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Excitotoxicity and compensatory upregulation of GAD67 in fetal rat hippocampus caused by prenatal nicotine exposure are associated with inhibition of the BDNF pathway

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ABSTRACT

Prenatal nicotine exposure (PNE) can cause hypersensitivity of hypothalamic-pituitary-adrenal (HPA) axis in offspring with intrauterine growth retardation. The purpose of this study was to explore the original mechanism of intrauterine development that mediates hypersensitivity of the HPA axis in offspring due to PNE. Pregnant Wistar rats were injected subcutaneously with 2 mg/kg·d of nicotine on the 9th to the 20th gestational day (GD9-GD20) and the fetuses were extracted at GD20. Compared with the control group, fetal rats by PNE showed increased hippocampal apoptosis, reduced synaptic plasticity and downregulation of the brain-derived neurotrophic factor (BDNF) pathway, whereas glutamic acid decarboxylase 67 (GAD67) expression was upregulated. Rat fetal hippocampal H19-7/IGF1R cell lines were treated with different concentrations of nicotine (1, 10 and 100 μM) for 3 days, the extracellular fluid glutamate (Glu) level increased and similar effects were observed as *in vivo*. Intervention treatments caused the opposite results. These results indicated that PNE downregulates the BDNF pathway and mediates the hippocampal excitotoxicity; then, the compensatory upregulation of GAD67 causes the imbalance of signal output in the fetal hippocampus. The negative feedback regulation of the paraventricular hypothalamic nucleus by the hippocampus is unbalanced, eventually causing hypersensitivity of the HPA axis of the offspring.

1. Introduction

A long-term adverse intrauterine environment will cause adaptive changes in the fetus, which is known as “intrauterine programming”. The adaptive changes may be beneficial in the intrauterine period because they are required for the fetus to adapt to the current unfavorable conditions, but such changes may eventually develop into harmful factors after birth and lead to increased susceptibility to certain diseases in adulthood (Seremak-Mrozikiewicz et al., 2014). Current research shows that the change in the HPA axis due to developmental programming is the core mechanism underlying susceptibility to adult chronic diseases in offspring caused by a poor intrauterine environment (Moisiadis and Matthews, 2014). Previous studies have suggested that an adverse intrauterine environment may cause changes in the developmental programming of HPA axis function in offspring, and the increased sensitivity to stress after birth is the main manifestation of HPA axis dysfunction (Ordjan et al., 2014; Wieczorek et al., 2015).

The prevalence of maternal smoking varies in different countries. According to the survey, the exposure rate of pregnant women to tobacco fog in China is between 38.9% and 75.1% (Zhang et al., 2015); In France, the rate is 69.57% (Blanquet et al., 2016); In Brazil, the smoking rate of pregnant women is 18% (Dias-Dame and Cesar, 2015); It has also been found that nearly 50% of non-smoking pregnant women in the United States have significantly elevated levels of cotinine (a nicotine metabolite), suggesting that they were exposed to smoke (Hawkins et al., 2014). Nicotine, a major harmful substance in cigarette smoke, is considered to interfere with fetal development (Yildiz, 2004). Moreover, active or passive smoking during pregnancy and prenatal nicotine exposure (PNE) can cause fetal developmental toxicity, such as intrauterine growth retardation (IUGR), and its harmful effects even extend from birth to adulthood, manifesting as increased susceptibility to multiple adult chronic diseases (neuropsychiatric diseases, metabolic diseases, etc.) (Xiao et al., 2010). Our previous studies also showed that PNE can result in reduced function of the HPA axis in the fetus and HPA axis hypersensitivity after birth and that the progenies are susceptible to metabolic syndrome and metabolic diseases (Liu et al., 2012; Xu

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et al., 2012, 2013).

The hippocampus is a crucial component of the HPA axis functional regulation centre. It has been reported that the hippocampus is a site that is sensitive to glucocorticoids and regulates the expression of corticotropin-releasing hormone (CRH) in the hypothalamus through negative feedback (Jacobson and Sapolsky, 1991). In addition, the hippocampus participates in the programming process of the perinatal HPA axis in response to stress (Bruin et al., 2010), and structural and functional impairment of the hippocampus will directly affect the activity of the HPA axis (Cerpa et al., 2015). The neurons emanating from the ventral hypoxia of the hippocampus receive inhibitory regulation of neuronal activity from the paraventricular hypothalamic nucleus (PVH) by participating in glutamate (Glu) - γ-aminobutyric acid (GABA) synaptic connections in the PVH-projecting region around the PVH (Jankord and Herman, 2008; Konishi et al., 2003). Several studies suggest (Chen et al., 2014; Gil-Mohapel et al., 2014; Lin and Wang, 2014; Wang et al., 2014) that multiple adverse intrauterine environments can cause hippocampal developmental damage and susceptibility to related neuropsychiatric disorders in offspring. A recent study in our laboratory has also shown that PNE can lead to the upregulation of glutamic acid decarboxylase 67 (GAD67) expression in the hippocampus of offspring (Cerpa et al., 2015), resulting in excessive decarboxylation of Glu to GABA under the action of GAD67. Then, local excitatory and inhibitory signal output in the fetal hippocampus becomes imbalanced, and the negative feedback regulation mechanism of the hippocampus on the HPA axis is impaired, which may be the intrauterine programming mechanism that mediates the postnatal HPA axis hypersensitivity in offspring induced by PNE.

Brain-derived neurotrophic factor (BDNF) is an important neurotrophic factor in the brain with the highest content in the hippocampus and cortex tissue. The binding of BDNF to its receptor tropomyosin-related kinase receptor B (TrkB) promotes the development and differentiation of various types of neurons and contributes the maintenance and promotion of neural growth by activating intracellular signaling cascades (Lee et al., 2002). Moreover, BDNF can protect hippocampal neurons from glutamate excitotoxicity (Melo et al., 2013). The expression of BDNF is regulated by the upstream signaling factor cAMP response element-binding (CREB) (Luo et al., 2017). Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channel proteins that mediate rapid synaptic signaling. Both α4β2 and α7 receptor subtypes are most abundant in the central nervous system, and nicotine can activate hippocampal nAChRs as a specific agonist of nAChRs (Gray et al., 1996). There is a study showing that the acute exposure to nicotine reduces BDNF levels in STC-1 cells, a type of enteric neuroendocrine cell. Mecamylamine, a nonselective nAChR antagonist, inhibits nicotine-induced downregulation of BDNF (Qian et al., 2016). In addition, mecamylamine can upregulate the expression of BDNF in the prefrontal cortex in a rat model of depression (Aboul-Fotouh, 2015). We speculated that PNE may mediate hippocampal developmental damage through the BDNF pathway, thereby affecting HPA axis hypersensitivity programming.

This study aims to investigate the molecular mechanism of fetal hippocampus developmental damage and the potential mechanism of the intrauterine origin of high-stress sensitivity programming in the HPA axis by establishing the IUGR model of nicotine exposure during pregnancy and the H19-7/IGF-IR rat hippocampal neuronal cell line cultured *in vitro*.

2. Materials and methods

2.1. Animals and treatment

Specific pathogen-free healthy Wistar rats, females (200 ± 20 g body weight) and males (250 ± 20 g body weight) were obtained from the Experimental Centre of the Hubei Medical Scientific Academy (Wuhan, China). Animal experiments were performed at the Centre for Animal Experiments of Wuhan University (Wuhan, China), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The Committee on the Ethics of Animal Experiments of the Wuhan University School of Medicine approved the protocol (Permit No. 201719). All animal experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee.

The animals were caged at 6 o'clock each night with 4 females and 2 males per cage (485 mm × 350 mm × 200 mm) after one week of adaptive feeding. GD0 was considered upon confirmation of mating by the observation of sperm under light microscopy. Pregnant rats were randomly divided into control and PNE groups. The animals were housed under standard conditions (room temperature: 18–22 °C; humidity: 40–60%) and had free access to standard food and water. From GD9 to GD20, rats in the nicotine group were subcutaneously administered 2.0 mg/kg nicotine (N3876; Sigma-Aldrich Co., Ltd., MO, USA) per day, while rats in the control group were administered the same volume of saline as the vehicle. On GD20, the randomly selected pregnant rats from the control and the PNE groups (n = 12) with 10–14 live fetuses were sacrificed for fetal experiments. Whole brain tissue of 1–2 fetal rats was extracted and fixed with 4% formaldehyde solution or embedded in OCT tissue embedding agent. The remaining fetal hippocampal tissue was separated and transferred to a −80 °C refrigerator for storage.

2.2. Transmission electron microscopy observation

Fetal rats were soaked in 4% formaldehyde fixative. The skull was cut from the top, and the whole brain was isolated and placed in formaldehyde. The fetal hippocampus was isolated and quickly placed in a glutaraldehyde fixative for electron microscopy. Ultrathin sections were made and embedded in epoxy resin and photographed under a transmission electron microscope (H-600; Hitachi, Japan).

2.3. Cell culture and treatment

Fetal rat hippocampal neuronal cells (H19-7/IGF1R cells) (No. CRL-2526™) were provided by the American Type Culture Collection (ATCC). H19-7/IGF1R cells were subcultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, BRL, USA) containing 10% FBS, 200 μg/ml G418, and 1 μg/ml puromycin in a 34 °C, 5% CO2 incubator. Then, the cells differentiated and matured in an incubator at 39 °C and 5% CO_2 in DMEM with N_2 supplements (Invitrogen, Carlsbad, CA, USA) prior to treatment. H19-7/IGF1R cells were treated with different concentrations of nicotine (0, 1, 10 and 100 μM) or with vecuronium bromide (ab120536; Abcam Inc., Cambridge, MA) or brain-derived neurotrophic factor (ab9794; Abcam Inc., Cambridge, MA) with or without nicotine for 3 days, and the culture medium was changed every other day. At the end of these treatments, cells and media were collected for subsequent experiments.

2.4. Cytotoxicity test

H19-7/IGF1R cells with good growth status were seeded onto 96-well plates at a density of 3.0×10^5 cells and treated with 0, 1, 10 and 100 μM nicotine for 3 days, or 100 μM nicotine for 1, 2, and 3 days. One-hundred microliters of medium was removed, and 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, USA) was added. Cells were incubated for 1–2 h in the dark at 37 °C and 5% CO_2. Absorbance values were read at 490 nm using an ultraviolet spectrophotometer (Thermo NanoDrop 2000c, Thermo Fisher Scientific, USA).

2.5. Reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR) analysis

TRIzol reagent (Invitrogen Co., CA, USA) was used to extract RNA from hippocampus and hypothalamus tissues, and single-strand cDNA was prepared from 2 μg of total RNA using the Takara RT reagent kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. Primers of α4, α7 and β2 nicotinic acetylcholine receptors (α4, α7 and β2 nAchR), cAMP response element-binding protein (CREB), brain-derived neurotrophic factor (BDNF), tropomyosin-related kinase B (TrkB), N-methyl D-aspartate 1 receptor submit 1 (NR1), N-methyl D-aspartate 1 receptor submit 2A (NR2A), N-methyl D-aspartate 1 receptor submit 2B (NR2B), synapsin 1 (Syn 1), Bcl-2-associated X (Bax), B-cell lymphoma-2 (Bcl-2), cysteinyl aspartate specific proteinase-3 (caspase-3), cell cycle protein A (Cyclin A), cyclin-dependent kinase 2 (CDK2), glutamate decarboxylase 67 (GAD67) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are presented in the supplementary material. Thunderbird SYBR qPCR mix (Toyobo, Japan) and an ABI PRISM 7300 instrument (Applied Biosystems, Carlsbad, CA, USA) were used for real-time PCR, with an initial denaturation at 95 °C for 5 min. All of the subsequent 40 cycles were performed at a temperature of 95 °C for 5 s and 72 °C for 30 s if the annealing temperature was below 60 °C. The different annealing conditions are presented in supplementary material.

2.6. Western blotting

Western blotting was used to detect the expression of BDNF and GAD67 in tissues and cell samples. The primary antibodies and dilution concentrations used were as follows: rabbit anti-BDNF (ab108319; Abcam Inc, Cambridge, MA) 1:2000, mouse anti-GAD67 (ab97739; Abcam Inc, Cambridge, MA) 1:500, rabbit anti-GAPDH (A10868; Abclonal, Boston, MA) 1:2000. Secondary antibodies goat anti-rabbit IgG Antibody HRP conjugate (222F6; Bionukes Biotechnology Co., Ltd., China) and goat anti-mouse IgG Antibody HRP conjugate (BL0001; Hefei Bio Sharp Co., Ltd., China) were diluted 1:5000. All the Western blotting results were tested for three times.

2.7. Annexin V-FITC/PI flow cytometry for apoptosis detection

H19-7/IGF1R cells with good growth status were seeded onto a 6-well plate at a density of 1.0×10^6 cells, treated with different concentrations of nicotine for 3 days, washed with PBS, and added to appropriate amounts of trypsinized cells to prepare cell suspensions according to the Annexin V-FITC. The remaining steps were performed with the Annexin V-FITC/PI detection kit (BB-4101; BestBio Biological Co., Ltd., China), and flow cytometry was performed after incubation at room temperature for 10 min in the dark.

2.8. Immunofluorescence analysis

H19-7/IGF1R cells were counted in 24-well plates (approximately 2×10^5cells per well). The sections were first incubated in a blocking solution containing 10% bovine serum and 0.1% Triton X-100 in PBS. After fixation with 4% paraformaldehyde for 15 min at 4 °C, blocking solution (10% goat serum-PBS) was added after antigen retrieval, and cells were blocked for 2 h at room temperature. Mouse anti-GAD67 antibody (diluted 1:500, ab97739; Abcam Inc, Cambridge, MA) was added and incubated overnight at 4 °C in a humid chamber. After washing five times with deionized water, FITC (bs-0295G-FITC; Bioss Inc., Beijing, China)-labeled goat anti-rabbit IgG and Cy3 (AS91111; Bioyear Inc., Wuhan, China)-labeled goat anti-mouse IgG secondary antibodies (diluted 1:200) were incubated at room temperature in the dark for 40–60 min. After rinsing with deionized water, DAPI (P0013B; Beyotime Biotechnology Co., Ltd., China) was added and incubated for 20 min at room temperature in the dark. Cells were rinsed with deionized water for 15 min and observed under an inverted fluorescence microscope, and photographs were taken. Immunofluorescence images were quantified using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) after merging.

2.9. Detection of the content of Glu in extracellular fluid

The cell culture medium was collected and extracellular medium Glu concentration was detected using the Glu content detection kit (A074, Nanjing Jiancheng Bioengineering Institute, China). The specific operation steps were performed according to the manufacturer's instructions.

2.10. Statistical analysis

SPSS 19 (SPSS Science Inc., Chicago, Illinois) was used for data analysis and Prism 6 (GraphPad Software, La Jolla, CA, USA) was used for making figures. Quantitative data were expressed as the mean ± SD. Student's two-tailed *t*-test was performed on one factor of prenatal nicotine treatment (control or nicotine). For the data from the *in vitro* study, a one-way ANOVA followed by a post hoc Dunnett *t*-test or a post hoc Bonferroni *t*-test were used to perform the multiple comparisons. Non-parametric statistics are used when the value of n is equal to 3. A Chi-square analysis was performed to test for a difference in the proportions of the categorical variables between groups, such as the ratio of Bax to Bcl-2. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Fetal rats

3.1.1. Alterations in ultrastructure, proliferation, apoptosis, and synaptic plasticity in the hippocampus

Transmission electron microscopy revealed that the cytoplasm of the hippocampal neurons in the control group was rich in organelles, and no structural changes were observed in mitochondria and endoplasmic reticulum. However, in the PNE group (2 mg/kg·d), the hippocampus of the fetal rats showed a reduction in the number of organelles, swelling of the mitochondria and expansion of the endoplasmic reticulum with degranulation (Fig. 1A). This finding suggests that PNE can cause ultrastructural damage to hippocampal neurons in fetal rats. The results demonstrated that compared with those in the control group, the expression levels of NR1, NR2A, and NR2B were significantly decreased in the PNE group ($p < 0.01$; Fig. 1B–D). CDK2 expression was not significantly different in the PNE group (Fig. 1E), but Cyclin A expression was significantly reduced ($p < 0.05$; Fig. 1F). Bcl-2 expression was significantly decreased in the PNE group ($p < 0.01$; Fig. 1G). The expression of Bax, caspase 3 and the ratio of Bax/Bcl-2 expression were significantly increased in the PNE group ($p < 0.01$; Fig. 1H–J). These results suggested that PNE can cause inhibition of hippocampal neuron proliferation, increased apoptosis, and changes in synaptic plasticity.

3.1.2. Alterations in CREB, BDNF and GAD67 expression in hippocampus

Compared with that in the control group, α4 and α7 nAchR gene expression was significantly upregulated in the PNE group ($p < 0.01$; Fig. 2A–C); CREB and BDNF gene expression was significantly reduced ($p < 0.01$; Fig. 2D and E); and GAD67 gene expression was significantly elevated ($p < 0.01$; Fig. 2F). The Western blotting results showed that compared with that in the control group, BDNF protein expression was markedly decreased in the PNE group, and GAD67 protein expression was significantly increased ($p < 0.01$; Fig. 2E–G). This result suggests that, in the hippocampus of fetal rats, PNE can induce increased expression of nAchRs, cause downregulation of the BDNF pathway and increase GAD67 expression.

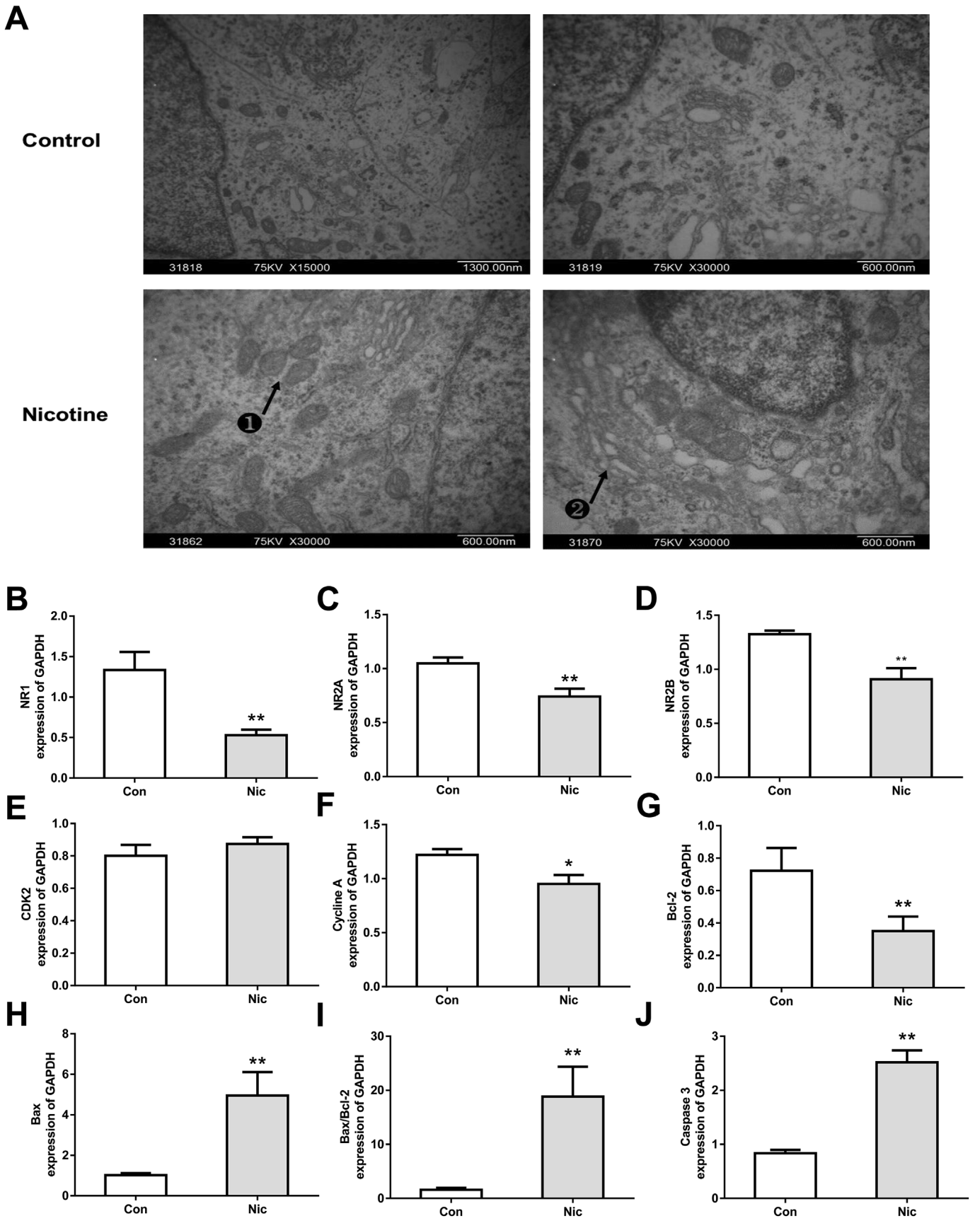

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Fig. 1. Effects of prenatal nicotine exposure on hippocampal ultrastructural, proliferation, apoptosis, and synaptic plasticity in fetal rats. A: Transmission electron microscopy × 30000, Control: Control group, Nicotine: Nicotine treatment group (2 mg/kg·d). ❶Cell mitochondrial swelling ❷Endoplasmic reticulum expanded and accompanied by degranulation; B–D: hippocampal synaptic plasticity gene expression, including N-methyl D-aspartate 1 receptor submit 1 (NR1), N-methyl D-aspartate 1 receptor submit 2A (NR2A), N-methyl D-aspartate 1 receptor submit 2B (NR2B); E, F: hippocampal proliferation related gene expression, including cyclin dependent kinase 2 (CDK2), cell cycle protein A (Cyclin A); G–J: hippocampus apoptosis related gene expression, including B-cell lymphoma-2 (Bcl-2), Bcl-2-Associated X (Bax), the ratio of Bax/Bcl-2 expression, cysteinyl aspartate specific proteinase-3 (caspase-3). Mean ± S.D., n = 12, each sample was pooled by fetal offspring's hippocampus from different pregnant rats. $*p < 0.05$, $**p < 0.01$ compared to the control group.

3.2. H19-7/IGF1R cell line

3.2.1. Alterations in proliferation, apoptosis and synaptic plasticity

The MTS proliferation assays showed that the cell viability of H19-7/IGF1R cells treated with 1–100 μM nicotine for 1–3 days had no notable changes (Fig. 3A and B). Compared with that in the control group, NR2A and NR2B expression was markedly downregulated after treatment with different concentrations of nicotine (1, 10, 100 μM) for 3 days ($p < 0.01$; Fig. 3C and D); the expression of CDK2 was significantly decreased after 3 days treatment with different concentrations of nicotine (1, 10, 100 μM) ($p < 0.01$; Fig. 3E); Cyclin A expression was markedly decreased in the 10 μM nicotine treatment group ($p < 0.05$; Fig. 3F). Furthermore, compared with those in the control group, Bax expression and the ratio of Bax/Bcl-2 expression were significantly upregulated, while Bcl-2 was observably decreased with the 10 and 100 μM nicotine treatment ($p < 0.01$, $p < 0.05$; Fig. 3G–I); The expression of caspase3 showed a statistically significant increase at 100 μM group ($p < 0.05$; Fig. 3J). In addition, Syn1 expression was significantly decreased in the 10 μM nicotine-treated group ($p < 0.05$; Fig. 3K). The results of cell flow-type apoptosis test showed that the proportion of apoptotic cells showed a concentration-dependent increase after 3 days treatment of different nicotine concentrations (1, 10, 100 μM) compared with that in the control group ($p < 0.05$; Fig. 3L, M). These results suggested that nicotine treatment inhibited the proliferation and synaptic plasticity of hippocampal neurons and increased their apoptosis.

3.2.2. Alterations in Glu release and GAD67 expression

The results showed that compared with that in the control group, the extracellular fluid Glu level significantly increased after 10 and

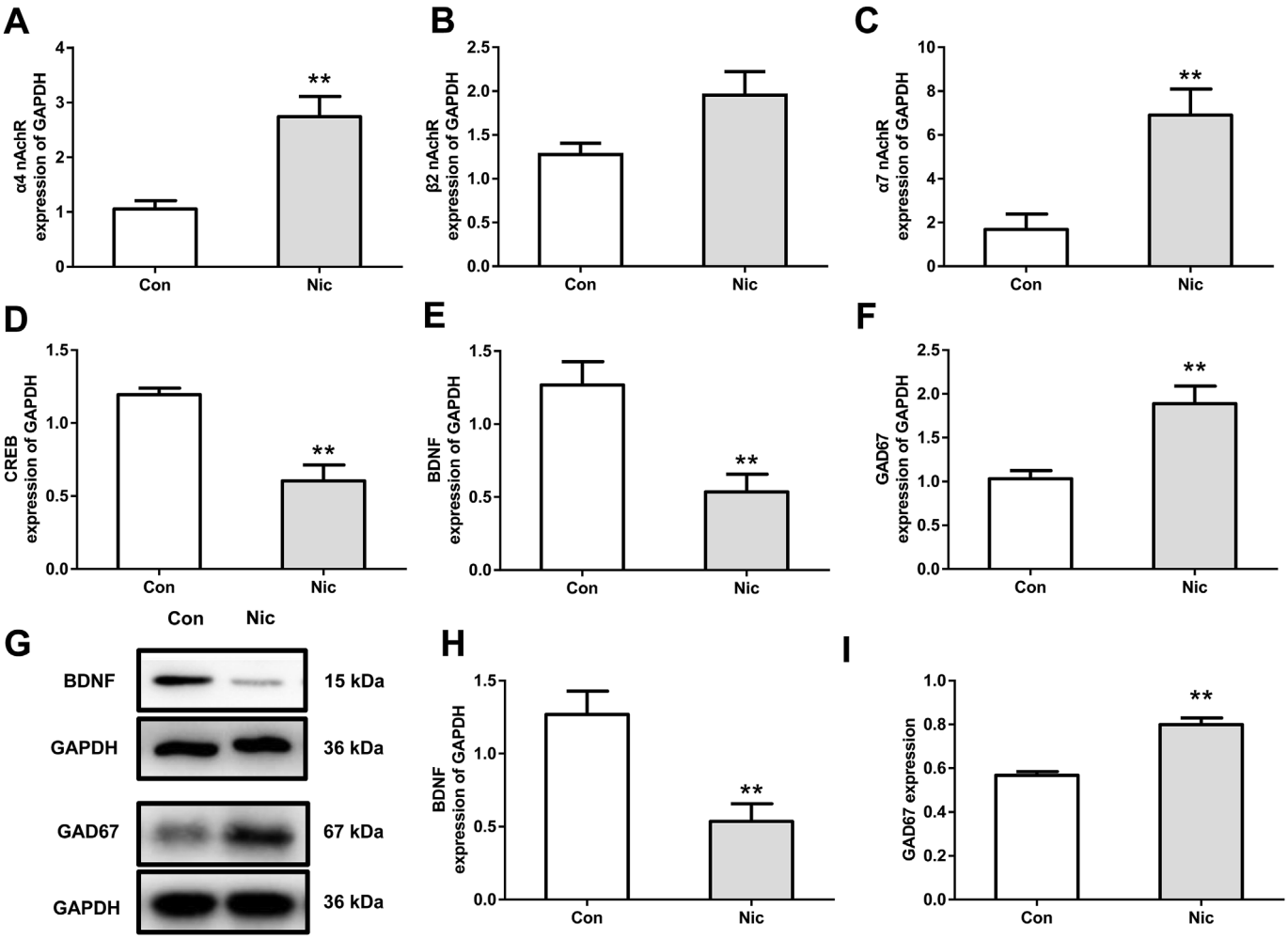

Fig. 2. Effects of prenatal nicotine exposure on cAMP response element-binding protein (CREB), brain-derived neurotrophic factor (BDNF) and glutamate decarboxylase 67 (GAD67) expression levels in fetal hippocampus. A–C: α4, β2, and α7 nicotinic acetylcholine receptor gene expression; D–F: CREB, BDNF and GAD67 gene expression; G: BDNF and GAD67 protein expression; H, I: quantitative analysis of BDNF and GAD67 protein expression. Mean ± S.D., n = 12 for RT-qPCR; n = 3 for Western blotting, each sample was pooled by fetal offspring's hippocampus from different pregnant rats and the Western blotting results were tested for three times. $**p < 0.01$ compared to the control group.

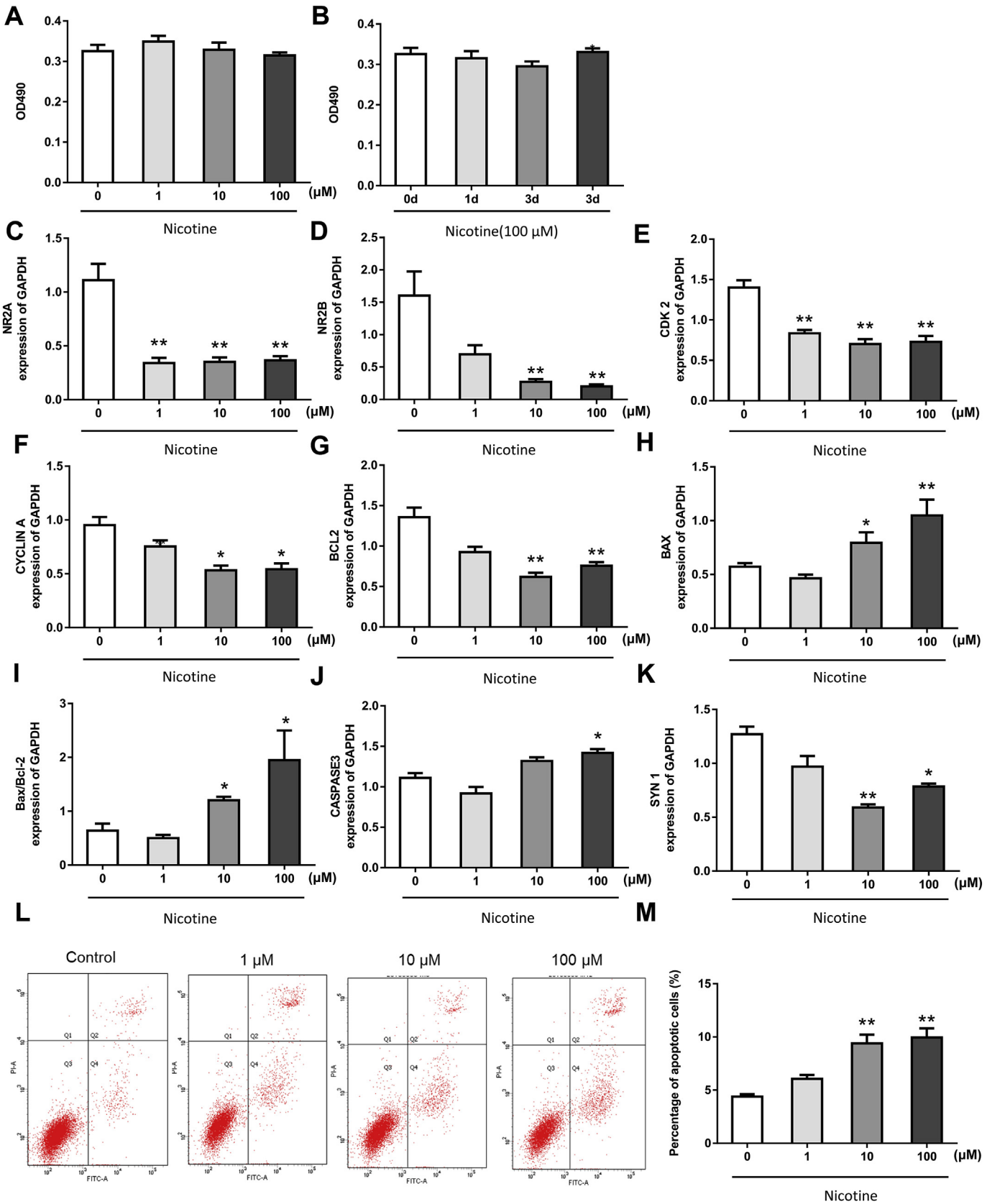

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Fig. 3. Cytotoxicity test and effects of nicotine treatment on proliferation, apoptosis, and synaptic plasticity of H19–7/IGF1R cell line. A, B: Cytotoxicity tests of H19-7/IGF1R cells treated with different concentrations of nicotine at different times, (A) Different concentrations of nicotine (1, 10, 100 μM) were administered for 3 days and (B) 100 μM of nicotine was administered for 1, 2, and 3 days; C, D: Neuronal cell synaptophysin-related gene expression after different concentrations of nicotine (1, 10, 100 μM) treatment for 3 days, including N-methyl D-aspartate 1 receptor submit 2A (NR2A) and N-methyl D-aspartate 1 receptor submit 2B (NR2B); E, F: Neuronal cell proliferation-related gene expression after different concentrations of nicotine (1, 10, 100 μM) treatment for 3 days, including cyclin-dependent kinase 2 (CDK2) and cell cycle protein A (Cyclin A); G–I: Neuronal cell apoptosis-related gene expression after different concentrations of nicotine (1, 10, 100 μM) treatment for 3 days, including B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X (Bax), the ratio of Bax/Bcl-2 expression and caspase3; J: The gene expression of synapsin 1 (Syn 1) after different concentrations of nicotine (1, 10, 100 μM) treatment for 3 days; L: Annexin V-FITC/PI apoptotic flow cytometry after treatment of different concentrations of nicotine (1, 10, 100 μM) for 3 days; M: The percentage of apoptotic cells through Annexin V-FITC/PI apoptotic flow cytometry. Mean ± S.D., n = 9. *$p < 0.05$, **$p < 0.01$ compared to the control group.

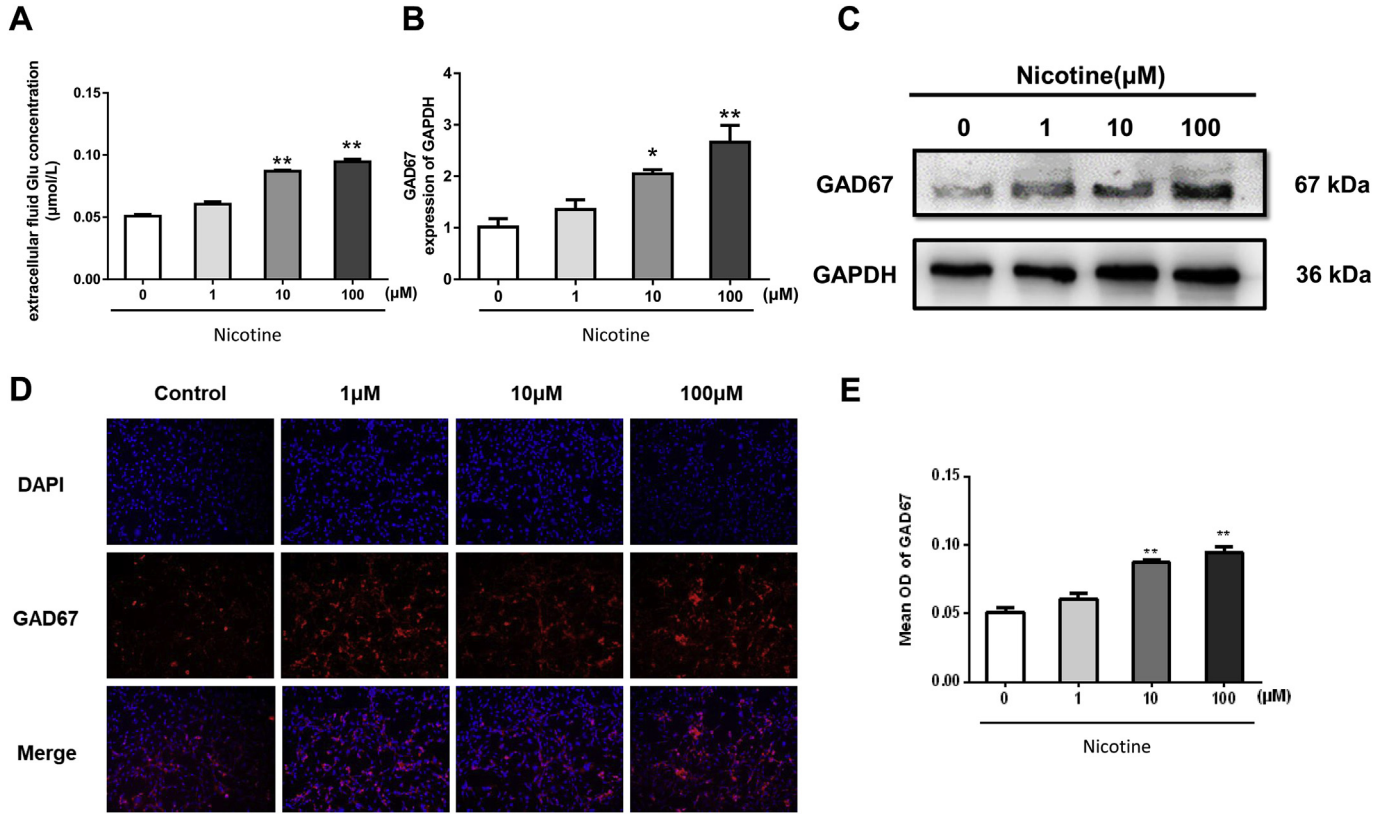

Fig. 4. Effects of nicotine treatment on glutamate (Glu) release levels and glutamate decarboxylase 67 (GAD67) expression in H19–7/IGF1R cells. A: Glu content in extracellular fluid changes after treatment with different concentrations of nicotine (1, 10, 100 μM) for 3 days; B–E: The mRNA and protein expression of GAD67 after treatment with different concentrations of nicotine (1, 10, 100 μM) for 3 days, (B) GAD67 gene expression, (C) GAD67 protein expression measured by Western blotting, (D) GAD67 protein expression measured by immunofluorescence, and (E) Quantitative immunofluorescence analysis for the expression of GAD67. Mean ± S.D., for testing the extracellular fluid Glu level and GAD67 gene expression, n = 9. For testing GAD67 protein expression *via* immunofluorescence, n = 9. For testing GAD67 protein expression *via* Western blotting, n = 3, and the Western blotting results were tested for three times. **$p < 0.01$ compared to the control group.

100 μM nicotine treatment for 3 days in H19-7/IGF1R cells ($p < 0.01$; Fig. 4A). Compared with the control group, GAD67 expression was markedly increased in 100 μM nicotine-treated cells for 3 days ($p < 0.01$; Fig. 4B), and the same time, the Western blotting results showed that GAD67 protein expression was increased in a concentration-dependent manner (Fig. 4C). Quantitative immunofluorescence analysis showed that after treatment with 10 and 100 μM nicotine for 3 days, GAD67 expression was increased in a dose-dependent manner ($p < 0.01$; Fig. 4D and E) compared to that in the control group. These results suggested that nicotine can cause the imbalance between excitation and inhibitory transmitters in neuronal cells.

3.2.3. Alterations in α4, β2, and α7 nAchR and BDNF pathway

Compared with the control group, the expression of α4 nAchR was significantly increased in cells treated with 1 μM nicotine for 3 days ($p < 0.01$; Fig. 5A); the expression levels of α7 nAchR and β2 nAchR were markedly upregulated after treatment with different concentrations of nicotine (1, 10, and 100 μM) for 3 days ($p < 0.01$; Fig. 5B and C); and CREB and BDNF expression levels were significantly reduced in cells treated for 3 days with 10 and 100 μM nicotine ($p < 0.05$, $p < 0.01$, respectively; Fig. 5D and E). The expression of TrkB was significantly reduced in cells treated with 100 μM nicotine for 3 days ($p < 0.01$; Fig. 5F). The Western blotting results showed a concentration-dependent reduction in BDNF protein expression in nicotine-treated cells compared to that in the control group (Fig. 5G). All of these results suggested that nicotine may inhibit the expression of the BDNF pathway by activating α4β2 nAchR and α7 nAchR in neuronal cells.

3.2.4. Alterations in the BDNF pathway and GAD67 expression with the intervention of vecuronium bromide (a nonspecific inhibitor of nAChRs) and BDNF

Compared with the control group, the expression of CREB was markedly decreased after treatment with 10 μM nicotine for 3 days

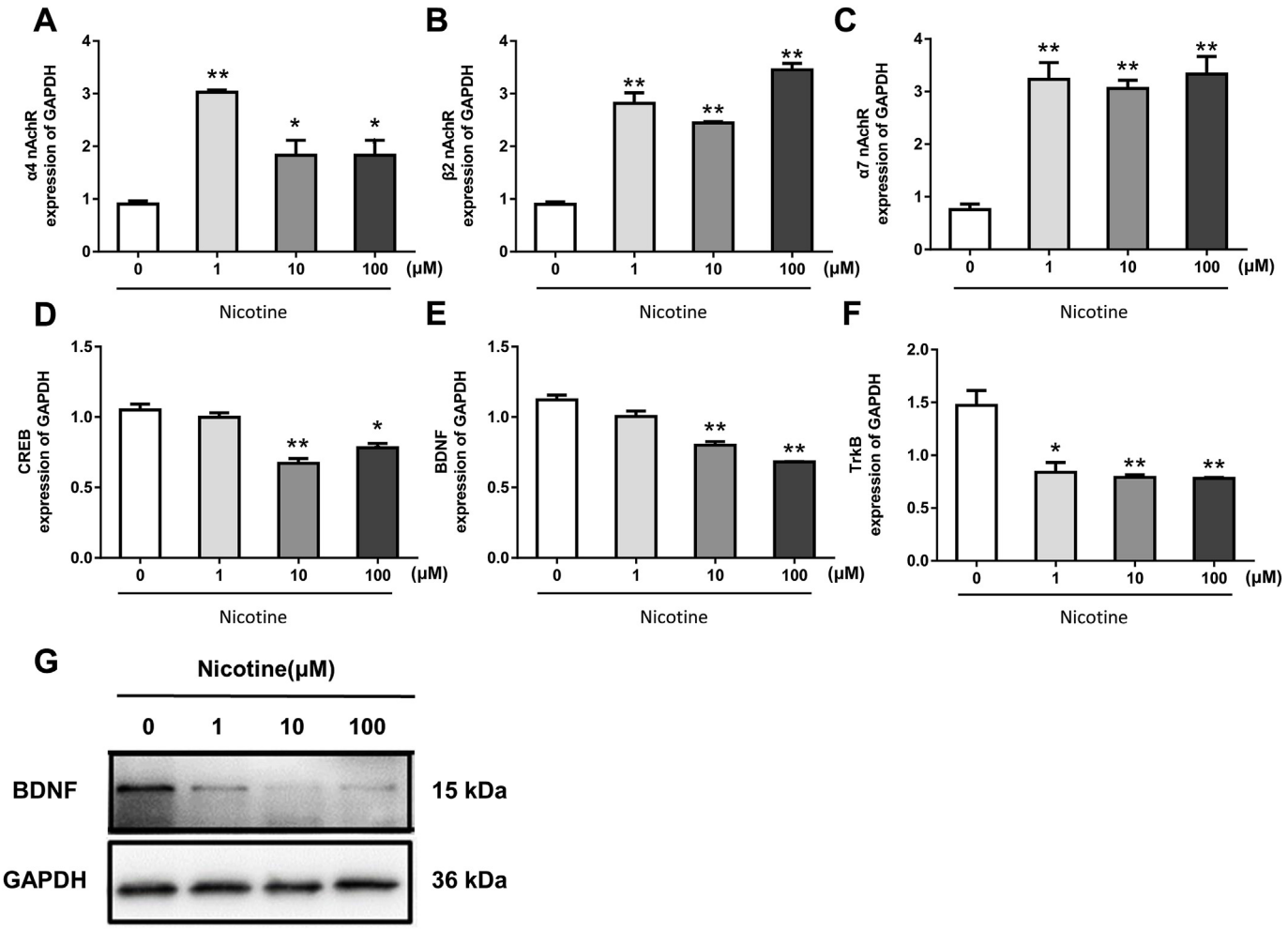

Fig. 5. Effects of nicotine treatment on α4, β2, and α7 nicotinic acetylcholine receptor (α4, α7 and β2 nAchR) expression and brain-derived neurotrophic factor (BDNF) pathway in H19–7/IGF1R cells. A–C: The expression of α4, β2, and α7 nAchR after treatment with different concentrations of nicotine for 3 days; D–F: The expression of BDNF pathway after treatment with different concentrations of nicotine for 3 days, including cAMP response element-binding protein (CREB), BDNF and tropomyosin-related kinase B (TrkB); G: BDNF protein expression after treatment with different concentrations of nicotine for 3 days. Mean ± S.D., n = 9 for RT-qPCR, n = 3 for testing BDNF protein expression and the Western blotting results were tested for three times. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the control group.

($p < 0.05$; Fig. 6A), and BDNF expression had a tendency to decrease (Fig. 6B). However, cotreatment with vecuronium bromide and nicotine could reverse the inhibitory effects of nicotine on the expression of CREB and BDNF ($p < 0.05$; Fig. 6A and B). In addition, TrkB expression was also significantly increased after cotreatment with vecuronium bromide and nicotine ($p < 0.05$; Fig. 6C). Compared with that in the control group, GAD67 expression was significantly upregulated after treatment with 10 μM nicotine for 3 days ($p < 0.01$; Fig. 6D). Compared with the 10 μM nicotine-treated group, both cotreatment with vecuronium bromide and nicotine and cotreatment with BDNF and nicotine reversed the upregulation of GAD67 expression caused by nicotine ($p < 0.01$; Fig. 6D). Furthermore, the cotreatment of vecuronium bromide, BDNF and nicotine also reversed the upregulation of GAD67 expression by nicotine ($p < 0.01$; Fig. 6D).

4. Discussion

4.1. The rationale for nicotine dose and concentration setting in this study

Studies have shown that intermittent inhalation of nicotine during smoking increases the nicotine peak at a constant plasma nicotine level (Benowitz et al., 1991), and long-term exposure can cause a relatively long brain residence time of nicotine (Ghosheh et al., 1999). It has also been reported that nicotine can accumulate in the brain as the number of injections increases (Ghosheh et al., 2001). Surprisingly, the mean brain/blood ratio was 4.6 after 10 consecutive injections of nicotine, and 5.2 after continuous infusion of 21 days of nicotine (Ghosheh et al., 2001). The above reports indicate that under long-term nicotine exposure conditions, the brain could have a significant accumulation of nicotine whether it is intermittent or continuous exposure. More importantly, the accumulation of nicotine is more pronounced in the intrauterine fetus as a closed whole, and cytochrome P450 2A6, the main metabolic enzyme of nicotine, is low in the fetal period (Machado Jde et al., 2011). These all determine that the fetal brain would have a higher concentration of nicotine. In addition, experiments have been conducted to imitate the exposure of pregnant women to cigarette smoke by injecting 6.0 mg/kg·d nicotine into pregnant rats (Lichtensteiger et al., 1988; Murrin et al., 1987). According to reports, each regular cigarette contained approximately 10 mg of nicotine (Hadidi and Mohammed, 2004), and the dose-conversion relationship between humans and rats is 1:6.17 (Reagan-Shaw et al., 2008), so we then deduced that the exposure of pregnant rats to nicotine (2.0 mg/kg·d) was equivalent to a 70 kg pregnant woman smoking less than 2.3 cigarettes per day. This is common in our daily life.

While there is a report suggesting that women with severe smoke have plasma nicotine content between 0.3 and 0.6 μM (Alkondon et al.,

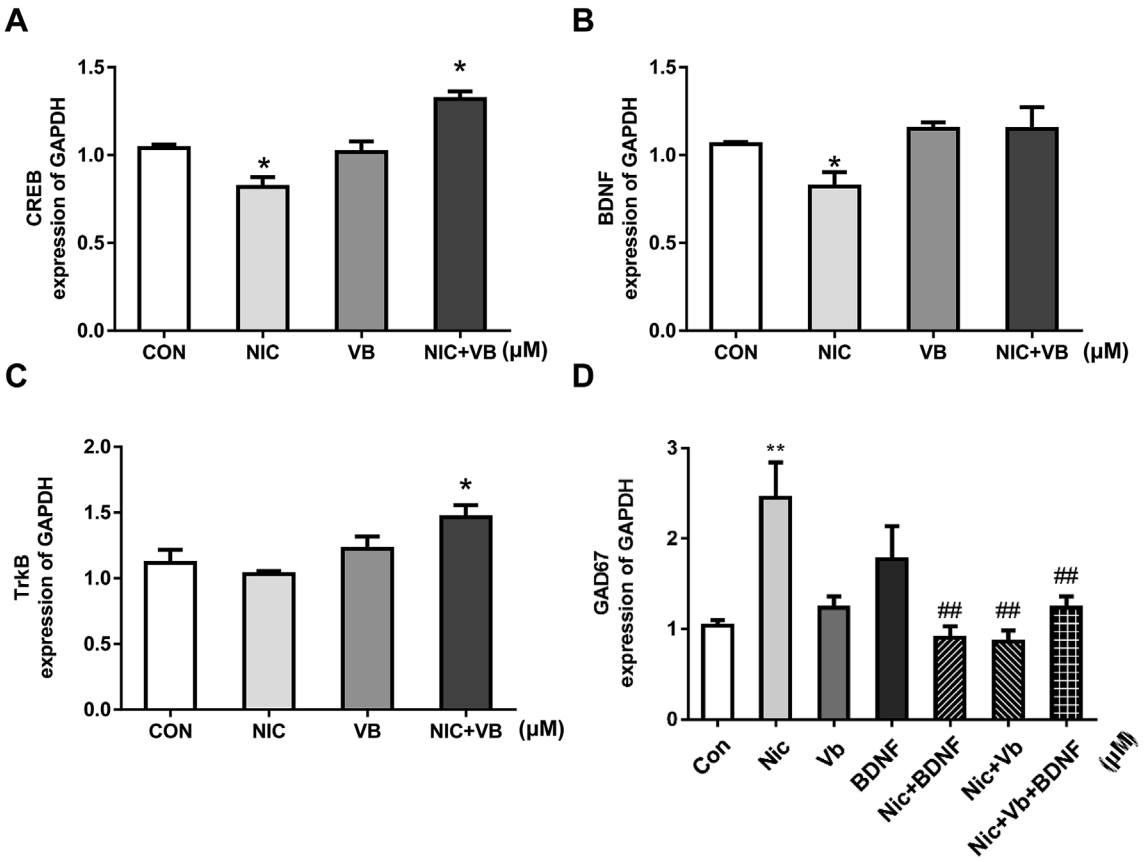

Fig. 6. Effects of vecuronium bromide (a nonspecific inhibitor of nicotinic acetylcholine receptors) and brain-derived neurotrophic factor (BDNF) intervention on BDNF pathway and glutamate decarboxylase 67 (GAD67) expression in nicotine-treated H19–7/IGF1R cells. A–D: The genic expression of BDNF pathway, including cAMP response element-binding protein (CREB), BDNF and tropomyosin-related kinase B (TrkB), and GAD67. A–C: Con: blank control group; Nic: nicotine group (10 μM); Vb: vecuronium bromide group (1 μM); BDNF: BDNF-treated group (10 μg/L); Nic + VB: cotreatment with vecuronium bromide (1 μM) and nicotine (10 μM). D: Con: blank control group; Nic: nicotine group (50 μM); Vb: vecuronium bromide group (5 μM); BDNF: BDNF treated group (10 μg/L); Nic + VB: cotreatment with vecuronium bromide (5 μM) and nicotine (50 μM). Nic + BDNF: cotreatment with nicotine (50 μM) and BDNF (10 μg/L); D: Nic + Vb + BDNF: the cotreatment of vecuronium bromide (5 μM), BDNF (10 μg/L) and nicotine (50 μM) combination group. Mean ± S.D., n = 9. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the control group. $^{\#\#}p < 0.01$ compared with the nicotine group.

2000), human fetus is exposed to higher nicotine concentrations than the smoking mothers (Luck et al., 1985). In the case of PNE, nicotine inhibits the expression of the efflux protein P-glycoprotein in the placenta, resulting in increased sensitivity of the fetus to nicotine (Wang et al., 2009); the brain's nicotine accumulation function and the low expression of the metabolic enzyme cytochrome P450 2A6 also aggravate the accumulation effect of nicotine in the fetal brain (Machado Jde et al., 2011). Moreover, our previous work found that the nicotine concentration in the fetal serum was 3.71 μM after the pregnant rats had been injected with 2.0 mg/kg·d of nicotine (Tie et al., 2016). Although we didn't detect the concentration of nicotine in the fetal brain, we speculate that the concentration of nicotine in the fetal brain is much higher than it in the serum considering the accumulation effect of the fetal brain. To this end, we set the nicotine concentrations in the in vitro experiments of this study to 1, 10, and 100 μM to cover all possible levels of nicotine exposure. Therefore, we believe that the effects of nicotine exposure in this study can provide an important reference for assessing population risk.

4.2. Developmental injury of fetal hippocampus by PNE

The CDK2-CyclinA complex plays an important role in regulating the cell cycle (Coudreuse and Nurse, 2010). Bcl-2 inhibits apoptosis, and Bax is an apoptosis-promoting gene in the Bcl-2 gene family. Overexpression of Bax can antagonize the protective effect of Bcl-2 and cause the apoptosis of cells. Therefore, the proportion of Bax/Bcl-2 is commonly used to reflect the apoptotic state (Haddad, 2007; Raisova et al., 2001). Caspase-3 is the major end-cleaving enzyme during apoptosis (Janicke et al., 1998; Liu et al., 1997) and plays an irreplaceable role in apoptosis. In the *in vivo* experiments of this study, we observed that the fetal hippocampus in the PNE group showed inhibited proliferation and increased apoptosis. Specifically, the expression of Cyclin A and Bcl-2 was decreased, but the expression of Bax and the ratio of Bax/Bcl-2 expression was also increased, and the upregulation of caspase3 expression may also indicate increased apoptosis. *In vitro* experiments also found that H19-7/IGF1R cells treated with different concentrations of nicotine also showed similar inhibition of proliferation and increased apoptosis. In addition, the results of transmission electron microscopy also directly indicated the damage of hippocampus. It is suggested that PNE can lead to inhibition of fetal hippocampal proliferation and increased apoptosis in offspring.

The vertebrate body cells and granulosa cells in the hippocampus express excitatory postsynaptic glutamate receptors highly, including AMPA receptors (AMPARs) and NMDA receptors (NMDARs). Among them, NMDARs include three different subunits, NR1, NR2, and NR3, which play crucial roles in synaptic transmission (Volianskis et al., 2015). Abnormal expression of NMDARs can induce impaired synaptic plasticity in hippocampal neurons. There is a study indicating that NR2A and NR2B subunits can play roles in hippocampal LTP and LTD to affect hippocampal bidirectional synaptic plasticity (Fox et al., 2006). In addition, chronic moderate stress can cause loss of dendritic spines and reduced NMDAR subunits in cultured rat hippocampal CA1

neurons (Pawlak et al., 2005). In the present study, we observed changes in synaptic plasticity gene expression in the hippocampus of PNE fetal rats, meaning decreased expression levels of NR1, NR2A and NR2B. *In vitro* experiments also showed that NR2A and NR2B expression was reduced following treatment with different concentrations of nicotine. It is indicated that PNE can cause alterations to synaptic plasticity in fetal hippocampal neurons.

4.3. Excitotoxicity damage and compensatory upregulation of GAD67 of fetal hippocampus by PNE

Tobacco contains a variety of chemical components and nicotine, which is highly fat-soluble and can penetrate the placenta to enter the fetal bloodstream and brain to accumulate in the fetus, representing a major risk factor that disrupts embryonic development (Dwyer et al., 2008; Huang et al., 2006). The most important excitatory and inhibitory neurotransmitters in the hippocampus are Glu and GABA, respectively. Glu plays a central role in many important processes of the central nervous system. During development, Glu has some protective and nutritive effects, but excessive release of Glu shows a variety of toxic manifestations (Tanaka, 2013). The excitotoxicity of Glu is the ultimate common mechanism leading to acute and chronic neuronal cell death, which causes the development of neuropsychiatric diseases (Parkinson's disease, epilepsy, Alzheimer's disease, etc.) (Li et al., 2004; Mattson, 1996; Romer et al., 2003). In this study, we observed that PNE can result in ultrastructural damage to hippocampal neurons in fetal rats, showing reduced organelles in the cytoplasm of the hippocampus, swelling of the mitochondria and expansion of the endoplasmic reticulum with degranulation. In addition, *in vivo* and in vitro experiments, we also observed the inhibition of proliferation, increased apoptosis, and damage to synaptic plasticity in the hippocampus under nicotine treatment. Moreover, in vitro experiments, after treatment of H19-7/IGF1R cells with different concentrations of nicotine, the content of extracellular fluid Glu increased significantly. These results indicate that PNE may give rise to massive release of Glu in the fetal hippocampus and mediate neuronal excitability.

The hippocampus not only suppresses the HPA axis but also negatively regulates the response of the HPA axis to acute stress (Tasker and Herman, 2011). The medial prefrontal cortex and hippocampal cortex control the HPA axis through GABAergic neuronal populations in the hypothalamus and basal forebrain that inhibit neurons in the PVH-projecting region around the PVH (Bowers et al., 1998; Herman et al., 1996). The PVH-projected GABAergic neurons are sites of stress inhibition (Radley and Sawchenko, 2015). GAD converts Glu to GABA by decarboxylation and is the rate-limiting enzyme for GABA synthesis (Alexopoulos and Dalakas, 2013). GAD has two isozymes in the brain: GAD67 and GAD65. GAD67 has high affinity for Glu and participates in the synthesis and transmission of GABA under pathological conditions (Modi et al., 2015). In addition, fetal hippocampus expresses only GAD67 (Bond et al., 1990). This observation suggests that GAD67 plays a key role in balancing Glu and GABA levels, adjusting signaling output and regulating HPA axis activity in fetal hippocampus (Dent et al., 2007). In vivo experiments in this study showed that the gene and protein expression of GAD67 was notably upregulated in PNE fetal rats. A previous study in our laboratory also found a significant increase in the number of GAD67-labeled GABAergic neurons by immunofluorescence (He et al., 2017). In vitro experiments also revealed that the gene and protein expression of GAD67 were significantly enhanced after 3 days of treatment with different concentrations of nicotine. Therefore, we hypothesize that in the case of fetal rat hippocampal Glu excitatory injury induced by PNE, the compensatory upregulation of GAD67 accelerates the conversion of Glu to GABA, thereby reducing the local Glu content in the hippocampus. However, excessive upregulation of GAD67 may mediate the imbalance of the output of Glu and GABA signals in the fetal hippocampus, resulting in the impairment of negative feedback regulation of hippocampus to PVH. Further, these effects are extended to the postnatal period and ultimately mediate the offspring's HPA axis hypersensitivity.

4.4. Inhibition of the BDNF pathway mediates excitotoxicity in fetal hippocampus by PNE

The nAChRs are ligand-gated ion channel proteins that mediate rapid signaling between synapses, and nicotine is its natural ligand. The nAChRs can be roughly divided into two types, central type and peripheral type, and 16 kinds of nAChR subunits have been confirmed so far. Different combinations of different subunits exhibit different physiological and pharmacological characteristics. The most widely distributed nAChR subunits in the central nervous system are the α4β2 and α7 receptor subtypes (Kishioka et al., 2014). BDNF is a crucial neurotrophic factor in the brain with the highest levels in the hippocampus and cortex. The expression of BDNF is regulated by the upstream signaling factor CREB (Luo et al., 2017). BDNF binding to its receptor TrkB activates multiple intracellular signaling cascades, which promote the development and differentiation of various types of neurons and maintain the growth of nerves. Moreover, BDNF also contributes to the recovery of neurons after injury and the prevention of neuronal degeneration. It has been reported that BDNF can protect hippocampal neurons from the damage of excitotoxicity by activating the extracellular signal-regulated kinase and phosphatidylinositol 3-kinase signaling pathways (Melo et al., 2013). In differentiated astrocytes, BDNF induces the expression of Glutamate transporter-1 and Excitatory Amino Acid Transporter 1. At the same time, the increase in Glu intake reduces the concentration of Glu (Amara and Fontana, 2002; Rodriguez-Kern et al., 2003). Studies have also suggested that topiramate can inhibit glutamate excitotoxicity in hippocampal neurons by activating the BDNF pathway (Mao et al., 2015).

In this study, we found that expression of α4 and α7 nAchR subunits were significantly upregulated in the PNE fetal hippocampus, while CREB and BDNF expression levels were significantly reduced. In vitro experiments showed that the BDNF pathway was markedly decreased after treatment with different concentrations of nicotine for 3 days. However, after administration of vecuronium bromide, a nonspecific antagonist of nAchRs, the downregulation of BDNF pathway was reversed. Under the condition that BDNF was given as compensation, the expression of GAD67 expression was also reversed. These results implied that PNE can induce excitotoxicity by inhibiting the BDNF pathway through activating nAChRs in the fetal hippocampus.

5. Conclusions

In summary, PNE can result in the inhibition of the BDNF pathway in the fetal hippocampus, which boosts glutamate excitotoxicity, and thus, the compensatory upregulation of GAD67 leads to an imbalance in the output of hippocampal excitatory and inhibitory signals, ultimately mediating the high sensitivity of the HPA axis in the offspring.

Declarations

The authors declare that they have no competing interests as defined by Food and Chemical Toxicology or other interests that might be perceived to influence the results and discussion reported in this paper. The authors have nothing to disclose.

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Appendix A. Supplementary data

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