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Investigation into the effects of prenatal alcohol exposure on postnatal spine development and expression of synaptophysin and PSD95 in rat hippocampus

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ABSTRACT

Ethanol is known as a potent teratogen responsible for the fetal alcohol syndrome characterized by cognitive deficits especially pronounced in juveniles but ameliorating in adults. Since the mechanisms of these deficits and following partial recovery are not fully elucidated, the aim of the present study was to investigate the process of synaptogenesis in the hippocampus over the first two months of life in control and fetal-alcohol rats. Ethanol was delivered to the pregnant dams by intragastric intubation throughout 7–21 gestation days at the daily dose of 6 g/kg generating a mean blood alcohol level of 246.6 ± 40.9 mg/dl on gestation day 20. The spine densities as well as the expression of pre- and postsynaptic proteins, synaptophysin (SYP) and PSD-95 protein, were evaluated for three distinct hippocampal regions: CA1, CA2+3, and DG and four postnatal days: PD1, PD10, PD30 and PD60, independently. Our results confirmed an intensive synaptogenesis within the brain spurt period (first 10 postnatal days), however, the temporal pattern of changes in the SYP and PSD-95 expression was different. The ethanol exposure during half of the 1st and the whole 2nd human trimester equivalent resulted in an overall trend toward lower values of synaptic indices at PD1 with a fast recovery from these deficits observed already at PD10. At PD30, around the age when the most pronounced behavioral deficits have been previously reported in juvenile fetal-alcohol subjects, no significant changes were found in either the hippocampal levels of synaptic proteins or in the spine density in principal hippocampal neurons.

1. Introduction

Today, both in the developed and developing countries, alcoholism is still a serious problem having a negative influence on the human health and countries' economy. The studies on perinatal (pre- and neonatal) ethanol exposure are especially important for better understanding alcohol-induced malformations and malfunctions during brain development.

The neuroteratogenic effects of alcohol cause brain damage resulting in a variety of cognitive and behavioral abnormalities. The most common alcohol-induced behavioral dysfunctions are impairments of motor (Abel, 1982; Abel and Reddy, 1997; Tran et al., 2000) and cognitive functions (Mattson et al., 2001). In animal experiments, learning deficits have been demonstrated especially in spatial tasks sensitive to hippocampal damage (Girard et al., 2000; Hamilton et al., 2003; Johnson and Goodlett, 2002; Nagahara and Handa, 1997; Neese et al., 2004; Lee and Rabe, 1999; Reyes et al., 1989). The endurance of adverse effects of fetal ethanol on both brain morphology and behavior is still an open issue. Generally the deficiencies in learning and memory were observed in juveniles ameliorating with maturation (Girard et al., 2000; Nagahara and Handa, 1997; Wozniak et al., 2004; Zimmerberg et al., 1991). These results have been confirmed in the previous studies carried out in our laboratory (Dursun et al., 2006; Elibol-Can et al., 2013).

In the light of these findings it has been of interest to investigate alcohol-induced anomalies in the hippocampus development that would correlate with learning and memory deficits frequently observed in juvenile fetal-alcohol subjects and also, if possible, to unveil the compensatory processes that could be responsible for behavioral recovery in young adults.

To answer these questions, in our previous study (Elibol-Can et al., 2013), we were examining changes in the hippocampal volumes, numbers of principal hippocampal neurons, and expression of doublecortin, a marker for neurogenesis, over a protracted postnatal period in fetal-alcohol and control rats. In the latter study, a moderate prenatal intoxication with ethanol showed a mild effect

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on hippocampus development limited to a marginally lower number of granular cells in DG on PD30 which correlated with but could hardly be responsible for deficient cognitive performance observed in juvenile fetal-alcohol rats. Obtained results suggested that, the juvenile behavioral deficits and the following functional recovery in fetal-alcohol subjects may be due to more subtle plastic changes within the hippocampal formation. Therefore, in the present study, the process of hippocampal synaptogenesis in control and fetal-alcohol rats has been investigated throughout the first two postnatal months.

Synapse density was shown to correlate with cognitive status (Chen et al., 1995; Dumitriu et al., 2010) and it can be also indirectly evaluated by measuring the levels of synaptic proteins such as synaptophysin (SYP) and postsynaptic density (PSD) 95 protein (Glantz et al., 2007; Siew et al., 2004;) as well as spine density (Dumitriu et al., 2010). Synaptophysin and other synaptic vesicle proteins together with proteins belonging to postsynaptic densities such as PSD-95 have been implicated in mechanisms of activity-dependent neuroplasticity underlying learning and memory formation (Head et al., 2009; VanGuilder et al., 2010). It has been suggested that SYP plays a role in formation and stabilization of synapses (Tarsa and Goda, 2002) and thus it is considered a reliable marker that can be used to determine distribution and density of synapses (Glantz et al., 2007; Li et al., 2010). On the other hand, PSD-95 was postulated to play a role in the organization of glutamate receptors and other constituents of the postsynaptic density (Gardoni et al., 2009; Kim and Sheng, 2004; Tang et al., 1999; Zhang et al., 2008; Zhao et al., 2005) and thus determining the size and the strength of synapses (Ehrlich and Malinow, 2004; Garner et al., 2000; Kennedy, 1998), formation of synapse assemblies (Garner et al., 2000) and spine-maturation (El-Husseini et al., 2000; Nikonenko et al., 2008). All these processes are part of cellular mechanisms underlying various forms of synaptic plasticity including learning and memory formation (Nyffeler et al., 2007).

The majority of excitatory synaptic inputs are located on the surface of dendritic spines and colocalization of synaptophysin, PSD-95 and dendritic spines have been well documented (Okabe et al., 2001). Therefore, in addition to mapping synaptic proteins the development and density of dendritic spines are likely good indicators of the extent of synapse formation and the synapse density.

In the present study, the spine densities as well as the expression of pre- and postsynaptic proteins, SYN and PSD-95, were evaluated in fetal-alcohol and control rats for three distinct hippocampal regions: CA1, CA2+3, and DG, during critical postnatal time points: on the first postnatal day (PD1), PD10 (roughly overlapping with the end of the growth spurt period), PD30 (a juvenile age), and PD60 (corresponding to the age of young adults).

2. Materials and methods

2.1. Subjects

Seventy-five adult, naive, female ($n = 60$) and male ($n = 15$) Wistar rats, obtained from the Gülhane Military Medical Academy (GMMA) Animal Breeding Facility (Ankara), were initially used in the present study. Throughout the experiments, rats were kept in the METU animal facility under the stable temperature of $22 \pm 1\,^{\circ}C$ and 12 h/12 h light/dark cycle, with ad libitum food and water supply. After mating, female rats were examined for the presence of the vaginal plug which was an evidence of successful fertilization and this day was marked as gestational day 0 (GD0). On GD7, pregnant dams were assigned (counterbalanced for initial body weight) to one of three treatment groups: Alcohol Group (A), pair-fed Intubated Control Group (IC), a control for possible intubation-induced stress effects, and Control Group (C).

Male pups belonging to the dams from each of treatment groups (A, IC, and C) were randomly assigned to 4 age subgroups sacrificed on PD1, PD10, PD30, and PD60 to be used for either SYP/PSD95 immunohistochemistry, estimation of synaptic protein levels, or spines assessment (PD1: $n = 22$, $n = 24$, $n = 22$, P10: $n = 19$, $n = 24$, $n = 22$, P30: $n = 23$, $n = 24$, $n = 21$, and P60: $n = 22$, $n = 24$, $n = 21$, respectively). To avoid the potential litter effect, no more than two pups from the same litter were assigned to the same group. All experimental procedures were approved by the Ethics Committee for the care and use of laboratory animals at of the Middle-East Technical University, Ankara, Turkey.

2.2. Ethanol treatment

Starting from the GD7 throughout GD20, dams from A group were daily administered 6 g ethanol/kg body weight by intragastric intubations. Ethyl alcohol 96.5% (v/v), Merck was used in the study. Animals in IC group (a control for intubation-induced maternal stress) received the same volume of fluid with sucrose substituted isocalorically for ethanol; they were given the same amount of food as that consumed by their weight-matched A group females. Animals in C group received ad libitum access to laboratory chow and water with no additional treatment. The protocol of alcohol administration was as described previously (Dursun et al., 2006). Briefly, the alcohol/isocaloric sucrose solution was delivered by intragastric intubations using stainless curved feeding needle (18 ga, 3 in, Stoelting Co., Wood Dale, IL). Daily portion of alcohol/sucrose solution was divided into two equal doses given to animals 1 h apart. The alcohol solution was prepared daily as a 25% (vol/vol) ethanol mixed with distilled water and stored at room temperature.

2.3. Determination of blood alcohol concentration

Blood alcohol concentration (BAC) was assessed on GD20 in a different group of pregnant dams ($n = 4$). Blood samples (1–2 ml) were taken from the rat-tail vein 3 h after the last intragastric intubation (Tran and Kelly, 2003). Blood samples were then centrifuged for 10 min at $1000 \times g$, blood plasma separated and stored at $-80\,^{\circ}C$ until BAC determination was carried out. BAC (mg/dl) was determined by an alcohol assay kit (Biolabo, France) at the Gülhane Military Medical Academy (Sag et al., 2006).

2.4. Tissue preparation and visualization for immunofluorescence

At different postnatal ages, rats belonging to different treatment groups were deeply anesthetized with a mixture containing ketamine hydrochloride (80 mg/kg Alfamine 10%, Alfasan International B.V. Holland) and xylazine (10 mg/kg Alfamine 2% Alfasan International B.V. Holland) (i.p.) and intracardially perfused first with saline and then with 4% paraformaldehyde. The brains were dissected, postfixed overnight in 4% paraformaldehyde, after that cryoprotected with 30% sucrose solution in 0.1 M PBS until sunk, quickly frozen in liquid nitrogen and then stored at $-80\,^{\circ}C$. Frozen brains were coronally cut on a Shandon Cryotome at 20-μm. The sampling rate was 1/24 (3 or 4 sections per rat).

Brain sections were mounted on polylysine-coated slides and subjected to the antigen retrieval by citrate buffer to uncover epitopes. After rinsing in PBS, the sections were incubated for 1 h at room temperature with blocking solution containing 5% normal goat serum (NGS). Afterwards, at $4\,^{\circ}C$, the sections were incubated for 48 h with primary antibodies either against SYP (cell signaling #4329, 1:100) or PSD95 (cell signaling #2507, 1:100). On the following day, sections were incubated for 2 h at room temperature

with fluorescent-conjugated secondary antibody (Alexa Fluor 488, goat anti-rabbit IgG, 1:100) and stained with DAPI nuclear counterstain (Kilic et al., 2010). Negative control was provided by omitting the primary antibody in antibody dilution buffer.

Since there was no significant difference in the expression of SYP and PSD-95 between the right and the left hippocampi, in the present study, the immunohistological data was considered only from the left hippocampus. Coronal brain sections of left hippocampus were visualized using a Nikon Microscope equipped with a fluorescent attachment. Corpus callosum which contains no presynaptic or postsynaptic protein was used as a background. The pictures of each hippocampal region (CA1, CA3, DG) (3–4 pictures) were taken under fluorescence microscope at 40× magnification. By using ImageJ software, relative optical density (ROD) measurements were done by converting the pictures in grayscale (0–255). For each image obtained from the cell layer studied (SR for CA1; SL for CA3; ML for DG), 5 representative areas (100 × 100 pixel each) have been chosen and averaged. The background value of corpus callosum was substracted and resulting value was considered as the optical density value of the sample.

2.5. Western blotting

Rats belonging to either A, IC, or C groups (8 pups intermixed between litters per age group) were sacrificed by decapitation, their left hippocampi were dissected and pooled (Kilic et al., 2012). Protein extraction was performed by homogenizing the hippocampi with lysis buffer (RIPA buffer Sc2498) in the presence of protease inhibitors. Tissue samples were centrifuged at 14,000 rpm for 20 min at 4 °C, and then supernatant was collected. Protein concentrations were determined by using Qubit assay kit and Qubit® 2.0 Fluorometer. Sample proteins were separated by polyacrylamide gel electrophoresis (XCell SureLock™ Mini-Cell Electrophoresis System, Invitrogen) and subsequently transferred to a nitrocellulose membrane. Then, blots on the membrane were washed with Tris buffered saline and incubated with blocking solution (2% ECL advance Blocking Reagent in Tris Buffered Saline) for 1 h at room temperature. Afterwards, the blots were incubated overnight with primary antibody (SYP and PSD95, cell signaling, 1:1000) at 4 °C and on the following day, with secondary antibody (HRP-linked anti-rabbit IgG, Amersham, 1:2500) for 1 h at room temperature. Blots were then incubated with substrate solution for visualization. Protein loading was controlled using a monoclonal mouse antibody against β-actin. Immunoreactive protein bands were quantified densitometrically using ImageJ analysis system (NIH, Bethesda, USA).

2.6. Golgi staining for dendritic spines

The pups belonging to either A, IC, or C group were deeply anesthetized and intracardially perfused with saline. The dissected part of the brain including whole hippocampus was fixed using FD Rapid GolgiStain™ kit according to manufacturer's descriptions. After fixation, the brain specimens were cut into 100 μm sections on a Leica cryostat at −22 °C. Sections were mounted on gelatin-coated microscope slides and stained according to manufacturer's descriptions. The stained cells were first traced with Neurolucida software (MBF Inc., Williston, VT) under a light microscope at 100× magnification and then analyzed with Neuroexplorer software (MBF Inc., Williston, VT). Spines were counted on dendrites longer than 10 μm in the second or third dendritic branch of pyramidal neurons and third dendritic branch of granular neurons. To count the spines, straight terminal branches that provided clear resolution of spines were selected, and spine density was calculated as the number of spines per 1 μm of dendrite length (sp/L).

2.7. Statistical analyses

Group means ± SEM were calculated from all measures. A two-way repeated-measures analysis of variance (ANOVA) was conducted on the dams' body weight data throughout GD7–20. Two-way ANOVA (age × treatment) was applied to the pups' weight, as well as biochemical and morphological data to evaluate the main effects of age and treatment as well as age × treatment interaction. Additionally, pups' weights were analyzed for each postnatal age separately by one-way ANOVA with treatment as independent variable. The between-group differences in immunofluorescence scores, protein levels, and spine density were analyzed by one-way ANOVA using treatment or pups' age as an independent factor. The post hoc comparisons of simple effects 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 $p \leq 0.05$.

3. Results

3.1. The dams and pups data

All dams gave births inside the normal range between 21st and 23rd day of pregnancy and no significant between-group differences were recorded in the length of gestation period. In all experimental groups, an increase in dams' body weight was observed throughout the gestational period. The repeated measure ANOVA yielded highly significant day effect ($F_{(13:741)} = 42.437$, $p \leq 0.001$) and insignificant main effect of treatment and treatment × day interaction.

Two-way ANOVA with age and treatment as independent variables applied to the pups' weights revealed the age effect ($F_{(3:335)} = 1703.000$, $p \leq 0.001$) and treatment effect ($F_{(2:335)} = 3.744$, $p = 0.025$) highly significant. The age × group interaction was insignificant. One-way ANOVA with treatment as independent factor revealed a significant group effect at birth and at PD10 ($F_{(2:92)} = 28.685$, $p \leq 0.001$; $F_{(2:85)} = 11.601$, $p \leq 0.001$ respectively) with the lower body weight in fetal-alcohol pups (5.2 ± 0.2 and 15.5 ± 0.4, respectively) as compared to IC (6.0 ± 0.2 and 17.4 ± 0.3, respectively) and C (7.0 ± 0.2 and 19.6 ± 0.9, respectively) groups ($p \leq 0.05$). The between-group difference in the pups' body weight disappeared at PD30.

3.2. Blood alcohol concentration (BAC)

The mean BAC in pregnant dams from A group, estimated 3 h after the second intubation on the GD20, was found as 246.6 ± 40.9 mg/dl. Since ethanol readily crosses placenta (Kesaniemi and Sippel, 1975) the fetal BAC is assumed to be close to the maternal BAC.

3.3. Immunohistochemistry results

Age-dependent changes in SYP and PSD95 immunoreactivity for three different hippocampal regions (CA1, CA3, and DG) in control rats are visualized in Fig. 1A and B, respectively. As seen from the presented microphotographs, SYP/PSD-95 immunostaining showed punctuated labeling pattern. The numerical data (IR measured as relative optical density) for all three treatment groups, three hippocampal regions, and four time points are presented in Fig. 2A for SYP and in Fig. 2B for PSD95. Two-way ANOVA with age and treatment as independent factors was carried out on SYP-IR scores separately for stratum radiatum (SR) of the CA1 region predominantly receiving Schaffer collateral innervation, stratum lucidum (SL) of the CA3 region receiving heavy mossy fiber input, and molecular layer (ML) of DG receiving heavy

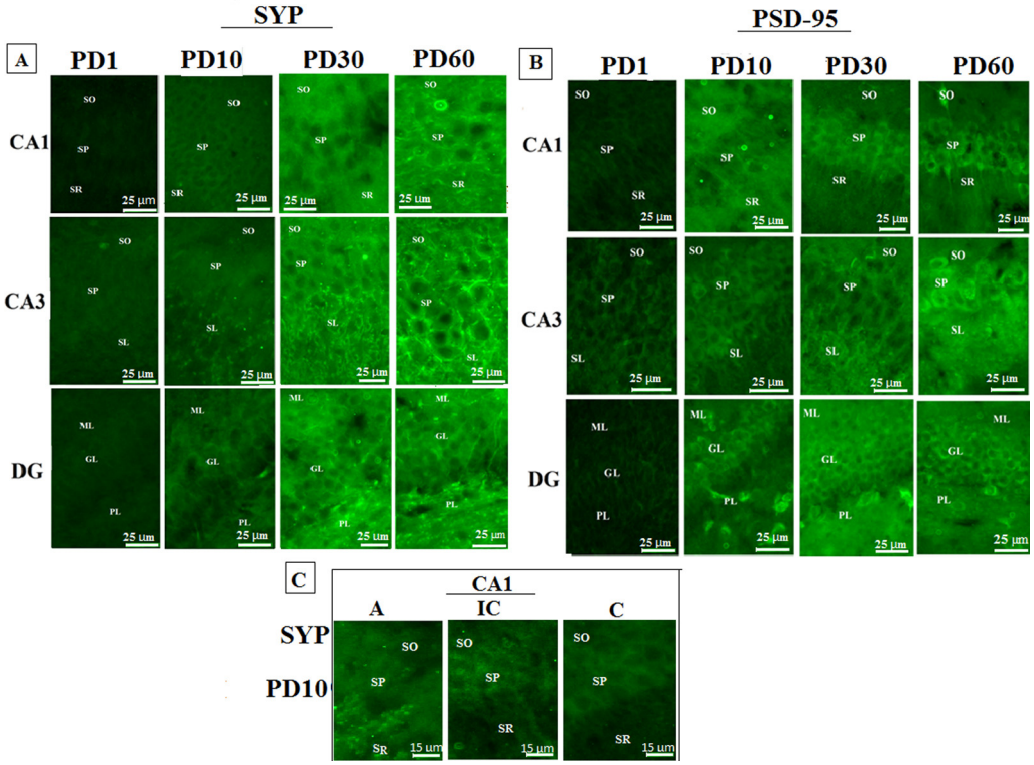

Fig. 1. (A) Representative photomicrographs showing SYP-immunoreactivity and (B) PSD95-immunoreactivity at postnatal days PD1, PD10, PD30, and PD60 in intact control group (C). Representative photomicrographs showing the between-group differences for SYP-immunoreactivity at PD10 in CA1. The microphotographs were taken under 40× magnification. *Abbreviations*: SO (stratum oriens), SP (stratum pyramidale), SR (stratum radiatum) SL (stratum lucidum). Scale bar: 25 µm.

innervation from the entorhinal cortex. In all hippocampal subregions studied, the main effect of age was yielded highly significant (CA1: $F_{(3;63)} = 17.116$, $p \leq 0.001$, CA3: $F_{(3;61)} = 26.780$, $p \leq 0.001$, DG: $F_{(3;63)} = 29.092$, $p \leq 0.001$), however, the main effect of treatment and age × treatment interaction remained insignificant. In all three regions, an increase in SYP-IR was observed between PD1–PD30 to reach a plateau at the juvenile age (Fig. 2A). In A group, there was an overall trend for relatively lower SYP-IR on PD1 and its increase was most rapid between PD1–PD10 ($p \leq 0.01$), while in the control groups the greatest increase in SYP-IR was observed between PD10-PD30 (Fig. 2A). Different temporal patterns of increase in SYP-IR in A and control groups resulted in a significant between-group difference in SYP-IR level at PD10 (Figs. 1C and 2A). One way ANOVA performed on SYP-IR data for each age independently revealed a significant main group effect on PD10 in CA1 and DG ($F_{(2;18)} = 10.118$, $p = 0.001$ and $F_{(2;18)} = 3.642$, $p = 0.050$, respectively). The group effect was marginally significant in CA3 ($F_{(2;16)} = 3.019$, $p = 0.077$). Post hoc comparison of simple effects confirmed significantly stronger SYP-IR in A group as compared to both controls ($p \leq 0.05$).

Two-way ANOVA with age and treatment as independent factors applied to PSD95 IR scores from each hippocampal region independently also revealed highly significant age effect (CA1: $F_{(3;67)} = 8.900$, $p \leq 0.001$, CA3: $F_{(3;71)} = 23.139$, $p \leq 0.001$, DG: $F_{(3;72)} = 8.240$, $p \leq 0.001$) yielding main treatment effect and the age × treatment interaction insignificant. Interestingly, the temporal pattern of postnatal changes in PSD95-IR levels was different than that of SYP-IR changes manifesting reverse U shape with maximum PSD-95 expression at PD10-PD30 followed by a decline in the overall PSD-95 concentration between PD30-PD60. Since two-factorial ANOVA yielded group effect insignificant, to analyze the temporal profiles of postnatal changes in SYP and PSD-95 expression, the IR data have been pooled across the groups (Fig. 3) and a two-way ANOVA (protein × day) has been performed on these data. The two-way ANOVA of pooled data performed for CA1, CA3, and DG regions independently yielded in all three regions highly significant age effect ($F_{(3,143)} = 20.741$, $p \leq 0.001$; $F_{(3,144)} = 42.748$, $p \leq 0.001$; and $F_{(3,150)} = 19.787$, $p \leq 0.001$, respectively) and significant protein × day interaction ($F_{(3,143)} = 7.996$, $p \leq 0.001$; $F_{(3,144)} = 16.028$, $p \leq 0.001$; and $F_{(3,150)} = 7.145$, $p \leq 0.001$, respectively) with protein effect significant in CA regions only ($F_{(1,143)} = 8.836$, $p = 0.003$ and $F_{(1,144)} = 19.125$, $p \leq 0.001$, respectively for CA1 and CA3). In CA regions, the overall levels of SYP were significantly higher than that of PSD-95. One-way ANOVA with age as independent factor applied to PSD95 data confirmed a significant increase ($p \leq 0.05$) in PSD95-IR in all hippocampal regions and in all treatment groups during PD1–PD10 and then a significant decrease between PD30-PD60 ($p \leq 0.05$).

3.4. Western blotting studies

In the Western blot analysis, SYP and PSD95 protein bands were detected at 38 kDa and 95 kDa, respectively (Fig. 2C and D). The results of Western blot analysis revealed similar pattern to that observed in immunohistochemistry studies. According to two-way ANOVA with age and treatment as independent factors, the main effect of age for both SYP and PSD95 was highly significant ($F_{(3;60)} = 30.011$, $p \leq 0.001$, $F_{(3;60)} = 10.196$, $p \leq 0.001$, respectively), while the main effect of treatment and the age × treatment interaction were insignificant.

In all treatment groups, one-way ANOVA revealed a significant main effect of age (A: $F_{(3;23)} = 6.140$, $p = 0.004$; IC: $F_{(3;23)} = 15.481$, $p \leq 0.001$; C: $F_{(3;23)} = 20.945$, $p \leq 0.001$). The post hoc comparison of particular time windows confirmed in all groups a significant increase in SYP expression between PD1–PD10 (A: $p = 0.015$, IC: $p = 0.009$, C: $p = 0.002$). This increase was most pronounced in A

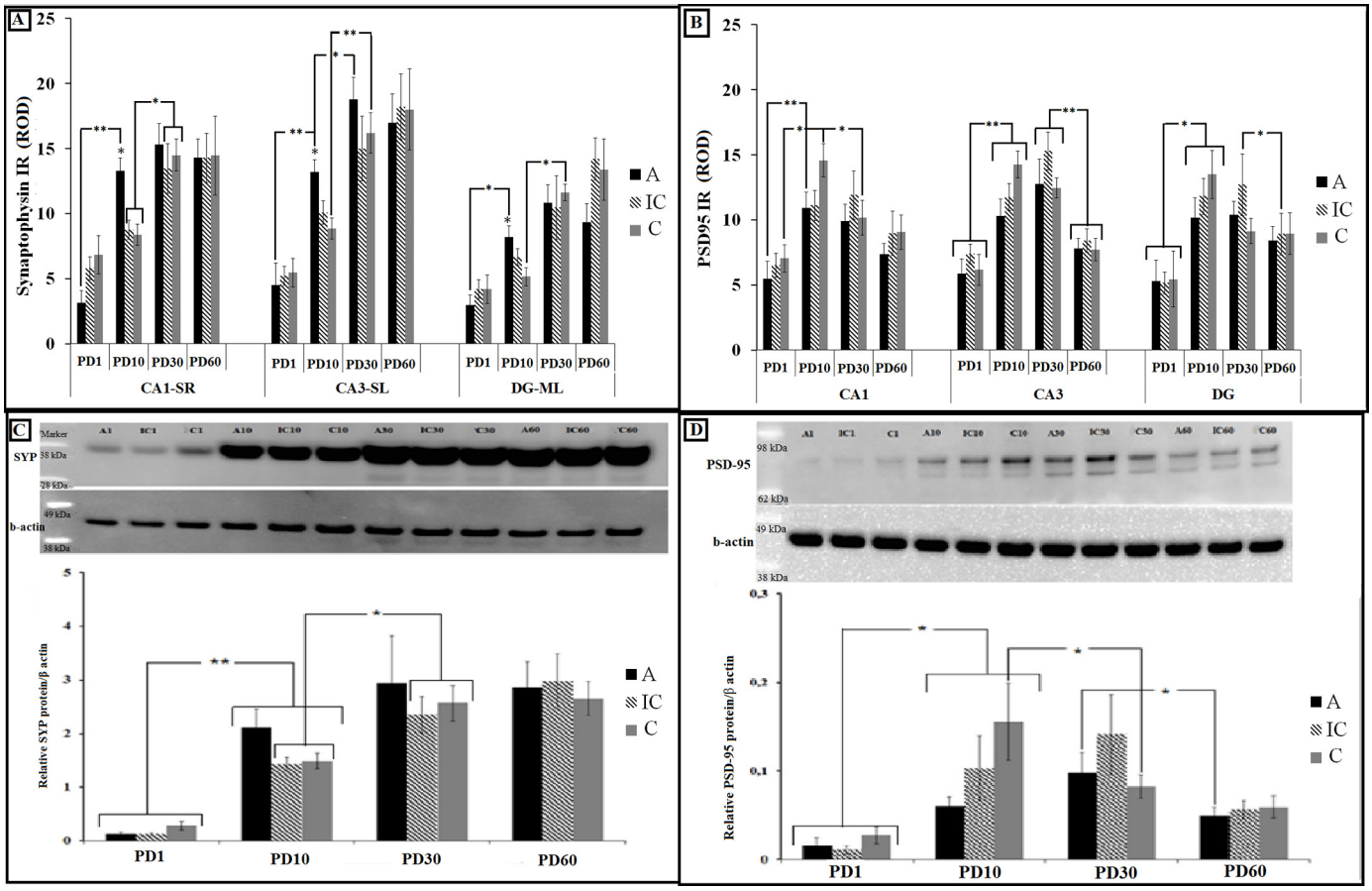

Fig. 2. (A) Mean immunoreactivity scores (±SEM) for synaptophysin and (B) PSD-95 protein in alcohol (A) and control (IC and C) groups at different postnatal ages for different regions of the hippocampus. ROD: relative optical density. (C) Immunoreactive protein bands representing synaptophysin (SYP) (38 kDa) and (D) PSD-95 protein (95 kDa) in alcohol (A) and control (IC and C) groups at the postnatal days PD1, PD10, PD30, and PD60. Band quantification is expressed as the mean (±SEM) of the relative intensity with respect to that of β-actin. Error bars denote ±SEM. Asterisks denote the level of significance: $p < 0.05^{*}$, $p < 0.01^{**}$, $p < 0.001^{***}$.

group (Fig. 2C). A significant increase in the hippocampal expression of SYP protein continued during PD10-PD30 period in both control groups (IC: $p = 0.051$, C: $p = 0.005$). One-way ANOVA with treatment as independent factor revealed marginally significant between-group difference in the expression of SYP protein on PD1 and PD10 ($F_{(2;17)} = 2.926$, $p = 0.085$, $F_{(2;17)} = 2.887$, $p = 0.087$, respectively). At PD1, in alcohol group, the level of SYP protein was lower and, at PD10, it was higher compared to both control groups (Fig. 2C).

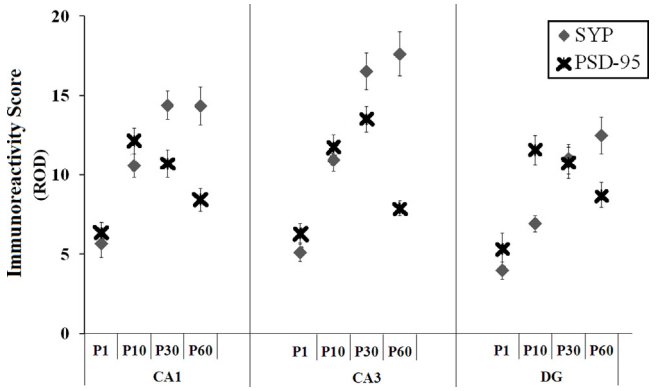

Fig. 3. Illustration of the temporal pattern of the changes in SYP and PSD-95 protein expressions in CA1, CA3, and DG hippocampal subregions throughout 2 postnatal months with immunoreactivity scores pooled across the treatment groups. Error bars denote ±SEM.

To analyze the temporal pattern of changes in PSD-95 levels during postnatal development in all three treatment group, one-way ANOVA with age as independent factor was applied to each treatment group, separately. According to the results of this analysis, a significant increase in the hippocampal PSD-95 concentration was observed in all groups (A: $F_{(3;19)} = 5.810$, $p = 0.007$, IC: $F_{(3;19)} = 3.700$, $p = 0.034$. and DG: $F_{(3;19)} = 4.996$, $p = 0.012$, respectively) between PD1–PD10. The expression of PSD95 protein reached a maximum level at PD30 in A and IC groups, and at PD10 in C group. Then, in all groups, a decrease in the overall hippocampal PSD-95 level was recorded. This decrease was yielded significant in C group between PD10-PD30, and in A group between PD30-PD60. In group IC, due to the high within-group variance, the decrease in PSD95 concentration did not reach the accepted significance level although, at PD60, it was markedly lower than at PD10 and PD30 (Fig. 2D).

3.5. Spine density studies

As seen from Figs. 4 and 5, the density of spines was increased with postnatal age. Two-way ANOVA with age and treatment as independent variables performed for spine density (spines per length) yielded a significant age effect ($F_{(3:143)} = 78.998$, $p \leq 0.001$ for CA1; $F_{(3:148)} = 58.388$, $p \leq 0.001$, for CA3; $F_{(3:68)} = 66.175$, $p \leq 0.001$, for DG). The main effect of treatment was shown to be insignificant while the age × treatment interaction was yielded significant for CA1 and CA3 pyramidal neurons ($F_{(6:143)} = 2.415$, $p = 0.030$; $F_{(6:148)} = 3.024$, $p = 0.008$, respectively) because of different temporal patterns of increase in spine density in intubated

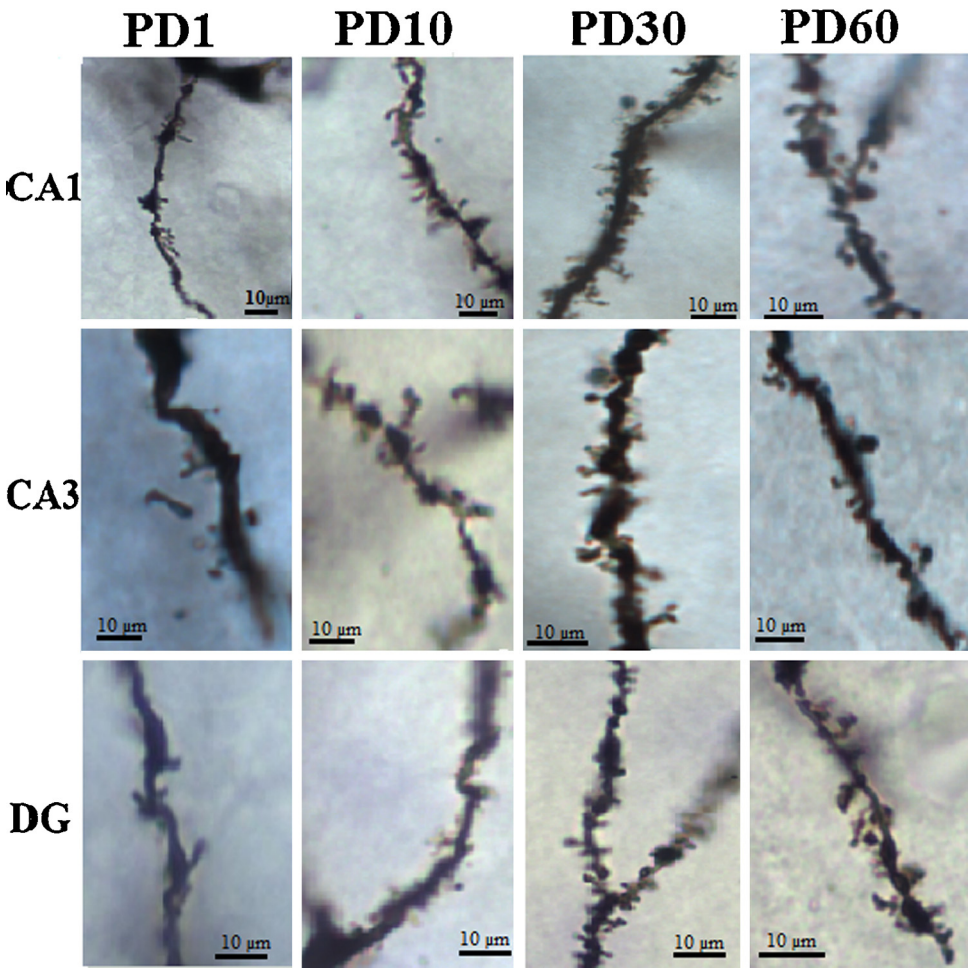

Fig. 4. Representative photomicrographs showing the spines of apical dendrites of pyramidal neurons at postnatal days PD1, PD10, PD30, and PD60 in intact control group under 100× magnification. Scale bar: 10 μm.

and control groups. As demonstrated in Fig. 5, in all three treatment groups, a significant increase in spine density was observed in CA1 region throughout the whole PD1–PD30 period while in CA3 and DG regions, it was most pronounced in PD1–PD10 time window. According to the results of one-way ANOVA with treatment as independent variable, at PD1, the total spine density of pyramidal neurons was lower in both A and IC groups compared to C group (CA1: $F_{(2:40)} = 11.852$, $p < 0.001$, CA3: $F_{(2:41)} = 10.843$, $p < 0.001$) and, at PD10, the total spine density of CA3 neurons was marginally higher in A group compared to C group ($F_{(2:41)} = 2.962$, $p = 0.063$) (Fig. 5).

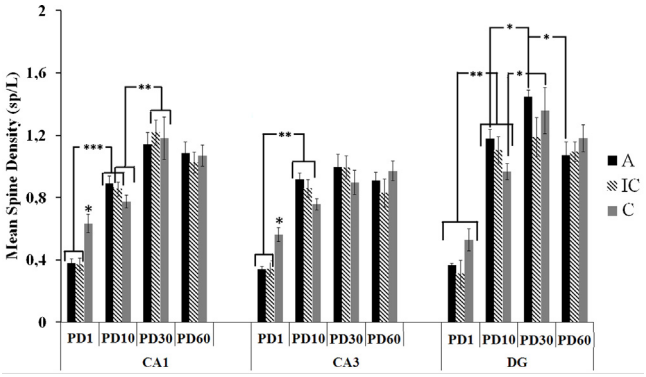

Fig. 5. Morphometric estimates of mean total spine density made for each postnatal age, each group, and each hippocampal region, independently. The degree of significance is denoted as $p \leq 0.05^{*}$, $p \leq 0.01^{**}$, $p \leq 0.001^{***}$. Error bars denote SEM.

4. Discussion

In the present study, the body weight gain by the pregnant dams was not affected by the intragastric administration of a moderate dose of ethanol (Majchrowicz, 1975) delivered during the gestation period equivalent to half of the first and the whole second human trimesters and resulting in the mean peak BAC of 246.6 ± 40.9 mg/dl. However, consistently with previous literature (Bâ, 2009; Dursun et al., 2006; Spong et al., 2001; Tran et al., 2000), on PD1 and PD10, the fetal-alcohol pups manifested significantly lower body weight compared to controls.

As expected, profound changes in the expression of synaptic proteins and spine densities were noted throughout the postnatal period studied. In all hippocampal subregions and treatment groups, a significant increase in SYP/PSD-95 expression paralleled by a significant increase in spine densities was observed during PD1–PD10 time window, known as a brain spurt period, the period of intensive synaptogenesis (Dobbing and Sands, 1979). Interestingly, the temporal patterns of postnatal changes in SYP and PSD-95 expression were quite different. The maximum, plateau level of SYP expression was recorded at PD30. In contrast, the expression of PSD-95 protein manifested reverse U shape reaching maximum level already at PD10 and showing a decline tendency between PD30 and PD60.

Our results indicate that in all three hippocampal subregions, both SYP and PSD-95 are produced mainly postnatally. The temporal pattern of PSD-95 expression during postnatal development of rat hippocampus coincides with postnatal synaptic development in human and nonhuman primate cortex where synaptic density was shown to rise after birth, reach a plateau in childhood and then decrease to adult levels by late adolescence (Glantz et al., 2007). It is also consistent with the results of other studies on rats (Chang et al., 2009). The decrease in PSD-95 levels between PD30-PD60 may be related to synaptic pruning resulting in refining the abundance of neural connections (Low and Cheng, 2006). In the present study, however, the potential decrease in the synapse counts was not reflected by the changes in SYP expression. Also, the declining trend in spine densities observed between PD30-PD60 was yielded statistically significant in DG for A group only.

In this study, the effect of prenatal exposure to a moderate dose of alcohol on synaptic markers and spine formation was relatively mild. In A group, there was an overall trend for relatively lower SYP expression on PD1 with a rapid increase between PD1–PD10 resulting in a significantly higher SYP-IR/concentration on PD10, as compared to intact control. Similar trend was also observed for spine density but not so much for PSD-95 expression. The fact, however, that at PD1, an overall expression of synaptic proteins and spine density were lower also in IC group points toward the potential effect of prenatal intubation-induced stress. The trend toward a lower expression of synaptophysin observed in this study shortly after the birth in the intubation groups, remains in line with significant decrease of synaptophysin in the rat pups' hippocampus after maternal stress reported previously by other authors (Afadlal et al., 2010; Barros et al., 2006; Biala et al., 2011; Xu et al., 2013). The endurance of this effect depends probably on the timing and severity of the stress. The previous studies examining effects of prenatal stress on PSD95 expression brought discrepant results. In some studies (Son et al., 2006), maternal stress did not significantly affect the postnatal expression of PSD-95, while in some other studies a decrease in PSD-95 levels was noted (Chutabhakdikul and Surakul, 2013). In contrast to that, consistently with our results, a reduction in the density of hippocampal spines has been frequently observed after maternal stress, however, this effect was reported to be sex-, age-, and region-specific (Bock et al., 2011; Martínez-Téllez et al., 2009). However, in the present study, the prenatal intubation stress should be less severe in A group because of sedative and anesthetic effects of ethanol. Despite of this, the trend for a decrease in synaptic proteins (especially SYP) was more pronounced in A group compared to IC indicating toward accumulation of two adverse effects, both stress and alcohol abuse. At PD10, in all hippocampal subregions of fetal-alcohol rats, the spine numbers and the expression of synaptic proteins caught up with and even overrode those in intact control, suggesting a compensatory rebound from the initial reduction in these indices caused by prenatal ethanol/stress exposure. The latter observation is parallel to previously reported by our group compensatory increase in the numbers of hippocampal neurons observed postnatally in fetal-alcohol and intubated control groups (Elibol-Can et al., 2013).

The mild effect of fetal ethanol on the expression of synaptic proteins noted in this study is in line with a previous report of a lack of changes in the postnatal levels of mRNA encoding synaptophysin-38 in the brains of rats exposed to ethanol during gestational days 8–21 (Maciejewski-Lenoir, 1993). Some other authors also did not find an adverse effect of gestational ethanol on the composition and levels of the PSD-95 associated NMDA receptor complexes (Hughes et al., 2001; Samudio-Ruiz et al., 2010). These results remain in contradiction to other studies reporting reduction in spine density (Ferrer et al., 1988) and changes in ligand binding, subunit expression, and function of NMDA receptors after perinatal ethanol exposure (for review see Costa et al., 2000). However, the discrepancies in the obtained results may arise from differences in ethanol doses applied and the peak BAC, as well as the way, duration, and timing of ethanol administration with respect to the developmental critical periods. According to the literature, in rats and mice, the early postnatal period (human third trimester equivalent) is the period of increased vulnerability to ethanol toxicity (Livy et al., 2003).

Exposure of neonate rats to ethanol was reported to significantly reduce hippocampal synapse and spine densities (Gonzàles-Burgos et al., 2006; Kuge et al., 1993; Whitcher and Klintsova, 2008). However, in some studies (Helfer et al., 2012) significant, ethanol-induced deficits such as a decrease in hippocampal long-term potentiation (LTP), were related to ethanol exposure during the 2nd rather than the 1st or the 3rd trimester equivalent. Interestingly, the reduction in hippocampal LTP considered a cellular model of memory was more pronounced in male than female rats prenatally exposed to ethanol and could not be explained by changes in NMDA receptor function or expression (Sickmann et al., 2013). Similarly, the lack of between-group differences in either expression of synaptic proteins including synaptophysin and PSP-95 or spine densities observed in the present study at PD30 did not correlate with deficits in behavioral memory frequently reported in juvenile fetal-alcohol rats (Elibol-Can et al., 2013; Girard et al., 2000; Nagahara and Handa, 1997; Wozniak et al., 2004; Zimmerberg et al., 1991). Our results are partially consistent with the previous reports of unchanged synaptic functions in *PSD-95* KO mice (Béïque et al., 2006) suggesting that overall PSD-95 levels may not always correlate with neural functions recorded at either cellular (LTP) or behavioral (learning and memory) level.

Taking together, our results confirm the existence of intensive synaptogenesis within the first 10 days after birth. The different pattern of temporal changes in SYP and PSD-95 IR and tissue levels observed in this study suggests that the postnatal dendritic remodeling is accomplished by the differential reorganization and redistribution of synaptic proteins. However, the obtained results suggest that changes in expression of a major postsynaptic protein, PSD-95, and spine numbers are not always parallel. In the present study, the exposure to ethanol during the last 2 weeks of gestation had a mild effect on hippocampal synaptogenesis and did not produce irreversible maturational deficits during hippocampal development. The damping effect of fetal alcohol/stress exposure on hippocampal synaptogenesis showed up as a trend toward lower synaptic indices at birth which, however, was compensated by PD10 through a rapid increase in both, the expression of synaptic proteins and spine density.

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