RESEARCH PAPER

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Prenatal ethanol intoxication and maternal intubation stress alter cell survival and apoptosis in the postnatal development of rat hippocampus

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It is well known that the fetal ethanol exposure and prenatal stress may have adverse effects on brain development. Interestingly, some morphological and functional recovery from their teratogenic effects that take place during brain maturation. However, mechanisms that underlie this recovery are not fully elucidated. The aim of this study was to examine whether the postnatal attenuation of fetal alcohol – and maternal stress-induced morphological and functional deficits correlates with compensatory changes in the expression/activation of the brain proteins involved in inflammation, cell survival and apoptosis. In this project, we investigated the hippocampus which belongs to the brain regions most susceptible to the adverse effects of prenatal ethanol exposure. Pregnant rat dams were administered ethanol (A) or isocaloric glucose solution (IC) by a gastric intubation during gestational days 7-20. The pure control group received *ad libitum* laboratory chow and water with no other treatment. The hippocampi of fetal-ethanol and control pups were examined at the postnatal day (PD)1, PD10, PD30 and PD60. Moderate fetal-ethanol exposure and prenatal intubation stress caused a significant increase in molecular factors relating to inflammation (iNOS) and cell survival/apoptosis pathways (PTEN, GSK-3 and ERK) at birth, with a rapid compensation from these developmental deficits upon removal of alcohol at PD10. Indeed, an increase in ERK1/2 and JNK1/2 activation at PD30 was observed with ethanol consumption. It indicates that the recovery process in A and IC brains started soon after the birth upon the ethanol and stressor withdrawal and continued until the adulthood.

Key words: fetal ethanol, maternal stress, hippocampal development, cell survival, apoptosis, inflammation

INTRODUCTION

Ethyl alcohol (ethanol) is a well-known teratogen that impairs brain development (Ward and Wainwright, 1989; Miller and Montgomery, 1992; Michaelis and Michaelis, 1994). The cells in the central nervous system are highly vulnerable to adverse effects of ethanol and fetal ethanol exposure may cause a massive wave of apoptotic neurodegeneration in many different regions of the developing human brain. (Ikonomidou et al., 2000; Light et al., 2002; Goodlett et al., 2005). The neocortex, hippocampus and cerebellum are especially sensitive to ethanol exposure (Tran and Kelly, 2003).

The ethanol-associated neuronal damage which is initiated by activation of the mitochondrial apoptotic pathway (Cartwright et al., 1998; Ikonomidou et al., 2000; Light et al., 2002; Ramachandran et al., 2003; Dikranian et al., 2005) may be related to several molecular mechanisms including induction of oxidative stress, activation of caspase-3, functional loss of neurotrophic factors and inhibition of survival factors (Kotch et al., 1995, Goodlett et al., 2005, Young et al., 2005, Ieraci and Herrera, 2007). Furthermore, in the developing brain, NMDA and $GABA_A$

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receptors play an important role in the appearance of the toxic effects of ethanol via apoptotic neurodegeneration (Costa et al., 2000; Hsiao et al., 2002; Nixon et al., 2004). Prenatal ethanol consumption reduces the concentration of brain-derived neurotrophic factor (BDNF) and alters its receptor, TrkB, which attenuates the function of intracellular cell survival signaling pathways such as MAPK/ERK and PI3-K/Akt (Guerri, 2002). Additionally, ethanol-related conditions might change the ratio of pro-apoptotic and anti-apoptotic proteins in a manner favoring cell death and make the developing brain more prone to neurodegeneration (de la Monte et al., 2000; Olney et al., 2002; Heaton et al., 2003).

In our previous investigation (Elibol-Can et al., 2014a), we observed behavioral deficits in fetal-ethanol juveniles (postnatal day (PD) 30) when compared to age-matched controls and to fetal-ethanol adult subjects (PD60), suggesting a functional recovery occurring with maturation in fetal-alcohol rats. Also, in this previous study, after a moderate prenatal intoxication with ethanol, a marginally lower number of granule cells was estimated in dentate gyrus (DG) on PD30, which correlated with a trend towards a lower number of DCX-immunoreactive neurons in the subgranular zone at PD10. Additionally, these changes were ameliorated in the adult subjects by the age of PD60. At the morphological level, moderate levels of fetal ethanol and the maternal intubation stress during gestation, resulted in a smaller soma size in granule cells, reduced dendritic parameters and lower spine density in pyramidal neurons which however was recorded at birth only with full recovery from these effects within the first 10 postnatal days (Jakubowska-Doğru et al., 2017). Our results suggest a fetal ethanol- and/or maternal stress- induced developmental delay rather than a permanent damage. Therefore, the results directed us toward the investigation of the regenerative capacity of young brain, specifically regarding protein changes related to cell survival and apoptosis. In addition, we intended to look deeper into the potential molecular correlates of developmental deficits and/or delays and potential compensatory processes after fetal ethanol exposure. To do so in the rat model, we examined hippocampal expression and activation of selected proteins related to cell survival, apoptosis and inflammation, which lack through investigation as related to prenatal ethanol exposure, over a protracted developmental period from birth to the adulthood, for the first time.

METHODS

Subjects

Adult (3-4 months old), naive, female (n=25) and male (n=15) Wistar rats, obtained from the Animal Breeding Facility of the Gülhane Military Medical Academy (Ankara), were initially used in the present study. The animals' care conditions during breeding and rearing were the same as in our previous studies (Dursun et al., 2006; Elibol-Can et al., 2014a, 2014b; Jakubowska-Doğru et al., 2017). Rats were mated and appearance of a vaginal plug was considered evidence of a successful fertilization. This day was marked as the gestational day 0 (GD0). On GD7, pregnant dams were assigned (counterbalanced for initial body mass) to one of three treatment groups: alcohol (A) group (n=10), intubated control (IC) group (n=8) and intact control (C) group (n=7). All experimental procedures were approved by the Committee for Animal Research Ethics in Middle East Technical University and all applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Ethanol Treatment

The dams from the ethanol-exposed (A group) were administered ethanol (96.5% v/v, Merck) at 6 g/kg of body mass, daily from GD7 to GD20 (Dursun et al., 2006; Elibol-Can et al., 2014a). Ethanol was diluted in 2-3 ml of water according to body mass and administered in two equal doses one-hour apart by intragastric intubations using a stainless curved feeding needle (18 ga, 3 in; Stoelting Co., Wood Dale, IL). The IC group received the same volume of fluid with sucrose substituted isocalorically for ethanol. In addition, to minimize differences in the food intake between the ethanol exposed group and control group, we provided the same amount of food to the IC group, which was calculated by determining the weight of food consumed on the previous day by group A females. The C group received *ad libitum* laboratory chow and water with no additional treatment. The protocol for ethanol treatment was described previously (Dursun et al., 2006). On GD20, the mean blood ethanol concentration in the pregnant rats from the A group which was estimated 3h after the second intubation by gas chromatography-mass spectrometry (GC-MS) was 246.6±40.9 mg/dl.

Pups

Male pups from each treatment group were randomly assigned to four age subgroups (n=7 for each treatment group) and euthanized at PD1 (n=21), PD10 (n=21), PD30 (n=21) and PD60 (n=21). The rats from each age group were intermixed between litters by assigning one or two pups from a single litter to each of the age groups in order to limit the effect of litter differences.

Western blotting

The rats were euthanized by decapitation and their right hippocampi were dissected. Protein extraction was performed by homogenizing the hippocampi with lysis buffer in the presence of protease and phosphatase inhibitors. Tissue samples were centrifuged at 14000 rpm for 20 min at 4^{0}C, and then supernatants were collected. After that, the hippocampal tissues were pooled by taking equal amounts of supernatant from each animal in the same group (Kilic et al., 2017). Protein concentrations were determined by a BCA assay kit in the Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific; Paisley, England). Sample proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Biorad; Hercules, USA) and subsequently transferred to polyvinyl fluoride (PVDF) membranes. Then, blots on the membrane were washed with Tris buffered saline and incubated with blocking solution (2% Blocking Reagent in Tris Buffered Saline) for 1 h at room temperature. Afterwards, the blots were incubated with primary antibodies; nNOS, iNOS, Hsp70, mTOR, Bax, Bcl2, Bcl-XL, PARP, PTEN, AKT, GSK3, ERK, JNK, and CREB overnight at 4°C and on the following day, with secondary antibodies (1:5000, HRP-linked anti-rabbit or anti-mouse IgG, Cell Signaling; Danvers, USA) for 1 h at room temperature. Blots were incubated with substrate solution by chemiluminescence labeling for visualization. Protein loading was controlled using β-tubulin antibody. At least three independent blots were performed to measure the expression of each protein. Immunoreactive protein bands were quantified densitometrically using ImageJ analysis system (NIH; Washington, USA).

Statistical analysis

Group means ± SEM were calculated for all of the measurements. A repeated-measures analysis of variance (ANOVA) was conducted on dam body mass data for GD7–20. Additionally, between-group differences in pup body mass were analyzed by one-way ANOVA using treatment as an independent factor for each age. Two-way ANOVA (treatment X age) was conducted to evaluate the main effects of age and treatment as well as age X treatment interaction. Following two-way ANOVA, the *post hoc* comparisons for multiple testing were conducted using Fisher's Least Significant Difference (LSD) test. The SPSS 15 statistical package was used for statistical analysis of the data. The criterion of statistical significance was $P \leq 0.05$.

RESULTS

In all experimental groups, an increase in dam body mass was observed throughout the gestational period. The repeated measure ANOVA showed a highly significant day effect ($F_{(10;140)}$=50.805, $P \leq 0.001$) and insignificant treatment X day interaction ($F_{(20;140)}$=1.234, P=0.236). There was no significant difference in dam's body mass between groups except for GD7 and GD8 (Fig. 1A). Gestational exposure to ethanol decreased the body mass of pups at birth ($F_{(2;24)}$=56.332, $P \leq 0.001$) and PD10 ($F_{(2;25)}$=31.602, $P \leq 0.001$). Pups in the IC group also had lower body mass at PD1 and PD10 ($P \leq 0.05$). This difference in the body masses disappeared at PD30 (Fig. 1B).

The present study investigated the effects of prenatal ethanol exposure on levels of proteins related to inflammation (iNOS, nNOS and Hsp70), apoptosis

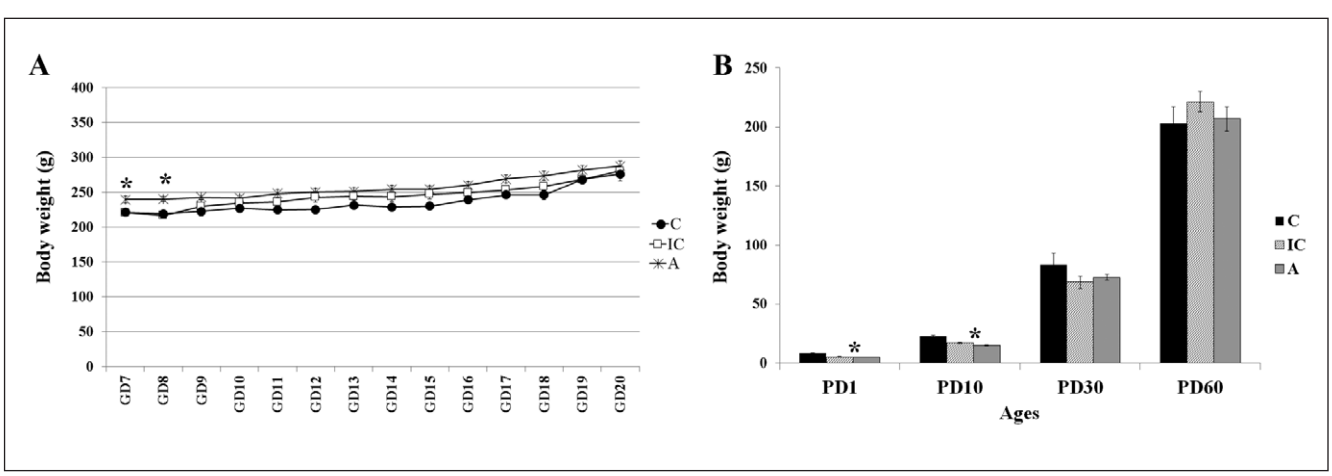

Fig. 1. Changes in the mean body masses of dams throughout the GD7-20 (A) and their offsprings at different postnatal ages (B) from C group, IC group and A group. The degree of significance was denoted as * for P≤0.05. Error bars indicate standard error of mean (SEM).

(Bcl2, Bax, Bcl-XL, and PARP) and cell survival (mTOR, PTEN, JNK, ERK, GSK, CREB and AKT).

In all treatment groups, two-way ANOVA (treatment X age) for iNOS protein levels showed a significant main effect of age ($F_{(3;35)}$=73.320, P<0.001) and a marginally significant main effect of treatment ($F_{(2;35)}$=3.150, P=0.061) with a significant age X treatment interaction ($F_{(6;35)}$=16.047, P<0.001). *Post hoc* analysis confirmed a significant increase in iNOS expression between PD10–PD30 (P<0.001) and a significant decrease between PD30-PD60 (P≤0.001) during normal development (Table I). At PD1, the level of iNOS protein was higher in the A group compared to control groups (P=0.030 for IC and P=0.008 for C) and, in both intubated groups (A and IC), it was lower at PD30 (P≤0.001) and higher at PD60 (P≤0.01) compared to C group (Fig. 2A).

In all three treatment groups, the results of two-way ANOVA for nNOS expression confirmed a significant main effect of age ($F_{(3;35)}$=32.615, P<0.001) with insignificant main effect of treatment and age X treatment interaction. According to *post hoc* analysis, there was an increase in nNOS levels during the PD30-PD60 period in all groups (P<0.01) (Table I). At PD30, nNOS expression was significantly higher in the IC group compared to the C group (P=0.028) and the A group (P=0.023) (Fig. 2B).

As estimated by a two-way ANOVA, the main effect of age ($F_{(3;35)}$=215.362, P<0.001), the main effect of treatment ($F_{(2;35)}$=2.944, P=0.072) and an age X treatment interaction ($F_{(6;35)}$=6.866, P<0.001) were also significant for Hsp70 expression. *Post hoc* analysis showed that, in all groups, it was observed a significant increase between PD10–PD30 (P≤0.001) and a significant decrease between PD30-PD60 (P≤0.01) in the hippocampal Hsp70 concentration (Table I). A significant treatment effect was observed at PD10 and at PD30. At PD10, the level of Hsp70 protein was higher in the IC group as compared to both A group (P=0.038) and C group (P=0.008). At PD30, the level of Hsp70 was significantly higher in the A group compared to both control groups (P<0.001 for IC and P=0.007 for C). These differences disappeared at PD60 (Fig. 2C).

The expression level of phosphorylated mTOR was presented in Fig. 2D. Two-way ANOVA confirmed

Table I. Age-related differences in the expression of proteins.

	Control (C) Group			Intubation Control (IC) Group			Alcohol (A) Group		
	PD1-PD10	PD10-PD30	PD30-PD60	PD1-PD10	PD10-PD30	PD30-PD60	PD1-PD10	PD10-PD30	PD30-PD60
iNOS	p=0.750	***p<0.001	***p<0.001	p=0.500	***p<0.001	p=0.194	**p=0.004	***p<0.001	p=0.019
nNOS	p=0.270	p=0.252	**p=0.004	p=0.575	**p=0.010	**p=0.013	p=0.735	p=0.316	***p<0.001
HSP70	p=0.403	***p<0.001	***p<0.001	*p=0.016	***p<0.001	**p=0.010	p=0.788	***p<0.001	***p<0.001
mTOR	p=0.192	***p<0.001	***p<0.001	***p<0.001	***p<0.001	***p<0.001	p=0.207	***p<0.001	***p<0.001
Bax	***p<0.001	p=0.593	*p=0.038	p=0.923	***p=0.001	p=0.307	p=0.983	p=0.722	p=0.101
Bcl-2	p=0.593	***p<0.001	*p=0.030	p=0.874	**p=0.002	**p=0.005	p=0.943	**p=0.004	*p=0.014
Bax/Bcl-2	p=0.298	***p<0.001	p=0.273	p=0.512	***p<0.001	p=0.408	p=0.520	***p<0.001	p=0.195
Bcl-XL	*p=0.011	***p<0.001	***p<0.001	***p<0.001	***p<0.001	p=0.869	**p=0.004	***p<0.001	***p<0.001
PARP	***p<0.001	***p<0.001	**p=0.005	***p=0.001	p=0.777	p=0.436	p=0.120	p=0.202	p=0.057
PTEN	***p<0.001	***p<0.001	p=0.355	***p<0.001	***p<0.001	p=0.908	***p<0.001	***p<0.001	**p=0.004
AKT	***p<0.001	p=0.509	***p<0.001	***p<0.001	**p=0.006	***p<0.001	**p=0.001	p=0.164	***p<0.001
GSK-3α	**p=0.010	***p<0.001	***p<0.001	*p=0.011	**p=0.005	***p<0.001	***p<0.001	***p<0.001	***p<0.001
GSK-3β	p=0.686	***p<0.001	***p<0.001	p=0.675	p=0.173	p=0.067	***p<0.001	***p=0.001	**p=0.006
ERK-1	p=0.950	p=0.053	*p=0.014	p=0.774	p=0.524	p=0.830	*p=0.025	***p<0.001	***p<0.001
ERK2	p=0.145	***p<0.001	***p<0.001	***p<0.001	**p=0.002	**p=0.007	***p<0.001	***p<0.001	***p<0.001
JNK-1	***p<0.001	*p=0.019	p=0.663	***p<0.001	p=0.223	p=0.518	***p<0.001	***p<0.001	p=0.170
JNK-2	p=0.723	***p<0.001	***p<0.001	***p<0.001	***p<0.001	p=0.550	*p=0.037	***p<0.001	***p<0.001

p-values refer to the difference between two consecutive age groups and are estimated by one-way ANOVA followed by *post hoc* LSD test; *** for p≤0.001, ** for p≤0.01, and * for p≤0.05.

a significant main effect of age on mTOR expression ($F_{(3;35)}$=122.104, P<0.001), a significant main effect of treatment ($F_{(2;35)}$= 31.562, P<0.001) and a significant age X treatment interaction ($F_{(6;35)}$=13.228, P<0.001). According to the *post hoc* analysis, a significant increase was observed in hippocampal mTOR concentration in all groups between the PD10–PD30 period, following a significant decrease in expression between PD30-PD60 (Table I). In the intubated groups, mTOR expression was lower on PD1 compared to the C group (P<0.001). At PD10 and PD30, it was still significantly lower in A group at PD10 and PD30 compared to both control groups (P<0.001). At PD60, the expression level of mTOR was higher in intubated groups compared to the intact control group, but was significant only for IC (P<0.001) (Fig. 2D).

In order to investigate the effect of prenatal ethanol intoxication and maternal stress on the Bcl-2 family in rat hippocampus, we measured the expression of Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) proteins and evaluated between-group differences and age-dependent changes in the Bax/Bcl-2 ratio (Fig. 3A, B and C, respectively). A significant main effect of age ($F_{(3;35)}$=17.005, P<0.001) and an age X treatment interaction ($F_{(6;35)}$=5.142, P=0.002) with an insignificant treatment effect ($F_{(2;35)}$=1.473, P=0.249) were revealed by

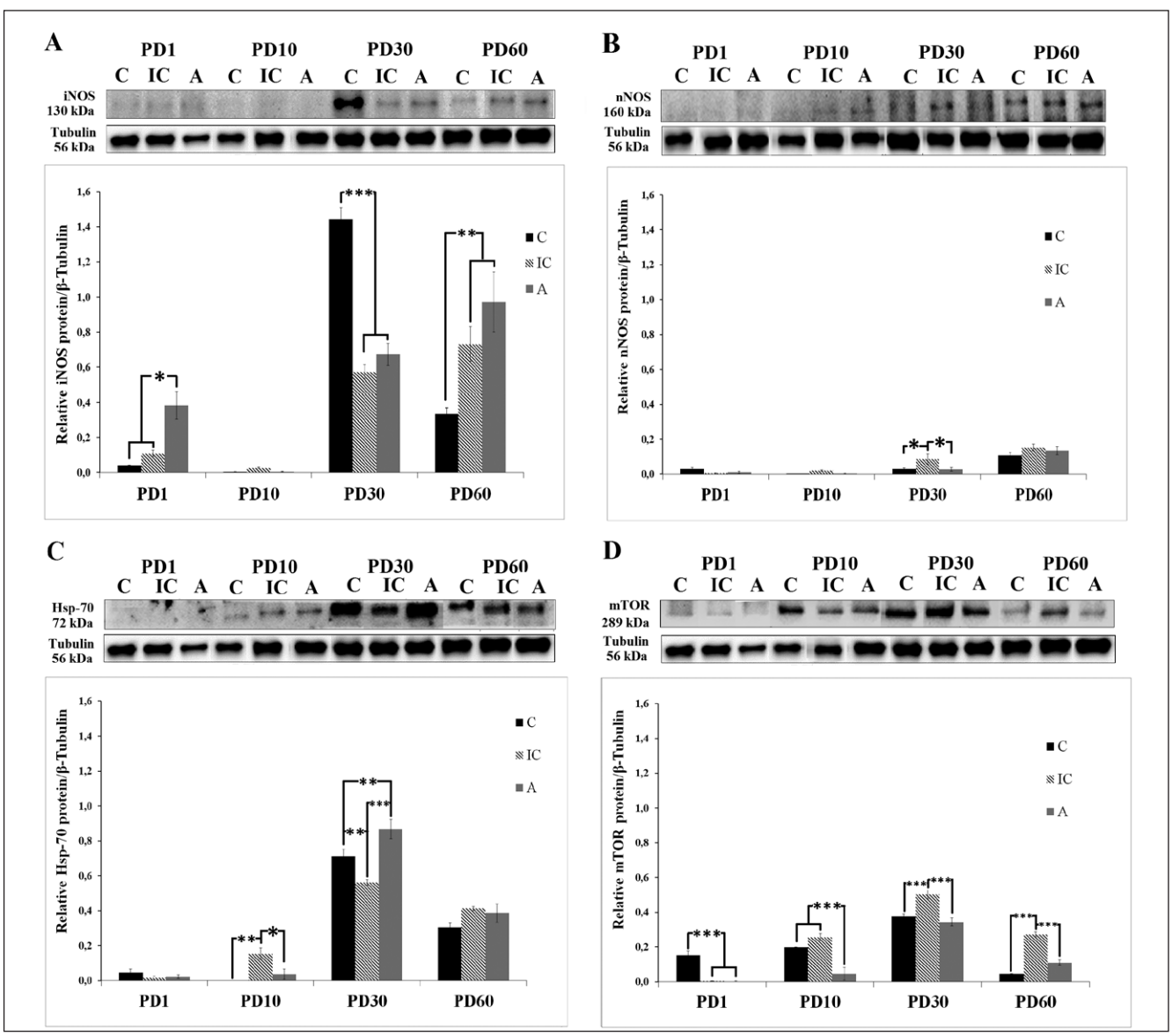

Fig. 2. Prenatal ethanol consumption has an effect on neuroinflammation. Relative amounts of iNOS (A), nNOS (B), Hsp70 (C), and mTOR (D) proteins analyzed by western blot. Normalization for proteins was made with tubulin. The degree of significance was denoted as * for P≤0.05, ** P≤0.01, *** P≤0.001. Error bars indicate standard error of mean (SEM).

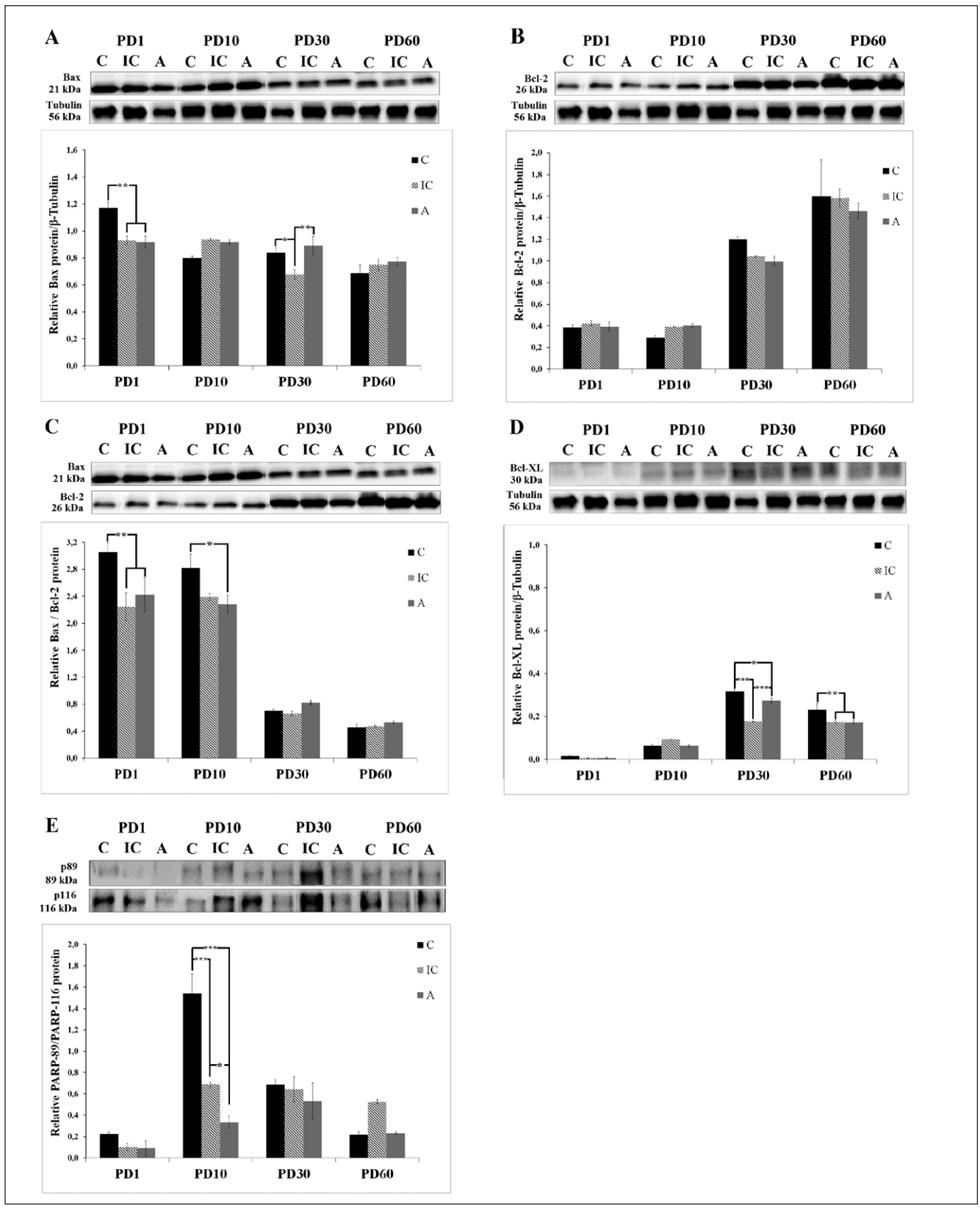

Fig. 3. Prenatal ethanol consumption affects apoptosis at adult age. Relative amounts of Bax (A), Bcl-2 (B), the ratio of Bax to Bcl-2 (C), Bcl-XL (D), and the ratio of cleaved PARP89 to the full-lenght PARP116 (E) proteins analyzed by western blot. Normalization for proteins was made with tubulin. The degree of significance was denoted as * for P≤0.05, ** P≤0.01, *** P≤0.001. Error bars indicate standard error of mean (SEM).

two-way ANOVA for Bax expression. The two-way ANOVA for Bcl-2 expression also revealed a significant main effect of age ($F_{(3;35)}$=60.541, P<0.001), while treatment effect and age X treatment interaction were rendered insignificant ($F_{(2;35)}$=0.223, P=0.802 and $F_{(6;35)}$=0.374, P=0.888, respectively). Two-way ANOVA for the Bax/Bcl-2 ratio revealed significant age ($F_{(3;35)}$=154.363, P<0.001) and treatment ($F_{(2;35)}$=4.516, P=0.022) effects with an insignificant age X treatment interaction. According to the pairwise comparison with *post hoc* LSD test, the expression of Bax significantly decreased between PD1-PD10 in the C group (P=0.001) and between PD10-PD30 in the IC group (P=0.001) with no age-dependent changes in the A group (Table I). In contrast to this, the expression of Bcl-2 significantly increased between PD10-PD30 and PD30-PD60 in all groups (P<0.001) (Table I). However, the expression of Bax in the A group did not change throughout the entire developmental period (Table I). Also, the Bax/Bcl-2 ratio significantly decreased between PD10-PD30 (P<0.001) in all groups. While there was no significant between-group difference in Bcl-2 expression, the expressions of both Bax and the Bax/

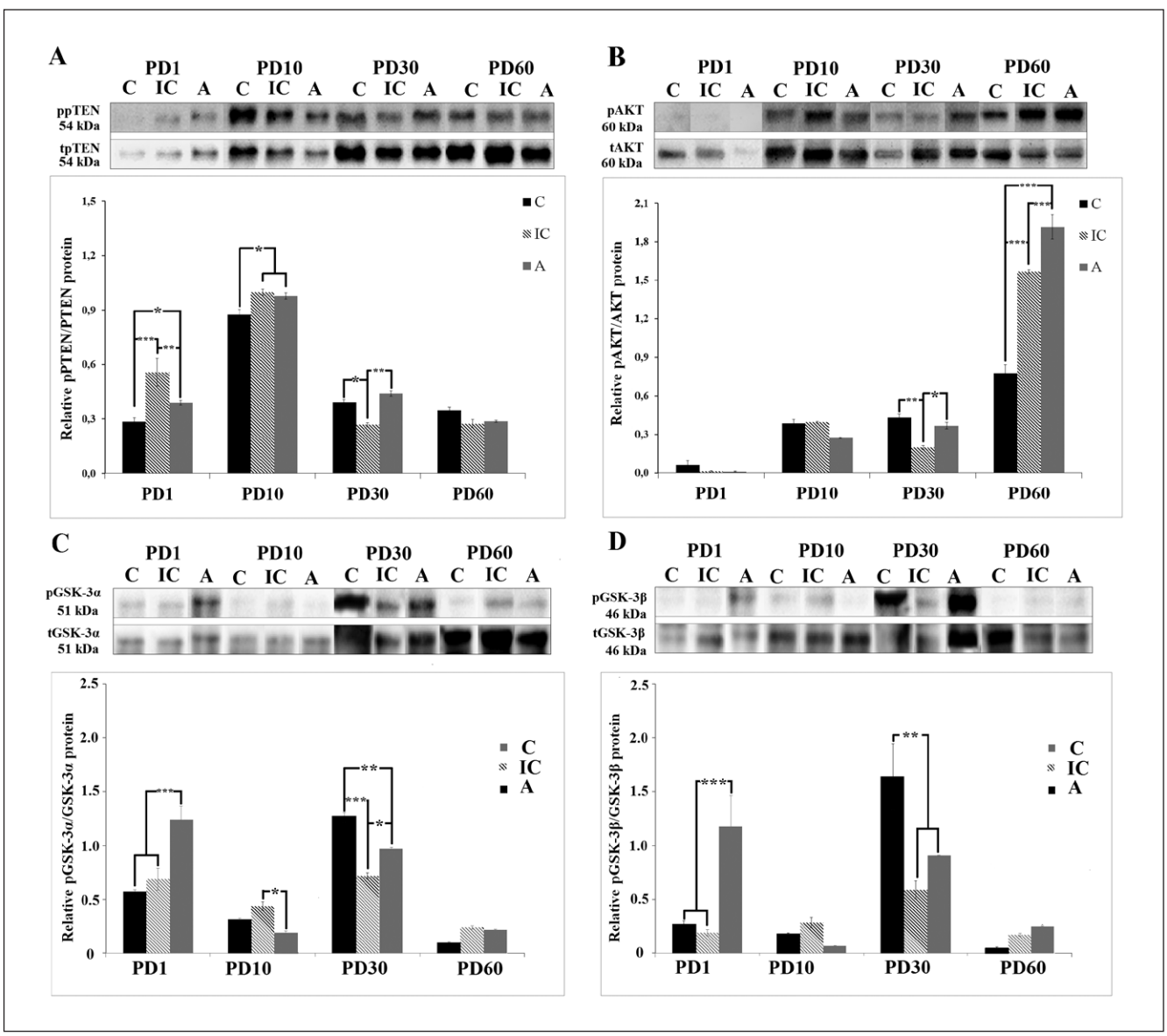

Fig. 4. Prenatal ethanol consumption affects cell survival through AKT pathway at birth. Relative amounts of (A) the ratio of phosphorylated PTEN to the total PTEN (pPTEN/PTEN), (B) the ratio of phosphorylated AKT to the total AKT (pAKT/AKT), (C) the ratio of phosphorylated GSK-3α to the total GSK-3α (pGSK-3α /GSK-3α), (D) the ratio of phosphorylated GSK-3β to the total GSK-3β (pGSK-3β/GSK-3β) proteins analyzed by western blot. Normalization for proteins was made with tubulin. The degree of significance was denoted as * for P≤0.05, ** P≤0.01, *** P≤0.001. Error bars indicate standard error of mean (SEM).

Bcl-2 ratio were lower at PD1 in the IC and A groups compared to C group ($P \leq 0.05$) (Fig. 3A and C). The Bax/Bcl-2 ratio continued to be significantly lower in the A group compared to the C group at PD10 ($P=0.023$) (Fig. 3C). In addition, another Bcl-2 family member, Bcl-XL, also showed similar changes to Bcl-2 during the study period, with a significant age effect ($F_{(3;35)}=244.785$, $P<0.001$), treatment ($F_{(2;35)}=13.220$, $P<0.001$) and age X treatment interaction ($F_{(6;35)}=10.254$, $P<0.001$). *Post hoc* analysis showed that there was a significantly higher expression of Bcl-XL protein in C group compared to both alcohol ($P=0.004$) and IC groups at PD30 and at PD60 ($P<0.001$) (Fig. 3D).

For the observation of cellular component disassembly, we also investigated the cleavage of PARP (the ratio of p89/p116), which serves as a marker of cells undergoing apoptosis (Fig. 3E). The two-way ANOVA for PARP expression demonstrated a significant main effect of age ($F_{(3;35)}=24.438$, $P<0.001$), a significant main effect of treatment ($F_{(2;35)}=12.075$, $P<0.001$) and a significant age X treatment interaction ($F_{(6;35)}=8.546$, $P<0.001$). According to *post hoc* analysis, the ratio of cleaved PARP (p89) to

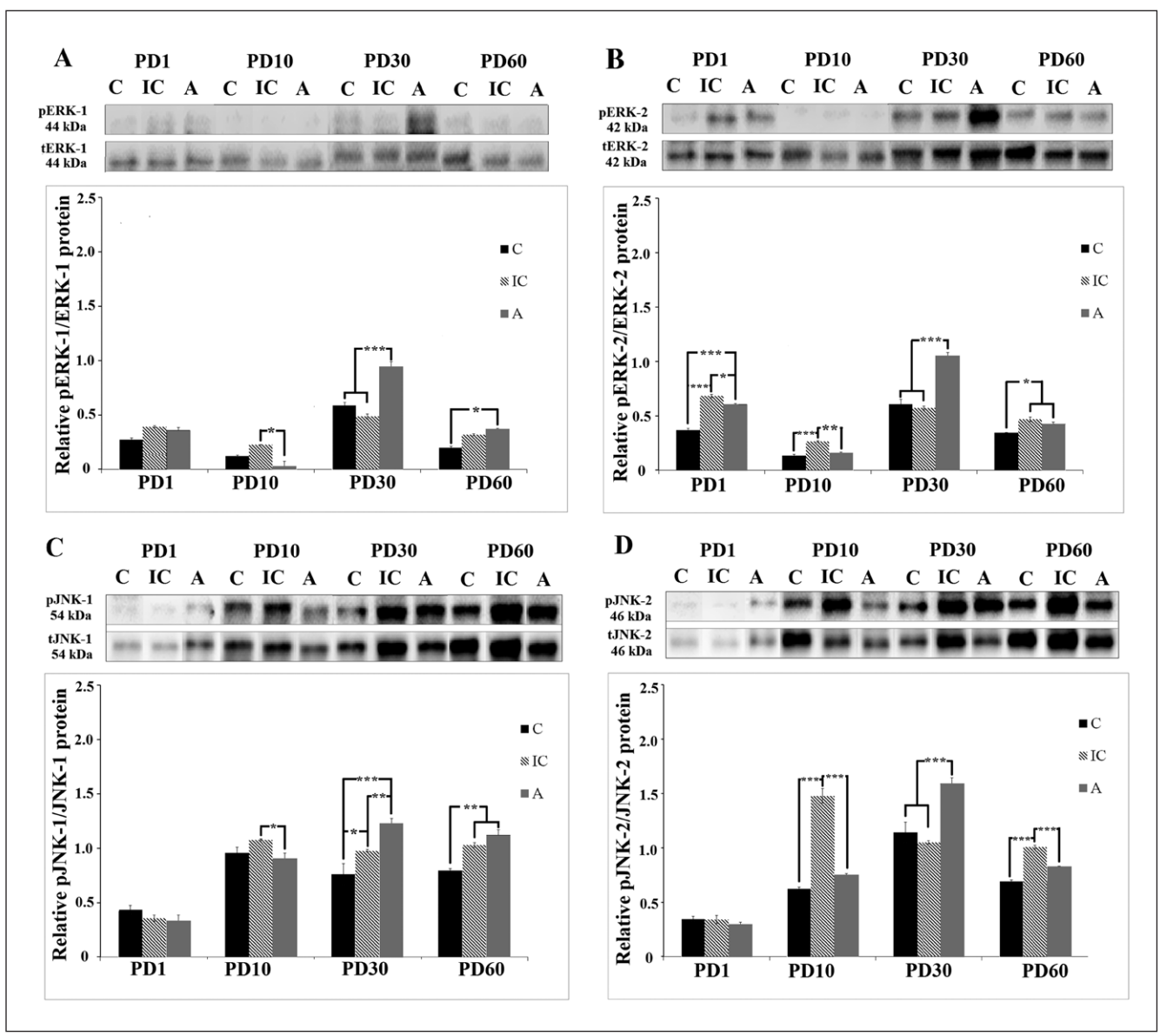

Fig. 5. Prenatal ethanol consumption affects cell survival through ERK7JNK pathway at juvenile age. Relative amounts of (A) the ratio of phosphorylated ERK1 to the total ERK1 (pERK1/ERK1), (B) the ratio of phosphorylated ERK2 to the total ERK2 (pERK2/ERK2), (C) the ratio of phosphorylated JNK1 to the total JNK1 (pJNK1/JNK1), (D) the ratio of phosphorylated JNK2 to the total JNK2 (pJNK2/JNK2), (E) the ratio of phosphorylated CREB to the total CREB (pCREB/CREB) proteins analyzed by western blot. Normalization for proteins was made with tubulin. The degree of significance was denoted as * for P≤0.05, ** P≤0.01, *** P≤0.001. Error bars indicate standard error of mean (SEM).

full length PARP (p116) significantly increased during the PD1-PD10 period ($P \leq 0.001$) and decreased during the PD10-PD30 ($P \leq 0.001$) and PD30-PD60 ($P=0.005$) periods in the C group, while no significant change was noted in the A group throughout the entire 2-months postnatal period (Table I). At PD10, the cleavage of PARP was significantly lower in the intubated groups compared to the C group ($P \leq 0.05$) (Fig. 3E).

A two-way ANOVA done for the ratio of the phosphorylated to the total PTEN revealed a significant main effect of age ($F_{(3;35)}=228.463$, $P \leq 0.001$) and age X treatment interaction ($F_{(6;35)}=8.309$, $P<0.001$) while the main effect of treatment remained insignificant ($F_{(2;35)}=2.767$, $P=0.083$) (Fig. 4A). In all groups, activation of PTEN increased between PD1-PD10, to decrease in the following period between PD10-PD30 ($P \leq 0.001$) (Table I). However, in the A group, a significant decrease in pPTEN/PTEN ratio continued also between PD30-PD60 ($P=0.004$). In the intubated A and IC groups, the activation of PTEN was significantly higher at PD1 ($P=0.044$ and $P \leq 0.001$, respectively) and at PD10 ($P=0.044$ and $P=0.017$, respectively) compared to control group. At PD30, the activation of PTEN was significantly lower in the IC group than in both C and A groups (Fig. 4A).

In addition, two-way ANOVA analysis evaluating between-group differences in phosphorylated AKT to total AKT ratio, a protein that is related to cell survival, showed a significant age effect ($F_{(3;35)}=511.570$, $P \leq 0.001$), treatment effect ($F_{(2;35)}=23.460$, $P<0.001$) and age X treatment interaction ($F_{(6;35)}=47.318$, $P<0.001$). As seen from Fig.4B and Table I, in all three groups, the ratio of pAKT/AKT showed an increase between the PD1-PD10 and PD30-PD60 periods reaching the highest value at PD60. According to *post hoc* analysis, the value of pAKT/AKT ratio was significantly higher in C group compared to A and IC groups at PD30, while at PD60, it became significantly higher in the A and IC groups than in the C group ($P \leq 0.01$) (Fig. 4B).

Next, we found a significant main effect of age for phosphorylated GSK-3α/total GSK-3α ($F_{(3;35)}=110.897$, $P \leq 0.001$) and for phosphorylated GSK-3β/total GSK-3β ($F_{(3;35)}=21.966$, $P \leq 0.001$). For both ratios, there were also a significant main effect of treatment ($F_{(2;35)}=4.689$, $P=0.019$, and $F_{(2;35)}=3.959$, $P=0.033$, respectively) and a age X treatment interaction ($F_{(6;35)}=16.759$, $P<0.001$, and $F_{(6;35)}=7.291$, $P<0.001$, respectively). In all groups, the activation of GSK-3α significantly decreased between the PD1-PD10 and PD30-PD60 periods and increased between the PD10-PD30 period ($P \leq 0.05$) (Table I). Activation of the β subunit of GSK-3 also significantly increased between the PD10-PD30 period and decreased between the PD30-PD60 period ($P \leq 0.05$). In the A group, the expression of both GSK-3α and GSK-3β was significantly higher at PD1 compared to both control groups ($P \leq 0.001$), and expression of GSK-3β in the A group was significantly lower at PD10 compared to IC group ($P=0.012$) (Fig. 4C and D). At PD30, a significant decrease in the expression of both GSK-3α and GSK-3β was observed in the intubated groups as compared to the C group ($P \leq 0.05$). No significant between-group differences were observed at PD60 for either GSK-3α or GSK-3β.

In the present study, the extracellular signal-regulated kinase (ERK) family members ERK1 and ERK2 underwent similar changes with age. A two-way ANOVA applied to these data revealed in both cases a significant effect of age ($F_{(3;35)}=10.792$, $P \leq 0.001$ and $F_{(3;35)}=136.315$, $P \leq 0.001$, respectively), significant treatment effect ($F_{(2;35)}=5.705$, $P=0.009$, and $F_{(2;35)}=64.916$, $P \leq 0.001$, respectively) and significant age X treatment interaction ($F_{(6;35)}=6.930$, $P<0.001$, and $F_{(6;35)}=35.044$, $P<0.001$, respectively) (Fig. 5A and 5B). *Post hoc* analysis confirmed a significant decrease in the activation of ERK1/2 between PD30-PD60 in the C group ($P=0.014$ and $P \leq 0.001$, respectively). However, ethanol treatment significantly changed the activation pattern of ERK1/2 during all studied time periods (Table I). At PD1, the phosphorylation ratio of ERK2 was significantly higher in intubated groups compared to control group ($P \leq 0.001$). In the A group, the ratio of pERK1/2 to total ERK1/2 was significantly higher at PD30 compared to both control groups ($P \leq 0.001$) and at PD60 compared to the C group (Fig. 5A and B).

Fig. 5C and 5D show changes in the phosphorylation ratio of c-Jun N-terminal kinase (JNK)1 and JNK2 in all three groups, throughout the two postnatal months. A two-way ANOVA analysis of pJNK1/JNK1 and pJNK2/JNK2 ratios confirmed for both proteins a significant age effect ($F_{(3;35)}=66.282$, $P \leq 0.001$ and $F_{(3;35)}=86.162$, $P \leq 0.001$, respectively), treatment effect ($F_{(2;35)}=9.132$, $P=0.001$, and $F_{(2;35)}=30.631$, $P \leq 0.001$, respectively) and age X treatment interaction ($F_{(6;35)}=7.113$, $P<0.001$, and $F_{(6;35)}=34.550$, $P<0.001$, respectively). *Post hoc* analysis revealed a significant increase in the activation of JNK1 between PD1-PD10 and in the activation of JNK2 between PD10-PD30 in C group ($P \leq 0.001$) which was followed by a decrease in the relative phosphorylation of JNK2 but not JNK1 in this group (Table I). Nevertheless, on PD30 and PD60, activation of JNK1 was significantly lower in the intact control (C group) compared to both IC and A groups ($P \leq 0.05$). However, in the A group, the ratios of phosphorylation of JNK1/2 significantly decreased compared to the IC group at PD10 ($P=0.043$ and $P \leq 0.001$, respectively). Relative phosphorylation of JNK2 was significantly higher in IC on PD10 and PD60 compared to both C and A groups ($P \leq 0.001$) and in A group on PD30 compared to both C and IC groups ($P \leq 0.001$). (Fig. 5C and D).

DISCUSSION

According to the previous studies by us and other authors, the adverse effects of fetal ethanol exposure and/or maternal stress observed either on behavioral, morphological, or molecular level tend to be more pronounced in juvenile subjects than in adult animals. This fact points either towards an ethanol- or a stressor-induced developmental delay or a recovery from ethanol-induced impairments due to some regeneration process. Therefore, in the present study, we investigated hippocampal expression/activation of some regulatory proteins that may potentially contribute either to the damage of the developing nervous system or to the recovery from the damage produced by the adverse external factors, here, fetal-ethanol exposure and maternal stress.

Since it has been previously reported that prenatal ethanol exposure among other multiple effects may also cause neuro-inflammation (Tiwari and Chopra, 2011; Komada et al., 2017), we included to the present study iNOS, and Hsp70 proteins known as inflammatory markers (Feldman et al., 1993; Borges et al., 2012). For comparison, we also examined nNOS levels. iNOS expression in the intact subjects (C group) underwent a fluctuation during the two-months postnatal period: low during the first 10 postnatal days, highly increased at the juvenile age (PD30) to decline in the young adults (PD60). Interestingly, at PD1, fetal ethanol exposure resulted in the elevated iNOS values which, however, dropped to the normal level already at PD10. High iNOS levels at birth overlapped with deficits in hippocampal neurons morphology which, however, disappeared by PD10 (Jakubowska-Doğru et al., 2017). Increased level of iNOS at PD1 is consistent with a previous study by Dizon and colleagues (2004) who also observed in the brains of newborn guinea pigs a transient increase in the concentration of iNOS. Parallel to this, previous studies by Blanco and colleagues (2004) showed that short-term ethanol treatment may up-regulate iNOS expression in the cortical astrocytes culture; the effect regulated via nuclear factor kappa B (NF-κB). Relatively low iNOS levels at PD30 in intubated groups were paralleled by elevated nNOS expression in pups exposed to the maternal stress *per se*. High iNOS level observed again in young adults from intubated groups is consistent with previous reports showing enhanced expression of microglial activation markers in the hippocampus of the adult prenatally stressed animals (Slusarczyk et al., 2015). The other NO producer, nNOS, which has a role in CNS development was affected by maternal intubation stress at PD30. During postnatal development, in all three groups, the highest amount of nNOS expression was noted at the adult age. Generally, nNOS expression in this study was very little affected by the applied treatments. According to the literature, NOS enzymes and their product NO may act as a double-edge sward, on the one hand mediating inflammation and apoptosis, on the other hand, showing some neuroprotective effects. The neuroprotective effect of NO were previously reported in the nervous system developing under ethanol intoxication (Chandler et al., 1997; de Licona et al., 2009). Bonthius and colleagues (2002) demonstrated that prolonged ethanol exposure enhanced NMDA receptor-stimulated NO formation followed by activation of cGMP-PKG pathway and an increase of neurons survival. On the other hand, decreased levels of nNOS expression were found to exacerbate brain injury by restricting brain growth after prenatal ethanol exposure (Kimura and Brien, 1998; Karacay et al., 2015). Looking at our results, we can speculate that prenatal ethanol administration may have compromised NO signaling in the hippocampus and led to a compensation mechanism by reversing NO signaling effect upon ethanol withdrawal at birth in the form of a quick recovery from morphological deficits.

In the intact group, during a transition period from infancy to the juvenile age (PD10-PD30), it was observed a significant increase in the hippocampal expression of heat shock protein 70 (Hsp70), one of highly conserved stress proteins which through inhibiting components of inflammatory signaling pathways, such as the NF-κB transcription factor, may also have an anti-inflammatory effect in organism's immune responses (Borges et al., 2012). The hippocampal concentration of Hsp70, showed a significant increase in IC group at PD10 and in A group at PD30. Previously, an increased level of hippocampal Hsp70 was found to be associated with duration of maternal ethanol consumption (Holownia et al., 1995). Hsp70 was also increased in neuronal cells under some other pathological conditions such as trauma, ischemia or seizures (Lemoine et al., 2003). We found that prenatal intubation stress affects Hsp70 expression at the brain growth spurt period. Previous *in vitro* studies indicated that stress rapidly increased the expression of heat shock proteins (Lallemandmezger et al., 1993), which can be produced by non-neuronal cells (Walker and Carlock, 1993).

The mammalian target of rapamycin (mTOR) pathway also regulates the NO system, producing both pro-inflammatory and anti-inflammatory effects (Weichhart et al., 2015). In the intact control group, the expression of mTOR significantly increased through the developmental PD1- PD30 period to drop at young adult age. This remains in line with a previous report by other authors who also observed changes in mTOR level with age (Choi et al., 2016). The relatively low level of mTOR at birth in intubated groups might be related

to the disruptive effect of prenatal stress and ethanol administration on the role of mTOR in the process of neuronal and glial differentiation during the third trimester (Lee, 2015). The mTOR level continued to be significantly lower in the fetal-ethanol pups throughout the whole brain spurt period when an intensive neurogenesis and cell growth still take place. This may be one of the factors contributing to a decreased count of granular cells in the gyrus dentatus in the fetal-ethanol group at PD30 (Jakubowska-Doğru et al., 2017). In A group, the mTOR expression reached its normal level at juvenile age while in IC group, a compensatory increase in mTOR concentration was recorded from juvenile age to the adulthood. However, the effect of maternal stress was observed as a significant increase in the mTOR expression at PD30 and PD60, supporting a role for mTOR in controlling the cell growth and metabolism in response to stress (Hall, 2008).

Previous studies showed that ethanol-induced mTOR activation may occur through the AKT-mTOR signaling pathway (Fu et al., 2016). The main functions of AKT downstream pathways are promoting cell proliferation, growth, and motility (migration) as well as regulation of apoptosis. According to the literature, effect of ethanol administration on AKT activation depends on the ethanol dose and the type of cells. Acute systemic administration of alcohol resulted in activation of AKT in the rodent nucleus accumbens (Neasta et al., 2011) while an inhibition of PI3K/AKT signaling by ethanol was recorded in primary cortical neurons (Liu et al., 2010). In our study, in the intact rat pups, a significant increase in phosphorylated AKT was observed between PD1-PD10 and PD30-PD60., while it was stable at PD10-PD30. Until PD30, ethanol administration decreased activation of AKT, in line with the previously described negative effect of alcohol on the survival of neurons, which may be attributable to this pathway (Akbar et al., 2006). Both maternal stress and prenatal ethanol exposure resulted in a significant increase in the amount of phosphorylated AKT in young adults when the neurodevelopmental processes were about to be completed. Apparently, prenatal ethanol did not affect activation of AKT during a critical first postnatal month. A transient decrease in AKT activity was observed only in juveniles exposed to maternal stress. A reduction in AKT activation was also observed in prenatally stressed animals at PD30 (Jia et al., 2016).

To better understand how prenatal stress and ethanol exposure affected the PI3K-AKT-mTOR signaling, we also investigated the activity of PTEN, the upstream effector and regulator molecule of AKT (Tseng et al., 2012). As expected, changes in the activation of PTEN and AKT were generally in the opposite directions: at birth, high activation of PTEN corresponded to relatively low AKT activation, and a relatively low activation of PTEN in young adults corresponds to high AKT activation. Significantly higher activation of PTEN and very low activation levels of both AKT and mTOR at PD1 in pups exposed to fetal ethanol and/or maternal stress corresponded to the deficiencies in morphological development in the hippocampal neurons (Jakubowska-Doğru et al., 2017). However, at the end of brain spurt period (PD10), when we already observed a normalization of the morphological parameters in hippocampal neuron, a burst in PTEN activation especially pronounced in IC and A groups was noted. It was accompanied by a small increase in the AKT activation. This is weakening a possibility that PI3K-AKT-mTOR signaling pathway contributes to the recovery from the morphological deficits caused by gestational ethanol and/or stress. A decrease in PTEN activation was observed through PD10-PD60 period with a burst in AKT activity at PD60 which was significantly bigger in IC and A groups compared to the intact control.

GSK3, another downstream molecule of PI3K-AKT--mTOR pathway, is also essential for neuroprotection. As seen from our results, treatment- and age-dependent activation of GSK-3α and GSK-3β, two isoforms of GSK3, are highly similar. GSK3 is constitutively active, but its activity can be inhibited by phosphorylation on Ser21 of GSK3α and Ser9 of GSK3β. GSK3 substrates negatively regulated by GSK3-mediated phosphorylation are involved in cell fate determination and proliferation, while substrates positively regulated by GSK3 are more involved in cell differentiation and functions controlling such neuronal processes as neurite outgrowth, synapse formation, and neurotransmission (Cole, 2012). According to the presented data, during normal development, both GSK-3α and GSK-3β reached peak levels at PD30. However, fetal ethanol exposure disrupted this pattern by an increase in the activity of both GSK-3 subunits at birth. Activation of AKT by ethanol in the present study caused phosphorylation of GSK-3β, which inactivates it (Cole, 2012). There is also an evidence that chronic fetal exposure to ethanol induces a dephosphorylation of GSK3β and increases its activity (Xu et al., 2003). This reduction in GSK-3 activity may be a compensatory mechanism for attenuation of axonal degeneration, as suggested in previous studies (Muyllaert et al., 2008). At PD30, a decrease in the phosphorylation of GSK-3 by ethanol paralleled a decrease in AKT phosphorylation. Apparently, gestational maternal stress did not have such an effect. However, at PD10, the significant increase in GSK-3 activity due to the decrease in phosphorylation was independent from AKT phosphorylation by ethanol. Increase in GSK3 activity during brain spurt period may contribute to a recovery from fetal ethanol- and maternal stress-induced

delay in morphological development of hippocampal neurons (Jakubowska-Doğru et al., 2017). In contrast, it was reported that ethanol-dependent dephosphorylation of GSK-3 causes ROS production and activation of Bax as well as caspase-3 in developing mouse brain (Liu et al., 2009; Luo, 2014).

PTEN also controls phosphorylation and activation of ERK1/2 pathways by deactivating these pathways via downstream effectors (Gu et al., 1998; Ning et al., 2004). ERK pathways were shown to be involved in many processes in the nervous system including development by affecting cell proliferation, differentiation, adhesion, migration and survival but also apoptosis which plays an important role during neural development (Alessandrini et al., 1999; Irving and Bamford 2002). In the present study, under fetal ethanol intoxication, an increase in ERK1 activation was observed around the second postnatal month while in ERK2 almost through the whole postnatal period followed. At PD30, activation of ERK1/2 reached a peak value. Prenatal stress affected activation of ERK2 only, with the greatest increase at birth. Previously, in contrast to our findings, prenatal acute and chronic ethanol administration in mice was shown to inhibit ERK expression and activation in thalamus and cerebral cortex (Kalluri and Ticku, 2002; Mooney and Miller, 2011). Deficit in NMDA receptor-dependent ERK1/2 activation was also reported in the dentate gyrus of adult C57BL/6J mice prenatally exposed to ethanol (Samudio-Ruiz et al., 2009). There is also an evidence that acute ethanol in binge-like drinking model may increase pERK1/2 activity compared to a vehicle control in brain regions known to control addictive behaviors and drug self-administration (Agoglia et al., 2015). ERK1/2 plays a pivotal role as a signaling molecule for differentiation of progenitor cells (Samuels et al., 2008). In our previous studies, at PD30, we observed a reduction in the number of granular cells (Elibol-Can et al., 2014a) and in NeuN protein expression (unpublished data) in hippocampus due to prenatal ethanol exposure. On the other hand, a drop in ERK1/2 (especially ERK2) activity at PD60 corresponded to the morphological and functional recovery (Elibol-Can et al., 2014a). Therefore, a significant increase in ERK1/2 activation in juvenile fetal alcohol pups may be the result of a compensatory mechanism against the reduction of neuronal differentiation in the hippocampus. The above mentioned discrepancies in the results may partially arise from the differences in the dose, timing, and the route of ethanol administration as well as animal species, brain region under examination, and timing of biochemical assays.

In addition to ERK1/2, the regulation of JNK1/2, another mitogen activated kinase (MAPK), is essential for neuroprotection against substance-induced neurotoxicity (Yuan et al., 2017). As it was observed in the case of ERK1/2, the activation of JNK1/2 also significantly increased at PD30 through PD60. Interestingly, in the IC group, an increase in JNK1/2 also referred to as stress-activated kinases was highly pronounced through the extended period between PD10 and PD60. Previous studies have shown an enhancing effect of ethanol on the phosphorylation of the proteins found in the MAPK pathway, including ERK1/2 and JNK1/2 (Zhao et al., 2015; Wang et al., 2017). Similarly to ERKs, upon activation, JNKs may phosphorylate and activate nuclear transcription factors to initiate neuronal regenerative mechanisms and apoptosis (Cavigelli et al., 1995; Xu et al., 1997). There is a notion that a parallel activation of the ERK1/2, just as it is in the present study, may oppose the apoptosis-inducing JNK activation both *in vitro* and *in vivo* (Xia et al., 1995; Xu et al., 1997).

Since the programmed cell death (apoptosis) during brain development is an important homeostatic mechanism adjusting the pool of progenitor cells required for proper morphogenesis of the nervous system and adjusting neuron populations to match their target fields (Kuan et al., 2000), it is not surprising that the highest expression of pro-apoptotic Bax protein and the highest ratio of Bax to anti-apoptotic Bcl-2 was recorded during the first 10 postnatal days (human 3rd trimester equivalent covering the brain growth spurt period). On the contrary, the highest levels of anti-apoptotic proteins such as Bcl2 and Bcl-XL were found during the second postnatal month when development has been largely completed. There is a great number of *in vivo, ex vivo* and *in vitro* studies suggesting that ethanol promotes apoptotic cell death in neural tissues and gives rise to the observed neuropathologies and behavioral impairments (Freund, 1994; Cartwright et al., 1998; de la Monte et al., 2000; Ikonomidou et al., 2000; Heaton et al., 2003; Luo, 2012) by affecting Bcl-2 family proteins (Ge et al., 2004; Heaton et al., 2011). The adverse effects of gestational ethanol intoxication and stress can be long-lasting showing up in different forms at different postnatal ages. One of such effects is an increased apoptosis and excessive loss of neurons. In our previous studies (Elibol-Can et al., 2014a), under the same experimental paradigm, we observed a transient decrease in the hippocampal neuron count at PD30. In the present study, however, both prenatal ethanol exposure and maternal intubation stress showed damping effect on the expression of apoptotic markers decreasing the Bax/Bcl-2 ratio. Inactivation of PARP-1, another death promoter molecule that senses DNA damage and its activity is quantifiable by subsequent increased cleavage of full length PARP-1 to its respective 89 kDa fragment, is also a hallmark of apoptosis (Soldani and Scovassi, 2002; Kim et al., 2005; Cherian et al., 2008). The peak of

PARP89/PARP116 in intact control was reached at PD10 and it was significantly higher than the values of this ratio in IC and A groups confirming the results of Bax/Bcl2 evaluation. Prenatal ethanol exposure disrupted the age-dependent change in PARP cleavage during the studied period, as suggested previously (Ramachandran et al., 2001; Cherian et al., 2008).

If not apoptosis, another reason of a decrease in neuron counts due to ethanol intoxication may be a slowed down proliferation of neural progenitor cells (Jacobs and Miller, 2001). In our study, this notion finds support in decreased levels of AKT, mTOR, and GSK-3 enzymes during the first postnatal month. All three enzymes are known as positive regulators of neurogenesis (Yu and Cui, 2016).

CONCLUSION

In summary, during the postnatal development, a wide range of changes in expression and activation profiles of proteins related to inflammation, cell survival and apoptosis was observed in fetal ethanol- and/or maternal stress- exposed rat pups. During the early postnatal period (PD1-PD10), in the treatment groups, it was seen an upregulation of proteins such as iNOS, Hsp, PTEN, GSP-3, ERK and JNK having apart from multiple other functions pro-inflammatory and pro-apoptotic effects. Interestingly, GSK3 activity and iNOS expression were more affected by fetal ethanol than the maternal stress. Despite of this, apoptotic indices Bax/Bcl2 and PARP cleavage were significantly decreased during the critical brain growth spurt period in both IC and A pups. It indicates that the recovery process in A and IC brains started soon after the birth upon the ethanol and stressor withdrawal. It is reflected by a fast upregulation of morphological features in the hippocampal neurons observed at PD10 in our previous study (Jakubowska et al., 2017). A significant increase in the activity of survival-related AKT pathway in A and IC groups during the second postnatal month shows that neural development and the compensatory processes continue until the adulthood. Indeed, these data together with our previous findings confirm that developmental shaping of the hippocampus at both the cellular and molecular level continues through the juvenile age or even longer.

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