Examination of Age-Dependent Effects of Fetal Ethanol Exposure on Behavior, Hippocampal Cell Counts, and Doublecortin Immunoreactivity in Rats

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ABSTRACT: **Ethanol is known as a potent teratogen having adverse effects on brain and behavior. However, some of the behavioral deficits caused by fetal alcohol exposure and well expressed in juveniles ameliorate with maturation may suggest some kind of functional recovery occurring during postnatal development. The aim of this study was to reexamine age-dependent behavioral impairments in fetal-alcohol rats and to investigate the changes in neurogenesis and gross morphology of the hippocampus during a protracted postnatal period searching for developmental deficits and/or delays that would correlate with behavioral impairments in juveniles and for potential compensatory processes responsible for their amelioration in adults. Ethanol was delivered to the pregnant dams by intragastric intubation throughout 7–21 gestation days at daily dose of 6 g/kg. Isocaloric intubation and intact control groups were included. Locomotor activity, anxiety, and spatial learning tasks were applied to juvenile and young-adult rats from all groups. Unbiased stereological estimates of hippocampal volumes, the total number of pyramidal and granular cells, and double cortin expressing neurons were carried out for postnatal days (PDs) PD1, PD10, PD30, and PD60. Alcohol insult during second trimester equivalent caused significant deficits in the spatial learning in juvenile rats; however, its effect on hippocampal morphology was limited to a marginally lower number of granular cells in dentate gyrus (DG) on PD30. Thus, initial behavioral deficits and the following functional recovery in fetal-alcohol subjects may be due to more subtle plastic changes within the hippocampal formation but also in other structures of the extended hippocampal circuit. Further investigation is required.** © 2013 Wiley Periodicals, Inc. Develop Neurobiol 74: 498–513, 2014

Keywords: **fetal alcohol; postnatal hippocampal development; rat; unbiased stereology; doublecortin immunoreactivity**

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INTRODUCTION

For thousands of years, ethanol has been the most widely abused drug in the world. Today alcohol is known as a potent teratogen. Exposure to ethanol *in utero* may cause a neurodevelopmental deficit called fetal alcohol syndrome (FAS) (Jones et al., 1973) or fetal alcohol spectrum disorders (Thomas et al., 2010; Warren et al., 2011), including brain damage resulting in a variety of cognitive and behavioral abnormalities.

Morphological, neurochemical, and electrophysiological studies suggest that among brain structures, the cerebellum and hippocampal formation are most vulnerable to the teratogenic effects of perinatal exposure to ethanol (Bonthius and West, 1990; Goodlett et al., 1997; Mihalick et al., 2001; Livy et al., 2003; Miki et al., 2003). This may be due to a particularly low content of biochemical antioxidants (i.e. Vitamin E) in these structures normally attenuating the potential effects of ethanol-induced oxidative stress (Abel and Hannigan, 1995). In light of these findings, it is not surprising that perinatal alcohol intoxication mostly affects motor and cognitive functions.

Generally, more pronounced deficits in learning and memory tasks were noted in juveniles as compared to adult subjects (Zimmerberg et al., 1991; Nagahara and Handa, 1997; Girard et al., 2000; Wozniak et al., 2004; Dursun et al., 2006). Amelioration of behavioral deficits with maturation may suggest an inherent ability to functional recovery in the young brain. The self-regenerative capability of a young brain after fetal exposure to ethanol is of considerable interest because it may contribute to human neurodevelopmental recovery also after other developmental insults. The importance of this issue has been recognized also by other researchers. Olney's group (Wozniak et al., 2004) has previously taken an attempt to investigate how behavioral deficits and potential recovery after neonatal alcohol insult relate to degenerative or regenerative changes in the brain.

The aim of this study was to re-examine the behavioral deficits in juvenile and young-adult fetal-alcohol rats and to compare developmental changes in the hippocampus of control rats and the rats exposed to ethanol during gestation to disclose developmental deficits and/or delays that would correlate with behavioral impairments in juveniles and if possible, to reveal a potential compensatory process that could underlie amelioration of cognitive deficits occurring with maturation. To do so, behavioral tests measuring locomotor activity anxiety and learning skills were applied to control and fetal-alcohol subjects from two age groups: juvenile and young-adult. In addition, age-dependent changes in the hippocampal volumes, counts of principal hippocampal neurons, and neurons expressing doublecortin (DCX), a marker for neurogenesis (Brown et al., 2003), were analyzed for different hippocampal regions throughout the first two postnatal months in rats exposed to ethanol intoxication during gestation days (GDs) 7–20.

MATERIALS AND METHODS

Subjects

A total of 120 adult Wistar rats (20 males and 100 females) obtained from the Gülhane Military Medical Academy, Animal Breeding Facility (Ankara, Turkey) were used for breeding in this study. The study consisted of three separate cohorts of animals: one used in behavioral experiments, second in stereological studies, and the third for DCX immunohistochemistry. Rats were housed in a secluded room with the temperature of $22 \pm 1^{\circ}C$ and under 12 h/12 h light/dark cycle commencing at 7:00 a.m. Throughout the experiment, animals had *ad libitum* access to food and water, except when stated otherwise (as described below). Female rats were individually housed in transparent Plexiglas cages. For mating, a male rat, picked at random, was placed into a female's cage for a maximum time period of 1 week. Each morning, female rats were examined for the presence of the vaginal plug, which was an evidence of successful fertilization, and this day was annotated as gestational day 0 (GD0). On GD7, pregnant dams were assigned (counterbalanced for initial body weight) to one of three treatment groups on average 15 dams per group: Alcohol group (A), pair-fed intubated control group (IC), a control for possible intubation-induced stress effects, and intact control group (C).

The day of birth was referred to as postnatal day 0 (PD0). At birth, the number of pups in each litter was counted. The body weight gains of dams and offspring were monitored on a daily basis. Until weaning at PD25, pups (except those killed earlier for stereological studies) remained with their natural dams. Afterward, pups were group-housed by litter and sex (on average four pups per cage) in transparent Plexiglas cages ($46 \times 24 \times 20$ cm). Because in most of the previous similar studies, the data have been analyzed for each sex separately; in this study, for more reliable comparison of our results with the literature, we used the male pups only. Male pups belonging to the dams from each of treatment groups (A, IC, and C) were randomly assigned to four age subgroups and killed for either stereological or DCX-IR studies at PD1($n = 19$ and $n = 22$), PD10 ($n = 23$, and $n = 19$), PD30 ($n = 23$ and $n = 23$), and PD60 ($n = 23$ and $n = 22$, respectively). In behavioral studies, only two age groups were used: PD30 ($n = 22$) and PD60 ($n = 20$). To limit the effects attributable to contributions from individual litters, the rats from each age/treatment group were intermixed between litters

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with no more than two pups from the same litter in a group. The pregnant dams and then the offspring were monitored with regard to body weight gain. All experimental procedures were approved by the Ethics Committee of the Middle East Technical University, Ankara, Turkey.

Ethanol Administration

Ethanol was administered by intragastric intubation (binge-like drinking model) allowing precise determination of the applied ethanol dose and ensuring higher peak blood alcohol concentration compared with the liquid diet (Bonthius and West, 1990). The protocol of ethanol administration was adopted from our previous study (Dursun et al., 2006). Starting from GD7 throughout GD20, dams from Group A were daily administered with 6 g of alcohol/kg body weight. Animals in Group IC received the same volume of fluid with sucrose substituted isocalorically for ethanol; they were also given the same amount of laboratory chow as the weight-matched dams from Group A. Animals in Group C received *ad libitum* access to laboratory chow and water with no additional treatment. The alcohol/isocaloric sucrose solution was delivered by intra-gastric intubations using a stainless curved feeding needle (18 ga, 3 in, Stoelting Co., Wood Dale, IL). The 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 (99.8% vol/vol, Merck) mixed with distilled water and stored at room temperature.

Determination of BAC

To avoid the potential effect of maternal stress induced by the blood collection on the pups, blood alcohol concentration (BAC) was assessed on GD20 in a separate group of pregnant dams ($n = 4$). Blood samples (1–2 ml) were taken from the rat-tail vein 2 h after the last intra-gastric intubation. The timing of blood collections was based on previous studies determining peak BAC in rat dams (Marino et al., 2002; Tran and Kelly, 2003). Blood samples were then centrifuged for 10 min at 1,000*g*, blood plasma separated, and stored at −80°C until BAC determination was accomplished. BAC (mg/dl) was determined by an alcohol assay kit (Biolabo, France) at the Gülhane Military Medical Academy as previously described (Uzbay et al.,2004; Sag et al., 2006).

Behavioral Testing

Behavioral tests were run at two ages: at P30 (juveniles) and at P60 (young-adults) in two controls (C30, $n = 8$ and C60, $n = 8$), two intubation controls (IC30, $n = 7$ and IC60, $n = 6$), and two fetal-alcohol groups (A30, $n = 7$ and A60, $n = 6$). Behavioral tests included open field (OF) (1 day), elevated plus maze (EPM; following 1 day), and Morris water maze (MWM; total 12 days), the latter test carried out in the presence and absence of allothetic (distal visuospatial) cues stimulus conditions. The OF (Hall and Ballachey, 1932; Denenberg, 1969; Prut and Belzung, 2003) and the EPM (Pellow et al., 1985; Lister, 1987) allow to test spontaneous locomotor activity and anxiety-like behavior in small rodents benefiting from these animals' innate tendency to avoid open, brightly lit, and/or elevated places. The MWM for long has been commonly used to assess hippocampus-dependent place learning corresponding to the episodic memory in humans (Morris, 1984).

OF Test. The OF apparatus constituted of a square box (120 × 120 cm) with 50 cm high side walls made of plain wood painted black and illuminated by a bright light from the ceiling. The rat was placed at the middle of one of the side walls facing the wall. Its locomotor activity was recorded by the computerized video tracking system (EthoVision System 3.1 by Noldus Information Technology, Holland). The OF was divided by virtual lines into 16 equal squares, 12 of which comprised the peripheral zone, and remaining 4, the central zone of the arena. The system recorded time spent and distance moved (ambulation) in each of the zones for 20 min in 5 min intervals.

EPM Test. The EPM was constructed of polyester and consisted of a central platform (10 × 10 cm), two open arms (50 × 10 cm), and two closed arms (50 × 10 cm) with dark, 30 cm high Plexiglas walls with no ceiling. The arms were arranged in a cross shape with the two open arms and two closed arms facing each other. The maze was elevated 80 cm above the floor. On a single testing session, each animal was placed in the center of the maze facing an open arm. Rats were allowed to explore the maze for 5 min. During this time, the number of entries with all four paws to the closed and open arms, the total time spent in closed and open arms separately, and total time spent on the central platform were recorded by the computerized video tracking system (EthoVision System 3.1). The EPM tests were carried out as described previously (Kayir and Uzbay, 2006).

Place Learning in the Morris Water Maze. MWM used to monitor spatial learning and memory in small rodents was a circular tank, 150 cm in diameter and 60 cm high. It was filled to the depth of 45 cm with water maintained at 23°C (±1) by an automatic heater. A nontoxic paint was used to make the water opaque. Computerized video tracking system (EthoVision System 3.1) was used to track the animal in the pool and to record data. The pool was virtually divided into four quadrants (NE, NW, SE, and SW). A movable platform (11 cm × 11 cm) made of transparent Plexiglas was located in the center of one of the quadrants. The top of the platform was 2 cm below the surface of the water such that the animal could not see it but could easily climb on it to escape from the water. Experimental room was furnished with several extra-maze cues immobile throughout the entire experimental period. These distal extra-maze cues were either available to the animals and could be used as a spatial reference frame in place learning (an allothetic paradigm defined as object-centered strategy

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of pathfinding), or eliminated by nontransparent curtains surrounding the pool. Prior to the place learning, animals were subjected to 1 day shaping training to learn swimming and climbing the platform. Shaping training was carried out with the pool surrounded by nontransparent curtains and the platform changing location between the trials (Dursun et al., 2006). It was applied to reduce the possible confounding effect of non-mnemonic factors arising from being introduced to a novel stressful situation. During following place learning, conducted both with and without allothetic reference frame, the platform was placed in the center of one of the quadrants (different for each stimulus conditions) where it remained throughout this stage of experiment. Rats were given four daily trials, for 4 consecutive days under allothetic paradigm and 6 consecutive days when the distal visuospatial cues were absent. Each rat was released into the water facing the pool wall at one of the four starting points (N, S, E, W), which were used in a pseudorandom order such that each start position was used only once during the daily experimental session. The trial was finished when the animal found the platform or 60 s passed. Later the rat was returned to its cage for a 5-min inter-trial interval. The video-tracking system was automatically recording the swim trajectory, swim velocity, escape latency, and the swim distance to reach the invisible platform.

On the completion of place learning, to assess the strength of the acquired place preference, a platform has been removed from the pool and a 60-s probe trial was carried out. On the computer screen, an imaginary annulus 40 (40 cm in diameter) was drawn around the place where originally platform was located. On the probe trial, the percentage time spent and the distance swum by the animal in the platform quadrant and in the annulus 40 were recorded.

Histological Procedures

Histological procedures included stereological cell counting and immunohistochemistry against DCX (DCX-IR). The morphology of the hippocampus from the control and fetal alcohol rat pups was examined under three treatment conditions (A, IC, and C) in four time windows: at PD1 (shortly after the birth), PD10 (at the end of the brain growth spurt period), PD30 (at the juvenile age, when the most prominent cognitive deficits used to be reported in fetal alcohol subjects), and PD60 (in young-adults). Total 12 groups were used in each study: A: $n = 6$; IC: $n = 6$; C: $n = 7$ for PD1, A: $n = 8$; IC: $n = 7$; C: $n = 8$ for PD10, A: $n = 8$; IC: $n = 7$; C: $n = 8$ for PD30, and A: $n = 8$; IC: $n = 8$, C: $n = 7$ for PD60 in stereological studies, and A: $n = 6$; IC: $n = 5$; C: $n = 9$ for PD1, A: $n = 8$; IC: $n = 6$; C: $n = 5$ for PD10, A: $n = 7$; IC: $n = 7$; C: $n = 8$ for PD30, and A: $n = 8$; IC: $n = 6$, C: $n = 8$ for PD60 in DCX-IR studies.

Fixation. Pups 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) (intraperitoneally) and perfused intracardially with 0.1 *M* phosphate buffer (pH 7.4) followed by 4% paraformaldehyde solution in 0.1 *M* phosphate buffer. The brains were removed from the skulls and postfixed overnight in 4% paraformaldehyde. After that, brains were cryoprotected with 30% sucrose solution in 0.1 *M* PBS until sunk, quickly frozen in liquid nitrogen and then stored at −80°C. The stereological studies on frozen sections have previously been successfully performed by other research groups (Goodlett et al., 1997; Bonthius et al., 2004; Fitting et al., 2010; Dursun et al., 2011; Boldrini et al., 2012; Vázquez-Roque et al., 2012).

Sectioning, Sampling, and Staining for Stereological Studies. The fixed brains were cut coronally on a Shandon Cryotome (Thermo Fisher Scientific Inc.) at the nominal setting of 50 μm and all sections that included the entire hippocampal formation (from the dorsal tip of the hippocampus, where the corpus callosum begins to form, and past the end of the ventral hippocampus) were collected. A systematic random sampling of one section out of every third in PD1, every fourth in PD10 and PD30 brains, and every fifth in the PD60 brains (16–22 section per rat) was carried out that comprised 2,000–2,500 μm of total hippocampal length at PD1, 3,000–3,500 μm at PD10, and 3,500–4000 μm at PD30 and PD60. Collection of the first section was random within the predetermined collection interval (Gundersen and Jensen, 1987). The sections were floated in 0.1 *M* PBS in 24-well plates, mounted on polylysine covered glass slides, dried at room temperature, and stained with cresyl fast violet (Nissl staining). The staining solution contained 1 g of cresyl violet acetate (Merck), 2–3 drops of glacial acetic acid, and distilled water. The mounted sections were dehydrated in increasing alcohol concentrations (70, 95, and 100%), defatted in xylene solutions, and eventually rehydrated in decreasing alcohol concentrations. Afterward, the slides were placed in the staining solution and differentiated in dilute acetic acid solution. Finally, the samples were dehydrated and cleared with xylene. Sections were then cover-slipped using Entellan (Merck) mounting medium (Dursun et al., 2013).

Stereological Cell Counting Procedure. The cell counts confined to the pyramidal cells in CA1 and CA2+3 regions, and granule cells in the DG from the left hippocampi were performed using unbiased stereological procedures. The unbiased stereology technique was applied using a commercial computer-assisted stereological workstation (StereoInvestigator, Microbrightfield, Williston, VT) including a high-resolution computer monitor DM5500 and a Leica light microscope equipped with a Leica DFC320 R2 digital firewire camera. Areal outlines and volumes were confined to the stratum pyramidale in regions CA1 and CA2+3 and the stratum granulosum in the dentate gyrus according to Paxinos and Watson (2007, figures 47–89) rat brain atlas and Paxinos et al. (2007, figures 63–78) developing mouse brain atlas. The

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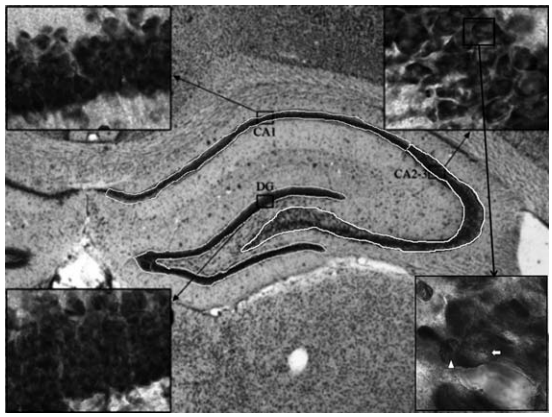

Figure 1 Photographs showing hippocampal regions at low (×10) and at high magnification (×100) lens, DG: dentate gyrus; CA1: Cornu Ammonis region 1, CA2: Cornu Ammonis region 2; CA3: Cornu Ammonis region 3. Arrow shows a neuron and the arrowhead shows a glial cell.

identification of the different hippocampal subdivisions was based on the previous anatomical reports (Blackstad, 1956; West et al., 1991; Tran and Kelly, 2003). Figure 1 presents a photomicrograph of a representative Nissl-stained coronal section through CA hippocampal region on which pyramidal and glial cells can be easily discriminated. The principal neurons of different hippocampal regions were clearly differentiated by their characteristic shapes, sizes, and densities, according to morphological criteria described by West et al. (1991). Nevertheless, in addition to principal neurons, the counts may also include basket interneurons that are difficult to discriminate. Basket cells, however, constitute only a very small fraction (less than 1%) of all neurons in granular and pyramidal cell layers (West et al., 1991). The neuroanatomical borders of the principal cell layers of the hippocampus were outlined under a low-power (4×) objective (Fig. 1) and the selected areas were systematically sampled with the aid of StereoInvestigator software (Microbrightfield, Williston, VT). The neuronal counts were carried out within these areas under a high-power oil immersion lens (100×, NA. 1.25), using motorized X–Y–Z stage controlled through the StereoInvestigator software package. The optical fractionator workflow extension of the StereoInvestigator software was used to quantify the total number of neurons. The counting frame size varied according to the neuron size in each region. For CA1 and CA2+3 regions, it was set to 25 × 25 μm with a grid (sampling step) size of 150 × 150 μm, and for DG region to 12 × 12 μm with a grid size of 120 × 120 μm. Counting was performed in each sampling step according to the rules of the unbiased counting frame and the optical dissector (West et al. 1991). A fixed dissector height of 10 μm was used in every counting step with a guard height of 2 μm from the top surface of each section to avoid errors when counting the cells at the cut surface. To calculate the mean section thickness [(t)], first, the thickness of each sampled section was estimated at every sampled dissector location and then the thickness estimates were averaged across the whole set of sampled sections. The thickness sampling fraction was estimated as the dissector height relative to the mean section thickness [$\text{tsf} = 10/(t)$]. An unbiased estimate of the total number of hippocampal pyramidal and dentate granular cells (N) was calculated by multiplying the sum of the neuronal counts over all sections ($\sum Q$) with the reciprocals of the sampling fractions as follows: $N = \sum Q^{-} \times (1/\text{ssf}) \times (1/\text{asf}) \times (1/\text{tsf})$, where ssf is the section sampling fraction (the actual number of sections sampled in relation to the total number of sections), asf is the areal sampling fraction (the area of the counting frame relative to the sampling area per each sampling step), and tsf is the thickness sampling fraction. Statistical evaluation and error determination of obtained estimates were determined by the coefficients of error (CE) (Gundersen et al., 1999).

Sectioning, Sampling, and Staining for DCX Immunohistochemistry Studies. To estimate the numbers of DCX-IR neurons on the hippocampal slices belonging to fetal-alcohol and control rat pups of four age groups, the fixed, frozen, and cryoprotected brains were cut coronally on a Shandon Cryotome (Thermo Fisher Scientific Inc.) at the nominal setting of 20 μm. In this study too, only left dorsal hippocampi were used with three to four section per rat (every 24th section) stained.

The sections used in DCX-IR cell counts were dried in an incubator for 20–25 min at 37°C. After rinsing with 0.1 *M* PBS once, the antigen retrieval was carried out by citrate buffer to uncover epitopes. Sections were kept inside the boiling citrate buffer for 15 min, then, they cooled down inside citrate buffer for 15 min. After rinsing in 0.1 *M* PBS (3 times, 5 min each time), the sections were incubated for 1 h at room temperature with blocking solution containing 5% normal goat serum (NGS) with 0.3% Triton-X-100 in PBS. Afterward, the sections were incubated at 4°C for 24 h with primary antibodies against DCX (cell signaling #4604, 1:200). The antibody dilution buffer contained 2% NGS dissolved in 0.3% Triton-X-100 in PBS. Upon the completion of incubation with the primary antibody, sections were rinsed in 0.1 *M* PBS (three times, 5 min each time), and incubated for 2 h at room temperature in a dark place with fluorescent-conjugated secondary antibody, Alexa Fluor 488, and goat anti-rabbit IgG (1:250) diluted with 2% NGS dissolved in 0.3% Triton-X-100 in PBS. The secondary antibody incubation was followed by washing the sections with PBS (three times, 5 min each time) and counterstaining the cell nuclei with 4′,6-diamidino-2-phenylindole (DAPI) (Kilic et al., 2010). After being washed with PBS, slides were cover slipped by fluoromount, a water soluble mounting media. Negative control was provided for each staining by omitting the primary antibody in antibody dilution buffer.

The obtained immunofluorescence sections were visualized using a Nikon Microscope equipped with a fluorescent attachment at 40× magnification. Three to seven pictures of each hippocampal region: CA1, CA3, subgranular zone (SGZ) of DG, and additionally of subventricular zone

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Table 1 Effects of Fetal Ethanol on the Survival of Rat Pups

Groups	Rate of Succesful Pregnancy (%)	Mean No. of Pups Per Litter	Survival Rate of Female Pups (%)	Survival Rate of Male Pups (%)
A	31.6	5.7	67.0	72.6
IC	45.3	6.1	95.4	89.2
C	70.0	6.7	93.3	97.9

(SVZ) (2300 pictures in total) were taken under fluorescence microscope at 40× magnification. To determine the number of DCX positive cells, the cell counter option of ImageJ software was used (Yamamura et al., 2011).

Statistical Analyses

Group means ± SEM were calculated for all measures. A repeated-measures analysis of variance (ANOVA) was conducted on the dams' body weight data throughout GD7–20 and on the behavioral data.

Pups' weights were analyzed for each postnatal age separately by one-way ANOVA with treatment as independent variable. The analyses of morphological data were performed for each hippocampal subregion independently and included cross-sectional comparisons of treatment effects at different ages and longitudinal comparisons of age effect for different treatment groups. In this study, the group sizes were similar with number of subjects per group varying between 6 and 8. The morphological data showed normal distribution as assessed by Kolmogorov-Smirnov normality test. Under these conditions, two-way ANOVA (treatment × age) was conducted to evaluate the main effects of age and treatment as well as age × treatment interaction. Additionally, the between-group differences in the estimates of volumes and cell counts for each hippocampal region at each postnatal age, and between different ages for the same hippocampal region in each treatment group separately 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 $P \leq 0.05$.

RESULTS

Dams and Pups Data

Gestational exposure to ethanol decreased the percentage of successful pregnancies and survival rate in neonates (Table 1). The litter size was affected less, however, the body weight at birth and PD10 was significantly lower ($P \leq 0.05$) in Group A (5.7 ± 0.1 and 14.6 ± 0.3, respectively) compared with both IC (6.1 ± 0.1 and 16.7 ± 0.7, respectively) and C (6.7 ± 0.2 and 15.9 ± 0.6, respectively) controls. This difference disappeared at PD30.

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 ($F_{(20:480)} = 35.020$, $P \leq 0.001$) and insignificant main effect of treatment and treatment × day interaction.

Blood Alcohol Concentrations

The mean maternal blood alcohol concentration estimated 3 h after the second intubation on GD20 was 244.8 ± 49.8 mg/dl. As ethanol readily crosses placenta (Kesaniemi and Sippel 1975), the fetal BAC is assumed to be close to the maternal BAC.

Behavioral Results

OF Test. Figure 2(A) presents the mean time spent in outer versus inner zone of the OF, an index of anxiety level. As seen from this figure, all rats regardless of treatment and age spent more time in the outer zone. Two way repeated measure ANOVA (treatment × zone) yielded significant zone effect ($F_{(1:19)} = 20291.32$, $P \leq 0.001$ for juveniles and $F_{(1:17)} = 20404.47$, $P \leq 0.001$ for young-adults) and significant zone × group interaction ($F_{(2:19)} = 13.20$, $P \leq 0.001$, and $F_{(2:17)} = 63.65$ $P \leq 0.001$ for juvenile and adult rats, respectively) with main group effect insignificant. However, one way ANOVA with group as an independent factor applied to each zone and each age group separately revealed significant between-group differences with intubated groups spending significantly less time in the inner zone compared with the intact control group ($F_{(2:19)} = 13.25$, $P \leq 0.001$ and $F_{(2:17)} = 64.15$ $P \leq 0.001$ for juvenile and adult rats, respectively).

Figure 2(B) shows the mean distance moved in two zones of the OF, by each of the treatment group, during the consecutive 5 min intervals of the total 20 min testing period. The distance moved is an index of animals' locomotor activity. Two-way repeated measure ANOVA (treatment × time) conducted for each age and zone independently confirmed a significant decline in the overall locomotor activity throughout the testing period in the outer zone of the arena ($F_{(3:57)} = 27.63$, $P \leq 0.001$ for juveniles,

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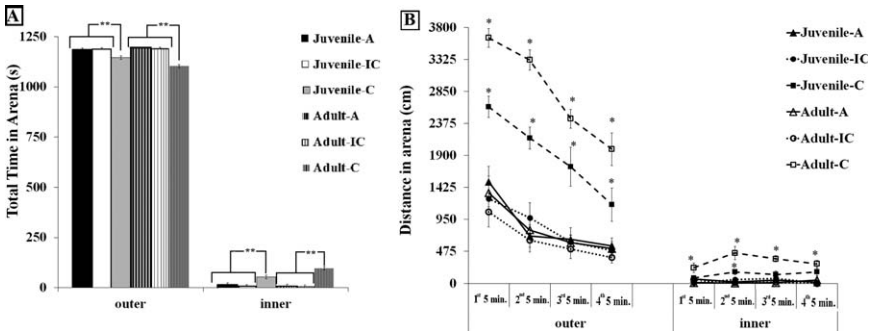

Figure 2 (A) The mean time (±SEM) spent in the different zones of the OF during the total 20 min testing period. (B) The mean distance (±SEM) moved in the outer and the inner zone of the OF, respectively, during the consecutive 5-min intervals of the total 20-min testing period for juvenile and young adult control and fetal-alcohol rats. Error bars denote SEM. The asterisks show the significant difference between intact control (C) and intubated groups (IC and A) for each postnatal age separately.

$F_{(3:51)} = 32.42$, $P \leq 0.001$ for young-adults). The main treatment effect was also significant in both juveniles and young-adults ($F_{(2:19)} = 21.36$, $P \leq 0.001$, $F_{(2:17)} = 104.31$, $P \leq 0.001$, respectively). One way ANOVA followed by the *post hoc* Fisher's LSD test performed for each zone, each age and each time interval independently revealed significantly lower locomotor activity in intubated groups (A, IC) compared with the intact control group ($P \leq 0.01$).

EPM Test. There was a trend among the fetal-alcohol rats, regardless of age, to spend relatively more time in the closed arms of the plus maze (Fig. 3); however, two-way ANOVA (treatment × age) performed for each arm independently yielded treatment effect insignificant with highly significant age effect ($F_{(1:36)} = 82.40$, $P \leq 0.001$ for open arms; $F_{(1:36)} = 76.86$, $P \leq 0.001$ for closed arms). A two-way repeated measure ANOVA (age × arm) also revealed a significant interaction between pups' age and their arm preference ($F_{(5:36)} = 22.77$, $P \leq 0.001$) with significantly higher preference of juveniles for closed and adults for open arms.

Morris Water Maze Test. In the course of training in the MWM, in all groups, a decrease in the swim distance to reach the hidden platform was observed [Fig. 4(A,D)]. In both paradigms (with and without allothetic cues), no significant difference in the task acquisition was noted between the adult groups. Two-way repeated measure ANOVA (treatment × day) yielded significant day effect only ($F_{(3:45)} = 12.61$ $P \leq 0.001$ in the allothetic paradigm; $F_{(5:80)} = 7.60$ $P \leq 0.001$ without distal visuo-spatial cues). The same analysis applied to the data from the juvenile groups revealed a significant day effect for both training conditions ($F_{(3:51)} = 24.83$ $P \leq 0.001$, $F_{(5:90)} = 10.44$ $P \leq 0.001$, respectively) and a significant main group effect in the training without allothetic cues only ($F_{(2:18)} = 9.53$ $P = 0.002$). One-way ANOVA performed for each training day and each age group independently, confirmed significantly worse performance in juvenile fetal alcohol pups compared with their age-matched controls on the first training day under allothetic cues ($F_{(2:17)} = 3.53$ $P = 0.05$) and on the four first training days without distal visuospatial cues ($F_{(2:18)} = 3.97$, $P = 0.037$; $F_{(2:18)} = 7.21$, $P = 0.005$; $F_{(2:18)} = 3.52$, $P = 0.05$; $F_{(2:18)} = 6.37$, $P = 0.008$, respectively).

On the probe trial, fetal-alcohol juvenile rats spent significantly less time in Annulus 40 under allothetic

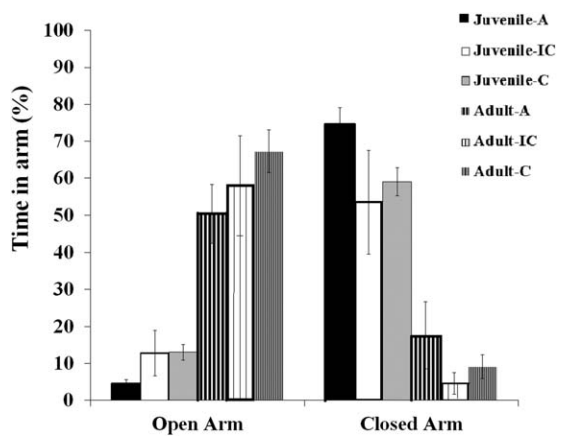

Figure 3 Comparison of the animal's behavior in the elevated plus maze test as a function of age (juvenile vs. young adult) and treatment (A, IC, and C). The bars represent mean time percent spent in open and closed arms of the plus maze. Error bars denote SEM.

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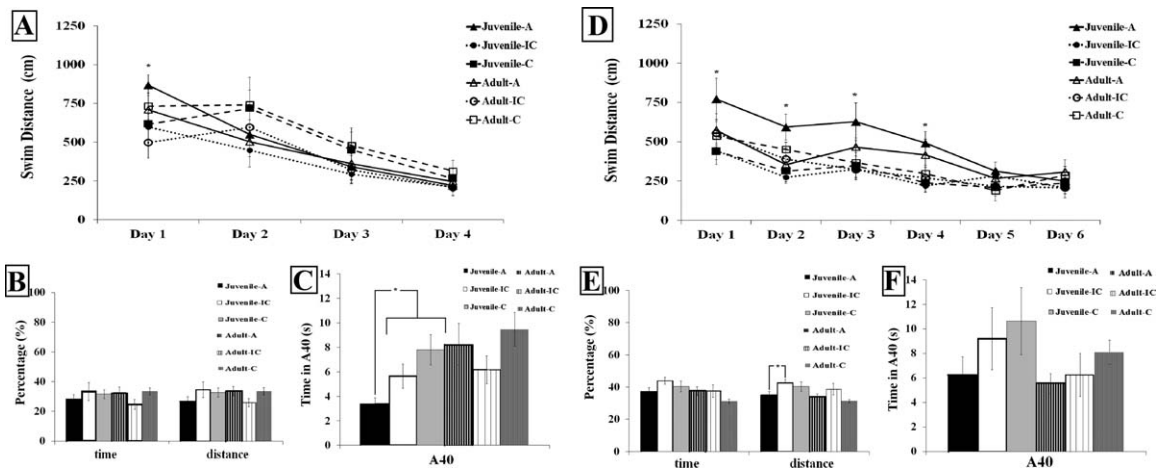

Figure 4 Mean swim distance (±SEM) calculated for the first 4 days of MWM training with allothetic cues (A) and six consecutive days of MWM training without distal visuospatial cues (D). Mean percent time (±SEM) spent and the distance swam in the platform quadrant on the probe trial under allothetic cues (B) and without allothetic cues (E). Mean time (±SEM) spent in the annulus 40 on the 60-s probe trial with (C) and without allothetic cues (*F*). Error bars denote SEM. Asterisk indicates significant difference at $P \leq 0.05$.

conditions [Fig. 4(C)] ($F_{(2:17)} = 5.83$, $P = 0.012$) and swam significantly shorter distance in the platform quadrant when trained without allothetic cues [Fig. 4(E)], $F_{(2:17)} = 3.13$, $P = 0.070$).

Estimates of Hippocampal Volumes. Table 2 presents volume estimates for each hippocampal region, each treatment group, and each postnatal age independently. Two-way ANOVA with age and treatment as independent variables performed on the volume data for each hippocampal region separately, yielded a significant main effect of age ($F_{(3:76)} = 272.33$, $P \leq 0.001$ for CA1; $F_{(3:76)} = 179.24$, $P \leq 0.001$ for CA2+3; and $F_{(3:26)} = 509.62$, $P \leq 0.001$ for DG). However, neither main group effect nor age × group interaction was significant.

Estimates of Total Neuron Numbers. Total numbers of neurons for each hippocampal region, each treatment group, and each postnatal age independently, are presented in the Figure 5 and in the Table 2. As seen from the Table 2, for all estimates, CEs were between 0.02 and 0.04 indicating sufficient accuracy in making estimates of total neuron number at the individual level (West et al., 1991). Table 2 also presents the coefficient of variance indicating interindividual variation for each group. The observed between-subject variation in the total number of granular and pyramidal cells was similar in the fetal alcohol and control groups.

Two-way ANOVA with treatment and hippocampal region as independent factors (3 × 3) performed on the estimates of neuron number at PD1 yielded the main effect of treatment and treatment × region interaction significant ($F_{(2:46)} = 2.84$, $P = 0.029$ and $F_{(4:46)} = 3.06$, $P = 0.026$, respectively) with region effect insignificant.

During the following two postnatal months, in all treatment groups and in all three hippocampal subregions, a significant increase in the number of principal neurons was observed. Two-way ANOVA with treatment and age as independent factors (4 × 3) performed for each hippocampal subregion independently revealed a significant effect of age in all three regions ($F_{(3:76)} = 171.24$, $P \leq 0.001$ for CA1; $F_{(3:76)} = 89.82$, $P \leq 0.001$ for CA2+3; and $F_{(3:26)} = 328.58$, $P \leq 0.001$ for DG). In all three hippocampal subregions, the greatest overall increase in the number of principal neurons was observed between PD1 and PD10 ($P \leq 0.001$). However, a slower but significant increase in neuron counts was found in all three hippocampal subregions also in PD10–PD30 and PD30–PD60 time windows (Fig. 5; Table 2).

The main treatment effect yielded by two-way ANOVA (treatment × age) was significant for CA1 region ($F_{(2:76)} = 3.45$, $P = 0.037$), marginally significant for CA2+3 region ($F_{(2:76)} = 2.706$, $P = 0.073$) and insignificant for DG region with age × group interaction significant for CA2+3 region only ($F_{(6:76)} = 2.67$, $P = 0.021$).

One-way ANOVA with treatment as independent variable performed for each postnatal age independently for CA1 region yielded a significant group effect on PD1 ($F_{(2:14)} = 3.837$, $P = 0.047$). On PD10, the main group effect approached ($F_{(2:20)} = 2.964$,

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Table 2 Mean Volumes and Total Neuron Number Estimates (±SEM) for Granular and Pyramidal Layers in DG and CA Subregions of the Hippocampus

		CA1				CA2+3				DG			
		Neuron Number (10^5)	CE	CV	Volume (mm^3)	Neuron Number (10^5)	CE	CV	Volume (mm^3)	Neuron Number (10^5)	CE	CV	Volume (mm^3)
PD1	A	1.7 ± 0.04	0.03	0.06	0.5 ± 0.02	2.0 ± 0.12	0.03	0.15	0.6 ± 0.04	1.8 ± 0.13	0.04	0.18	0.3 ± 0.02
	IC	1.9 ± 0.08	0.03	0.09	0.5 ± 0.02	2.1 ± 0.09	0.03	0.11	0.6 ± 0.03	1.8 ± 0.12	0.04	0.16	0.3 ± 0.02
	C	1.6 ± 0.08	0.03	0.12	0.4 ± 0.02	1.5 ± 0.11	0.03	0.19	0.6 ± 0.16	1.9 ± 0.14	0.04	0.20	0.3 ± 0.02
PD10	A	2.6 ± 0.05	0.03	0.05	1.1 ± 0.04[a]	2.6 ± 0.10	0.03	0.11	1.5 ± 0.07[a]	5.1 ± 0.11	0.03	0.06	0.9 ± 0.03[a]
	IC	2.8 ± 0.09	0.03	0.09	1.1 ± 0.04[a]	2.8 ± 0.05	0.03	0.05	1.6 ± 0.07[a]	5.3 ± 0.12	0.03	0.06	1.0 ± 0.03[a]
	C	2.6 ± 0.05	0.03	0.06	1.0 ± 0.03[a]	2.6 ± 0.06	0.03	0.06	1.5 ± 0.03[a]	5.0 ± 0.27	0.03	0.15	1.0 ± 0.03[a]
PD30	A	2.9 ± 0.07	0.03	0.07	1.2 ± 0.05[c]	2.9 ± 0.07	0.03	0.07	1.9 ± 0.07[a]	7.0 ± 0.28	0.03	0.11	1.4 ± 0.06[a]
	IC	3.0 ± 0.09	0.02	0.08	1.3 ± 0.06	2.8 ± 0.13	0.03	0.12	1.8 ± 0.04[c]	7.9 ± 0.32	0.03	0.11	1.4 ± 0.04[a]
	C	2.9 ± 0.09	0.03	0.09	1.3 ± 0.06[b]	2.9 ± 0.12	0.03	0.12	2.0 ± 0.11[b]	7.8 ± 0.35	0.03	0.13	1.4 ± 0.06[a]
PD60	A	3.4 ± 0.09	0.03	0.08	1.5 ± 0.04[a]	3.2 ± 0.11	0.03	0.10	2.3 ± 0.11[b]	10.6 ± 0.59	0.02	0.16	1.7 ± 0.07[a]
	IC	3.6 ± 0.15	0.03	0.12	1.5 ± 0.07[b]	3.4 ± 0.11	0.03	0.09	2.3 ± 0.10[a]	10.0 ± 0.45	0.03	0.13	1.7 ± 0.06[a]
	C	3.5 ± 0.13	0.03	0.10	1.6 ± 0.05[a]	3.3 ± 0.13	0.03	0.12	2.4 ± 0.13[b]	10.4 ± 0.46	0.03	0.12	1.8 ± 0.05[a]

CE, coefficient of error; CV, coefficient of variation.

[a]$P \leq 0.001$; [b]$P \leq 0.01$; [c]$P \leq 0.05$, p-values refer to the difference between two consecutive age groups.

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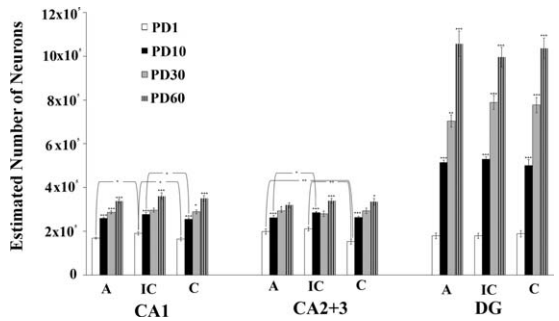

Figure 5 Comparison of mean total neuron numbers (±SEM) within hippocampal CA1, CA2 + CA3, and DG regions in fetal alcohol (A) and control (IC and C) rat pups at different postnatal ages: PD1, PD10, PD30, and PD60, respectively. Error bars denote SEM. Asterisks indicate significant difference between the two consecutive age groups (PD1 vs. PD10, PD10 vs. PD30, and PD30 vs. PD60): $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, respectively.

$P = 0.075$) but did not reached the accepted significance level of $P \leq 0.05$. *Post hoc* analyses revealed a significantly higher number of neurons in Group IC compared with Group C on both PD1 and PD10 ($P = 0.020$ and $P = 0.035$, respectively). In addition, on PD1, the total number of neurons in Group IC was significantly higher than that in Group A ($P = 0.046$). On PD10, the number of neurons in Group IC was also higher compared with Group A, but this difference did not reach the required level of significance remaining at $P = 0.064$.

A subsequent analysis for CA2+3 region using one-way ANOVA revealed a significant main effect of treatment on PD1 ($F_{(2:16)} = 7.925$, $P = 0.004$) with significantly higher number of neurons in both, Groups A and IC compared with Group C ($P = 0.009$ and $P = 0.002$, respectively). On PD10, a higher number of neurons was recorded in Group IC compared with both Groups A and C, however, the main group effect approached but did not reach the significance ($F_{(2:20)} = 2.798$, $P = 0.085$) (Fig. 5).

In DG region, on PD30, fetal-alcohol rats showed a trend toward having fewer granular cells compared with IC and C controls but these differences failed to reach significance ($P = 0.078$, and $P = 0.114$, respectively). The difference between Groups IC and C was insignificant.

Estimates of DCX-IR. Figure 6 shows representative images of immunostaining against DCX in CA1, CA3, SGZ, and SVZ regions, at four postnatal ages (PD1, PD10, PD30, and PD60), for the control group, while Figure 7 presents the numbers of DCX-IR neurons, for each region, postnatal age, and treatment

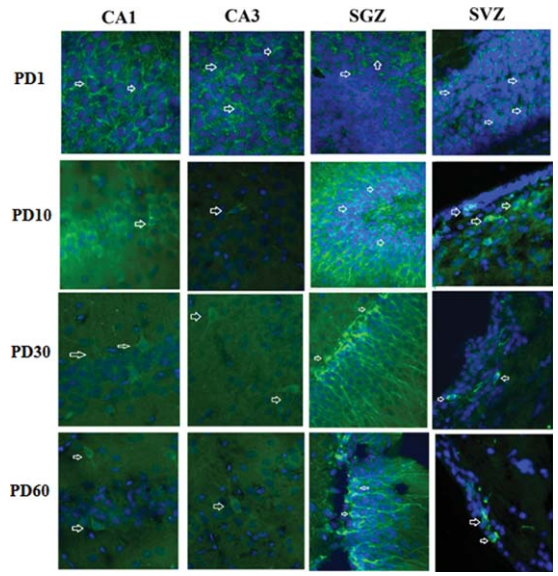

Figure 6 Representative photomicrographs showing DCX-immunoreactivity in CA1, CA3, SGZ, and SVZ regions for all experimental ages in the control group. Magnification, ×40; Arrows shows the DCX-positive cells; green: DCX; blue: DAPI (the nuclear stain).

group, separately. Two-way ANOVA with age and treatment as independent factors carried out on these data revealed highly significant effect of age in all four regions ($F_{(3:70)} = 77.79$, $P \leq 0.001$ in CA1 $F_{(3:71)} = 82.84$, $P \leq 0.001$ in CA3, $F_{(3:70)} = 26.88$, $P \leq 0.001$ in SGZ, and $F_{(3:65)} = 55.40$, $P \leq 0.001$ in SVZ). The main effect of treatment and age × treatment interaction were significant in CA1 region only ($F_{(2;70)} = 3.669$, $P = 0.031$ and $F_{(6;70)} = 2.468$, $P = 0.032$, respectively) with an overall number of DCX-IR neurons lower in the intubated control compared with Groups A and C.

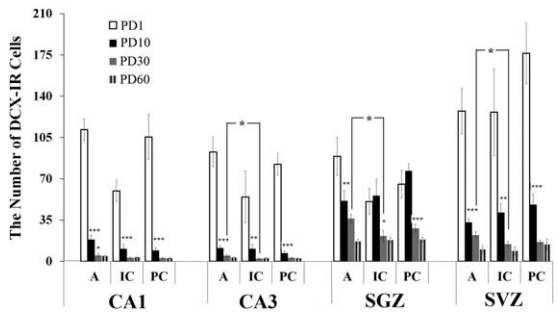

Figure 7 Comparison of the numbers DCX-IR neurons in alcohol (A) and control (IC and C) groups at different postnatal ages for CA1, CA3, SGZ, and SVZ regions of the left hippocampus. Error bars denote ±SEM. Asterisks denote the level of significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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In all hippocampal regions and all treatment groups, the estimates of DCX-IR cell counts were the highest on PD1, showing a decline during the following postnatal period. This decline was stepwise in SGZ and relatively rapid in the CA regions and SVZ (Fig. 7). Regardless of treatment, in all regions, except SGZ, the greatest decrease in DCX-IR cell counts was recorded between PD1 and PD10, while in SGZ, the greatest decrease was observed between PD10 and PD30 (Fig. 7).

According to the results of one-way ANOVA with treatment as independent variable, no significant between-group differences were found in the numbers DCX-IR neurons at birth and PD10. On PD30, the main treatment effect was yielded marginally significant ($F_{(2;21)} = 3.265$, $P = 0.060$; $F_{(2;21)} = 3.364$, $P = 0.056$; and $F_{(2;21)} = 3.100$, $P = 0.068$, for CA3, SGZ, and SVZ, respectively). The output of *post hoc* tests suggested, in Group A, a trend toward having a higher number of DCX-IR neurons compared with both Groups IC and C in CA3 ($P = 0.025$, $P = 0.069$, respectively) and in SVZ ($P = 0.029$, and $P = 0.074$, respectively). In SGZ, a significant difference was noted between Groups A and IC only ($P = 0.018$); however, there was no statistically significant difference between control groups.

DISCUSSIONS

To our knowledge, this is the first study examining behavior, changes in the hippocampal neuron numbers, and the expression of DCX (a neurogenesis marker) throughout the first two postnatal months in the same laboratory strain of control and fetal-alcohol rats. This study is complementing previous similar studies on the effect of neonatally applied ethanol on animal behavior and gross morphology of hippocampus and related structures (Wozniak et al., 2004).

Effects of Fetal-Alcohol on Behavior

In this study, both juvenile and young-adult fetal-alcohol rats manifested significantly lower locomotor activity and higher anxiety-like behavior. Although locomotor hyperactivity linked to the deficits in response inhibition has often been shown as a characteristic feature of FAS in human (Abel, 1982; Driscoll et al., 1990; Westergren et al., 1996), in the animal studies brought contradictory results: increase (Bond, 1981; Ulug and Riley, 1983; Meyer and Riley, 1986; Vorhees and Fernandez, 1986) or no change (Wigal and Amsel, 1990; Westergren et al., 1996; Randall and Hannigan, 1999; Carneiro et al., 2005; Dursun et al., 2006). These discrepancies may be due to differences in the experimental protocols used, and especially the differences in the ethanol dose (Bond, 1981) and timing of the exposure relatively to the developmental stage (Kelly et al., 1987; Tran et al., 2000; Tran and Kelly, 2003; Smith et al., 2012). On the other hand, increased anxiety was shown to suppress exploratory behavior and thus spontaneous locomotor activity in a novel environment (Osborn et al., 1998). This may explain lower activity scores in the OF observed in Group A, in this study. The increased anxiety levels in subjects exposed to fetal-alcohol have been reported previously (Weinberg et al., 1996; Ogilvie and Rivier, 1997; Osborn et al., 1998; Dursun et al. 2006; Gabriel et al., 2006) and linked to decreased sensitivity of $GABA_A$ receptor's to endogeneous anti-anxiety neurostereoids such as allopregnanolone (Zimmerberg et al., 1995) and/or increased activation of the hypothalamic-pituitary-adrenal axis making animals hyper-responsive to stressors (Austin et al., 2005; Kapoor et al., 2006).

However, the effects on locomotor activity and anxiety were not secular to the alcohol groups but were observed also in the Group IC which points toward the intubation-induced prenatal stress rather than alcohol effects *per se*.

Consistently with previous literature (Gianoulakis, 1990; Nagahara and Handa, 1997; Girard et al., 2000; Wozniak et al., 2004; Dursun et al., 2006), only juvenile fetal-alcohol rats demonstrated impaired learning and memory retention suggesting amelioration of learning deficits taking place with maturation in the animals exposed *in utero* to ethanol.

Hippocampal Volumes and Neuron Number Estimates

No significant between-group differences in the postnatal increase of hippocampal volumes were noted. The fastest volume increase in the CA2+3 and the slowest in the DG region could contribute to the relatively lower cell density in CA2+3 area and relatively high cell density in the DG area.

On PD1, no significant difference was found in the neuron counts between the three hippocampal subregions in fetal alcohol and control rats. Consistently with the literature data (Dobbing and Sands 1979; Goodlett et al., 1990; Bonthius and West, 1991), the greatest overall increase in the neuron numbers was observed during so-called brain growth spurt period (PD1-PD10). This increase was much faster (threefold) in DG as compared to CA subregions

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(approximately by 50%) resulting in a significant difference in the neuron counts between DG and CA regions already at PD10. However, during the following period, PD10-PD60, slower but still significant increase in the total neuron counts was recorded not only in DG known for its well-documented life-long neurogenesis (Bayer et al., 1982; Kaplan and Bell, 1983; Veena et al., 2011) but also in the Ammon's horn. The latter finding is at odds with some previous reports according to which neurogenesis in CA region is completed by the end of the first postnatal week (Bayer et al., 1993; Bandeira et al., 2009). There are, however, very few studies examining changes in the numbers of hippocampal neurons throughout an extended postnatal period and the discrepancies in the obtained results may arise from the differences in the cell quantification methods such as optical fractionator versus isotropic fractionators. Isotropic fractionator technique estimates neuron numbers by counting nuclear antigen (NeuN) marked isolated nuclei in homogenous suspension (Bandeira et al., 2009), which may produce underestimated results due to previously reported developmental delay in acquisition of NeuN by neurons (Lyck et al., 2007). On the other hand, however, another recently published study (Morterá and Herculano-Houzel, 2012) using the isotropic fractionator method, reported a continuous increase in neuron numbers in varies brain regions including hippocampus, throughout the period from birth to adolescence. In addition, results similar to ours were also reported by some other authors who used the optical fractionator technique for the quantification of total cell numbers (Gokcimen et al., 2007; Smith et al., 2008).

Counts of DCX Expressing Neurons

As expected, in all groups and brain regions, the highest numbers of DCX-expressing neurons were found at PD1 with the highest overall count of DCX-IR neurons in SVZ and no differences between the hippocampal subregions. During the postnatal development, a decline in the number of DCX-IR neurons was sharp in CA areas and step-wise in subgranular and subventricular proliferative zones. However, at more advanced postnatal ages (PD30 and PD60), DCX-IR was still detected not only in SGZ and SVZ but also in CA regions. This finding is in line with an increase in the estimates of neuron counts observed in CA regions between PD10–PD60. These data suggest a possibility of limited neurogenesis still taking place during a protracted postnatal period in the brain areas beyond DG and SVZ (Rietze et al., 2000; Inta et al., 2008).

Effects of Intubation-Induced Prenatal Stress and Fetal Alcohol on Hippocampal Neuron Counts and DCX Expression

Interestingly, pups born from intubated dams manifested a trend toward higher neuron numbers during the neonatal period, which in turn indicates towards an increased neurogenesis during the late gestation period in these groups. In contrast to this, a substantial body of evidence indicates that alcohol and stress inhibits rather than stimulates neurogenesis, and thus, adversely affects neuron counts in the hippocampus (Lemaire et al., 2000; Mirescu and Gould, 2006; Redila et al., 2006; Gil-Mohapel et al., 2010; Sliwowska et al., 2010). However, most of the experimental data on the effects of prenatal stress on hippocampal neuron counts were collected from adult animals. On the other hand, there is an evidence that the effects of prenatal stress on neurogenesis in hippocampus are age- (Koehl et al., 2009), gender- (Schmitz et al., 2002), and strain-dependent (Darnaudéry and Maccari, 2008; Lucassen et al., 2009). Interestingly, it was also reported that moderate ethanol intake, may increase rather than decrease neurogenesis (Miller, 1995; Aberg et al., 2005) and that the prenatal ethanol exposure may ameliorate the stress effects on hippocampal neurogenesis (Sliwowska et al., 2010). All these findings suggest that both developmental and adult neurogeneses are highly regulated processes.

The expected adverse effect of fetal ethanol *per se* on the postnatal estimates of hippocampal neuron numbers was very mild and confined to a marginal ($P \leq 0.078$) reduction in DG granular neurons at PD30 which correlated with spatial learning and memory deficits in juvenile fetal-alcohol rats. However, it is known that the severity of ethanol-induced hippocampal damage depends on several factors including developmental time point when alcohol was administered. Some previous reports demonstrated a reduction in neuron numbers only in rats treated with alcohol during the third trimester-equivalent but not prenatally (Maier and West, 2001; Livy et al., 2003; Tran and Kelly, 2003; Gonzàles-Burgos et al., 2006).

According to our data, during the neonatal period (PD1–PD10), neither dentate nor SVZ neurogenesis as assessed by the numbers DCX-IR neurons was significantly affected by the fetal-ethanol exposure and/or prenatal stress. However, in fetal-alcohol pups as compared to intact control, there was a trend toward a lower count of DCX-IR neurons in SGZ at PD10, and a general tendency towards higher number of DCX-IR at PD30 which correlated with relatively lower count of granular cells recorded in Group A at

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PD30 and an increase in the granular cells count in this group at PD60. The latter finding is consistent with the studies reporting a significant increase in the number of immature neurons in the DG in fetal-alcohol juvenile but not adult rats (Singh et al., 2009; Gil-Mohapel et al., 2010; Chang et al., 2012). These changes in the numbers of migratory neurons in DG suggest a delayed adverse impact of fetal-alcohol on the dentate neurogenesis and then escape from fetal ethanol-induced inhibition representing an intrinsic compensatory process occurring along with functional recovery from cognitive deficits.

Taken together, our results suggest an extended postnatal neurogenesis in both DG and CA hippocampal subregions with the time course of postnatal increase in neuron counts being region specific. The mild overall effect of fetal-ethanol exposure on hippocampal neurogenesis, total neuron counts and regional volumes proves lower vulnerability of the brain to detrimental ethanol effects during the second trimester equivalent relatively to the neonatal brain growth spurt period (Olney et al., 2002; Wozniak et al., 2004). In this study, in pups prenatally exposed to ethanol, a marginally significant reduction in neuron number was found on PD30 in DG only, which correlated with but could hardly be shown as the only reason of poorer cognitive performance observed in juvenile pups. Additional studies are needed to better understand which morphological and/or functional anomalies in postnatal development of hippocampus but also the other structures of the extended hippocampal circuit are responsible for the behavioral deficits observed in juvenile subjects prenatally exposed to alcohol abuse and which processes are responsible for their amelioration.

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