Adverse early life environment induces anxiety-like behavior and increases expression of FKBP5 mRNA splice variants in mouse brain

Xingrao Ke, Qi Fu, Amber Majnik, Susan Cohen, Qiuli Liu, and Robert Lane

INTRODUCTION

Perinatal adverse early life environment (AELE) affects offspring health later in life (1, 4). More specifically, clinical studies reveal a strong association between an AELE and psychiatric disorders (1, 15). The most prevalent psychiatric disorders are anxiety disorders, affecting about one-third of the population during their lifetime (3). Anxiety disorders disproportionately affect those in low social economic status (SES) communities (6, 29). Furthermore, a low SES community and environment often limit families to a diet that is high in saturated fat, cholesterol, and sugar (Western diet), as well as exposing them to concerns for safety that lead to environmental stress and predisposition toward offspring anxiety disorders (28).

Ke X, Fu Q, Majnik A, Cohen S, Liu Q, Lane R. Adverse early life environment induces anxiety-like behavior and increases expression of FKBP5 mRNA splice variants in mouse brain. Physiol Genomics 50: 973–981, 2018. First published September 21, 2018; doi: 10.1152/physiolgenomics.00054.2018.—Adverse early life environment (AELE) predisposes adult offspring toward anxiety disorders. Anxiety disorders are associated with prenatal injuries in key regions of the brain including prefrontal cortex (PFC), hippocampus (HP), and hypothalamus (HT). Injuries in these brain regions result in an impaired hypothalamus-pituitary-adrenal axis (HPA axis) and stress response. An important regulator of the stress response is FK506-binding protein 5 (FKBP5). FKBP5 is a cochaperone of the glucocorticoid receptor (GR) and inhibits GR-mediated regulatory feedback on the HPA axis in response to stress. Human studies have shown that polymorphisms of FKBP5 are associated with higher FKBP5 levels. Increased FKBP5 leads to GR resistance and impaired negative feedback, which is associated with anxiety disorders. FKBP5 and its mRNA splice variants in the aforementioned brain regions have not been reported. We hypothesized that AELE will increase expression of FKBP5 and its mRNA splice variants in PFC, HP, and HT as well as increase anxiety in adult mice. AELE increased expression of FKBP5 and its mRNA variants in PFC, HP and HT at postnatal day 21. Additionally, AELE caused anxiety and increased GR abundance in association with these changes in FKBP5 expression. We speculate that these changes in FKBP5 mRNA variants affect HPA axis function and contributes to subsequent anxiety-like behavior later in life in AELE mice.

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Anxiety disorders are associated with hypothalamus-pituitary-adrenal (HPA) dysfunction (11, 20). Correlational studies have shown associations between SES and salivary cortisol levels. This is suggestive of a causal link between childhood poverty and activity of the stress-sensitive HPA system (14). HPA function is regulated by prefrontal cortex (PFC) (25, 36), hippocampus (HP) (21), and hypothalamus (HT). These brain regions are vulnerable to perinatal stress-induced insults (8, 9, 16, 31). Injuries in these brain regions result in a dysfunctional HPA axis (11).

The HPA axis is regulated in part by the interaction between FK506-binding protein 5 (FKBP5) and the glucocorticoid receptor (GR) in the brain (10). FKBP5 is a target gene of GR. GR is activated by cortisol binding and translocates to the nucleus. GR then binds to GR response elements in the promoter or enhancer regions of FKBP5, which activates FKBP5 transcription. FKBP5 acts as a co-chaperone protein of GR (5). Increased levels of FKBP5 protein inhibit GR activity, creating a short negative feedback loop whereby GR activation limits further GR activation (5, 38). Disruptions of this feedback loop have been associated with dysfunction of the HPA axis and anxiety disorders (11). Furthermore, recent studies have shown that increased FKBP5 expression is associated with anxiety disorders and may be a predictor for the disease state (5, 22).

One important level of FKBP5 regulation is transcriptional alternative splicing. The human FKBP5 gene contains 13 exons whereas the mouse FKBP5 gene contains 17 exons. Exons 1–6 and 10 are leading sequences. Alternative splicing of these exons generates six mRNA variants and two protein isoforms in mice (Fig. 1). Recent studies have focused on the interactions between FKBP5 genetic variants and stressors, which have been linked with anxiety disorders (18, 19). However, the effect of an AELE on FKBP5 mRNA alternative splice variants has not been elucidated.

Therefore, we hypothesized that AELE will affect the expression of FKBP5 and its variants in the key brain regions that program HPA axis and lead to anxiety later in life in mice. To test our hypothesis, we used a mouse model of AELE that is composed of Western diet-fed dams that experience environmental stress from the third trimester of pregnancy to weaning. We measured gene expression of FKBP5 and its mRNA variants in juvenille PFC, HP, and HT, as well as anxiety-like behavior in adult mice. The goal of this study was to determine if AELE affects FKBP5 splice variant expression and the development of anxiety-like behavior in mice.
**Methods**

**Animals.** All experiments were conducted according to the Public Health Services Policy on Human Care and Use of Laboratory Animals, and all procedures were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee (2). The mouse model of AELE used in this study has been previously described (9). In brief, 3 wk old C57Bl/6 female mice were subject to either a control diet (10% fat, Research Diet, New Brunswick, NJ; product #D14020502) or a Western diet (40% fat, comprising increased saturated fat, cholesterol, and sugar; Research Diet, product #D12079B) for 5 wk before impregnation. Dams in the control diet group experienced a normal environment designated as Control, while dams fed a Western diet experienced a “stressed” environment designated as AELE. The stressed environment consisted of prenatal unpredictable random environmental changes (e.g., altered light cycle, frequent cage changing, and a novel object in the cage) from embryonic day (E)13 to E17. As well as a static change in the maternal environment consisting of 1/3 of the standard amount of bedding from E13 to postnatal day 21 (P21). Minimal bedding induces disorganized postnatal caregiving as described by Rice et al. (32). Dams delivered spontaneously, and litters were culled to six. At P21, pups from both control and AELE groups were either weaned to control diet to grow older for behavior test or anesthetized and killed, respectively. Brains were quickly removed. Body weights and brain weights from both older for behavior test or anesthetized and killed, respectively. Brains were quickly removed. Body weights and brain weights from both control and AELE groups were collected. PFC, HP, and HT were dissected and flash-frozen in liquid nitrogen and stored in −80°C for mRNA and protein extraction. For immunohistochemistry studies, animals were individually fixed via intracardiac perfusion with ice-cold 0.9% normal saline (VWR, Radnor, PA), followed by ice-cold 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 5 min each for a total volume of 10–15 ml fixative. Whole brains were removed and postfixed at 4°C overnight. Brains were then transferred to 70% ethanol, paraffin-embedded, and sectioned coronally at 4 μm per section. We used the same number of males and females in each group and analyzed them by sex for all experiments. No differences in sex were found, so all data shown are combined between equal numbers of male and female mice.

**RNA isolation and real-time RT-PCR.** Total RNA was extracted from PFC, HP, and HT with the NucleoSpin RNA II kit (MACHEREY-NAGEL, Bethlehem, PA) and quantified with the BioTek®Epoch® Microplate Spectrophotometer (Fisher Scientific, Pittsburgh, PA). RNA was treated with DNase I (Ambion, Austin, TX). Gel electrophoresis was used to confirm the integrity of the samples. In brief, cDNA was synthesized from 1 μg of DNase-treated total RNA. cDNA, probe, and primers were added to TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA), and samples were run on an ABI ViiA7 (Thermo Fischer Scientific). Relative quantification of PCR products was based on value differences between the target and internal control by the comparative CT method (TaqMan Gold RT-PCR manual; PE Biosystems, Foster City, CA). Cycle parameters were 50°C 2 min, 95°C 10 min, and then 40 cycles at 95°C 15 s, 60°C 60 s. For each set of reactions, samples were run in quadruplicate. mRNA levels of FKBP5 (Mm.PT.58.10937155, Integrated DNA Technologies), and its mRNA variants were calculated relative to hypoxanthine phosphoribosyltransferase 1 (HPRT1, Mm.PT.39a.22214822, Integrated DNA Technologies), which was used as an internal control. FKBP5 variant primer and probe sequences are listed in Table 1.

**Protein isolation and immunoblot.** Tissue from PFC, HP, and HT were homogenized in ice-cold RIPA lysis buffer (Ambion, Austin, TX) for 5 min at 4°C. Proteins were quantified using the Bradford method (Bio-Rad, Hercules, CA). Samples were mixed with SDS-PAGE sample buffer (Thermo Fischer Scientific), and after boiling, proteins were separated via 12% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% nonfat dry milk in TBS and probed with rabbit polyclonal antibodies raised against full-length FKBP5 (1:500, Santa Cruz Biotechnology) and β-actin (1:1000, Santa Cruz Biotechnology) for 2 h at room temperature, after which membranes were washed in TBS-T and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, Santa Cruz Biotechnology) for 1 h. Membranes were washed again and then developed using ECL or Clarity Western ECL substrate (Bio-Rad). Band intensities were measured using ImageJ (NIH).

**Table 1. Primers for real-time RT-PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
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<tr>
<td>FKBP5 V201</td>
<td>5’GGGAGAAGCGTCTTCTACTCT</td>
<td>5’AGAATACATCTTGAAGTTCA</td>
<td>6FAM-AGAACCGGAACTACTGAGGGTGAGT</td>
</tr>
<tr>
<td>FKBP5 V202</td>
<td>5’AGGAGGATGTTGTTTCGTTTACAAAGGA</td>
<td>5’GATGCGGCAATTGATGAGTATG</td>
<td>6FAM-AATGACTACTGATGAGGGCA</td>
</tr>
<tr>
<td>FKBP5 V203</td>
<td>5’ATCTGGCAGAACATGCTGTCT</td>
<td>5’CTTCGCTGTTGTTCTGGTGTGTA</td>
<td>6FAM-AGATCACAAGAAGGAAAGGGGAGA</td>
</tr>
<tr>
<td>FKBP5 V204</td>
<td>5’GGATTGGGAGGATGTTGTTTCA</td>
<td>5’CCAGCGAGAAGGCTGAGGGTT</td>
<td>6FAM-CTGCGTTGAGGTGCG</td>
</tr>
<tr>
<td>FKBP5 V205</td>
<td>5’ATTGGGAAGGATGTTGTTTCA</td>
<td>5’GATGCGGCAATTGATGAGTATG</td>
<td>6FAM-AGAACCGGAACTACTGAGGGTGAGT</td>
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**Fig. 1. Schematic representation of the FKBP5 gene and splice variants in mice.** Mouse FKBP5 has 17 exons. Open boxes represent noncoding exons, while closed boxes represent coding exons. Alternative splicing generates 3 mRNA variants (V201, V202, and V206), which are translated into a single protein isoform. Variant V205 is translated into a second protein isoform. Variants V203 and V204 are noncoding transcripts. Additionally, exons 2 and 3 have short (S) and medium (M) variants that only have a portion of each respective full-length exon. Horizontal black bars represent the exon junctions spanned by real-time RT-PCR primers for variant detection. The arrow represents the direction of transcription.
OH) with protein inhibitors. After centrifugation at 10,000 rpm at 4°C for 15 min, the supernatants were stored at −80°C until use. Protein concentrations were determined by the BCA method (Pierce, Rockford, IL). Antibodies against FKBP5 (Santa Cruz Biotechnology, Cat. #sc-13983) and GR (Santa Cruz Biotechnology, Cat. #sc-8992,) at 1:50 dilution for both were used to determine protein abundance and Vinculin (Cell Signaling, Cat. #13901) at 1:10,000 dilution was used as a loading control. Immunoblot analyses were performed by a capillary immunoblot analysis method with the ProteinSimple Wes automatic system according to the manufacturer’s instructions (ProteinSimple, Santa Clara, CA). The FKBP5 antibody targets amino acids 358–457 at the COOH terminus. FKBP5 was validated before use in our assay. In brief, marker, lysate, antibody diluent, primary antibody, corresponding secondary antibody, detection mixture, and washer buffer were sequentially loaded onto Wes separation module 12–230 kDa prefilled plate (Cat. # SM-W004) and run in a sized-based assay with Wes (Protein Simple). Western blot image and data analysis were generated automatically with Protein Simple Compass software.

**Immunohistochemistry and Immunofluorescence.** Immunohistochemistry (IHC) was used to localize FKBP5 expression in PFC, HP, and HT brain regions as previously described (23, 24). Immunofluorescent triple labeling was used to colocalize FKBP5 either with the neuronal marker NeuN and oligodendrocyte marker CNPase or with the microglia marker coronin 1a and astrocyte marker GFAP. Briefly, adjacent coronal sections from the same brains were deparaffinized, rehydrated, and subject to antigen retrieval treatment. Sections were then blocked with Protein Block (DAKO, Carpinteria, CA; Cat. #X090930-2) for 30 min at room temperature (RT) and followed by incubation with either a mixture of rabbit anti-FKBP5 (Santa Cruz Biotechnology, Cat. #sc-13983) 1:200, mouse anti-NeuN (EMD Millipore, Billerica, MA; Cat. #MAB377) 1:2,000, and chicken anti-CNPase (Neuromics, Edina, MN; Cat. #CH23013) 1:50, or a mixture of rabbit anti-FKBP5 1:200, mouse anti-GFAP (Affymetrix eBioscience, Cat. #14-9892-82), and chicken anti-coronin 1a (Neuromics, Cat. #CH23017) for 60 min at RT. After being washed in Tris-buffered saline + Tween 20 (TBST) twice, sections were then exposed to a mixture of donkey anti-rabbit-Cy5 1:750, donkey anti-mouse AF488 (Jackson ImmunoResearch, Cat. #115-545-205) 1:750, and donkey anti-chicken Cy3 1:750 for 45 min at RT. After being washed in TBST twice, sections were counterstained for nuclei with DAPI. Sections were washed and mounted with Prolong Gold anti-fade mounting media. Images were captured by confocal microscopy (Carl Zeiss LSM510, Jena, Germany).

**Serum corticosterone ELISA.** Serum was collected at P21 from Control and AELE pups. In brief, whole blood was collected at the same time of day from each animal immediately following its death. Serum was collected by centrifuging whole blood at 2,000 rpm at 4°C for 10 min. This was done in a consistent manner on three individual groups of AELE and control animals. Serum corticosterone levels were measured with a corticosterone rat/mouse ELISA kit (Cat. # 07DE-9922, MP Biomedicals) and following the manufacturer’s instructions.

**Open field test.** Mice at 12 wk old were observed in an open field arena as a measure of anxiety-like behavior and general locomotion activity. The open field arena used in this study was 120 × 120 × 30 cm. The arena was painted white and divided into 16 equal quadrants (30 × 30 cm) by black lines. For testing, mice were placed in the central quadrant and left to explore for 5 min. Mice were monitored and recorded by an overhead camera (Hitachi 2500A) ~2 m above the open field box. A 10% acetic acid solution was used to clean the apparatus between tests. The mean number of entries and time spent in peripheral and central quadrants as well as the mean speed and total distance traveled were measured with Stoelting ANY-maze software (Wood Dale, IL).

**Statistics.** All data presented are expressed as means ± SD. ANOVA (Fisher’s protected least significant difference), and Mann-Whitney test determined statistical significance. Significance was set as P < 0.05.

**RESULTS**

**AELE decreased pups body weight at P21.** AELE significantly decreased body weight of the pups at P21 compared with the controls group (Fig. 2A). However, AELE did not affect brain weight at this age (Fig. 2B). Additionally, there were no sex differences in these body or brain weights.

**AELE increased the expression of FKBP5 and mRNA variants in PFC, HP, and HT brain regions at P21.** In PFC, AELE significantly increased total FKBP5 mRNA levels (Fig. 3A) and protein levels (Fig. 3D). In addition, AELE significantly increased mRNA levels of FKBP5 variant V201, V203, and V204 (Fig. 3G).

In HP, AELE did not change total FKBP5 mRNA levels (Fig. 3B) but significantly increased FKBP5 protein levels (Fig. 3E) and mRNA levels of variant V201 and V203 (Fig. 3H).

In HT, AELE significantly increased total FKBP5 mRNA levels (Fig. 3C) and protein levels (Fig. 3F). Moreover, AELE significantly increased mRNA levels of all five FKBP5 variants examined (Fig. 3I).

Variant V201 was the predominant variant expressed in all brain regions measured. (Fig. 3, G–I).

**FKBP5 expression was detected in neurons and microglia.** IHC results showed FKBP5 expression in all three brain regions (Fig. 4, A–J), and FKBP5 expression was not confined to a particular HP subfield (Fig. 4, E–J). Coexpression of FKBP5 and cellular markers for neurons, microglia, oligodendrocytes, and astrocytes was used to examine cell type-specific expression of FKBP5. FKBP5 expression was detected in NeuN+ cells (PFC, Fig. 5, A–C) and coronin 1a+ cells (subcortex white matter, Fig. 5, G–I) but not in either CNPase+ cells (subcortex white matter, Fig. 5, D–F) or GFAP+ cells (subcortex white matter, Fig. 5, J–L). Colocalization
Fig. 3. FKBP5 mRNA and protein levels at P21. Total FKBP5 mRNA levels at prefrontal cortex (PFC, A), hippocampus (HP, B), and hypothalamus (HT, C). FKBP5 protein levels at PFC (D), HP (E), and HT (F). FKBP5 mRNA variant levels at PFC (G), HP (H), and HT (I). n = 6, *P < 0.05.
staining indicated that FKBP5 colocalization with either NeuN or coronin 1a is the same in other brain regions as prefrontal cortex or subcortex white matter. Representative images were chosen from these two regions.

**AELE increased GR abundance in PFC, HP, and HT brain regions at P21.** AELE significantly increased GR protein levels in PFC (Fig. 6A), HP (Fig. 6B), and HT (Fig. 6C). However, AELE did not affect serum corticosterone levels at P21 (Fig. 7).

**AELE increased anxiety-like behavior in adult mice.** The open field test demonstrated AELE significantly increased the total distance traveled (Fig. 8A) in adult offspring. AELE also significantly increased mobile time in the outer zone (Fig. 8B). An increase in total distance traveled indicates an increase in

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![Figures](https://example.com/figures.png)

Fig. 4. Immunohistochemistry staining of FKBP5 in P21 mouse brain regions. Prefrontal cortex (PFC): controls (A) and AELE (B); hypothalamus (HT): controls (C and AELE (D); hippocampus (HP): controls (E–G) and adverse early life environment (AELE, H–J) in subfield CA1 (E, H), CA3 (F, I), and dentate gyrus (DG; G, J). Black squares inserted at top left corner of each image represent the locations of high-magnification images were from. Brown staining indicates FKBP5 positive. Scale bar = 100 μm; n = 4.

Fig. 5. Cell-type expression of FKBP5. Representative immunofluorescence of NeuN (neuronal marker, red, A), FKBP5 (white; B, E, H, and K), CNPase (oligodendrocyte marker, green; D), coronin 1a (microglia marker, red; G), and GFAP (astrocyte marker, green, J). DAPI (blue) was used for nuclei counterstaining. Representative colocalization of FKBP5 and NeuN in prefrontal cortex (C), FKBP5 and CNPase (F), FKBP5 and coronin 1a (I), and FKBP5 and GFAP in subcortex white matter (L). Scale bar = 20 μm.
locomotion activity, while an increase in mobile time in the outer zone indicates decreased exploratory behavior and increased anxiety (12).

DISCUSSION

The data presented here illustrate two important findings. That is, that AELE increased expression of the stress response gene FKBP5 and its mRNA variants in critical brain regions that influence HPA axis function and contributes to anxiety later in life in juvenile mice. Additionally, AELE caused anxiety and increased GR abundance in association with the aforementioned changes in FKBP5 mRNA variants. These findings may suggest that early life stress and diet endured by the mother can affect offspring stress response and subsequently lead to anxiety behavior later in life.

Anxiety disorders are associated with impaired HPA axis and dysregulated expression of stress response gene FKBP5 in the brain (5, 30). In rodents, a single prolonged stress has been shown to acutely induce FKBP5 mRNA in PFC, HP, and amygdala (30). Furthermore, increased expression of FKBP5 in PFC, HP, and striatum of chronically dexamethasone-treated mice was associated with increased anxiety-like behavior (35). Experiments in FKBP5 knockout mice have shown that these mice are less vulnerable to stress than wild-type controls (17). Under basal conditions, exploratory drive, locomotor activity, anxiety-related behavior, stress-coping, and depression-like behavior did not differ between young adult FKBP5 −/− mice and littermate controls. However, after different acute stressors, lack of FKBP5 led to better coping behavior (37). In our study, we found that AELE increased FKBP5 expression in PFC, HP, and HT brain regions. While these aforementioned studies directly exposed the mice to an acute insult, in our study the insult was to the dam or during the perinatal period. We speculate that AELE reprograms the HPA axis and stress response by altering FKBP5 expression in the critical brain regions and leads to anxiety later in life. Future studies include assessment of FKBP5 mRNA variant expression and HPA axis function in adulthood as well as FKBP5 epigenetic characteristics.

While FKBP5 genetic variants or polymorphisms have been found to be associated with many psychiatric disorders including anxiety disorders (13, 26, 27), fewer studies focus on FKBP5 mRNA splice variants. Genetic variation in FKBP5 has been showed to be associated with the extent of stress hormone
dysregulation in patients suffering from major depression (26). In this study, the authors observed a significant interaction between disease status and FKBP5 risk allele carrier status (minor allele T) on GR-stimulated FKBP5 mRNA expression. Patients carrying the risk T allele, but not the CC genotype, showed a reduced induction of FKBP5 mRNA. Additionally, FKBP5 isoform 2, but not isoform 1, has been shown to be upregulated in lymphocytes of patients with melanoma (33). FKBP5 isoform 2 is codified by the transcript variant 4 in humans (NM_001145777.1 mRNA), which is equivalent to variant V205 in mouse, which was increased in PFC and HT brain regions in our model. In our study, we also found that AELE increased variants V201 and V203 in all three brain regions with all variants examined increased in HT. Variant V201 is the predominant variant expressed in the brain. To our knowledge, this is the first evidence of AELE affecting the expression of FKBP5 mRNA variants in a region-specific manner in the brain. It has been shown that polymorphisms of FKBP5 are associated with higher FKBP5 levels, leading to GR resistance and impaired negative feedback (5). Therefore, we speculate that increased mRNA variant expression of V201 and V203 contributes to FKBP5 upregulation that may impact frontal-hippocampal-hypothalamus connectivity via their effects on glucocorticoid activity in this model.

FKBP5 expression in the PFC, HP, and HT in our model is consistent with a previous report in mouse brain (34). Furthermore, FKBP5 was shown here to colocalize with microglia cell marker in the mouse brain. To our knowledge, this is the first evidence of FKBP5 expression in microglia. Our previous work (9) using this AELE model demonstrated an increase in microglia count in the juvenile HP. Increased microglia number may contribute to increased FKBP5 expression in our model.

Since FKBP5 is a target gene of the GR, we then investigated GR expression in these three brain regions. As expected, AELE significantly elevated GR protein levels in all three brain regions examined. We speculate that AELE increases GR expression and leads to FKBP5 upregulation in the key brain regions related to HPA axis regulation that contribute to anxiety-like behavior in our model.

The finding that AELE did not change serum corticosterone levels at P21 in our model is similar to a mouse model of exposure to dim light at night during early development (7). Dim light exposure in early life increases adult anxiety-like responses without changing corticosterone levels at P21 (7). We speculate that exposure to these stressors may not elicit an enduring physiological stress response early in life but may reprogram stress response later. In addition, increased expression of stress response genes without changing serum corticosterone levels in our model indicates an impaired GR negative feedback loop and dysregulation of HPA axis function.

The limitation in this study is that we did not measure cell type-specific expression of FKBP5 mRNA variants but, rather, used whole brain regions. However, our IHC results indicate that FKBP5 is mainly expressed in neurons and microglia. Moreover, we acknowledge that it is impossible to perfectly replicate a low SES environment in an animal, considering the diversity and complexity of the human experience.

In this study, we have identified AELE increases expression of specific FKBP5 mRNA splice variants in juvenile mouse brain regions related to regulation of HPA axis function. We speculate that increased expression of specific FKBP5 mRNA splice variants in the critical brain regions affects HPA axis function and subsequent anxiety-like behavior later in life in AELE mice.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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