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Original Article

# Pregnancy at Advanced Maternal Age Affects Behavior and Hippocampal Gene Expression in Mouse Offspring

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## Abstract

There is growing evidence that advanced maternal age is a risk factor for neurological and neuropsychiatric disorders in offspring. However, it remains unclear whether the altered brain programming induced by advanced maternal age is mediated by pre- or postnatal factors. Here, a mouse model was used to investigate whether pregnancy at advanced age may provoke behavioral and brain gene expression changes in offspring. Swiss Albino mice conceived by 3-month-old males and either 15–18-month-old ($n$ = 11) or 3-month-old control females ($n$ = 5), were delivered by cesarean section, fostered after birth by 3-month-old dams and subjected to a battery of behavioral tests. Furthermore, genome-wide mRNA expression was analyzed in the hippocampi of 4-month-old males offspring using microarrays. Offspring conceived by old mothers exhibited increased ultrasound vocalization activity during separation from the foster mother, increased anxiety-like behaviors in adult life, and altered patterns of hippocampal gene expression, compared to controls. These effects were not reversed by the postnatal maternal care provided by the young foster mothers, suggesting that the altered brain programming is already established at birth, consistent with prenatal effects related to maternal aging.

**Keywords:** Delayed motherhood—Maternal effects—Brain disorders

The current trend toward delayed motherhood is increasing across developed countries. In the United States, the proportion of first births to women aged older than 35 years has increased nearly eight-fold since 1970. In 2014, the birth rate for women aged 35–39 and 40–44 was 50.9 and 10.6 births per 1,000 women, respectively, with an increase of 3% and 2% compared to 2013 (1). Similar trends have been observed in Europe. For example, in the United Kingdom, the percentage of live births to mothers aged older than 35 years rose from 8.7% in 1990 to 19.3% in 2004. In 2009, 1 million babies in the European Union were born to mothers at advanced and very advanced ages (2). Nevertheless, the risks of negative pregnancy outcomes, such as miscarriage, congenital malformations, low birth weight, perinatal mortality, preterm delivery, and placental defects, are increased in women aged older than 35 years (3–7).

Advanced maternal age (AMA) not only promotes pregnancy complications and adverse perinatal outcomes but it is also associated

with adverse long-term health outcomes in offspring. For instance, neurological and neuropsychiatric diseases, including cerebral palsy, epilepsy, anxiety, autism, dyslexia, psychotic conditions, and minor neurodevelopmental disorders have been observed with higher frequency in children and adults born to elderly women (8,9). It has been suggested that negative, long-term, AMA-associated outcomes may be caused by gestational and perinatal complications that have been implicated in the risk for neurodevelopmental disorders (10,11). Aging is also associated with a decline in oocyte quality, including increased chromosomal and epigenetic aberrations (12) that are transmitted to the progeny and may negatively affect offspring outcomes. On the other hand, older women usually have a secure financial state, have achieved educational and career goals, and have age-related attributes such as emotional maturity, wisdom, and life experience: social advantages that may mitigate the biological disadvantages (9,13,14). Overall, both prenatal conditions (oocyte- and pregnancy-related) and postnatal factors associated with delayed motherhood may potentially influence children's brain and behavioral development. However, dissecting the causative roles of prenatal AMA-related circumstances on offspring health outcomes, while controlling for postnatal confounding factors, is challenging in human populations.

Mouse models have been used to understand the genetic and environmental contributions to neuropsychiatric disorders, exploring the interacting effects of genetic background, mutations, and prenatal factors on specific behavioral outcomes (15,16). We previously reported that, similar to humans, mice conceived by aged fathers exhibit behavioral abnormalities that resemble the core symptoms of autism, demonstrating that advanced paternal age produces comparable effects in both the mouse and human species (17,18). In the present study, we used a mouse model to investigate whether pregnancy at AMA may arouse behavioral and brain gene expression changes in the offspring. Mice conceived by differently aged females were delivered by cesarean section to rule out delivery complications in the analysis and were all nursed by young dams to control for possible age-related differences in postnatal maternal behaviors. Offspring were subjected to a wide range of behavioral tests aimed at assessing several endophenotypes related to different human psychiatric conditions. Furthermore, genome-wide mRNA expression was analyzed using microarrays in the hippocampi of adult male offspring.

## Methods

### Animals

All experiments were performed in accordance with the European Community regulation 86/609 and were approved by the Third Local Ethical Committee in Warsaw. Swiss-Webster outbred mice were used for all experiments. In the first pilot experiment, virgin females either at the age of 3 months (young maternal age [YMA] group, $n$ = 10) or 15–18 months (AMA group, $n$ = 10) were bred together with randomly selected 3 months virgin males. Reproductive and pregnancy outcomes were then evaluated. In a second experiment, breeding pairs were established using an independent cohort of virgin females, aged 15–18 months ($n$ = 11) or 3 months ($n$ = 5), which were paired with 3 months virgin males. After pregnancy was detected by the presence of the vaginal plug, males were removed and each female was singly housed; if no plug was detected after overnight male–female encountering, the female was changed to avoid possible effects of longer time to conception. Pregnant females were sacrificed 18.5 days post-coitum (dpc) and cesarean section was then performed. After cesarean section, pups conceived by AMA or YMA females were nurtured by 3-month-old foster mothers that had delivered the same day or 1 day before. Litters were reduced to the final number of six to eight pups, consisting of a maximum of 50% alien pups. After weaning, mice were housed in sex-specific groups composed of four to five siblings, in 30 × 13 × 11 cm cages.

### Experimental Procedure

Examination of body developmental landmarks, including weight, fur appearance, eye/ear opening, and incisors' eruption, was carried out daily from postnatal day 2 (P2) to weaning, which took place at P21. Behavioral tests were carried out as previously described (18). Isolation-induced ultrasound vocalizations (USVs) were analyzed on P4, P8, and P12. Assessment of righting reflex ability was performed at P6 and P12. In adults, each behavioral test was conducted starting at P75, in the following order: open field (OF) (P75), elevated plus maze (EPM) (P83), tail suspension (P91), and startle reflex (P100). All behavioral tests were made blind to experimental conditions and videotaped for further detailed analysis. An independent cohort of male offspring not subjected to behavioral tests was sacrificed at P120 and their hippocampi were collected for gene expression analysis. Because male individuals are generally more prone to develop neuropsychiatric disorders, the analysis of gene expression was performed only in males (19).

### Behavioral Tests

Offspring were subjected to a screening battery of tests to evaluate several behavioral endophenotypes, including early postnatal reflexes and isolation-induced ultrasound vocalization in pups, as well as adult startle reflex and prepulse inhibition, and anxiety- and depression-like behaviors. To test righting reflex ability, each animal was turned on its back and given a maximum of 60 seconds to return to the four paws position. Time of success was recorded for three consecutive trials for each pup. To test isolation-induced USV activity, pups were isolated one by one from their mother and littermates. Vocalizations were recorded for 5 minutes trial, using an ultrasound-sensitive microphone (Mini-2 Bat Detector - Summit, UK) tuned in the range of 60–80 kHz. Audacity software (http://audacity.sourceforge.net/) was used to record and digitalize the sound samples. The total number of USV emissions and their amplitudes were analyzed using Avisoft SASLab software (Avisoft Bioacoustics, Germany). Anxiety-related behaviors were evaluated in adult offspring using the OF and the EPM tests, which measure spontaneous locomotion and motivational responses to unfamiliar aversive places. Depression-like behavior was evaluated with the tail suspension test. Each mouse was suspended by its tail for 6 minutes and the total duration of immobility was analyzed. Finally, acoustic startle reflex and prepulse inhibition were evaluated using the Startle Box and Startle software (Med. Associates).

### Sample Preparation

The animals were sacrificed through decapitation and the brains were rapidly removed from the skulls. The hippocampi were dissected on ice-cold glass, inserted into freezing vials, snap-frozen in liquid nitrogen then stored at −80°C until further analysis. Total RNA was isolated from hippocampal samples using the Universal RNA Purification Kit (Eurx, Poland). RNA isolation included a DNase treatment step. The quantity and quality of RNA samples were estimated using a Nanodrop spectrophotometer (Nanodrop) and Bioanalyzer 2100 microcapillary electrophoresis device (Agilent). All RNA samples were of high quality (RNA Integrity Number > 9; 260/280 = 2.09 ± 0.01).

### Microarray Analyses

RNA samples isolated from the hippocampi of offspring (nine animals per group) randomly selected among all AMA and YMA litters were pooled for microarray analysis. Three poolings per experimental group, each containing three RNA samples, were prepared using equal amounts of RNA from each hippocampus for the final amount of 100 ng/pooling. Three AMA versus YMA cohybridization experiments were conducted on three separate microarrays. Experiments were performed in Agilent's Whole Mouse Genome Microarray 4x44K v2 (Agilent), according to standard Agilent protocols. Statistical analysis of microarray data was carried out with the use of the Limma package, a part of the Bioconductor project (www.bioconductor.org). Data were preprocessed, applying background correction (20), within-array normalization (method: "loess"), and between-arrays normalization (method: "Aquantile") (21). Next, data were filtered according to Limma guidelines and a linear model and test for nonsignificance using empirical Bayes method were applied to identify the genes that were significantly differentially expressed in AMA compared to YMA hippocampi (22). Furthermore, the Benjamini and Hochberg method for controlling false discovery rate was used for *p*-value adjustment. Genes were considered to be differentially expressed if their absolute values of logged fold changes (base 2 logarithm) reached 0.5 and adjusted *p* value was lower than .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/) (23). These terms described within multiple databases included: (i) ontologies, (ii) transcriptional regulators, (iii) biochemical pathways, and (iv) hub proteins. Enrichr calculates *p* values of enrichment using Fisher exact test. Only terms showing statistically significant enrichment of at least .05, after adjustment for multiple testing (Benjamini–Hochberg method), were considered to be genuinely enriched and included in the results.

### Confirmation of Microarray Data by Quantitative PCR

To validate the reliability of the results from the microarray experiment, quantitative real-time polymerase chain reaction (qPCR) was performed for seven genes (*Ada, Arl4d, Bag3, Egr2, Gem, Hspa1a*, and *Manf*), randomly selected from the 60 most deregulated transcripts of the microarray data set. Eighteen individual samples ($n$ = 9/group, randomly selected among all litters) were analyzed. The primer sets were designed using the Primer-BLAST tool (NCBI) (www.ncbi.nlm.nih.gov/tools/primer-blast/). The sequences of the oligonucleotide primers used for real-time PCR and detailed information are described in Supplementary Table 1. The reactions based on SYBR Green I were carried out in a 96-well optical plates on the Light Cycler 96 instrument (Roche Applied Science, Germany). All samples were analyzed in triplicate and negative controls without the cDNA template were included for all genes. All runs included a fivefold dilution series of cDNA and the generated standard curves gave a mean inter-run correlation coefficient of 0.996 (± 0.004, *SD*) and mean inter-run efficiency of 99.3% (± 7.6%, *SD*). Raw $C_t$ values were calculated by Light Cycler 96. For each sample, the relative expression ratio ($R$) was calculated according to the Pfaffl model (24). Quantitative real-time PCR was performed according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines." at the bottom of the paragraph "Confirmation of Microarray Data by Quantitative PCR.

### Statistical Analyses

When continuous data were normally distributed and homogeneity of variance assumptions were met, a standard $t$ test was used to compare groups. When data were not normally distributed and/or when the homogeneity of variance assumption was not met, a Mann–Whitney–Wilcoxon test was used. Differences in the isolation-induced ultrasound vocalization were analyzed across days using one-way analysis of variance, followed by Bonferroni post-test. The values were expressed as mean ± standard error of the mean and $p$ less than .05 was accepted as a level of significance. All individual data points were used as statistical units of the analysis. Litter effects on behavioral variables were assessed using a generalized linear mixed model, entering litter ID as a random effect. The model included random intercepts. Intraclass correlation coefficient (ICC) was measured to explore the cluster effect of the litter. To estimate the effect size of AMA on behavioral variables, the Cohen's $d$ index was calculated based on adjusted means and standard deviations.

For real-time PCR, $R$ values from each analyzed gene were checked for normal distribution (Shapiro–Wilk test) and equality of variances (Levene's test using median). Next, statistical tests were used to calculate $p$ values: (i) if $R$ ratios of a given gene were normally distributed and showed equal variance between experimental groups, a standard Student's $t$ test was used; (ii) if $R$ ratios of a given gene were normally distributed but did not show equal variance between experimental groups, a Student's $t$ test for groups with unequal variances was used; (iii) if $R$ ratios were not normally distributed, a Mann–Whitney–Wilcoxon test was used. All values are reported as mean ± standard error of the mean. Differences were considered statistically significant at $p < .05$.

Exhaustive information about animal management and experimental procedures, as well as detailed methodology used for the behavioral tests, microarray analysis, processing of microarray data, functional analysis, and quantitative real-time PCR are described in Supplementary Methods.

**Table 1.** Reproductive and Pregnancy Outcomes of Young and Aged Female Mice

| Group | Dam's Age (months) | Pregnant Females ($n$) | Spontaneous Delivery ($n$) | | Litter Size (number of pups) | Cannibalized or Stillborn (% of pups) |
|---|---|---|---|---|---|---|
| | | | At 19.5 dpc | At 22.5 dpc | | |
| YMA ($n$ = 10) | 3.2 ± 0.12 | 10 | 10 | — | 9.4 ± 0.7* | 3.2% |
| AMA ($n$ = 10) | 15.04 ± 0.31 | 5 | 0 | 3 | 2.8 ± 0.48 | 100% |

*Note:* AMA = advanced maternal age; YMA = young maternal age. Dam's age and litter size are expressed as mean ± SEM.
*$p$ = .0028 comparing YMA and AMA with $t$ test.

**Table 2.** Litter Sizes and Weights of Offspring Conceived by Young and Aged Females and Delivered by Cesarean Section 18.5 d Postcoitum

| Group | Dam's Age (months) | Litter Size (number of pups) | | Weight (grams) | |
|---|---|---|---|---|---|
| | | At Cesarean Section | At Weaning | 2 d Old | 21 d Old |
| YMA ($n$ = 5) | 3.4 ± 0.41 | 10 ± 0.89* | 9.6 ± 0.81 | 1.77 ± 0.03** | 8.42 ± 0.12 |
| AMA ($n$ = 11) | 16.18 ± 1.17 | 2.4 ± 0.45 | 2.1 ± 0.39 | 1.47 ± 0.07 | 8.29 ± 0.28 |

*Note:* AMA = advanced maternal age; YMA = young maternal age. Values are expressed as mean ± SEM.
*$p$ < .0001 comparing YMA and AMA with $t$ test. **$p$ = .018 comparing YMA and AMA with $t$ test.

## Results

### Reproductive and Pregnancy Outcome of Aged Females and Preweaning Development of Offspring

In a pilot experiment, we first sought to evaluate the effects of advanced female age on reproductive and pregnancy outcomes. To this end, virgin females, either aged 15–18 months (AMA, $n$ = 10) or 3 months (YMA, $n$ = 10), were paired with males aged 3 months and were allowed to deliver spontaneously. Table 1 shows the reproductive and pregnancy outcomes of young and aged females evaluated in this preliminary experiment. Only 50% of AMA females became pregnant within 1 month of encountering a male, whereas all control YMA females were pregnant within 10 days. The pregnancy outcome of old females was markedly compromised. While control YMA females delivered at a regular gestational age (19.5 dpc), three out of the five females of the AMA group spontaneously delivered late at 22.5 dpc and the remaining two females did not deliver spontaneously and were subjected to cesarean section at 24.5 dpc. The litter size of AMA females (2.8 ± 0.48) was significantly reduced ($p$ = .0028) compared to controls (9.4 ± 0.7). All pups delivered by AMA females were stillborn or were cannibalized soon after birth.

In a subsequent experiment, breeding pairs were established using females aged 15–18 months (AMA group) or 3 months (YMA control group) but cesarean sections were performed at 18.5 dpc in both pregnant AMA ($n$ = 11) and control ($n$ = 5) females, to overcome delivery complications. Furthermore, pups were nurtured by young foster mothers aged 3 months to rule out possible influences of postnatal maternal care on offspring behavior. At cesarean section, litter sizes were significantly different between groups (YMA: 10 ± 0.89; AMA: 2.4 ± 0.45; $p$ < .0001; Table 2). The weight of 2-day-old pups conceived by old mothers was significantly decreased compared to controls (YMA: 1.77 ± 0.03 g; AMA: 1.47 ± 0.07 g; $p$ = .018). However, during middle and late preweaning development, the body weight of the pups was not affected by maternal age, since there were no significant differences between groups in this measurement on P10 and P20. The survival rate at weaning was comparable between groups. Moreover, we did not find any effect of AMA when the offspring were subjected to a battery of preweaning developmental assays that included righting reflex ability (see Supplementary Results) and observation of developmental landmarks, such as incisor appearance, eye opening, fur appearance, and ear-pinnae detachment, which developed normally in all groups.

### Altered Responses to Social Isolation in Pups Conceived by Old Females

Social distress and communication deficits are key symptoms of several psychiatric diseases. To assess emotional responses and communicative ability during early postnatal life, pups were subjected to social isolation from mother and littermates and the number and intensity of emitted ultrasound vocalizations were examined. Pups conceived by older females tended to vocalize at higher rates compared to pups conceived by young mothers; in particular, differences were statistically significant when comparing 8-day-old pups ($p$ < .05; Figure 1a). The effect of AMA on USV rate at P8 was very strong ($d$ = 4.44). Analysis of USV amplitude, measured as the percentage of high-intensity calls, did not revealed any significant differences among groups (Figure 1b). After using a generalized linear mixed model, we did not find cluster effects of litter on P4 nor P8 (ICC < 0.03), whereas an effect of litter was observed on P12 (ICC = 0.992). Because fostering may affect offspring emotionality (25,26), we compared USV activity in adopted versus biological siblings conceived by young females. Analysis of USV rates and amplitude did not show any significant difference between fostered pups and those nurtured by their biological mothers (see Supplementary Results).

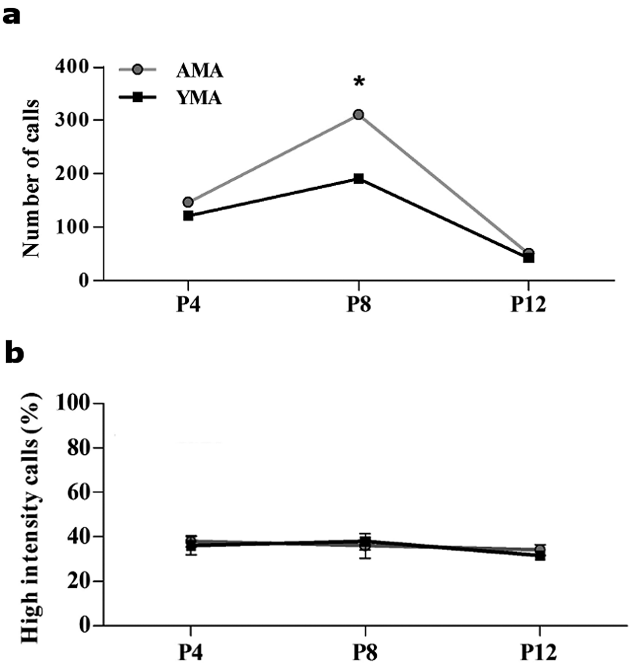


**Figure 1.** Advanced maternal age affects isolation-induced ultrasound vocalization (USV) activity in young offspring. (**a**) Mean number of USVs emitted by pups conceived by old (AMA) and young (YMA) females, on postnatal day 4 (P4), P8, and P12 in response to 5 minutes of maternal separation. Pups conceived by older females tended to vocalize at higher rates compared to pups conceived by young mothers; in particular, differences became statistically significant comparing 8-day-old pups. (**b**) Mean percentage of high intensity calls did not reveal any significant difference among groups. No significant sex differences were observed, thus data were collapsed across sexes. AMA = advanced maternal age, $n$ = 15; YMA = young maternal age, $n$ = 25. *$p$ < .05. Data are expressed as mean ± SEM.

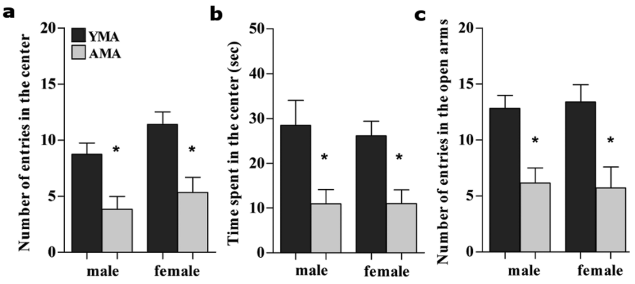


**Figure 2.** Advanced maternal age affects anxiety-related behaviors in adult offspring. Male and female mice conceived by old mothers displayed (**a**) fewer entries, as well as (**b**) decreased time spent in the center of an open field, and (**c**) fewer entries in the open arms of an elevated plus maze, compared to offspring conceived by young females. AMA = advanced maternal age, n = 13/sex; YMA = young maternal age, n = 20/sex. *$p < .05$. Data are expressed as mean ± SEM.

### Increased Anxiety-Like Responses but Normal Startle Reflex and Depression-Like Behavior in Adult Offspring Conceived by Old Females

Anxiety, depression, and defects in sensory-motor reflexes are common symptoms of several psychiatric disorders, including autism, mood disorders, and schizophrenia. To assess anxiety-like behavior, we analyzed the spontaneous locomotion and motivational responses of adult offspring using the OF and the EPM tests. Both male and female offspring conceived by aged mothers displayed fewer entries, as well as decreased time spent, in the center of an OF (Figure 2a,b), and fewer entries in the open arms of an EPM (Figure 2c), compared to offspring conceived by young females (Figure 2), suggesting an effect of AMA on anxiety-like behaviors. The effect of AMA on the EPM and OF measurement was fairly large ($1.325 < d < 0.88$). No significant differences in general locomotor activity were found among groups (see Supplementary Results). Furthermore, no effect of AMA was found on startle reflex and prepulse inhibition, used to measure sensory-motor reflexes and gating, nor in the time of immobility in the tail suspension test, used to measure depression-like behaviors (see Supplementary Results). No effect of litter was found in any of the measurements analyzed (ICC < 0.00003).

### AMA Affects Gene Expression Patterns in Hippocampi of Adult Male Offspring

To examine potential transcriptomic signatures associated with maternal age and behavioral abnormalities, we investigated hippocampal gene expression patterns in 4-month-old male offspring using microarrays. Among more than 39,000 probes tested on the microarray, 298 genes showed statistically significant changes in gene expression associated with maternal age. One hundred and fifteen—115—transcripts showed lower expression in the hippocampi of AMA compared to control mice, while 183 genes were upregulated (for a complete list of differentially expressed genes, see Supplementary Table 2). To identify the patterns of gene expression that elucidate biological processes, biochemical pathways, or transcriptional regulators affected by maternal age in the offspring hippocampus, we applied Gene Set Enrichment Analysis on the differentially expressed gene list, using the Enrichr tool (http://amp.pharm.mssm.edu/Enrichr/). Differentially expressed genes were significantly overrepresented in 39 functional categories belonging to biological processes and 7 categories belonging to molecular functions of the Gene Ontology database (for a complete list, see Supplementary Table 3). Out of 46 biological processes and molecular functions identified as enriched, 18 (39%) were related to protein homeostasis/modifications (Table 3). Accordingly, deregulated genes were significantly overrepresented in 14 biochemical pathways concerning the Heat Shock Factor 1 (HSF1) functions and the unfolded protein response (UPR), which control protein homeostasis and cellular stress responses (see Supplementary Table 4). Next, we asked which hub proteins interact with the peptides codified by the differentially expressed transcripts resulting from our analysis, as well as the transcription factors that regulate them. Overall, target genes for 70 hub proteins and 237 transcription regulators were overrepresented (for complete lists, see Supplementary Tables 5 and 6). Genechip data were validated by quantitative real-time PCR (qPCR). Genes were randomly selected from the 60 most deregulated transcripts of the microarray data set. All of the genes analyzed by qPCR displayed differences in gene expression when comparing offspring conceived by old versus young females (Figure 3). Pearson's correlation was used to confirm the reliability of microarray results. The correlation coefficient was 0.84 with a significance of $p = .018$, which gave us grounds to assume a positive, statistically significant relationship between the results of the qPCR and microarray experiments. The data sets supporting the results of this article are available in the GEO repository, GSE82325 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE82325).

## Discussion

The present study shows that AMA exerts negative effects on mouse pregnancy outcome and is associated with behavioral and brain gene expression changes in the offspring. Previous reports demonstrated that aged female mice have similar pregnancy rates and numbers of implantation sites at the first week of pregnancy, compared to younger females. However, in old pregnant dams, litter size diminishes significantly during gestation due to an increased resorption rate (27–29). Likewise, we observed decreased litter size and low birth weights in old females. Possibly, these effects are due to the unfavorable uterine environment provided by old females, which may display dysregulation of epigenetic and gene expression patterns and a redox imbalance status in uterine tissues (30–32). Interestingly, when pregnancy was successfully conducted to term, old mothers displayed increased infanticide, with 100% of newborns cannibalized soon after birth, which is in agreement with previous reports indicating increased cannibalism in old rat dams (33). Conversely, in the second experiment, pups conceived by old females were not cannibalized by their young foster mothers, indicating that infanticide behavior in old dams was mostly driven by maternal changes rather than a decreased fitness of the pups. Possibly, an altered maternal hormonal milieu might underlie this phenomenon (34,35).

Offspring conceived by old dams displayed increased USVs during social isolation. Likewise, mutant mice bearing deletions for autism-linked genes also exhibit similar changes in USV activity when separated from the mother during early life (36–38) and an increased rate of isolation-induced USV calls has been observed in the pups of the BTBR T+tf/J inbred strain, which is considered the elective mouse model for idiopathic autism (39). However, the increased vocalization upon separation from the mother may also relate to anxiety, rather than sociability. Accordingly, adult mice conceived by aged females exhibit increased anxiety-like behaviors, which is in agreement with a recent study indicating the association between maternal age and anxiety traits in a human population-based study (40). Although anxiety is not essential for an autism diagnosis, an

**Table 3.** Significantly Over-Represented Gene Ontologies Involved in Protein Homeostasis and Post-Translational Modification

| Ontologies | Overlap (number of genes) | Overlap (%) | Adjusted *p* Value |
|---|---|---|---|
| Protein folding | | | |
| Response to topologically incorrect protein (GO:0035966) | 14/143 | 9.79 | 5.9417E–06 |
| Response to unfolded protein (GO:0006986) | 14/135 | 10.37 | 5.9417E–06 |
| Protein folding (GO:0006457) | 15/221 | 6.79 | 0.00010617 |
| Unfolded protein binding (GO:0051082) | 9/101 | 8.91 | 0.00169644 |
| Activation of signaling protein activity involved in unfolded protein response (GO:0006987) | 6/64 | 9.38 | 0.02561105 |
| Chaperone-mediated protein folding requiring cofactor (GO:0051085) | 3/11 | 27.27 | 0.04352825 |
| Endoplasmic reticulum unfolded protein response (GO:0030968) | 6/83 | 7.23 | 0.04519259 |
| Cellular response to unfolded protein (GO:0034620) | 6/86 | 6.98 | 0.04819287 |
| Protein post-translational modification | | | |
| Positive regulation of protein kinase activity (GO:0045860) | 18/456 | 3.95 | 0.00786698 |
| Positive regulation of kinase activity (GO:0033674) | 18/480 | 3.75 | 0.01216718 |
| Negative regulation of kinase activity (GO:0033673) | 11/202 | 5.45 | 0.01376533 |
| Chaperone-mediated protein folding (GO:0061077) | 6/47 | 12.77 | 0.01376533 |
| Regulation of transmembrane receptor protein serine/threonine kinase signaling pathway (GO:0090092) | 10/186 | 5.38 | 0.02308394 |
| Negative regulation of protein kinase activity (GO:0006469) | 10/189 | 5.29 | 0.02359045 |
| Negative regulation of protein modification process (GO:0031400) | 15/407 | 3.69 | 0.02460899 |
| Negative regulation of phosphorylation (GO:0042326) | 13/325 | 4.00 | 0.02561105 |
| Regulation of protein serine/threonine kinase activity (GO:0071900) | 15/416 | 3.61 | 0.02561105 |
| Negative regulation of protein phosphorylation (GO:0001933) | 11/260 | 4.23 | 0.03916214 |

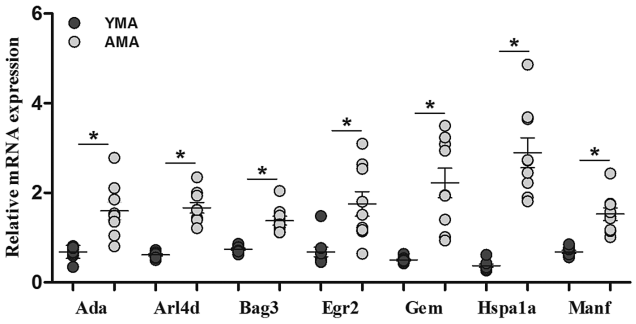


**Figure 3.** Advanced maternal age produces gene expression changes in the offspring hippocampus. Selected genes, which showed differential mRNA expression in microarray analysis, were analyzed by real-time quantitative polymerase chain reaction (qPCR). Genes were randomly selected from the 60 most deregulated transcripts of the microarray data set and relative (to the reference gene *Pgk1*) mRNA expression was compared between AMA and YMA male offspring. All genes analyzed by qPCR displayed differences in gene expression according to microarray results. Each dot represents an individual animal. AMA = advanced maternal age, $n = 9$; YMA = young maternal age, $n = 9$. $^*p < .001$. Data are expressed as mean ± SEM.

estimated 40% of children with autism fulfill diagnostic criteria for anxiety disorders and as many as 84% have subclinical anxiety symptoms (41,42). In this context, our data suggest that AMA might lead to an autism-like behavioral phenotype in mice. Nonetheless, further work is necessary to better elucidate the adult social phenotype of mice conceived by females at AMA, as well as their repetitive/stereotypic behaviors. Overall, although the behavioral alterations observed in mice conceived by old females do not entirely mirror the neuropsychiatric outcomes of offspring conceived by aged women, the present study provides compelling evidences regarding the contribution of pre- and postnatal factors mediating the increased risk of neurodevelopmental disorders in AMA offspring. Other mouse studies have reported that offspring conceived by old females display behavioral alterations, including decreased learning ability, decreased locomotor activity, and increased anxiety-like behavior (43,44). The mice analyzed in those experiments were reared by their biological mothers, which may have influenced their postnatal behavioral development. For instance, Lerch and colleagues reported that the increased age of mouse dams is associated with changes in maternal behavior and that those changes may have the potential to alter behavioral features in their offspring (44). Conversely, in the current study, the offspring included in the behavioral and gene expression analyses were conceived by young or old females but after delivery were all nursed by young foster mothers. Therefore, the effects of AMA had not been reversed by the postnatal environment provided by young foster mothers, demonstrating that the brain programming induced by AMA is already established at birth, consistent with prenatal effects and independent of postnatal conditions.

At the molecular level, we found that male offspring conceived by aged females displayed changes in hippocampal gene expression, compared to mice conceived by young females. Several genes, which showed differential mRNA expression, are involved in hippocampal regulation of anxiety-related behaviors in rodents, including the transcription factors *Arc*, *Egr1*, *Fos*, and *Fkbp5*, which are also important for synaptic plasticity and connectivity in the hippocampus (45–47). Moreover, prenatal and early postnatal adverse conditions have been shown to have long-term influence on the hippocampal expression of the *Arc*, *Egr1*, and *Fos* genes (48–50). Similarly, the prenatal environment provided by aged dams may inadequately support the fetus and could alter developmental programming of the expression of these genes in the hippocampus. Remarkably, some of the differentially expressed genes have been previously associated with neurodevelopmental disorders. Mutations involving the *Ophn1* and *Serpine1* genes have been linked with autism or schizophrenia (51–54). Single-nucleotide polymorphisms of *Ada*, *Pcdh8*, *Zbtb16*, and the *Plaur* promoter were proposed to influence the development of autism and have been associated with the disorder in population studies (54–57). In addition, the *Egr2* protein was found to be differentially expressed in frontal cortices from autistic human brain versus control and also showed differential gene expression between

lymphoblastoid cell lines derived from pairs of twins discordant with respect to severity of autism (58,59). In the current study, only male offspring were subjected to the transcriptomic analysis. Although the patterns of gene expression observed in males cannot be directly extrapolated to females, we can assume that both sexes may display transcriptomic changes in the brain, since both males and females showed similar behavioral abnormalities. However, further investigation will be needed to explore sex-specific effects of AMA on brain gene expression.

The functional characterization of gene expression profiles pinpointed biological processes and molecular functions that regulate cellular stress responses controlling protein homeostasis. Several genes involved in the unfolded protein response and the heat shock response, including *Xbp1*, *Manf*, *Atf3*, and different heat shock proteins (ie, *Hsp90b1*, *Hspa1a*, *Hspa5*, *Hspb1*, *Hspe1*, and *Hsph1*), were upregulated in the hippocampi of male offspring conceived by old dams, indicating a more active neuronal responses to cellular stress in these mice. Impaired functions of unfolded protein response and heat shock response have been implicated in normal brain aging and also in several age-related neurodegenerative disorders that are characterized by increased accumulation of oxidized and modified proteins and protein aggregates (60). Therefore, the increased expression of genes involved in unfolded protein response and heat shock response suggests that oxidative processes leading to neuronal aging might initiate earlier in the brain of offspring conceived by old females compared to controls. This phenomenon can be explained by the occurrence of maternal age-induced oxidative damage to oocyte mitochondria. For instance, it has been reported that oocytes from older women are more likely to contain mitochondrial DNA mutations than oocytes from younger women, including deletions of the *ATPase8* and *ND5* genes (61,62). Moreover, preovulatory oocytes from middle-aged women show increased mitochondrial numerical density, mitochondrial surface-to-volume ratio, and mitochondrial profile area, suggesting subtle but generalized changes in the oxidative phosphorylation capacity (63,64). Therefore, since mitochondria are almost exclusively maternally inherited, the offspring of older females may be predisposed to an oxidative-prone phenotype and in turn to early activation of oxidative stress responses in the brain. This hypothesis is supported by several epidemiological and animal studies, which demonstrated that AMA at birth is associated with a shorter life span in the offspring (65,66).

The biological mechanisms underlying the association between AMA and abnormal offspring outcomes remain unknown. Based on our results, the behavioral and gene expression changes observed in mice offspring may be related to downstream prenatal conditions associated with maternal age, such as chromosomal and epigenetic aberrations present in the oocytes (29), uterine and placental dysfunctions (30,32), and/or altered maternal hormonal profiles (34), rather than maternal behavior. Moreover, aged females are more prone to develop metabolic chronic diseases, such as obesity, gestational diabetes, and hypertension, which have been associated with negative offspring outcome (67,68). One molecular mechanism by which maternal age could influence the health of offspring is through epigenetic programming of fetal development. Support for an epigenetic effect includes reports of age-related changes of epigenetic signatures in the oocyte (12), which could lead to the inheritance of an altered maternal chromatin state (69,70). Furthermore, aberrant gestational/uterine environments of old females might affect the establishment of proper epigenetic marks in the developing brain. Epigenetic processes are involved in developmental events such as neuronal migration and connectivity formation and have been linked to several neuropsychiatric disorders (71), thus representing an intriguing path for future research.

In summary, this study demonstrates that AMA causes changes in offspring behavior and hippocampal gene expression in mice, which are not reversed by the postnatal maternal environment provided by young foster mothers. This suggests that the brain programming induced by AMA is already established at birth, consistent with prenatal effects and, at least in part, independent of postnatal conditions. However, the present study has some limitations. First, given the human-specific nature of many key symptoms and the lack of biomarkers and objective diagnostic tests, modeling of human neuropsychiatric disorders in animals still retains some translational disadvantage. Second, the use of maternal fostering limits the ecological validity of the present model in respect to humans, concealing the potential postnatal advantages connected to women aging. Nevertheless, mouse models of AMA are useful for exploring the contributions of the gestational uterine environment provided by aged females to the developing offspring and those related to downstream abnormalities carried by their oocytes (72). A better understanding of the prenatal mechanistic pathways may unveil novel targets for developing interventions aimed at the mother during pre-pregnancy and gestation that may effectively prevent or reverse the negative health outcomes of offspring conceived at AMA (73,74).

## Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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## Conflict of Interest

The authors do not have any conflicts of interest relevant to this paper.

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