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Title page

Prenatal ethanol exposure induces an intrauterine programming of enhanced sensitivity of the hypothalamic-pituitary-adrenal axis in female offspring rats fed with post-weaning high-fat diet

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The novelty of the work: “Intrauterine programming” involve in the intrauterine origin of prenatal ethanol exposure-induced enhanced sensitivity of the hypothalamic-pituitary-adrenal axis in female offspring rats fed with post-weaning high-fat diet

Abbreviations: ACTH, adrenocorticotropic hormone; AVP, arginine vasopressin; CA3, cornu ammonis 3; CORT, corticosterone; CRH, corticotrophin-releasing hormone; DG, dentate gyrus; GAD65, glutamic acid decarboxylase 65; GC, glucocorticoid; GD, gestational day; GR, glucocorticoid receptor; HFD, high-fat diet; HPA, hypothalamic-pituitary-adrenal; IUGR, intrauterine growth retardation; Mash1, mammalian achaete-scute homolog-1; MR, mineralocorticoid receptor; NR2B, N-methyl-D-aspartate-subtype glutamate receptor 2B; Pax6, paired box 6; PEE, prenatal ethanol exposure; PSD95, post-synaptic density 95; PW, postnatal week; Reln, Reelin; Tbr2, TGF-β receptor II; UCS, unpredictable chronic stresses; VGluT2, vesicular glutamate transporter 2; α-CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II-α.

Abstract

Our previous study demonstrated prenatal ethanol exposure (PEE) enhances the sensitivity of the hypothalamic-pituitary-adrenal (HPA) axis in adult offspring rats. This study was aimed to investigate the underlying mechanism. PEE treated female offspring rats were fed with high-fat diet and subjected to the unpredictable chronic stresses (UCS) in adulthood. For adult offspring, PEE group exhibited increased expression of hypothalamic corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) as well as elevated gain rates of serum adrenocorticotropic hormone (ACTH) and corticosterone after UCS. Meanwhile, PEE significantly decreased the expression of glutamic acid decarboxylase 65 (GAD65) and Reelin (Reln), and the expression ratio of hypothalamic vesicular glutamate transporter 2 (VGluT2)/GAD65 was enhanced in the adult PEE offspring. These changes were also accompanied by the enhanced expression of glucocorticoid receptor (GR), N-methyl-D-aspartate-subtype glutamate receptor 2B and the decreased expression ratio of mineralocorticoid receptor (MR)/GR in hippocampus. Furthermore, the abnormal hippocampus neurons were observed especially in the cornu ammonis 3 (CA3) and dentate gyrus subfields. For fetuses, PEE significantly decreased the expression of mammalian achaete-scute homolog-1 (Mash1) as well as GAD65 and Reln. Both VGluT2/GAD65 expression ratio and GR expression were increased while the MR/GR expression ratio was decreased in PEE group. PEE also caused ultrastructural injury in CA3. Our findings suggest that PEE causes the persistent remodeling alterations of impaired morphology and decreased MR/GR expression ratio in hippocampus, as well as the imbalanced glutamatergic/GABAergic afferent inputs in hypothalamus. All of these would contribute to the enhanced sensitivity of the HPA axis in adult offspring.

Keywords: Prenatal ethanol exposure; Intrauterine programming; Hippocampus; Stress sensitivity; Hypothalamic-pituitary-adrenal axis; Glucocorticoid

Introduction

Epidemiological studies have demonstrated that people with intrauterine growth retardation (IUGR) are more likely to develop metabolic disorders (*e.g.* obesity, hypertension and type 2 diabetes) in adulthood[1]. This association between IUGR and adult metabolic disorders are address to the "programming" hypothesis that adverse factors during gestation could have permanent effects on the structure and functional alterations of the fetal tissues and cause dysfunctions and diseases in adulthood[1,2]. The hypothalamic-pituitary-adrenal (HPA) axis is an important neuroendocrine axis involved in the stress response. Intrauterine programming of the HPA axis might have life-long effects on neuroendocrine functions[3]. Epidemiological, clinical and animal studies have consistently shown that an adverse intrauterine environment usually results in an enhanced sensitivity of the HPA axis in adulthood[3-5]. Prenatal treatment with the betamethasone (a synthetic GC), sensitizes the HPA axis response to the painful stress of a heel-stick among full-term infants[5], and also increase the reactivity to standardized psychosocial stress in those 10 years old children[6]. Animal studies also show that the male and female BALB/c mice as well as the female C57BL/6 mice that were exposed to alcohol vapors prenatally exhibited significantly higher shock-induced plasma ACTH levels[7]. Prenatal l-alpha-acetylmethadol exposure (an opiate analgesic) increases the HPA axis reactivity to immunological stressors in adult offspring rats[8]. Furthermore, several studies have demonstrated that the enhanced sensitivity of HPA axis is linked to the development of metabolic or mental diseases mediated by intrauterine events, such as diabetes, non-alcoholic fatty liver disease (NAFLD) and depression[9-13].

Ethanol consumption is common in daily life. A recent survey showed an obvious increasing trend of ethanol abuse in young women over recent decades[14] and the prenatal ethanol exposure (PEE) rate for newborns is increasing each year[15]. Approximately 50% of women of reproductive age are heavy drinkers in North America and 10%-15% of pregnant women drink alcoholic beverages on a daily basis[16]. Ethanol consumption during pregnancy is a definitive cause of fetal developmental toxicity.

Numerous studies have shown that PEE increases the incidence of health problems in the offspring. These health problems include IUGR, neuropsychiatric disorders and adult metabolic syndrome[17-22]. Our previous work of introduced a mechanism of "HPA axis-associated neuroendocrine metabolic programming alteration" to explain the increased susceptibility to metabolic diseases found in IUGR offspring rats with PEE[23]. We also found that PEE causes IUGR by inhibiting the development of fetal HPA axis and altering the peripheral glucose and lipid metabolism[21,23]. These changes were maintained until adulthood and are characterized by low baseline and enhanced sensitivity of the HPA axis in adult male offspring rats fed with high-fat diet (HFD)[23,24]. The HFD is proved to be able to enhance the HPA responses[25]. The dysfunction of the HPA axis can further cause adult metabolic diseases such as NAFLD by interfering with peripheral glucose and lipid metabolism[23,24]. However, the specific mechanism responsible for the intrauterine programming of hypersensitivity of the HPA axis in the PEE offspring rats remains unclear.

The hypothalamic paraventricular nucleus (PVN) controls the activity of the HPA axis[26].When facing to stress stimulus, corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) are secreted from parvocellular neurons in PVN to stimulate the secretion of adrenocorticotropic hormone (ACTH) from pituitary gland. The pituitary gland subsequently promotes the release of glucocorticoids (GC, cortisol in humans and corticosterone (CORT) in rodents) from the adrenal cortex. GC binds to its receptors including glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) in the hippocampus and activates the feedback regulation on PVN then suppresses the HPA axis. Therefore, the hippocampus restores the stress state of the HPA axis to baseline levels[27]. It is reported that female animals possess a more sensitive HPA axis[28-30]. We employed the unpredictable chronic stress (UCS) to examine the sensitivity of HPA axis in female PEE-induced IUGR offspring rats with post-weaning HFD. We also explored the intrauterine programming mechanism by measuring the potential excitatory ability of hypothalamus and the feedback capacity of hippocampus. These findings may contribute to elucidate the intrauterine programming of chronic

adult diseases associated with the dysfunction of HPA axis.

Results

Adult offspring rats

Changes in birth weight, IUGR rate and HPA axis activity

PEE significantly decreased the birth weight of female offspring rats to 66.9% of the control (4.37 g ± 0.12 g vs. 6.53 g ± 0.21 g, $P<0.01$), and increased the incidence of IUGR from 14.30% ± 0.65% to 94.74% ± 2.57% ($P<0.01$) at PD1.

The serum ACTH levels of adult PEE rats were lower than control ($P<0.01$, Fig. 1A), while the CORT presented a decreasing trend ($P=0.062$, Fig. 1B) in the PEE group before UCS. However, serum ACTH and CORT concentrations were all significantly increased after UCS when compared to their corresponding levels before UCS ($P<0.01$, $P<0.01$, Fig. 1A, 1B) or to the control groups after UCS ($P<0.01$, $P<0.01$, Fig. 1A and 1B). This result was also supported by the higher gain rates of serum ACTH and CORT concentrations in the PEE group ($P<0.01$, $P<0.05$, Fig. 1C). Furthermore, the mRNA expression levels of hypothalamic CRH and AVP, reflecting the HPA axis activity, were decreased in the PEE group before UCS when compared to their respective controls ($P<0.05$, $P<0.05$, Fig. 1D, 1E). However, the CRH and AVP were significantly increased after UCS when compared to their respective controls after UCS ($P<0.01$, $P<0.01$, Fig. 1D, 1E).

Changes in potential excitatory ability of hypothalamus

Vesicular glutamate transporter 2 (VGluT2) is a key transporter of glutamate and the main excitatory neurotransmitter in the nervous system. Post-synaptic density 95 (PSD95), Ca2+/calmodulin-dependent protein kinase II-α (α-CaMKII) are marker proteins of glutamatergic neuron. The expression of VGluT2, PSD95 and α-CaMKII were not changed after PEE treatment (Fig. 2A, 2B and 2C). Glutamic acid decarboxylase 65 (GAD65) is the major enzyme involved in

gamma aminobutyric acid (GABA) synthesis, while Reelin (Reln) involved in synaptic transmission and plasticity. Both GAD65 and Reln were markedly decreased in PEE group before UCS and after UCS ($P<0.05$, $P<0.01$, Fig. 2D and 2E). Further, the expression ratios of VGluT2/GAD65, were increased in PEE group both before UCS and after UCS ($P<0.05$, $P<0.05$, Fig. 2F) and were also significantly elevated after UCS when compared with their corresponding levels before UCS ($P<0.01$, Fig. 2F).

Functional and morphologic changes of hippocampus

Hippocampal GR but not MR mRNA expression levels in PEE group were increased before UCS ($P<0.01$, Fig. 3A, 3B). Thus, the MR/GR expression ratios were significantly decreased ($P<0.01$, Fig. 3C). After UCS, the hippocampal expression ratios of MR/GR were still decreased in PEE group ($P<0.05$, Fig. 3C). The expression levels of MR, GR and N-methyl-D-aspartate-subtype glutamate receptor 2B (NR2B) were up-regulated ($P<0.05$, $P<0.01$, $P<0.01$, Fig. 3A, 3B, 3D).

The morphologic observation showed that hippocampal neurons of the control group were orderly arranged with clear and integral structures before UCS. There were only a few nuclei of neurons in the cornu ammonis 3 (CA3) of PEE group that were dense and darkly stained. No obvious morphological change was observed in the hippocampus from the control after UCS. However, the neurons in the hippocampus of the PEE group were irregularly arranged with lots of dense and darkly stained nuclei, especially in the CA3 and dentate gyrus (DG) subfields.

Fetal offspring rats

Changes of body weight, the IUGR rate and serum CORT concentration

PEE decreased the body weight of the female fetal offspring rats to 80.59% of the control ($P<0.01$, Fig. 4A). There was an increase in the IUGR rate in the PEE group on gestational day 20 (GD20) ($P<0.01$, Fig. 4B). The PEE group showed a significantly higher level of serum CORT than the

control (P<0.01, Fig. 4C).

Expression changes of genes reflecting HPA axis activity and potential excitatory ability of hypothalamus

The expression levels of hypothalamic CRH and AVP were decreased in PEE fetal rats (P<0.05, P<0.01, Fig. 5A and 5B). Paired box 6 (Pax6), TGF-β receptor II (Tbr2) and mammalian achaete-scute homolog-1 (Mash1) are important transcriptional factors in governing glutamatergic/GABAergic differentiation during fetal neuronal development[31,32,33]; transient expressions of Pax6, Tbr2 and Mash1 *in uterus* induced the persistent expression changes of glutamatergic neuronal proteins of VGluT, PSD95, α-CaMKII and GABAergic neuronal proteins of GAD and Reln, respectively[31]. PEE significantly decreased the expression of GABAergic transcriptional factor Mash1 in fetal rats' hypothalamus, as well as the GAD65 and Reln (P<0.01, Fig. 5H, 5I and 5J)，but not Pax6, Tbr2, VGluT2, PSD95 and α-CaMKII (Fig. 5C, 5D, 5E, 5F and 5G). The expression ratios of VGluT2/GAD65 were also significantly increased in PEE group (P<0.05, Fig. 5K).

Functional and structural changes of hippocampus

The MR/GR expression ratios in PEE fetal hippocampus were decreased (P<0.01, Fig. 6C) due to the elevated GR expression levels (P<0.01, Fig. 6B), but not the changes of MR expression (Fig. 6A). The mRNA expression levels of NR2B were also significantly increased in the PEE group (P<0.01, Fig. 6D). Further observations of the fetal hippocampal ultrastructure showed that neurons in CA3 of the control group had 1 or 2 large and round nuclei with evenly distributed chromatin and abundant endoplasmic reticulum, ribosomes, mitochondria, lysosomes and other organelles. However, visible hypertrophy of the Golgi body and dilatation of the endoplasmic reticulum were observed in neurons from fetal hippocampal CA3 of the PEE group. Varied thickness of the abnormal vessel walls,

disorganized layers of smooth muscle cells and crimped intima were indicated (Fig. 6E).

Discussion

The sensitivity of HPA axis was enhanced due to the increased excitatory ability of hypothalamus in PEE female offspring

Epidemiological investigations have shown that mothers with alcohol syndrome consume 13.6 bottles of alcoholic liquor weekly, which is equivalent to 3–4.3 g/kg of ethanol[34]. It is also reported that the average blood ethanol concentration ranged from 20 mM to 170 mM in the alcoholics after drinking 3 to 5 bottles of liquor[35]. Moreover, after a daily administration of 4 g/kg of ethanol, the serum ethanol levels of pregnant rats and fetus from PEE group were 87 and 58 mM, respectively[23]. These findings indicated that the ethanol dosage in the present study was approximately equivalent to a small amount of alcohol consumption.

GC is not only a critical metabolic hormone regulating fetal growth, development and maturity *in utero*, but also a key factor in the development of IUGR[36,37]. Previous studies have demonstrated that prenatal dexamethasone exposure leads to IUGR[38-40]. These findings suggest that GC may play an important role in the pathogenesis of fetal IUGR. This result is supported by our previous findings that PEE causes fetal over-exposure to maternal GC[21] and induces a low basal activity and an enhanced stress sensitivity of the HPA axis in male offspring rats with HFD[24]. In the present study, the serum CORT level of female fetus on GD20 and the rate of IUGR were increased. Additionally, we observed reduced birth weights in the female PEE offspring rats. This finding is consistent with previously described[24]. Furthermore, we found that the concentrations and the gain rates of serum ACTH, CORT, and the expression levels of hypothalamic CRH and AVP were significantly increased after UCS. This result indicated the sensitivity of the HPA axis to UCS was enhanced in the female PEE-induced IUGR offspring rats fed with HFD.

The imbalance of excitatory/inhibitory signal in the brain have been proposed as one of the main

pathological features for many diseases related to HPA axis dysfunction[31,41,42]. Numerous studies also demonstrated an important role of the dynamic balance of hypothalamic glutamate (Glu) and GABA levels in the regulation of HPA axis activity[43,44]. In response to a stressor, the glutamatergic afferent fibers stimulate CRH neurons in the PVN of hypothalamus, which subsequently activates the HPA axis and increases the presynaptic Glu release. The release of GABA is then stimulated through the Glu-GABA synaptic transmission to maintain the balance of local amino acids and prevent excessive activation of hypothalamus[45]. Therefore, imbalance of glutamatergic and GABAergic afferent inputs contributes as an underlying mechanism to the altered excitatory/inhibitory signal in the hypothalamus PVN[46].

In the present study, PEE significantly decreased the expressions of GABAergic neuronal proteins - GAD65 and Reln, but not glutamatergic neuronal proteins - VGluT2, PSD95 and α-CaMKII, in the female offspring rats both before and after UCS. We further observed an increased expression ratio of VGluT2/GAD65 in the female offspring rats both before and after UCS. What's more, we observed enhanced sensitivity of the HPA axis, which is shown as elevated serum ACTH and CORT concentrations, enhanced gain rates of ACTH and CORT, and increased hypothalamic CRH and AVP mRNA expression levels. These findings suggest that PEE increase the potential excitatory ability of hypothalamus in the female adult offspring. This effect might be the trigger of the enhanced sensitivity of the HPA axis.

The attenuated hippocampal feedback regulation leads to the enhanced excitation of hypothalamus in PEE female offspring

Two types of GC receptors, namely the MR and the GR, are widely distributed in hippocampus and these receptors determine the regulation of hippocampus on the HPA axis. The affinity of MR to GC is 10 times higher than GR[47]. Therefore, when the circulatory GC level is low, the local GC is readily to bind to MR but not GR. This binding modulates the basal activity of the HPA axis. However, when the GC level rises, the hippocampal MR becomes to be fully occupied and the excessive GC could

activate GR in the hippocampus. The activated GR further increases the release of glutamate[48] and subsequently activates the hypothalamic GABA neurons and inhibits CRH neurons in PVN region to suppress excessive activation of the HPA axis[49,50]. Therefore, the balance in the MR- and GR-mediated effects on the stress system is of critical importance to the set point of HPA activity[51]. Meanwhile, the balance between the differential activation of MR and GR in the hippocampus, is one of major factors modulating local potential excitatory ability in the hypothalamus and plays a key role in the regulation of the HPA axis.

GR is known as a major trigger responsible for the susceptibility of hippocampal neurons to GC. Excessive GC induces Ca^{2+} influx-mediated neuronal death and degeneration via over-activating hippocampal GR[52,53]. Pyramidal neurons in CA3 and granular cells in DG seem to be particularly vulnerable[54,55]. It is reported that N-methyl-D-aspartate (NMDA) may act at the downstream of GR and participate in the neuronal degeneration[56]. The N-methyl-D-aspartate-subtype glutamate receptor 2A (NR2A) and NR2B subunits are considered to be the main types of functional NMDA channels in the neurons of central nervous system[57]. NR2B preferentially links to signaling cascades involved in central nervous system injury by promoting neuronal death and neurodegeneration. The over-activation of NR2B increases the Ca^{2+}permeability of neurons and triggers neuronal death[58,59]. Moreover, dexamethasone is a GR agonist that increases the expression and phosphorylation of NR2B. Conversely, mifepristone is a known GR antagonist that significantly down-regulates NR2B expression[60].

In the present study, we found that GR and NR2B expression were increased while the expression ratios of MR/GR were decreased in the hippocampus of female offspring rats with PEE. The structural damage observed in pyramidal neurons from CA3 and granule cells from DG in the hippocampus of PEE adult rats were further aggravated by UCS. Our findings suggest that the PEE-induced functional and structural remodeling of the hippocampus might be responsible for the attenuated feedback regulation on HPA axis and the enhanced potential excitatory ability of the

hypothalamus.

Increased potential excitatory ability of hypothalamus induced by hippocampus-hypothalamus interaction originated *in utero*

An increasing number of studies suggest that multiple chronic diseases in adulthood are related to intrauterine programming induced by a prenatal adverse environment[61-63]. Our previous study demonstrated that excessive maternal GC induced by PEE could cross the placenta barrier and enter into the fetus to inhibit the development of HPA axis[21]. In the present study, increased serum CORT and decreased CRH and AVP mRNA expression were observed in PEE fetal rats. These results suggest there is an inhibition of fetal HPA axis, which is consistent with our previous study. Further, PEE significantly decreased the expression of Mash1, as well as GAD65 and Reln in the hypothalamus of fetal rats, but not Pax6, Tbr2, VGluT2, PSD95 and α-CaMKII. Increased VGluT2/GAD65 ratio was also observed in the fetal hypothalamus of PEE animals. These results indicate that PEE induced maternal GC exposure may up-regulate the hypothalamic excitatory/inhibitory signal, mainly through the inhibition of GABAergic differentiation. Moreover, the increased expression of GR and NR2B, decreased expression ratios of MR/GR and histological injury were observed in fetal hippocampus. Our findings indicated that the increased potential excitatory ability of hypothalamus induced by PEE was programmed *in utero*. These changes may be attributed to the remodeling of hippocampus, that is, the structural damage of the fetal hippocampus and the subsequent attenuated feedback regulation caused by the over-exposure to maternal GC. The excessive GC permanently changed the hypothalamic set point and further enhanced the sensitivity of the HPA axis to UCS in adulthood.

Numerous studies have suggested that epigenetic modifications play an important role in the intrauterine programming of susceptibility of adult disease[64-66]. Recent studies indicate that high level of GC may affect the epigenetic modifications of target genes through the altered epigenetic regulators (including DNA methyltransferase, Dnmts)[67,68]. During the early development, the

epigenetic modification of hippocampal GR is vulnerable to GC[69-71]. Several reports also support that epigenetic changes of hippocampal GR may underlie the IUGR-associated HPA axis programming alteration[72-75]. In our study, PEE can lead IUGR fetal rats over-exposed to maternal GC, meanwhile, the expression of GR in fetal hippocampus was also increased. We speculated that high level of maternal GC may act on the epigenetic regulators such as Dnmts, and induce the epigenetic changes in hippocampal GR, which further onset the high sensitivity of HPA axis in offspring rats.

Materials and Methods

Chemicals and reagents

Ethanol (analytical and chromatographic purity) was obtained from Zhen Xin Co., Ltd. (Shanghai, China). The rat ACTH radioimmunoassay kit was purchased from North Institute of Biological Technology (Beijing, China). The rat CORT ELISA kit was provided by Assaypro LLC (Saint Charles, USA). The TRIzol reagent was obtained from Life Technologies Co., Ltd (NY, USA). The reverse transcription and real-time RT-PCR kits were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The oligonucleotide primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The RNA-Solv Reagent and the HiBind™ PCR DNA extraction kits were provided by Omega Bio-Tek Inc. (Norcross, GA, USA). A 2.5% glutaral solution was purchased from Yuanmu Biotechnology Co., Ltd. (Shanghai, China). A 1% osmium acid solution was obtained from Jingying Chemical Technology Co., Ltd. (Guangzhou, China). The other chemicals and agents were of analytical grade.

Animals and treatment

Specific pathogen free (SPF) female Wistar rats weighing 180 g ~220 g and male rats weighing 260 g ~300 g were obtained from the Experimental Center of Hubei Medical Scientific Academy (No. 2009-0004, Hubei, China). The animal experiments were performed in the Center for Animal

Experiment of Wuhan University (Wuhan, China), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). All experimental procedures involving animals were approved by Medical Ethics Committee of the Basic Medical School of Wuhan University and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee and the Guide for the Care and Use of Laboratory Animals (eighth edition) by the National Research Council of the United States National Academies.

After one week of acclimation (room temperature: 18℃-22℃; humidity: 40%-60%), two females were mated with one male overnight. The confirmation of mating was performed by the appearance of sperm in a vaginal smear. This day was designated as GD0. Pregnant rats were randomly divided into either the control group or the PEE group. The schematic procedure of animal treatment is shown in Fig. 7.

As described in our previous study[23], the pregnant rats were treated with 4 g/kg·d of ethanol by oral gavage from GD11 to GD20. The control group received an equal volume of saline. For fetal rat experiments, eight pregnant rats from each group were euthanized on GD20 using isoflurane anesthesia. Pregnant rats with a litter size of 8~14 pups were considered to be qualified. The fetuses were quickly removed and the females were collected and weighed. IUGR was diagnosed when the body weight of fetus was two standard deviations lower than the average body weight from the control group[76]. Fetal blood samples were collected to prepare the serum. The fetal hippocampus and hypothalamus were isolated under a dissecting microscope. Fetal samples from the same pregnant rat were pooled as an independent sample. All samples were stored at -80 ℃ or fixed in 2.5% glutaraldehyde for transmission electron microscopic (TEM) analysis.

For adult rat experiments, eight pregnant rats from each group were allowed to deliver at full term (GD21~GD22). On postnatal day 1 (PD1), the litter size was normalized to eight pups per litter to assure adequate and standardized nutrition until weaning (postnatal week 4, PW4). After weaning,

two female pups were randomly selected from each pregnant rat in both control and PEE groups. All the female offspring were fed with HFD (providing 18.9% kcal from protein, 61.7% kcal from carbohydrate and 19.4% kcal from fat)[77] ad libitum. On PW17, one of the two pups from the same mother was sacrificed by anesthesia. The hippocampus, hypothalamus and blood samples were collected. The remaining rats were all exposed to a 3 weeks UCS procedure since PW17. The stressors included food deprivation for 24 h, water deprivation for 24 h, tail pinch (2 cm from the distal tail) for 5 min, heat in an oven at 45 ℃ for 5 min, cold swimming at 4 to 8 ℃ for 4 min and then towel-drying, and social isolation (one rat per cage) for 24 h. The stressors were randomly imposed once a week and once daily per rat between 8:30 AM and 10:30 AM except for the 24-h stressors. The serum was obtained before and after UCS and subsequently stored at -80 ℃. The hippocampus and hypothalamus tissues were also rapidly collected after UCS and stored at -80 ℃ until further analysis. Three whole brains from each group were fixed in 4% paraformaldehyde solution and for histologic examination using hematoxylin-eosin (HE) staining.

Light microscopy analysis

Hippocampus tissues stained with H&E was processed by standard procedures in gradient alcohols and xylene, paraffin embedded. Sections were observed and photographed with an Olympus AH-2 light microscope (Olympus, Tokyo, Japan).

TEM analysis

1 mm^3 tissue blocks of hippocampus samples were placed in 3% glutar-aldehyde/1.5% paraformaldehyde solution with 0.1 mol/L PBS. Samples were postfixed for 1.5 h in 1% osmium tetroxide/1.5% potassium ferrocyanide solution and washed in 0.1 mol/L PBS, dehydrated in graded concentrations of ethanol, and embedded in Epon 618. Epoxy blocks were sliced on an ultratome (LKB-V, LKB, Stockholm, Sweden, 70 nm), stained with uranyl acetate and lead citrate, and examined using a Hitachi H600 transmission electron microscope (Hitachi, Co., Tokyo, Japan). Digital images were computationally acquired.

Real-time quantitative RT-PCR

Total RNA was collected from hippocampus or hypothalamus tissue using TRIzol reagent. Next, single-stranded cDNA was obtained using a First Strand cDNA Synthesis Kit. The primers were designed using the NCBI BLAST database and Primer Premier 5.0 software (Premier Biosoft, CA, USA). The StepOne thermal cycler (Life Technologies) and Takara RT-PCR kits were used for the RT-PCR assay according to the following procedure: 10 s at 95 ℃ for pre-denaturation and 5 s at 95 ℃ for denaturation, annealing (gene-specific annealing conditions are listed in Table 1) and 15 s at 72 ℃ for elongation (for the β-actin and AVP reactions). The last 2 or 3 steps were performed for 40 cycles. The relative quantification of the following target genes was determined using their respective standard curves: CRH, AVP, Pax6, Tbr2, VGluT2, PSD95, α-CaMKII, Mash1, GAD65, Reln, MR, GR, NR2B, and the housekeeping gene β-actin. The primers and PCR condition details are listed in Table 1. The relative expression levels of all the target genes were obtained using β-actin expression level to standardize the comparison. For the adult samples, the relative mRNA levels were further standardized with the control to eliminate the baseline differences between two different batches of rats (with or without UCS).

Serum ACTH and CORT detections

The serum ACTH concentration was measured by radioimmunoassay kit following the manufacturer's protocol. The serum CORT concentration was determined by ELISA assay. Additionally, the gain rates of serum ACTH and CORT were calculated as described below and presented as a percentage (%).

$$Serum\ ACTH(CORT)con.\ gain\ rate(\%) = \frac{ACTH(CORT)con.\ after\ UCS - ACTH(CORT)con.\ before\ UCS}{ACTH(CORT)con.\ before\ UCS} \times 100$$

Histological and ultrastructural examination

The hippocampus tissues of adult rats randomly selected from each group were fixed in 4% paraformaldehyde solution for 24 h. The tissue was then paraffin-embedded and sliced (5-6 μm). The HE staining of hippocampus was performed following the standard procedures. The hippocampus

tissues from fetal rats were sequentially fixed in 2.5% glutaral solution and 1% osmium acid solution. The sections of each hippocampal region were embedded in Epon 812 and sectioned with a LKB-V ultramicrotome (Bromma, Kista, Sweden). The ultrastructures of the hippocampus were observed using an H-7500 TEM (Hitachi, Tokyo, Japan).

Statistical analysis

SPSS 17 (SPSS Science Inc., Chicago, Illinois) and Prism (GraphPad Software, La Jolla, CA, USA) were used for data analysis. Quantitative data were expressed as the mean ± S.E.M. and evaluated with Independent Samples t-test. The mean weights for each litter were calculated and used for statistical analysis. For enumeration data, the body weight growth rate as well as IUGR rate was arcsine square-root transformed before t-test evaluations[78]. Statistical significance was defined as $P<0.05$.

Conclusions

The intrauterine programming of enhanced sensitivity of the HPA axis in female offspring rats induced by PEE is shown in Fig. 8. The fetal over-exposure to maternal GC induced by PEE, can inhibit the regulation of fetal hippocampus on HPA axis and induce the programming alterations of potential excitatory ability of fetal hypothalamus, embodies in the persistent remodeling alterations of impaired morphology and decreased MR/GR expression ratio in hippocampus, as well as the imbalanced glutamatergic/GABAergic afferent inputs in hypothalamus. All of these would contribute to the enhanced sensitivity of the HPA axis in adult offspring.

Conflict of interest statement

All authors have no conflicts of interest.

Acknowledgments

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Table 1

Rat oligonucleotide primers and reaction conditions used in quantitative real-time PCR.

Genes	Forward primers	Reverse primers	Products (bp)	Annealing
β-actin	GTTGCCAATAGTGATGACCT	GGACCTGACAGACTACCTCA	208	54 ℃, 20 s
CRH	AGAACAACAGTGCGGGCTCA	GCTCCGGTTGCAAGAAATTCA	196	60 ℃, 30 s
AVP	AAGAGGGCCACATCCGACA	AGGGCAGGTAGTTCTCCTCCTG	160	58 ℃, 20 s
Pax6	AAGCAAAATAGCCCAGTATAAACG	TAATGGGTCCTCTCAAACTCTTTC	450	58 ℃, 20 s
Tbr2	CCCCAACAGAGCGAAGAGGT	GGGAAGACAGGTGGGCTCATT	290	58 ℃, 20 s
VGluT2	TCCACCGGGGTGGCAAAGTT	TGCGATGTATCCGCCCGGAA	128	60 ℃, 30 s
PSD95	TATGTAACGAAGATCATCGAAGGA	GAGAATACGAGGTTGTGATGTCTG	229	58 ℃, 20 s
α-CaMKII	GCATCTGCCGCTTGTTGAA	AGTGTAGCACAGCCTCCAAG	192	58 ℃, 20 s
Mash1	GAAGATGAGCAAGGTGGAGACG	CGGAGAACCCGCCATAGAGT	169	60 ℃, 30 s
GAD65	TGCAGCCTTGGGGATCGGAA	CCCCAAGCAGCATCCACATGCA	237	60 ℃, 30 s
Reln	CAGCAATGGGCTCGTGGTTT	TGTGGGTCTTGTCCTTCTTTT	233	58 ℃, 20 s
MR	TGCATGATCTCGTGAGTGA	AAGTTCTTCCTGGCCGGTAT	190	62 ℃, 30 s
GR	CACCCATGACCCTGTCAGTC	AAAGCCTCCCTCTGCTAACC	156	61 ℃, 30 s
NR2B	TGGAATGGCATGATCGGTGAG	AGCCACCGCAGAAACAAT	240	60 ℃, 30 s

CRH, corticotrophin-releasing hormone; AVP, arginine vasopressin; Pax6, paired box 6; Tbr2, TGF-β receptor II; VGluT2, Vesicular glutamate transporter 2; PSD95, post-synaptic density 95; α-CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II-α; Mash1, mammalian achaete-scute homolog-1; GAD65, glutamic acid decarboxylase 65; Reln, Reelin; MR, mineralocorticoids receptor; GR, glucocorticoids receptor; NR2B, N-methyl-D-aspartate-subtype glutamate receptor 2B.

Figures

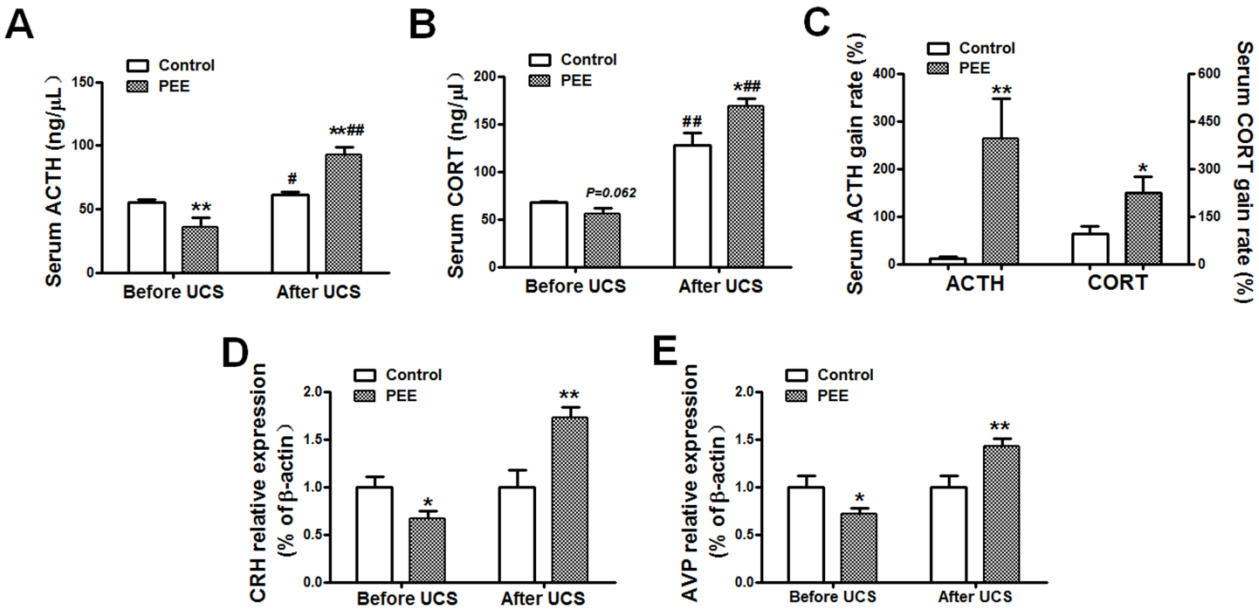

Figure 1. Effects of prenatal ethanol exposure (PEE) on the sensitivity of hypothalamic-pituitary-adrenal axis of female adult offspring rats fed with high-fat diet before and after unpredictable chronic stress (UCS). A: Serum adrenocorticotropic hormone (ACTH) concentrations; B: Serum corticosterone (CORT) concentrations; C: Gain rates of serum ACTH and CORT concentrations; D, E: Hypothalamic corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) mRNA expression levels. Mean±S.E.M., n=8 offspring from 8 litters. $^{*}P<0.05$, $^{**}P<0.01$ vs. control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs*. before UCS.

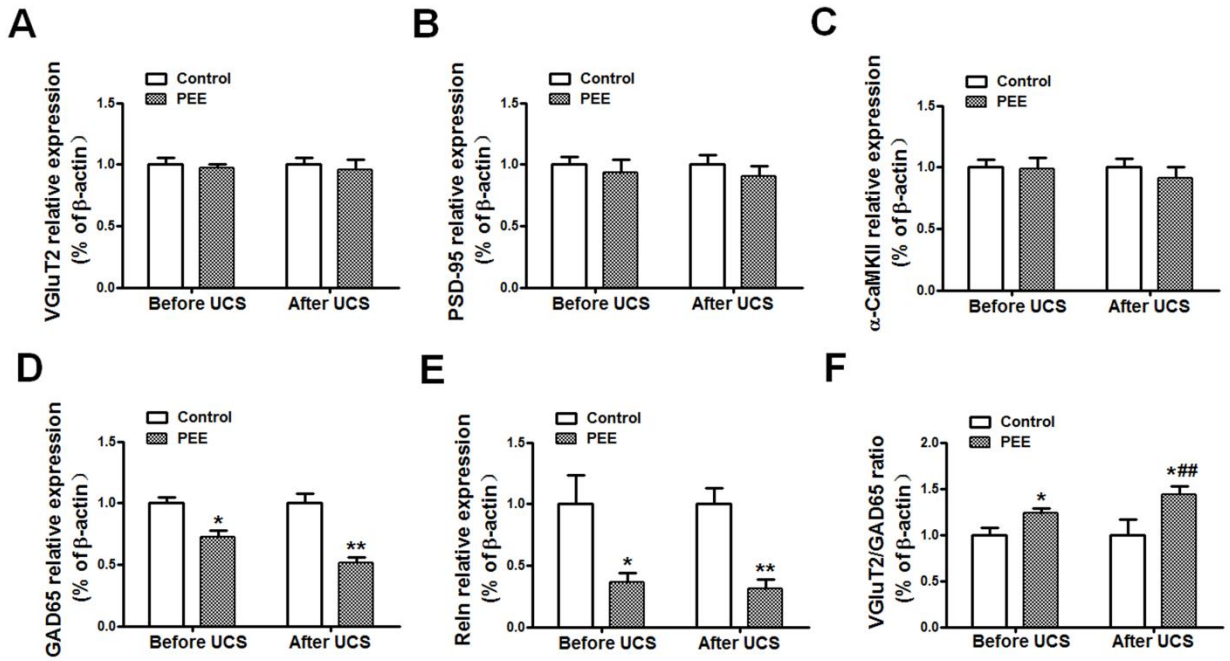

Figure 2. Effects of prenatal ethanol exposure (PEE) on the potential excitatory ability of hypothalamus of female adult offspring rats fed with high-fat diet before and after unpredictable chronic stress (UCS). A-E: Hypothalamic vesicular glutamate transporter 2 (VGluT2), post-synaptic density-95 (PSD-95), Ca2+/calmodulin-dependent protein kinase II-α (α-CaMKII), glutamic acid decarboxylase 65 (GAD65) and Reelin (Reln) mRNA expression levels; F: Hypothalamic VGluT2/GAD65 mRNA expression ratios. Mean±S.E.M., n=8 offspring from 8 litters. $^{*}P<0.05$, $^{**}P<0.01$ vs. control; $^{\#\#}P<0.01$ *vs.* before UCS.

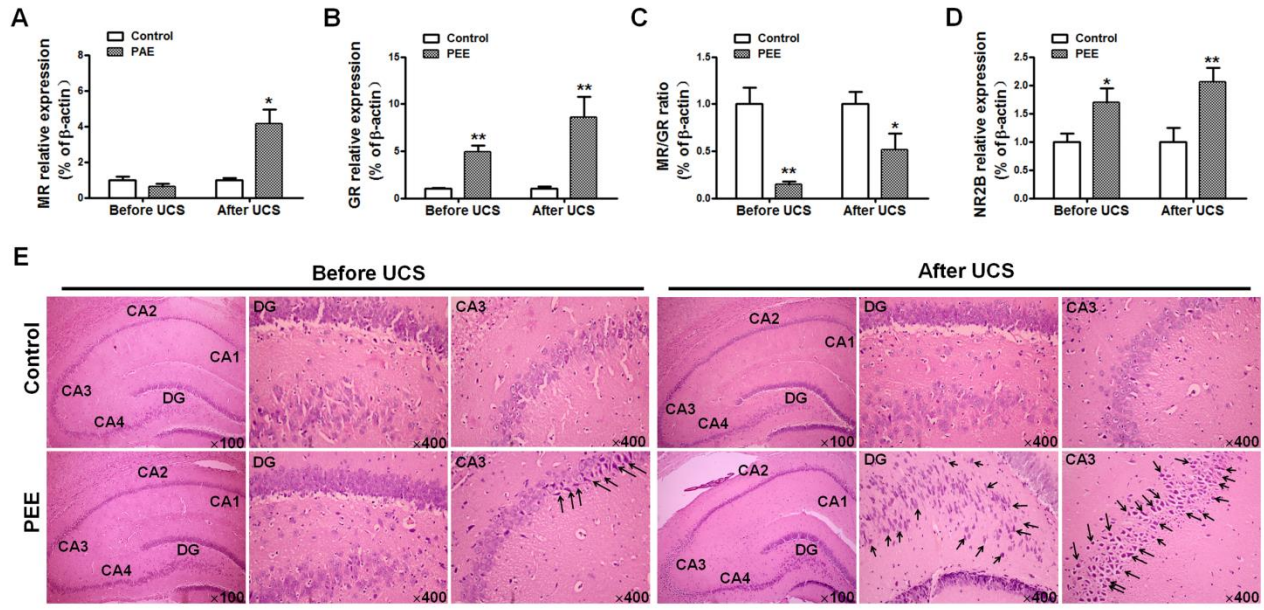

Figure 3. Effects of prenatal ethanol exposure (PEE) on the gene expression levels and morphology of hippocampus in the female adult offspring rats with high-fat diet before and after unpredictable chronic stress (UCS). A, B: Hippocampal mineralocorticoid receptor (MR) and glucocorticoids receptor (GR) mRNA expression levels; C: Hippocampal MR and GR mRNA expression ratios; D: Hippocampal N-methyl-D-aspartate-subtype glutamate receptor 2B (NR2B) mRNA expression levels; E: Morphologic changes of the whole hippocampus (hematoxylin-eosin (HE), ×100), as well as the granular cells in dentate gyrus (DG) (HE, ×400) and the pyramidal cells in cornu ammonis 3 (CA3) (HE, ×400). Mean ± S.E.M., n=8 offspring from 8 litters. $^{*}P<0.05$, $^{**}P<0.01$ vs. control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs*. before UCS.

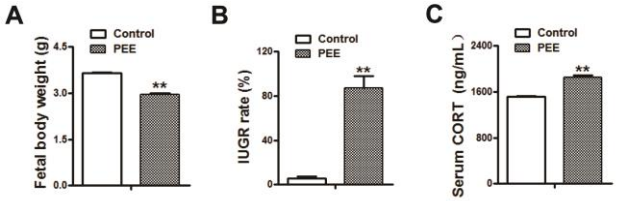

Figure 4. Effects of prenatal ethanol exposure (PEE) on body weight and serum corticosterone (CORT) concentration in the female fetal rats on gestational day 20. A: Fetal body weight; B: intrauterine growth retardation (IUGR) rate; C: Serum CORT concentrations**.** Mean±S.E.M., n=8

litters. $^{**}P<0.01$ *vs*. control.

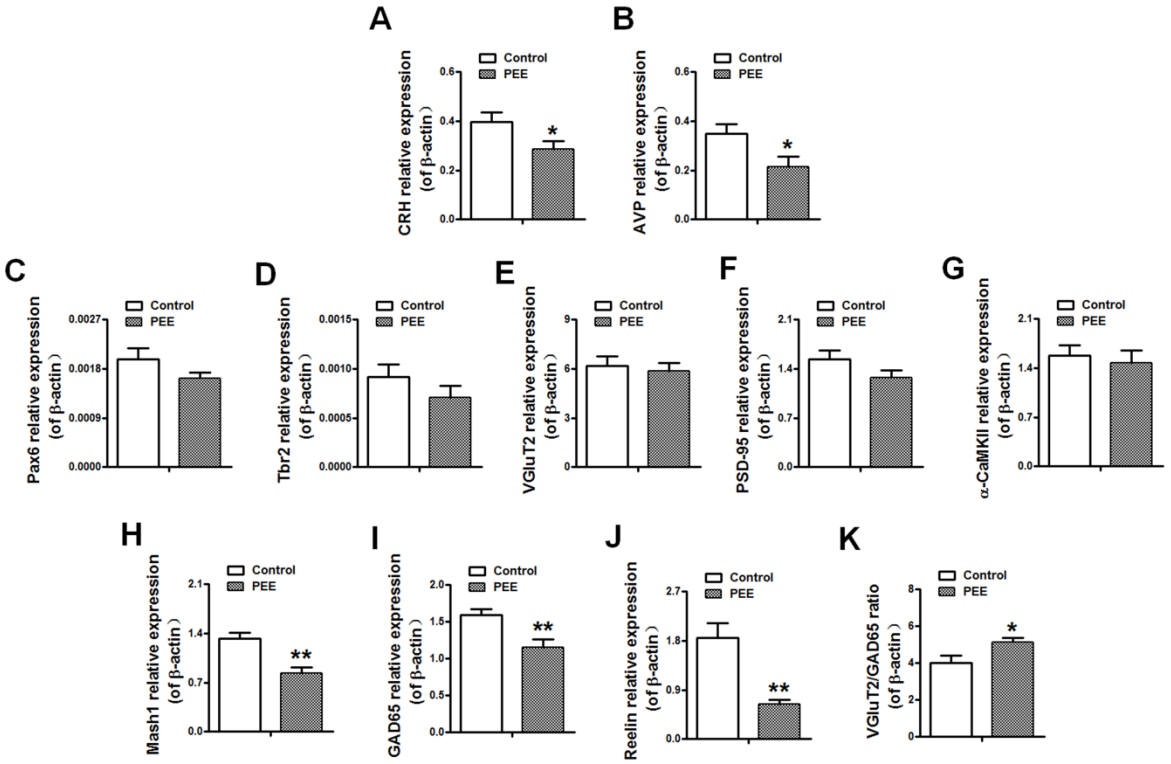

Figure 5. Effects of prenatal ethanol exposure (PEE) on expression of hypothalamic corticotropin-releasing hormone (CRH), vasopressin (AVP), paired box 6 (Pax6), TGF-β receptor II (Tbr2), vesicular glutamate transporter 2 (VGluT2), post-synaptic density 95 (PSD95), Ca2+/calmodulin-dependent protein kinase II-α (α-CaMKII), mammalian achaete-scute homolog-1 (Mash1), glutamic acid decarboxylase 65 (GAD65) and Reelin (Reln) of the female fetal rats on gestational day 20. A-J: hypothalamic CRH, AVP, Pax6, Tbr2, VGluT2, PSD95, α-CaMKII, Mash1, GAD65 and Reln mRNA expression levels; K: Hypothalamic VGluT2 and GAD65 mRNA expression ratios. Mean±S.E.M., n=8 litters. $^{*}P<0.05$, $^{**}P<0.01$ *vs*. control.

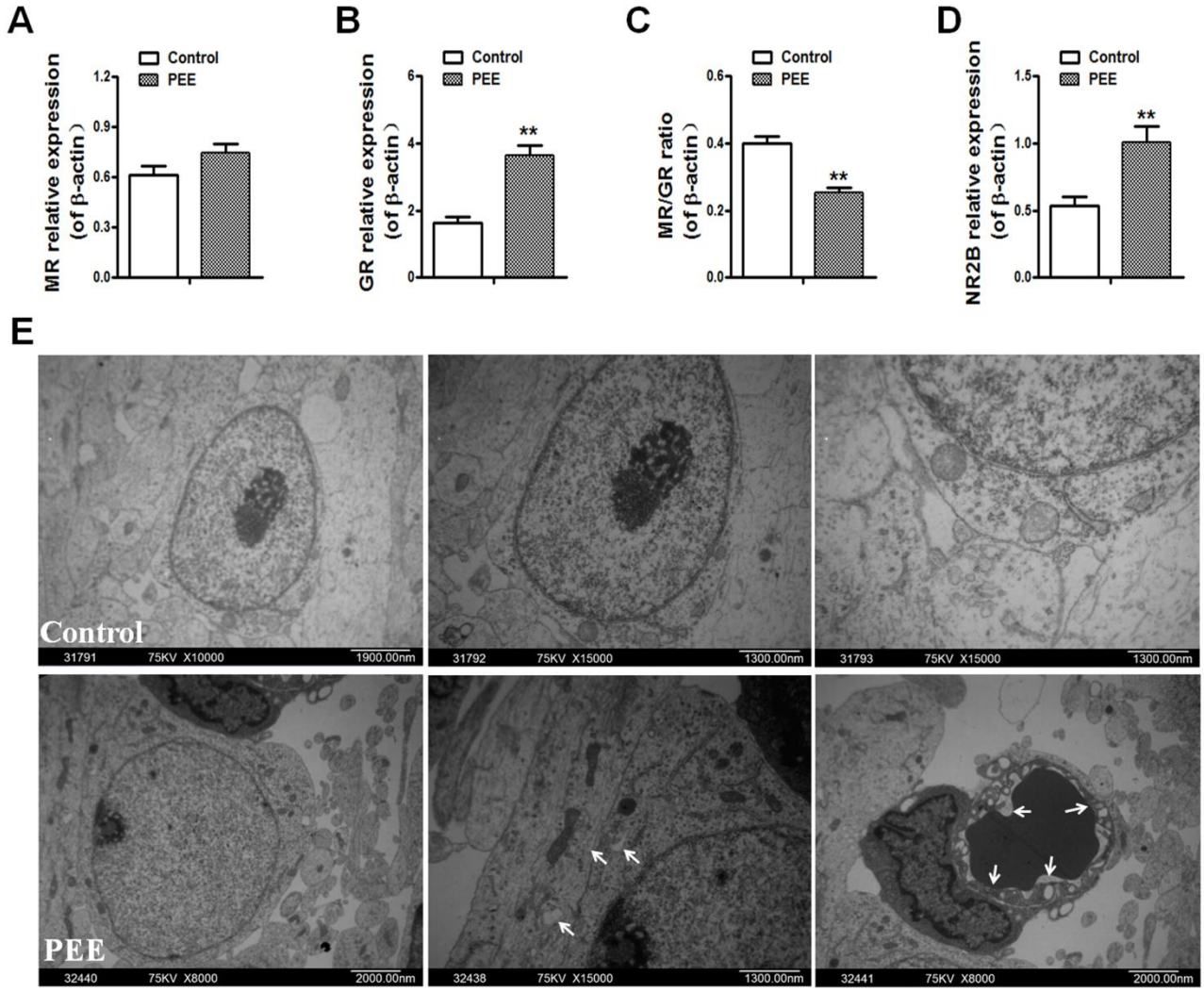

Figure 6. Effects of prenatal ethanol exposure (PEE) on expression of hippocampal mineralocorticoid receptor (MR), glucocorticoid receptor (GR), N-methyl-D-aspartate-subtype glutamate receptor 2B (NR2B), and the ultrastructure of cornu ammonis (CA3) area in female fetal rats on gestational day 20. A, B: Hippocampal MR and GR mRNA expression levels; C: Hippocampal MR/GR mRNA expression ratios; D, Hippocampal NR2B mRNA expression levels; E, The ultrastructure of fetal hippocampus (Transmission electron microscopy, ×8000, ×10000, ×15000). Mean±S.E.M., n=8 litters. $^{**}P<0.01$ *vs*. control.

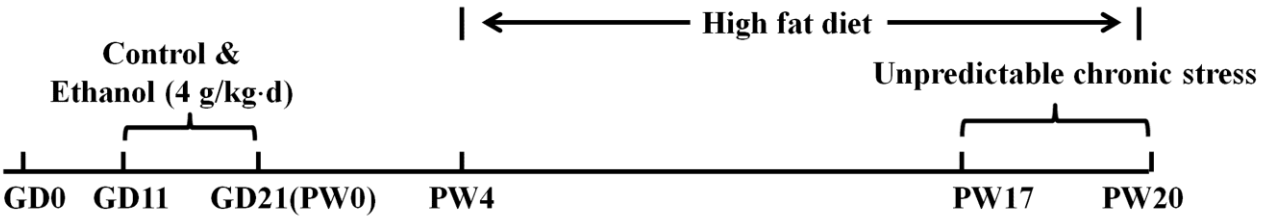

Figure 7. Schematic procedure of animal treatment from gestational day (GD) 0 to postnatal week (PW) 20.

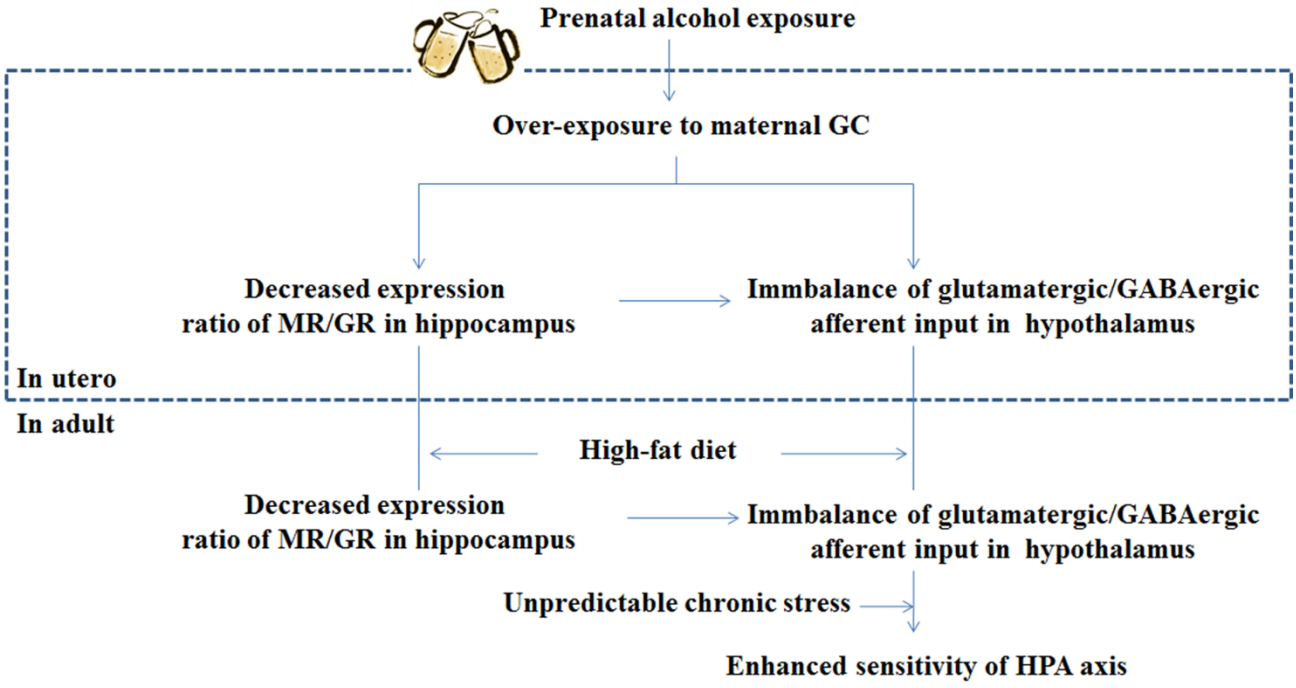

Figure 8. The intrauterine programming mechanism of enhanced sensitivity of hypothalamic-pituitary-adrenal (HPA) axis in female adult offspring rats with prenatal ethanol exposure. GC, glucocorticoid; MR, mineralocorticoid receptor; GR, glucocorticoid receptor; VGluT2, vesicular glutamate transporter 2; GAD65, glutamic acid decarboxylase 65.