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Prenatal gene-environment interactions mediate the impact of advanced maternal age on mouse offspring behavior

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Autism spectrum disorders encompass diverse neurodevelopmental conditions marked by alterations in social communication and repetitive behaviors. Advanced maternal age is associated with an increased risk of bearing children affected by autism but the etiological factors underlying this association are not well known. Here, we investigated the effects of advanced maternal age on offspring health and behavior in two genetically divergent mouse strains: the BTBR T^{+} Itpr3^{tf}/J (BTBR) mouse model of idiopathic autism, and the C57BL/6 J (B6) control strain, as a model of genetic variability. In both strains, advanced maternal age negatively affected female reproductive and pregnancy outcomes, and perturbed placental and fetal growth, and the expression of genes in the fetal brain tissues. Postnatally, advanced maternal age had strain-dependent effects on offspring sociability, learning skills, and the occurrence of perseverative behaviors, varying between male and female offspring. These findings disentangle the relationship between genetic determinants and maternal age-related factors in shaping the emergence of autism-like behaviors in mice, highlighting the interplay between maternal age, genetic variability, and prenatal programming, in the occurrence of neurodevelopmental disorders.

Keywords Advanced maternal age, Pregnancy, Behavior, Autism, BTBR mice

Autism spectrum disorders (ASD) include a heterogeneous group of neurodevelopmental conditions characterized by deficits in social communication and the presence of repetitive/perseverative behaviors[1]. ASD has a multifactorial etiology, comprising both genetic and environmental contributions, which independently and/or in combination may influence neurodevelopmental outcomes and the manifestation of behavioral symptoms. Hundreds of genes have been associated with ASD development; however, numerous studies indicate only a moderate genetic contribution and an extensive environmental etiology[2–7]. Recent studies have suggested that the maternal environment provided before birth may have a substantial role in ASD development[8–10]. Nevertheless, none of the identified prenatal environmental stressors seems to be sufficient alone to arouse ASD in the offspring, suggesting that individual variations in ASD risk may result from genetic diversity, which may lead to either resilience or vulnerability to environmental perturbations[11–13].

Epidemiological studies have suggested the association between advanced maternal age (AMA) and the occurrence of ASD in the offspring. Aging negatively affects females' reproductive outcomes and increases the risk of pregnancy complications (i.e., miscarriage, placental defects, preterm delivery, etc.), which per se are associated with adverse neurodevelopmental outcomes in the offspring[14–19]. Moreover, advanced maternal age has been associated with brain and behavioral alterations in the offspring, including ASD in humans and ASD-like behaviors in mouse models[17,18,20–22]. An interplay between age-related oocyte and embryonic abnormalities with systemic and uterine aging is known to play an important role in offspring developmental programming, health, and survival[16,23–27]. Experimental models have shown that gene-environment interactions occurring during pregnancy shape fetal neurodevelopmental trajectories and affect long-term programming of offspring health and behavior[28–31]. Therefore, an interaction between genetic vulnerability and a suboptimal uterine environment may occur in aged females and may affect neurodevelopmental outcomes. Mouse models have been widely employed to study genetic and environmental risk factors contributing to ASD development. The

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BTBR T + Itpr3^{tf}/J (BTBR) strain has been identified as the most validated model of idiopathic ASD, because its behavioral endophenotypes resemble the core symptomatology of autism, including altered communication, social deficit, and higher levels of repetitive behaviors, as compared to other strains[32]. Besides its unique phenotypic features, the BTBR mouse is characterized by a complex genetic architecture involving multiple genetic loci rather than a single gene mutation, making this strain an excellent model for studying the heterogeneous genetic origins of ASD[33]. A growing number of studies have investigated the influences of environmental risk factors for ASD in the BTBR strain in comparison with the control C57BL/6 J (B6) strain, demonstrating that maternal-related environmental factors may be associated with either exacerbation or alleviation of ASD-like behaviors of BTBR mice[30,31].

Here, we examined the effects of advanced maternal age, a risk factor for ASD, on prenatal development, fetal head, and placental transcriptomes, as well as on postnatal offspring growth and autism-like behaviors of two genetically divergent mouse strains, the BTBR model of idiopathic autism and the B6 control strain. B6 and BTBR offspring conceived by old and young females were fostered at birth to a young mother of a different strain to control postnatal confounding variables related to maternal care. This design enabled the assessment of the specific effects of an adverse prenatal environment, associated with maternal aging, on the postnatal behavioral phenotype of offspring, and allowed for the exploration of strain-specific genetic vulnerabilities.

Materials and methods

Experimental design

Four experimental groups were established: (i) B6 YMA (Young Maternal Age), offspring conceived by young B6 females; (ii) B6 AMA (Advanced Maternal Age), offspring conceived by old B6 females; (iii) BTBR YMA, offspring conceived by young BTBR females; (iv) BTBR AMA, offspring conceived by old BTBR females (n = 4–16 pregnant females per group depending on the experiments). In one cohort of animals, pregnancies were interrupted at embryonic day 12.5 (E12.5) to examine placental and fetal growth and gene expression in male and female conceptuses. A second cohort of pregnant females delivered the offspring, which were postnatally fostered to a young females of a not-related strain to control postnatal variability in maternal care. Male and female offspring were analyzed for their social communication capabilities, sociability, learning skills, and cognitive flexibility.

Animals

BTBR and B6 mice were purchased from Jackson Laboratory (USA) and maintained under inbreeding in the animal facility of the Institute of Genetics and Animal Biotechnology PAS in Jastrzębiec (Poland). Young (10–23-month-old, YMA group) and old (35–65-month-old, AMA group) females were mated with young (10–25-month-old) strain-matched males to obtain offspring. Young 3–5-month-old females of a not-related strain (e.g. derived from Swiss Webster mice by inbreeding in the Institute of Genetics and Animal Biotechnology PAS) were used as foster mothers. Mice were housed in 265 × 207 × 140 mm transparent polycarbonate cages in groups of 3–5 subjects per cage, and maintained under 12 h light–dark cycle, 20–22 °C, and 40–60% humidity, with food (Labofeed H, Kcynia, Poland; caloric value 13 MJ/kg) and water ad libitum. All procedures applied to animals were performed in accordance with Polish and European regulations following the ARRIVE guidelines, and have been reviewed and approved by the II Local Ethics Committee for Experiments on Animals in Warsaw (Approval no.: 62/2015, 25.06.2015).

Placentas and fetuses collection and analyses

Fetuses with their placentas were collected from pregnant BTBR and B6 old (42–65 weeks old, AMA) and young (10–23 weeks old, YMA) females. Mice were ethically euthanized by cervical dislocation 12 days after plug detection. The uterus was isolated from the abdominal cavity followed by dissection of the conceptuses by separating the fetus from the placenta. Then, placentas and fetuses were weighed and dissected: the decidua was removed from the placenta, and the fetal head was separated from the rest of the body. The obtained placentas and fetal heads were then immediately snap-frozen in liquid nitrogen and stored at -80 °C until further analyses. Developmentally arrested conceptuses with evident signs of hemorrhages were classified as resorbed fetuses, and not included in the further analyses.

Extraction of nucleic acids

RNA for transcriptomic analyses was extracted from placentas and fetal heads following the manufacturers' instructions. Total RNA extraction was conducted using the Universal RNA Purification Kit (Eurx, Poland), and included a DNase treatment (Eurx, Poland). The quantity and purity of isolated nucleic acids were validated using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity of selected samples was additionally validated using a Bioanalyzer 2100 microcapillary electrophoresis device (Agilent, USA). To determine the sex of the collected fetuses DNA samples were analyzed by standard PCR for the presence of male-specific locus *Sry*, as previously described[34]. DNA was isolated from fragments of fetal bodies homogenized in vials containing 2 steel beads (~ 3 mm) and extracted using the Wizard DNA Purification kit (Promega), by including a RNAse treatment (Qiagen, Germany). PCR reactions for sexing purposes were carried out using DreamTaq Green PCR mix (Thermo Fisher Scientific, Massachusetts, USA).

Microarrays

Genome-wide transcriptomic analysis was performed using SurePrint G3 Mouse GE v2 8 × 60 K microarrays (GPL21163, Agilent Technologies, USA) and Agilent Technologies Reagent Set according to the manufacturer's instructions. 100 ng of RNA of each sample (n = 4 male fetuses and placentas *per* maternal age group *per* tissue, obtained from different litters) was labeled using Low Input QuickAmp Labeling Kit One-Color (Agilent

Technologies, USA) and hybridized on the microarrays according to the manufacturer's protocol. Statistical analysis of raw data was carried out using the default protocol of the Partek Genomics Suite. Each probe data was logged, normalized using quantile normalization, and annotated using the "GPL21163_noParents.an.txt" annotation file from the Gemma database[35]. Results were adjusted for multiple comparisons using the FDR step-up method. Genes were considered to be differentially expressed if their absolute values of logged fold changes (base 2 logarithms) reached 1.5 and the adjusted p-value was lower than 0.05.

Functional Analysis

The list containing names of differentially expressed genes was annotated with over-represented (enriched) biological terms using the Enrichr tool (http://amp.pharm.mssm.edu/Enrichr/)[36]. These terms described within multiple databases included: ontologies, molecular functions, biological processes, and cellular components. Enrichr calculates *p* values of enrichment using Fisher exact test. Only terms showing statistically significant enrichment of at least 0.05, after adjustment for multiple testing (Benjamini–Hochberg method), were considered to be genuinely enriched and included in the results.

Quantitative PCR of selected genes

Microarray data were validated by quantitative Real-Time PCR performed on the LightCycler 96 thermocycler (Roche, Switzerland). The genes Gabra5, Gabrb1, Gabrb3, Ndn, and Pianp were selected for the fetal head, while Pla2g2f, Pla2g5, Prl7a1, and Tpbpa were selected for the placenta, based on their involvement in relevant pathways resulting from the functional analysis. The sample set used for qPCR analyses was expanded to 34 individual offspring by including samples originating from BTBR and B6 old and young fetuses and placentas of both sexes. 1000 ng of total RNA from each sample were retrotranscribed to cDNA using First Strand cDNA Synthesis Kit (Roche) and quantitative real-time PCR was run using the LightCycler FastStart SYBR Green Master (Roche). Primers were designed using the Primer-BLAST NCBI tool and are listed in Supplementary Table 1. The resulting amplicons covered all mRNA variants of each gene and were positioned on two different exons. Primers were verified by temperature gradient PCR (55–65°C) followed by gel electrophoresis. Analyses were run in triplicate (3 independent replicates per sample) and contained a negative control (without cDNA) as well as a 5-fold dilution series of cDNA to determine PCR efficiency. Melting curve analysis was performed to verify the presence of one gene-specific peak and the absence of primer-dimer peaks using the dedicated Light Cycler 96 software (ver. 1.1, Roche, Switzerland). Raw Ct values were calculated by the Lightcycler, while relative expression ratios were calculated according to the Pfaffl method[37]. The stability of 4 candidate reference genes (Gapdh, Hmbs, Pgk1, Tbp) was analyzed using geNorm (https://genorm.cmgg.be/) to select the optimal gene for each tissue: Hmbs was selected for the fetal head, while Gapdh for the placental tissues. Quantitative real-time PCR was performed according to the MIQE guidelines[38].

Offspring postnatal fostering and development

Virgin B6 and BTBR, young (11–23 weeks) and old (35–40 weeks), females were mated overnight with young (10–20 weeks) strain-matched males. Females of a not-related strain (e.g. derived from Swiss Webster mice by inbreeding in the Institute of Genetics and Animal Biotechnology PAS) were mated one day before to obtain foster mothers. On E18.5 pregnant females were singly housed and monitored twice a day from E19.5 to E21.5 for signs of labor. When delivery was successful and a dam had gathered all pups to the nest, then foster and biological mothers were separated from the respective litters. Experimental B6 and BTBR pups were gently placed on the bedding material of the foster mother's cage together with the foster siblings, reducing the size of the litter to a maximum of 10 pups. Finally, the mixed litter was transferred to the foster mother's nest followed by the return of the foster mother in its home cage.

Analyses of offspring health and behaviors

Pregnancy and delivery rates, litter size, body weight at postnatal day 4 (P4) and P8, and survival to P24 were monitored. The offspring obtained were subjected to a behavioral screening as described previously[18], including one test conducted in pups, and two tests conducted in both male and female adults.

Isolation-induced ultrasound vocalization (USV) activity

Tests for isolation-induced USV were done on P4 and P8. Dams were removed from their home cages 15 min before the beginning of the test. Afterward, each pup was isolated from its littermates and moved into a plastic container (120 × 90 × 60 mm) filled with clean bedding materials situated in a sound-attenuating chamber under a controlled temperature (25 °C). An Ultrasound Microphone (Avisoft UltraSoundGate condenser microphone capsule CM16, Avisoft Bioacoustics, Germany) positioned 200 mm above the pup was used to capture the USVs, which were recorded using the Avisoft Recorder software (ver. 4.2, Avisoft Bioacoustics, Germany). After 3 min of recording, the tested pup was carefully transferred onto a scale and its body weight was recorded. After testing, pups were marked by non-toxic marker for identification and placed back into their home cage, then the next pup was tested sequentially. Acoustical analysis of recorded USVs was performed with Avisoft SASLab Pro software (ver. 5.3.2–37, Avisoft Bioacoustics, Germany). USV call detection was provided by an automatic whistle-tracking algorithm. Additionally, the accuracy of USV calls detection was checked manually by an operator blinded to experimental groups. Analyzed parameters included the number, mean duration, and mean peak frequency of USV calls emitted, during the three-minute isolation.

Three-chamber social test

The three-chamber social test was performed on offspring aged 3 to 4 months within a glass box measuring 600 × 300 × 300 mm, partitioned by two plexiglass walls into three chambers. The tested mouse was allowed

to freely move among the chambers through openings measuring 60 × 60 mm. At the beginning of the test, the mouse was placed in the central chamber and allowed to freely explore the whole apparatus for 10 min for habituation. Subsequently, the mouse was removed from the arena and briefly placed in a cage with clean bedding materials. An unfamiliar 2–5-month-old 129/SvImJ male (social stimulus), which was previously habituated to the procedure, was placed inside a small wire cage in one of the side chambers, and an identical empty wire cage was placed in the opposite chamber as a non-social object. Then, the subject was again moved to the central chamber and allowed to freely explore all three chambers for 10 min. Measurements were taken using EthoVision XT (ver. 13, Noldus Technologies, Netherlands), which automatically calculated time spent in each chamber and time spent on sniffing social stimulus or object. Furthermore, the preference index for sociability, called social score was defined as time spent in the chamber containing the social stimulus divided by total test time.

Water T-maze

Mice were tested in a plexiglass t-shaped maze consisting of a start arm (450 × 100 mm) and two goal arms (300 × 100 mm) surrounded by 200 mm high walls and immersed into a circular 900 mm diameter container filled with 20–22 °C water to a depth of 150 mm. Moreover, a transparent movable plexiglas platform (70 × 100 mm) was submerged 10 mm below the surface of the water at the end of one goal arm. On the first day of the test, the testing mouse was placed in the starting arm of the apparatus without the platform for 60 s and the first arm entered by the mouse was noted as a preferred. Then, the platform was located at the end of only one goal arm, opposite to the arm selected in the previous pre-training session, and the testing mouse was placed in the starting arm and allowed up to 60 s to find the submerged platform. Ten trials per day were performed, for four consecutive days of habit acquisition, then mice were challenged in a reversal learning paradigm under ten trials per day, for 3 consecutive days. On each trial, an error was recorded when the mouse entered the arm without the platform or entered and then left the arm with the platform. If mice were unable to find the platform within 60 s, they were gently guided to the location and gently convinced to stay on the platform for 10 s. Mice were trained until they achieved a learning criterion of eight out of ten trials correctly performed for at least two consecutive days, for a maximum of 4 days. In the reversal learning session, the platform was switched to the opposite arm of the T-maze. The training procedure was the same as the habit-acquisition session. The total number of errors per day was accounted for throughout habit acquisition and reversal learning trials.

Statistics

Statistical analyses were performed using GraphPad Prism software (ver. 6, GraphPad Software, Inc., United States), STATISTICA software (ver. 7.1, StatSoft Inc., United States), and R (https://www.r-project.org/). All data were screened for normal distribution to determine the appropriate statistical test using D'Agostino-Pearson or similar tests. Percentages were compared with Fisher's exact test. Differences in continuous variables among multiple experimental groups were measured using one-way ANOVA or Kruskal–Wallis tests, followed by post hoc tests. Differences between two selected experimental groups were conducted using the Mann–Whitney or t-student tests, according to the distribution and homogeneity of data variance. Correlations between continuous variables were calculated using Pearson's test. Correlation significances were established with Fisher's test using the cocor R tool (http://comparingcorrelations.org/)[39]. Multifactorial two-way and three-way ANOVAs based on trimmed means were used to analyze the impact of the maternal age, strain, and offspring sex factors, as well as their interactions, on dependent variables. Multifactorial analyses were conducted using the WRS2 R package, along with its dedicated lincon posthoc test for pairwise factors comparisons[40]. The correlation between qPCR and microarray gene expression results was calculated using the function wincor of the WRS2 package (https://rdrr.io/cran/WRS2/src/R/wincor.R). All data are expressed as the mean ± standard error (SEM). Differences were considered statistically significant at $p < 0.05$.

Results

Advanced maternal age (AMA) negatively affects fetal and placental development in B6 and BTBR conceptuses

Mid-pregnancy outcomes and fetal/placental development were heavily affected by advanced maternal age in both strains (Table 1). AMA was associated with reduced fetal developmental rates at E12.5 in both strains, with females aged 42–65 weeks displaying smaller litters (B6, $p < 0.01$; BTBR, $p = 0.08$) and an increased frequency of resorbed fetuses (B6, $p < 0.0001$; BTBR, $p < 0.05$) compared to strain-matched 10–23-week-old control females. Fetal weight was reduced in old females of both strains compared to strain-matched young controls ($p < 0.0001$), while the placental weight was reduced only in B6 old females compared to young strain-matched controls ($p < 0.0001$) (Fig. 1A). Two-way ANOVA showed a significant effect of maternal age ($F_{(1,115)} = 23.39$, $p < 0.0001$), strain ($F_{(1,115)} = 32.16$, $p < 0.0001$), and their interaction ($F_{(1,115)} = 8.87$, $p = 0.0035$) on fetal/placental weights ratio, which was reduced in old vs. young mothers in both strains. The fetal-to-placental weight ratio was significantly higher in the conceptuses of young BTBR mothers as compared to the other experimental groups, including young B6 females ($p < 0.0001$). However, a low fetal/placental ratio was observed in old BTBR mothers compared to strain-matched young controls ($p < 0.0001$) (Fig. 1B). The correlation between fetal and placental weights was positive in young mothers, whereas it was negative in old females (Fig. 1C), with a statistically significant difference between maternal age groups in both strains ($p = 0.01$). Overall, these results indicate that advanced maternal age is associated with a suboptimal pregnancy environment leading to perturbation in fetal growth and low fetal survival in both strains (Fig. 1D).

	Plug + females [N]	Females with conceptuses [N (%)]	Collected conceptuses [N]	Litter size at 12.5 dpc [mean ± SEM]	Resorbed fetuses [%]
B6 YMA	7	7 (100%)[ab]	52	7.4 ± 0.5[de]	12.1%[g]
B6 AMA	28	7 (25%)	24	3.4 ± 0.06	51.3%
BTBR YMA	13	6 (46.1%)[c]	67	9.6 ± 1.3[f]	6.3%[h]
BTBR AMA	25	4 (16%)	17	4.3 ± 2.4	22.7%

Table 1. Pregnancy outcomes in young (YMA) and old (AMA) B6 and BTBR females at embryonic day 12.5. [a]$p < 0.001$ comparing B6 YMA vs B6 AMA with Fisher's exact test. [b]$p < 0.05$ comparing B6 YMA vs BTBR YMA with Fisher's exact test. [c]$p = 0.06$ comparing BTBR YMA vs BTBR AMA with Fisher's exact test. [d]$p < 0.01$ comparing B6 YMA vs B6 AMA with Mann Whitney test. [e]$p = 0.07$ comparing B6 YMA vs BTBR YMA with Mann Whitney test. [f]$p = 0.08$ comparing BTBR YMA vs BTBR AMA with Mann Whitney test. [g]$p < 0.0001$ comparing B6 YMA vs B6 AMA with Fisher's exact test. [h]$p < 0.05$ comparing BTBR YMA vs BTBR AMA with Fisher's exact test. YMA: 10–23 weeks old mothers. AMA: 42–65 weeks old mothers.

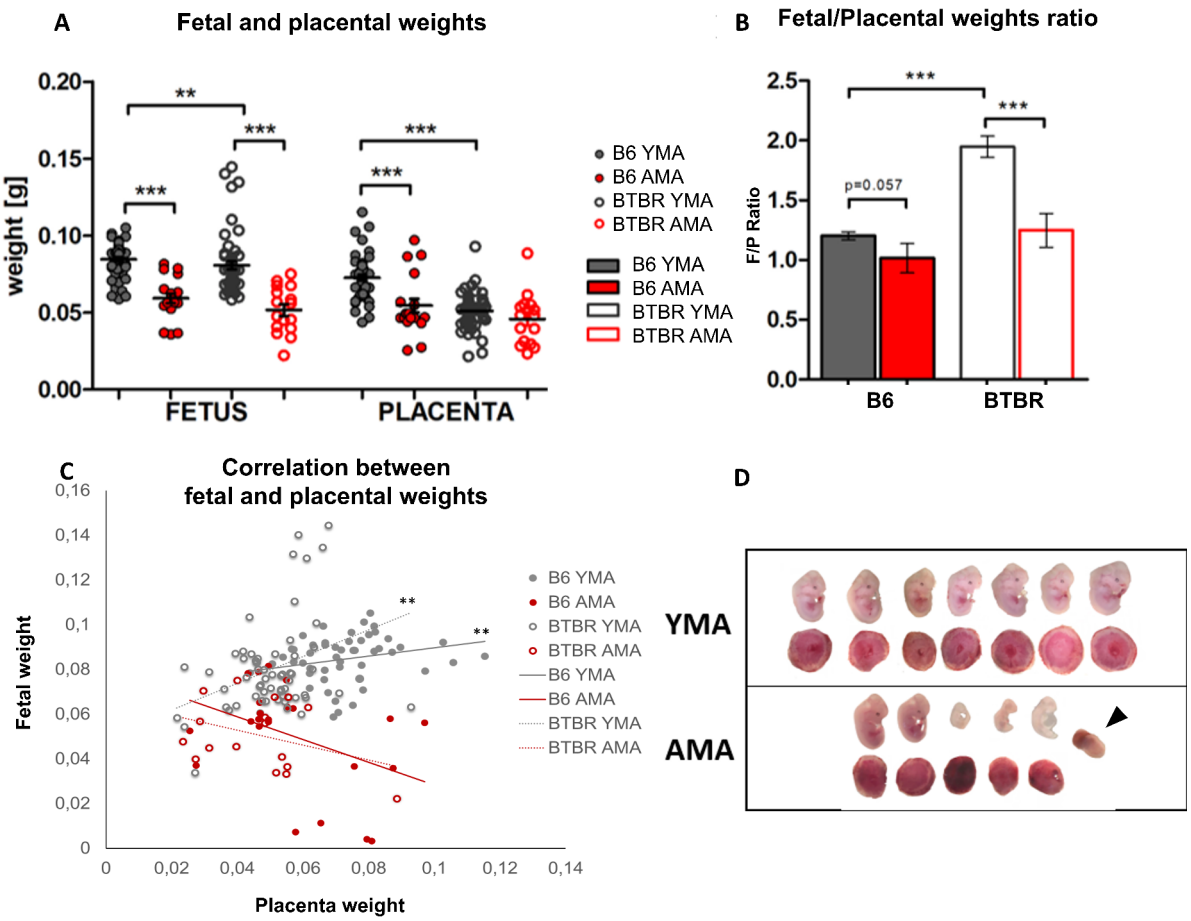


Fig. 1. Advanced maternal age disrupts fetal and placental development in BTBR and B6 mice. (**A**) Fetal and placental weight; $^{**}p < 0.01$, $^{***}p < 0.0001$ with Mann Whitney test. (**B**) Fetal/placental weight ratio; $^{**}p < 0.01$, $^{***}p < 0.0001$ with Mann Whitney test. (**C**) Correlation between fetal and placental weights were significantly different comparing AMA with YMA; $^{**}p < 0.01$ with Fisher's test. B6 YMA n = 51, B6 AMA n = 23, BTBR YMA n = 60, BTBR AMA n = 17. (**D**) Representative images depicting fetuses and placentas collected from old and young females at embryonic day 12.5; the black arrowhead indicates a resorbed conceptus.

Advanced maternal age affects mRNA expression of relevant neurodevelopmental genes in the fetal brain of BTBR and B6 conceptuses

To investigate the mechanisms underlying the adverse maternal age effects on prenatal development we conducted a gene expression analysis in fetal heads and placentas. Conceptuses were collected at E12.5 from old and young pregnant females of the B6 strain and processed for microarray analyses. The most significant differences

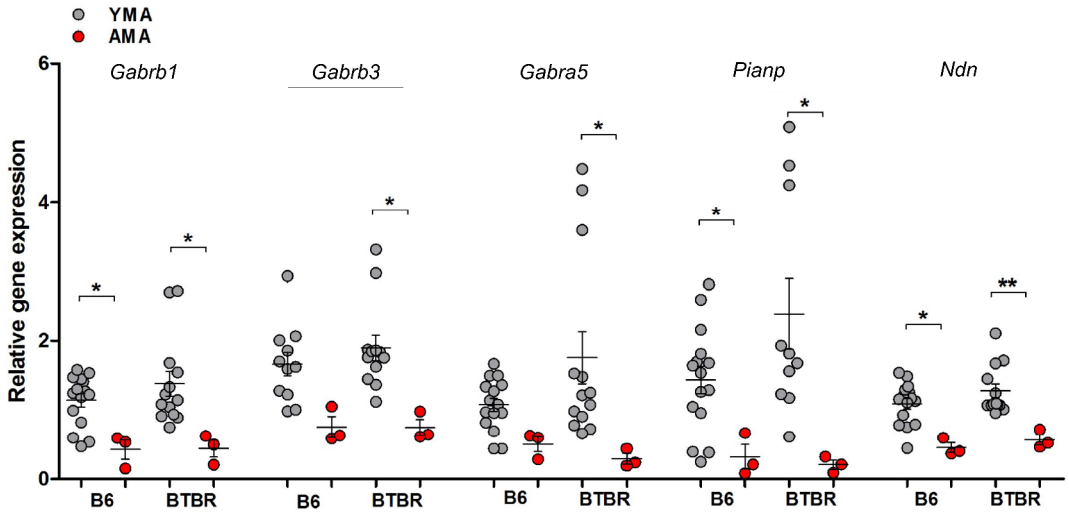


Fig. 2. Advanced maternal age induces changes in gene expression of fetal heads and placentas in BTBR and B6 mice. (**A**) Relative mRNA expression of several neurodevelopmental-relevant genes was decreased in AMA vs YMA fetal heads in both strains; *$p < 0.05$ with one-way ANOVA.

	Plug + females [N]	Delivery [N (%)]	Litter size at delivery [mean ± SEM]	Obtained offspring [N]	Body weight at P4 [mean ± SEM]	Body weight at P8 [mean ± SEM]	Survival at P24 [N (%)]
B6 YMA	19	16 (84.2%)[a]	6.7 ± 0.4[cd]	101	2.36 ± 0.03[f].	3.62 ± 0.07[f].	91 (90.1%)
B6 AMA	24	9 (37.5%)	4.7 ± 0.5	38	2.56 ± 0,06	4.08 ± 0.12	33 (86.8%)
BTBR YMA	18	13 (72.2%)[b]	8.5 ± 0.6[e]	111	2.39 ± 0.05[g]	3.50 ± 0.09	96 (86.5%)
BTBR AMA	14	3 (21.4%)	4.0 ± 1.2	12	2.75 ± 0.06	3.19 ± 0.07	8 (66.7%)

Table 2. Delivery outcomes of young and old B6 and BTBR females, and pups' survival to weaning. [a]$p < 0.01$ comparing B6 YMA vs B6 AMA with Fisher's exact test. [b]$p < 0.05$ comparing BTBR YMA vs BTBR AMA with Fisher's exact test. [c]$p < 0.05$ comparing B6 YMA vs B6 AMA with Unpaired t-student test. [d]$p = 0.051$ comparing B6 YMA vs BTBR YMA with Mann Whitney test. [e]$p < 0.05$ comparing BTBR YMA vs BTBR AMA with Mann Whitney test. [f]$p < 0.01$ comparing B6 YMA vs B6 AMA with Unpaired t-student test. [g]$p < 0.01$ comparing BTBR YMA vs BTBR AMA with Unpaired t-student test. YMA: 11–23 weeks old mothers. AMA: 35–40 weeks old mothers.

between old and young females were found in gene expression of the fetal heads (398 differentially expressed genes, DEG) as compared with the placenta (51 DEG). The full list of DEGs is reported in Supplementary Table 2 and the raw data sets are available in the GEO repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE276507). Gene ontology analyses did not reveal any relevant pathway enrichment among the placental DEG, whereas, in the fetal head, DEGs were enriched in several pathways, biological processes, and molecular functions related to neurodevelopment (Supplementary Table 3). Subsets of DEGs were selected for quantitative PCR validation based on their involvement in enriched pathways and the bulk difference between the maternal age groups. Selected genes were then analyzed in an independent cohort of B6 and BTBR fetuses ($n = 15$ YMA and $n = 3$ AMA per strain). There were no significant differences in fetal head gene expression of selected genes comparing male and female fetuses (Supplementary Fig. 1), thus both sexes were collapsed for further analyses. Three-way ANOVA showed a significant effect of maternal age on fetal head gene expression of the majority of the selected genes with no interaction among strain, maternal age, and offspring sex factors (Supplementary Table 4). In both strains, GABA receptors, Pianp, and Nnd genes were significantly less expressed in the fetuses conceived by old compared to young females ($p < 0.05$) (Fig. 2). These results suggest that AMA perturbs important neurodevelopmental transcriptional pathways already at prenatal stages in both strains, possibly paving the way for the occurrence of behavioral phenotypes in the offspring.

Pups conceived by old B6 and BTBR mothers display altered growth patterns

Given the profound negative effects of AMA on pregnancy and fetal health, we analyzed offspring postnatal outcomes at delivery in a separate cohort of old mothers (35–40 weeks old) compared to young strain-matched control females aged 10–23 months. After birth, newborns were fostered to young females of a not-related strain to rule out eventual influences of strain and maternal age on the postnatal environment. Litter size at delivery was significantly lower in old compared to young females in both strains ($p < 0.05$) (Table 2). B6 and BTBR pups conceived by old females displayed increased body weight on postnatal day 4 (P4) compared to strain-matched

young controls ($p < 0.01$), indicating a postnatal catch-up recovery from the fetal growth restriction experienced prenatally. Similar differences in body weight between AMA and YMA pups were still significant at P8 in the B6 strain ($p < 0.01$), whereas BTBR AMA pups were smaller at P8 compared to the other experimental groups ($p < 0.05$). Under these experimental conditions, only 66% of BTBR pups born from old females survived to postnatal day 24, whereas other experimental groups had more than 85% survival rate (Table 2).

Advanced maternal age affects offspring sociability and learning skills in a strain- and sex-dependent manner

The obtained offspring of young and old mothers were subjected to a battery of behavioral tests aimed at examining communication, sociability, learning skills, and cognitive flexibility. Ultrasound vocalizations (USV) were analyzed as an early landmark of social communication in pups isolated from their mothers and littermates. As previously reported[41,42], pups of the BTBR strain emitted a higher number of USV calls, as compared to B6 pups ($p < 0.0001$) (Fig. 3A). BTBR displayed an increased duration of USV emissions than B6 pups at both P4 and P8 ($p < 0.0001$) (Fig. 3B), while the USV mean frequency was significantly lower in BTBR than B6 only at P8 ($p < 0.0001$) (Fig. 3C). These marked strain differences were not influenced by maternal age. Accordingly, two-way ANOVA showed an extremely significant effect of the strain on the number of USV emitted, both on P4 ($F_{(1,105)} = 95.35$, $p < 0.0001$) and P8 ($F_{(1,104)} = 67.68$, $p < 0.0001$), with no significant effects of maternal age. However, there was a significant interaction between the maternal age and the strain factors on P4 ($F_{(1,105)} = 4.9$, $p = 0.029$). These results demonstrate that strain-specific features of USV are innate in the two strains examined, and are not affected by advanced maternal age, nor by postnatal fostering.

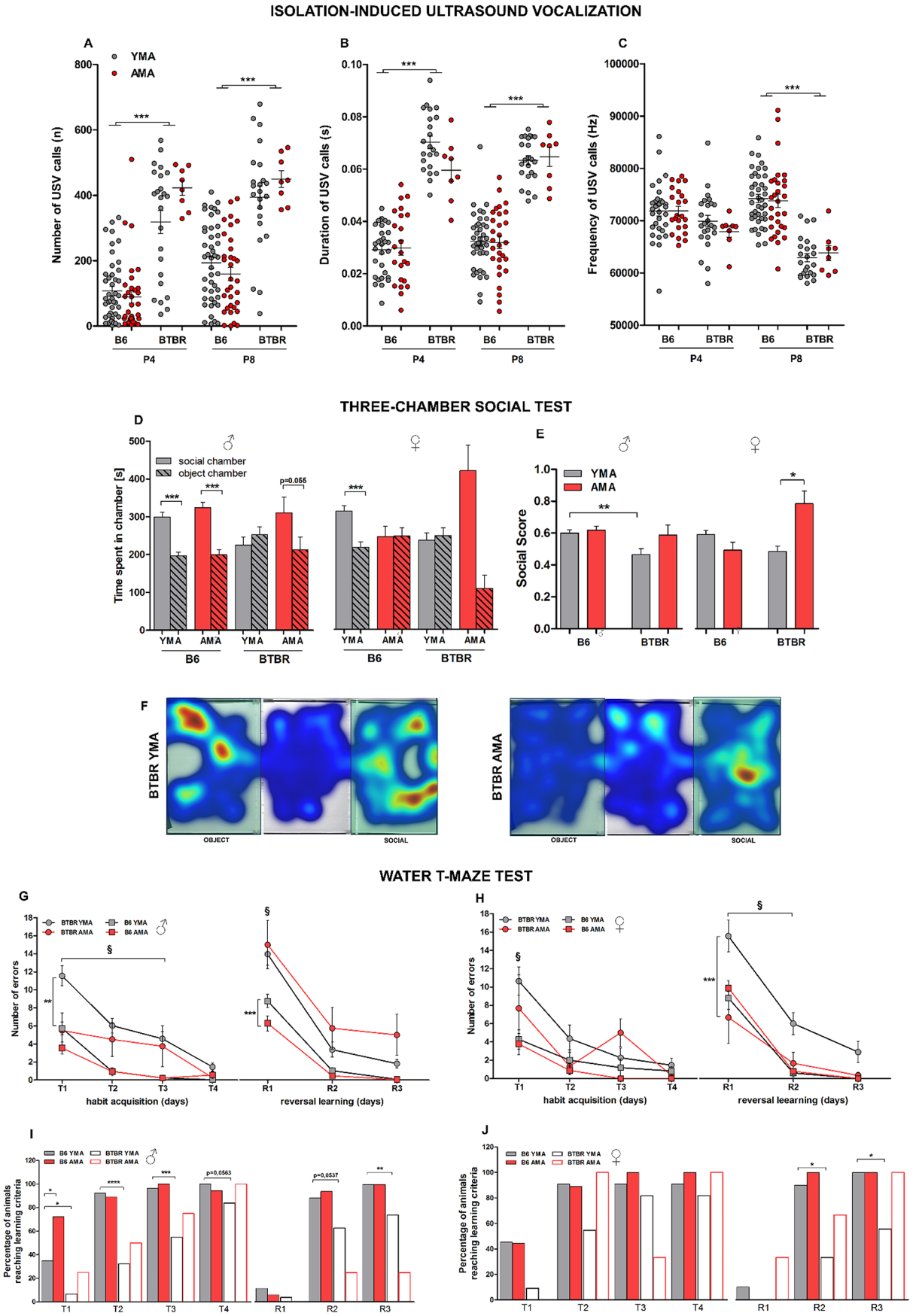
Sociability was examined by the three-chamber social test. B6 females conceived by old mothers displayed decreased sociability compared to strain- and sex-matched control offspring, while the sociability of B6 males was not affected by maternal age. Conversely, BTBR male and female offspring conceived by old females showed a non-significant increase in sociability compared to strain-matched controls (Fig. 3D–E). Two-way ANOVA revealed a significant effect of the strain factor on the social score of male offspring ($F_{(1,93)} = 1.42$, $p = 0.0475$), as well as a significant interaction between the strain and the maternal age factors on the social score of female offspring ($F_{(1,51)} = 8.71$, $p = 0.0048$) (Fig. 3D-F). Three-way ANOVAs showed a significant effect of the strain over the maternal age factors on the social score, sniffing score, and time spent into the social chamber, although there was a significant interaction between the maternal age and strain factors in all sociability outcomes analyzed (Supplementary Table 5). These results indicate that offspring sociability depends on a complex interaction of strain-, maternal-, and sex-related factors.

Finally, offspring were subjected to the water T-maze test to examine spatial learning and cognitive flexibility. The number of errors made by the mouse was used as a measure of spatial learning in the habit acquisition phase and as a score for cognitive flexibility in the reversal learning phase. Learning outcomes were mostly dependent on the strain, with BTBR mice making more errors than B6 in both training and reversal learning trials ($F_{(1,84)} = 4.42$, $p < 0.01$, $F_{(1,72)} = 8.34$, $p < 0.01$, on T1 and R1, respectively) (Fig. 3G–H). The effects of maternal age on learning performances were strain- and sex-specific, with offspring conceived by old mothers making generally fewer errors compared to strain- and sex-matched controls. In particular, this effect was observable in BTBR males in the habit acquisition phase ($P < 0.001$) and in B6 males in the reversal learning phase ($P < 0.0001$), as well as in BTBR females in the reversal learning phase ($p < 0.01$). Accordingly, the percentage of mice reaching learning criteria was lower in BTBR compared to B6 offspring conceived by young mothers in both the habit acquisition and the reversal learning phases, with significant differences observed in both males and females (Fisher's exact test—males: T1 $p < 0.05$, T2 $p < 0.0001$, T3 $p < 0.001$, R3 $p < 0.01$; females: R2 $p < 0.05$, R3 $p < 0.05$) (Fig. 3I–J). However, these strain differences were not significant when comparing the B6 and BTBR offspring conceived by old females. Moreover, a higher percentage of male B6 offspring conceived by old females reached learning criteria on testing day 1 as compared to the offspring conceived by young B6 females (Fig. 3G). Three-way ANOVAs showed a significant effect of maternal age on the number of errors made on day T1 and R1, as well as a highly significant effect of the strain factor on number of errors made on days T1, T2, R1, and R2 (Supplementary Table 5). An interaction among the maternal age, strain, and sex factors was significant in the reversal learning days analyzed. These results indicate that advanced maternal age positively affects the development of social, learning, and cognitive skills of the offspring and that these effects are strain- and sex-specific.

Discussion

The present study aimed to investigate the etiology of ASD by dissecting the contribution of genetic and maternal factors to fetal neurodevelopment and postnatal behaviors. To this aim, the impact of maternal age on prenatal and postnatal offspring health and behavioral outcomes was examined in two genetically divergent mouse strains: the BTBR model of idiopathic autism, and the inbred control B6 strain. A growing number of studies have investigated the influences of environmental risk factors for ASD in the BTBR strain in comparison with the control B6 strain, demonstrating that maternal-related adverse events occurring during pregnancy may influence neurodevelopmental and behavioral outcomes in BTBR mice, either exacerbating or alleviating the ASD-like behaviors characterizing this strain[28,30,31].

In the present study, AMA did not affect strain-specific features of pups' USV activity, with BTBR pups vocalizing at higher rates and higher duration of USV calls compared to B6 (as previously reported[41,42]), regardless of maternal age. Noteworthy, BTBR *vs* B6 differences in USV emissions remained significant even after postnatal fostering, suggesting that the BTBR USV features are innate and independent of maternal age and the postnatal maternal environment. Conversely, there were strain- and sex-specific effects of maternal age on offspring sociability, with B6 female offspring displaying a social deficit and BTBR showing higher sociability when conceived by old mothers. A similar effect was observed in a mouse model of gene-environmental interaction


ISOLATION-INDUCED ULTRASOUND VOCALIZATION
A
YMA
AMA
Number of USV calls (n)
B
Duration of USV calls (s)
C
Frequency of USV calls (Hz)
B6
BTBR
P4
P8
THREE-CHAMBER SOCIAL TEST
D
social chamber
object chamber
Time spent in chamber [s]
p=0.055
E
Social Score
F
BTBR YMA
BTBR AMA
OBJECT
SOCIAL
WATER T-MAZE TEST
G
H
BTBR YMA
B6 YMA
BTBR AMA
B6 AMA
Number of errors
habit acquisition (days)
reversal learning (days)
I
J
Percentage of animals reaching learning criteria
p=0,0563
p=0,0537

◀**Fig. 3**. The effects of maternal age on postnatal autism-like behavior are heterogeneous, depending on the genetic background and the offspring's sex. (**A**) Number of calls on postnatal days 4 and 8. BTBR pups emit a higher number of USVs compared to B6, while no significant differences were found comparing maternal age groups. (**B**) Mean duration of USV calls. (**C**) Mean frequency of USV calls. *** denotes $p < 0.0001$ with the Kruskal–Wallis test. Number of pups tested: B6 YMA n = 44, B6 AMA n = 33, BTBR YMA n = 24, BTBR AMA n = 8. (**D**) Time spent in the chambers containing the social and the object stimuli by male and female offspring; *** denotes $p < 0.0001$ with Kruskal–Wallis test. (**E**) 3-chambered test's social score; * denotes $p < 0.05$ with Kruskal–Wallis test. (**F**) Representative heatmaps of the time spent in the different locations of the 3-chambers apparatus by BTBR female offspring conceived by old and control mothers. (**G**) Number of errors made by male offspring on each testing day of the habit acquisition and the reversal learning tests. (**H**) Number of errors made by female offspring on each testing day of the habit acquisition and the reversal learning tests; ** denotes $p < 0.001$ and *** denotes $p < 0.0001$, comparing AMA vs YMA BTBR and B6 mice in each separate day, with Kruskal–Wallis test; § denotes a significant effect of the strain factor with 2-way ANOVA. (**I**) Percentage of male offspring reaching criteria of at least 80% correct trials in each testing day of the two learning phases; * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, and **** denotes $p < 0.0001$ comparing B6 with BTBR mice with Fisher's exact test. (**J**) Percentage of female offspring reaching criteria of at least 80% correct trials in each testing day of the two learning phases; * denotes $p < 0.05$ comparing B6 with BTBR mice with Fisher's exact test. Number of adult animals tested: males, B6 YMA n = 26, B6 AMA n = 18, BTBR YMA n = 31, BTBR AMA n = 4; females, B6 YMA n = 26, B6 AMA n = 18, BTBR YMA n = 31, BTBR AMA n = 4.

investigating the interactions between the *Cntnap2* gene knockout and the prenatal valproic acid exposure, with *Cntnap2*-KO mice displaying higher sociability when exposed to the prenatal valproic acid challenge[28]. Similar conclusions were obtained from previous research concerning the effects of early life stress on BTBR and B6 mice, which did not show worsening of ASD-like behaviors in the BTBR strain, while the early life stress was negatively affecting behavioral outcomes in the control strain[31]. Conversely, a recent study demonstrated that BTBR mice are relatively more vulnerable to the effects of prenatal maternal immune activation compared to the B6 strain, indicating that specific environmental perturbances may exacerbate some ASD-like behaviors in a predisposed strain like BTBR[30]. The results reported here show a positive effect of advanced maternal age on the social deficit of the BTBR offspring, especially in females, which expressed a higher degree of sociability when conceived by old mothers. The sex-dependent differences in sociability across experimental groups may reflect different affiliative social behaviors towards same- vs opposite-sex of social stimuli, which may have affected differently the sociability outcomes in males and females. Nevertheless, this evidence suggests that a combination of genetic vulnerability and environmental risk factors mediates the development of ASD-related social deficits and may not always result in the exacerbation of symptoms in a disease-predisposed individual. Interestingly, mice conceived by old mothers displayed better performance in spatial and reversal learning tasks than control mice conceived by young mothers. Whereas, BTBR mice conceived by young mothers displayed poor cognitive flexibility in the water T-maze test as compared to B6. This strain difference was consistent in both the habit acquisition and reversal learning trials, in both male and female offspring, which aligns with other studies[43,44]. Overall, AMA positively affected water T-maze learning performances, but, similar to the sociability outcomes, this effect was strain- and sex-dependent.

The impact of maternal care on offspring behavioral phenotypes is known as critical in shaping developmental trajectories[45]. In previous studies involving the Fmr1-KO mouse model of Fragile X Syndrome, cross-fostering has been instrumental in examining the role of maternal care on ASD-like behaviors. For instance, maternal environmental enrichment, which includes enhanced sensory and social stimulation provided by the foster mother, can significantly impact the behavioral and neurobiological outcomes of the offspring[46]. Suggesting that environmental factors, particularly those related to maternal behavior, can modulate the effects of genetic mutations associated with autism. In the present study, we transferred offspring from their biological old or young mothers to a young foster female of a different strain, immediately after delivery. This strategy allowed us to control confounding variables related to the postnatal maternal environment, and investigate gene-environment interactions occurring prenatally. However, mice with divergent genetic backgrounds may respond differently to the same treatment, such as postnatal care, particularly in the context of neurodevelopmental disorders[47].

Although the effects of maternal age on the offspring behavioral phenotype varies depending on the genetic background, the AMA-induced fetal growth restriction and decreased survival to-term were observed in both strains suggesting a broad multi-system disorder consequent to maternal aging. Also, the effects of AMA on fetal brain transcriptome, especially the expression of important neurodevelopmental genes, were consistent in both strains analyzed. Deregulated genes including GABA receptors, Pianp, and Ndn are involved in neurodevelopmental pathways[48]. GABAergic interneurons are generated in the ventral telencephalon and later migrate into the developing cortex and hippocampus between embryonic day 10 and 16[49]. Activation of the GABA receptor promotes the migration of neuroblasts from the ventricular zones to the intermediate zone (IZ) in the fetal brain and provides a stop signaling for early neuronal migration[50]. Furthermore, Pianp deficiency interacts with GABA receptors, and it is involved in the control of ASD-relevant behaviors[51]. Our data highlight the dysregulation of the GABA gene expression as a vulnerable pathway associated with maternal health issues such as advanced maternal age, although differences in mRNA expression of Pianp and GABA receptor genes may be a consequence of retarded development characterizing the fetuses conceived by old females. Summarizing, the observed dysregulation of early fetal gene expression pathways may play an important role in shaping neurodevelopment in the offspring of old females. Nevertheless, these early-life alterations resulted

in the improvement of autism-like behavioral phenotype in the adult BTBR mouse, suggesting the potential involvement of genetic factors and/or postnatal adaptations in the development of autism-like behaviors in mice.

Overall, the results demonstrate that the effects of AMA on fetal and placental growth and transcriptomics are similar in both strains, but they result in distinct postnatal consequences, with the offspring's behavioral phenotype depending on interactions among different genetic and maternal factors (e.g. the maternal age, the genetic background, and the sex of the offspring).

Many evidences suggest that the etiological causes of ASD must be examined in the context of the whole maternal–fetal compartment[52–54]. The present study sheds light on the complex prenatal origin of ASD in relation to maternal and genetic factors contributing to neurodevelopmental programming during prenatal life. The study reinforces the idea that the intricate relationship between genetic vulnerability and the suboptimal uterine environment provided by old mothers determines neurodevelopmental outcomes, elucidating the potential roots of conditions like ASD in disruptions within the fetal brain, thus retaining translational value in future development of prophylactic and therapeutic strategies to counteract the development of ASD in pregnancy at risk.

Data availability

The data supporting the findings of this study are provided within the manuscript and its supplementary materials, as well as at the GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE276507), and are available on request from the corresponding author (Silvestre Sampino, s.sampino@igbzpan.pl).

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Author contributions

MZ: investigation, validation, data curation, and Writing draft; AJ: investigation; AS: investigation, methodology, validation, data curation, and formal analyses; SS: conceptualization, methodology, formal analyses, resources, data curation, writing draft and final version of the manuscript, visualization, supervision, project administration, and funding acquisition. All authors reviewed the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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