Done F1. **(e)** Body weights of Veh-F2 and Done-F2 offspring between 2 and 8 weeks. The data are shown as the mean ± s.e.m. The numbers within each column represent the number of animals tested

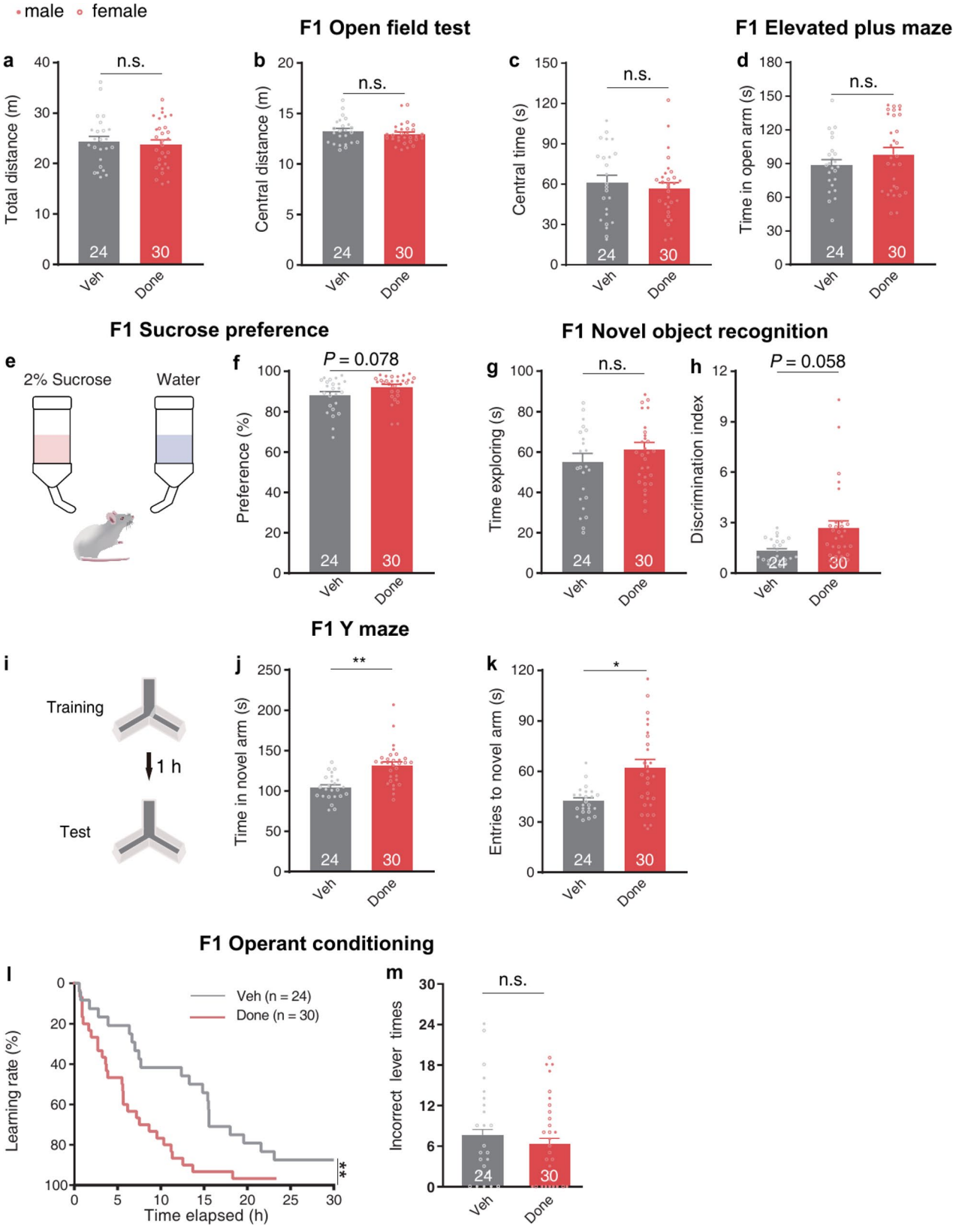

Fig. 3 (See legend on next page.)

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Fig. 3 Chronic maternal donepezil exposure may improve learning and memory in F1 offspring. **(a-c)** Results of open field test. The total distance traveled **(a)**, distance traveled in the central area **(b)**, time spent in the central area **(c)** of F1 offspring are shown. **(d)** Elevated plus maze test of F1 offspring, the time spent exploring the open arm was shown. **(e-f)** Schematics **(e)** and results of sucrose preference test. The ratio of sucrose to water consumption **(f)** of F1 offspring are shown. **(g-h)** Results of novel object recognition test. The total time spent exploring objects during the test phase **(g)**, discrimination index **(h)** of F1 offspring is shown. **(i-k)** Schematics **(i)** and results of Y maze test. The time spent exploring the novel arm **(j)**, entries to novel arm(k) of F1 offspring are shown. (l-m) Results of operant conditioning. Learning rate (l), incorrect lever times **(m)** of F1 offspring operant behavior are shown. The data are shown as the mean ± s.e.m. The numbers within each column represent the number of animals tested. Filled circles, data from each male; open circles, data from each female

59]. These compounds, known for their irreversible inhibition of acetylcholinesterase, have been used extensively in agriculture for decades. Even at low levels of exposure that do not cause acute intoxication, OP insecticides have been linked to reproductive toxicity [60] and adverse effects on head circumference and the behavior of offspring [61–66]. The present research adds to the growing body of evidence suggesting that acetylcholinesterase inhibitors, whether they act in the CNS or periphery, can have lasting impacts on cognitive function and neurological health in future generations. Therefore, further investigations concerning cholinergic system-targeting substances are warranted, considering the significant public health implications of these findings.

The different effects of central and peripheral acetylcholinesterase inhibitors, such as whether they both directly affect germ cells or whether the transgenerational effects of donepezil are hereditary effects of improved cognition rather than direct impacts of the drug, have also sparked thoughts about their mechanisms of transgenerational inheritance. In our previous research, we found that chronic voluntary seeking of cocaine in male rats, rather than cocaine exposure per se, renders offspring vulnerable to developing high cocaine-seeking behavior [10]. There are also reports concerning the epigenetic inheritance of depressive emotional states via both paternal and maternal alleles [67, 68]. Another plausible way is to test the potential transgenerational effects of additional nootropics, including racetams, monoaminergic modulators, etc., as well as other cholinergics, such as galantamine. Mechanistic studies concerning how brain activity affects gametes could shed new light on this topic.

Our data provide preliminary evidence that, in a rat model, paternal exposure to donepezil before conception could enhance learning and short-term memory in offspring, potentially through alterations in gene expression related to extracellular communication, possibly in nonneuronal cells. The extracellular matrix (ECM) in the brain is a complex network of proteins and carbohydrates that surrounds neurons and glial cells, providing structural support and playing a pivotal role in the regulation of cellular communication, synaptic plasticity, and ultimately, memory and cognitive function [69, 70]. By influencing the availability and diffusion of growth factors and guidance cues, the ECM helps shape the synaptic architecture that underlies cognitive flexibility and stability [71]. The ECM is integral to the neurovascular unit, influencing blood–brain barrier function and participating in neurovascular coupling, thereby regulating the delivery of nutrients and oxygen to active neurons—a process critical for the energy supply of neurons. ECM components, especially proteoglycans, modulate the formation, maturation, and elimination of synapses, which are the fundamental units of neural communication and the basis for learning and memory [71, 72]. In PCR validation of key genes, together with our transcriptomic sequencing, we observed down-regulation of *RhoA* and *Rac1*, along with the upregulation of matrix metalloproteinase 14 (*Mmp14*), *Micb* (MHC class I polypeptide-related sequence B), and *Cdh20* (Cadherin 20). These intricate changes highlight the complex mechanisms through which ECM dynamics can enhance cognitive functions, promoting effective learning and memory. As is shown in previous research, RhoA and Rac1 are essential for cytoskeletal dynamics and synaptic stability [73, 74]; their down-regulation may facilitate changes that promote synaptic plasticity, thereby enhancing the ability of neurons to adapt and form new connections essential for learning and memory. MMP14 plays a crucial role in ECM remodeling by degrading various ECM components [75], thereby could possibly enhance the dynamic remodeling of the ECM and allowing for more plastic neural connections. CDH20 contributes to cell adhesion, potentially strengthening synaptic connections [76]; its upregulation, alongside down-regulated RhoA and Rac1, may support greater synaptic stability and communication. Furthermore, it would be of interest to test whether the lack of gene expression changes could be linked to the lack of behavioral effects in the F2 generation. In our study, despite the fact that donepezil is an AChE-targeting drug, ECM stood out to be the primarily altered pathway. While this result reiterates the importance of the ECM for learning memory functions, it also suggests that the mechanism of germ cell epigenetic marker alteration due to paternal donepezil dosing deserves further consideration.

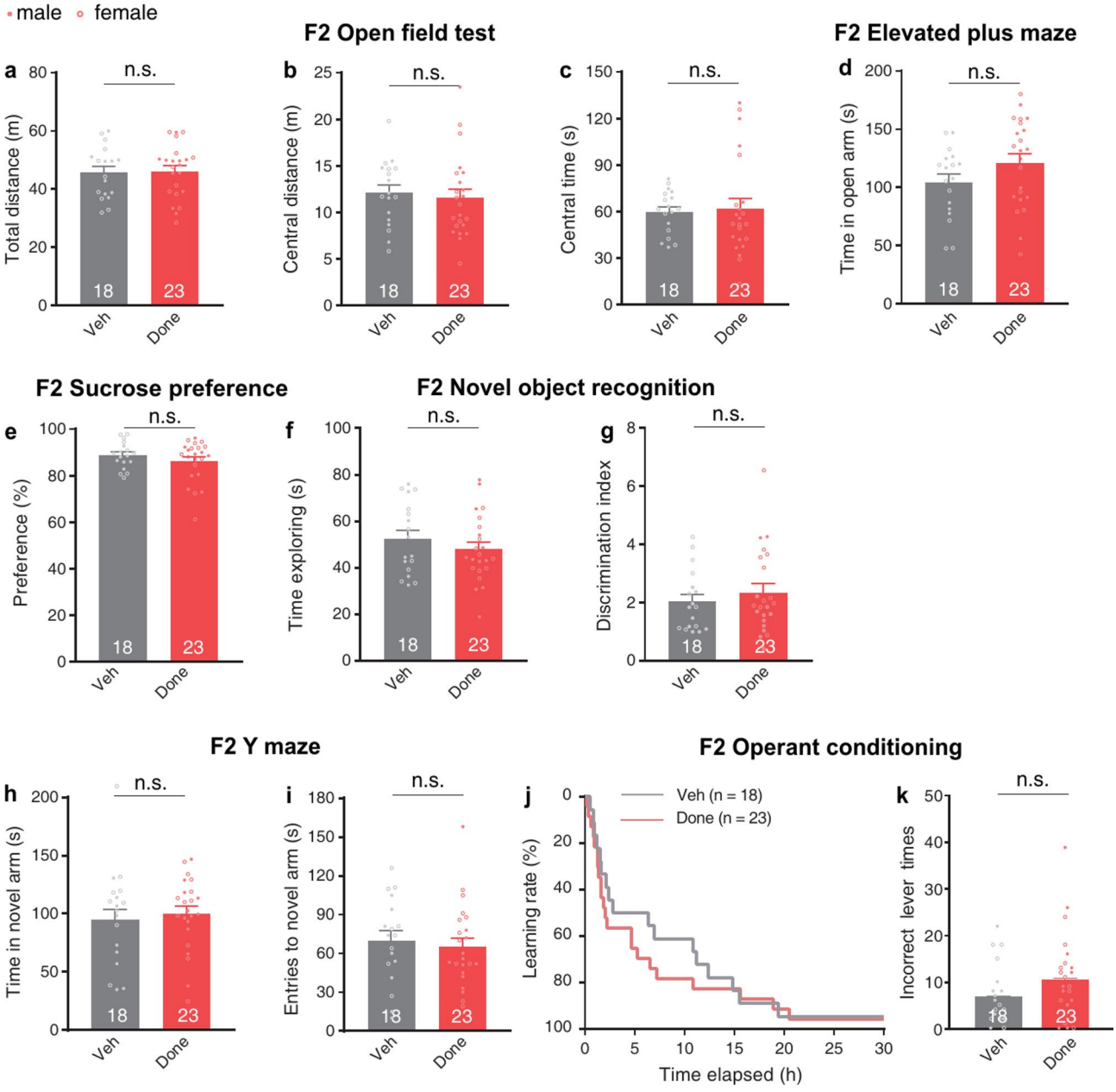

Fig. 4 Chronic paternal donepezil exposure had no significant impact on learning and memory in F2 offspring. **(a-c)** Results of open field test. The total distance traveled **(a)**, distance traveled in the central area **(b)**, time spent in the central area (c) of F2 offspring are shown. **(d)** Elevated plus maze test of F2 offspring, the time spent exploring the open arm was shown. **(e)** Sucrose preference test of F2 offspring, the ratio of sucrose to water consumption was shown. **(f-g)** Results of novel object recognition test. The total time spent exploring objects during the test phase **(f)**, and discrimination index **(g)** of F2 offspring is shown. **(h-i)** Results of Y maze test. The time spent exploring the novel arm **(h)**, entries to novel arm **(i)** of F2 offspring are shown. **(j-k)** Results of operant conditioning. Learning rate **(j)**, incorrect lever times **(k)** of F2 offspring operant behavior are shown. The data are shown as the mean ± s.e.m. The numbers within each column represent the number of animals tested. Filled circles, data from each male; open circles, data from each female

Conclusions

Paternal 21-day oral donepezil administration led to enhanced performance in learning and short-term memory tests in both male and female F1 offspring, but not in F2 offspring.

Transcriptomic analysis revealed significant alterations in genes associated with the extracellular matrix in the hippocampal tissue of the F1 generation, with potential core genes identified that may be involved in the observed behavioral enhancements.

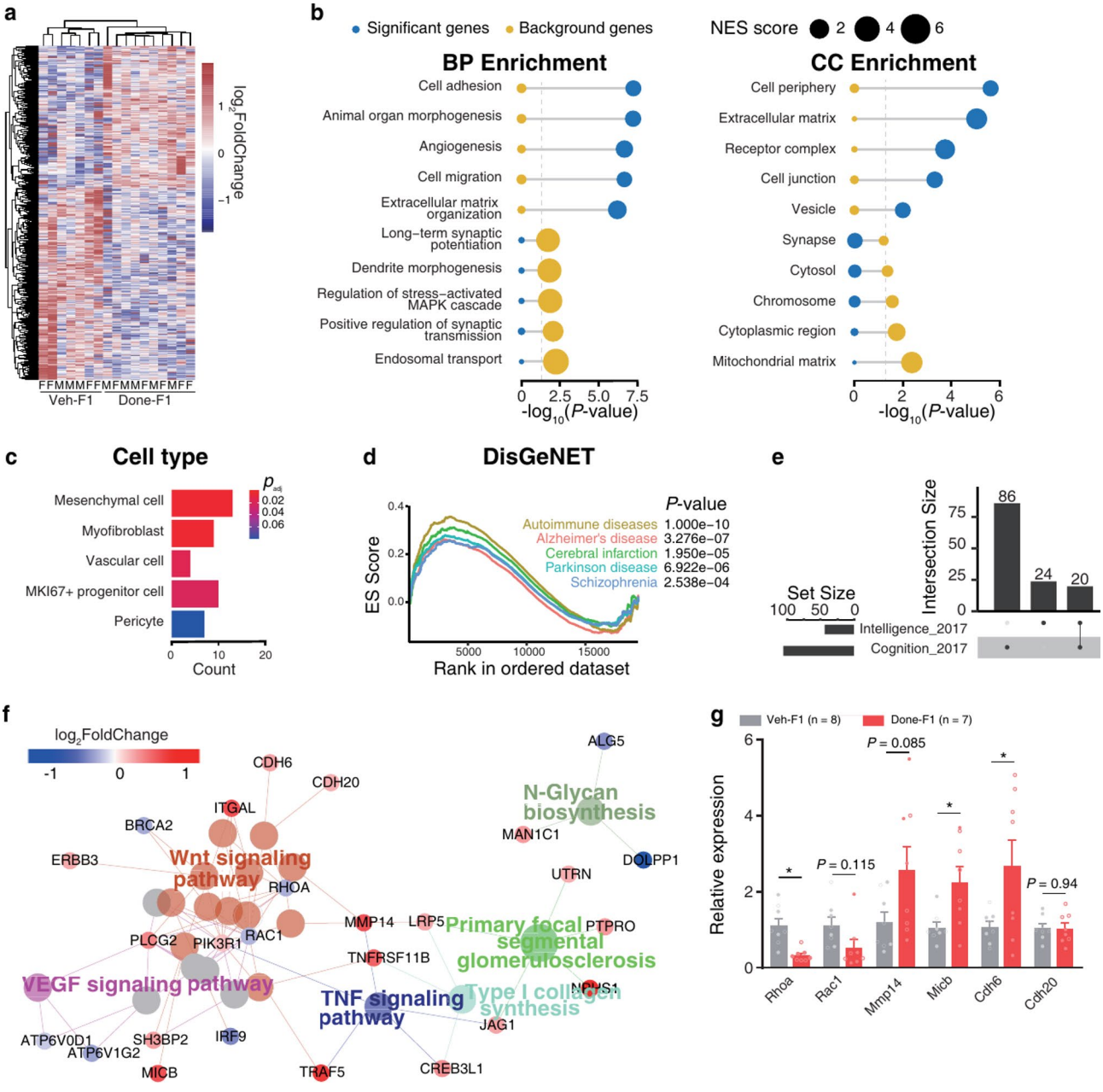

Fig. 5 Impact of paternal metformin exposure on the hippocampal transcriptome of the F1 generation. **(a)** Heatmap showing significant differences in the hippocampal transcriptome between Done-F1 and Veh-F1. Done-F1, females, $n = 5$; males, $n = 5$; Veh-F1, females, $n = 4$; males, $n = 3$. **(b)** Gene Ontology functional enrichment analysis. Significant functional annotations of differentially expressed genes vs. the background (read counts ≥ 10). Left, biological process enrichment; right, cellular component enrichment. **(c)** Top file cell type annotations of significantly differentially expressed genes using Cell Marker 2.0. **(d)** Disease type annotations of significantly differentially expressed genes using DisGeNET, along with gene set enrichment analysis results. **(e)** Number of intelligence- and cognition-related genes among the significantly differentially expressed genes from the UK Biobank whole-genome association study. There were 44 out of 1110 intelligence-related genes and 106 out of 4998 cognition-related genes. **(f)** Network clustering of significantly differentially expressed genes related to cognition and intelligence using ClueGO. Different colors represent signaling pathway clusters annotated by different Wiki pathways. **(g)** Quantitive real-time PCR validation on the expression of *RhoA*, *Rac1*, *Mmp14*, *Micb*, *Cdh20*, and *Cdh6* in hippocampal tissue of F1 generation. Done-F1, females, $n = 4$; males, $n = 4$; Veh-F1, females, $n = 4$; males, $n = 4$. The data are shown as the mean ± s.e.m. Filled circles, data from each male; open circles, data from each female

Abbreviations

AChE	Acetylcholinesterase
ANOVA	Analysis of variance
ASD	Autism spectrum disorder
CNS	Central nervous system
PCR	Polymerase chain reactions
SD	Sprague-Dawley
SSRIs	Selective serotonin reuptake inhibitors
REML	Restricted maximum likelihood
SEM	Standard error of mean

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12993-024-00252-z.

Supplementary Material 1. Supplementary Fig. 1 Pedigree diagram of animals used.

Supplementary Material 2. Supplementary File 1 R code to reproduce results for mixed model analysis.

Supplementary Material 3. Supplementary Table 1 Primers table.

Supplementary Material 4. Supplementary Table 2 Data and statistics.

Author contributions

GF, TP, XJ: project administration, methodology, investigation, and writing-review and editing. CJ, FW, XL: conceptualization, funding acquisition, and writing-review and editing. QL: conceptualization, project administration, supervision, funding acquisition, writing-original draft, and writing-review and editing. LM: conceptualization, supervision, funding acquisition, and writing-review and editing. All authors read and approved the final manuscript.

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Data availability

Data is provided within the Supplementary Table 1. R code to reproduce results for mixed model analysis is provided as Supplementary File 1. RNA-sequencing data derived from male and female F1 offspring from vehicle-treated fathers (Veh) and from donepezil-treated fathers (Done) were deposited in the SRA at PRJNA1093504[https://www.ncbi.nlm.nih.gov/bioproject/1093504].

Declarations

Ethical approval

The animal treatments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Shanghai Medical College of Fudan University.

Competing interests

The authors declare no competing interests.

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