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Effects of prenatal binge-like ethanol exposure and maternal stress on postnatal morphological development of hippocampal neurons in rats.

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Highlights

- Postnatal development of hippocampal neurons lasted until weaning and was region-dependent.
- Prenatal ethanol and maternal intubation stress showed similar effects manifested as a small delay in the development of some morphological features.
- Dendritic arbor and spine development appeared to be most affected.

Abstract

BACKGROUND: Alcohol is one of the most commonly used drugs of abuse negatively affecting human health and it is known as a potent teratogen responsible for fetal alcohol syndrome (FAS), which is characterized by cognitive deficits especially pronounced in juveniles but ameliorating in adults. Searching for the potential morphological correlates of these effects, in this study, we compared the course of developmental changes in the morphology of principal hippocampal neurons in fetal-alcohol (A group), intubated control (IC group), and intact control male rats (C group) over a protracted period of the first two postnatal months.

METHODS: Ethanol was administered to the pregnant Wistar dams intragastrically, throughout gestation days (GD) 7–20, at a total dose of 6 g/kg/day resulting in the mean blood alcohol concentration (BAC) of 246.6±40.9 mg/dl. Ten morphometric parameters of Golgi-stained hippocampal neurons (pyramidal and granule) from CA1, CA3, and DG areas were examined at

critical postnatal days (PD): at birth (PD1), at the end of the brain growth spurt period (PD10), in juveniles (PD30), and in young adults (PD60).

RESULTS; During postnatal development, the temporal pattern of morphometric changes was shown to be region-dependent with most significant alterations observed between PD1-30 in the CA region and between PD10-30 in the DG region. It was also parameter-dependent with the soma size (except for CA3 pyramids), number of primary dendrites, dendrite diameter, dendritic tortuosity and the branch angle demonstrating little changes, while the total dendritic field area, dendritic length, number of dendritic bifurcations, and spine density being highly increased in all hippocampal regions during the first postnatal month. Moderate ethanol intoxication and the maternal intubation stress during gestation, showed similar, transient effects on the neuron development manifested as a smaller soma size in granule cells, reduced dendritic parameters and lower spine density in pyramidal neurons at PD1. Full recovery from these effects took place within the first 10 postnatal days.

CONCLUSIONS; This study showed regional and temporal differences in the development of different morphometric features of principal hippocampal neurons in intact subjects over a protracted 2-months postnatal period. It also demonstrated an overlap in the effects of a moderate fetal ethanol intoxication and a mild maternal stress produced by the intragastric intubation, a commonly used method of ethanol administration to the pregnant dams. Fast recovery from the adverse effects on the soma size, dendritic arborization and spines density observed at birth indicates towards the fetal ethanol/stress induced developmental retardation.

Keywords: prenatal ethanol intoxication; maternal stress during gestation; hippocampus; neuronal morphometry; rat

1. Introduction

Alcohol is one of the most commonly used drugs of abuse having serious teratogenic effects threatening public health, and thus being of a great clinical interest. Morphological, neurochemical, and electrophysiological studies carried out using animal models suggest that the hippocampal formation is one of the brain structures most vulnerable to the teratogenic effects of perinatal exposure to ethanol (Alfonso-Loeches and Guerri, 2011). Previous studies have shown that perinatal ethanol administration in the hippocampus results in dysregulated chemical signaling (Puglia and Valenzuela, 2010; Goodlett et al., 2005), inhibits LTP induction (Helfer et al., 2012),

affects hippocampal neurogenesis (Gil-Mohapel et al., 2010), increases apoptotic neuronal death (Olney et al., 2014), alters neural wiring (West et al., 1981), and decreases dendritic spine density (Berman and Hannigan, 2000). Along with these functional and structural changes cognitive deficits including attention, memory and learning impairments are commonly reported consequences of prenatal ethanol exposure (Sutherland et al., 2000; O'Leary et al., 2015).

The persistence of negative effects of prenatal exposure to ethanol in both humans and animals is still a matter of discussion. Our own studies (Dursun et al., 2006; Elibol-Can et al., 2014a) as well as some previous studies by other research groups (Nagahara and Handa, 1997; Wozniak et al., 2004) showed more profound behavioral deficits (attention deficit, hyperactivity, or cognitive impairment) in juveniles compared to the adult subjects. Attenuation of some behavioral disorders linked to the fetal-alcohol syndrome (FAS), which has been often observed along with maturation, may indicate the potential for recovery in the young brain, which in turn is of considerable interest also from the clinical perspective. To address this issue, in our previous investigations, we examined changes in the hippocampal volumes, numbers of principal hippocampal neurons, expression of doublecortin (DCX, a marker for neurogenesis), and the expression levels of synaptophysin and PSD95 protein (indirect markers for synapse density) over a protracted postnatal period in fetal-alcohol and control rats. In these studies, a moderate prenatal intoxication with ethanol showed a mild effect on hippocampal development limited to a marginally lower number of granular cells in DG on postnatal day (PD) 30 which correlated with a trend towards a lower count of DCX-immunoreactive neurons in subgranular zone at PD10 (Elibol-Can et al., 2014a). 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 the levels of synaptic proteins in either cornu ammonis (CA) or dentate gyrus (DG) of the rat hippocampus (Elibol-Can et al., 2014b). In the present study, we attempted to examine whether behavioral deficits and the following functional recovery previously observed in the juvenile fetal-alcohol subjects (Dursun et al., 2006; Elibol-Can et al., 2014a) may be due to some other subtle plastic changes within the hippocampal formation such as abnormalities in cellular morphology, which is known to be important for establishment of network connectivity and activity patterns (Krichmar et al., 2002; Schaefer et al., 2003). For this purpose, we have monitored morphological development of hippocampal principal neurons in three distinct hippocampal subregions: CA1, CA3, and DG, throughout the first two postnatal months in control and fetal-alcohol rat pups.

2. Materials and Methods

2.1. Subjects

Adult, naive, female (n=50) and male (n=15) Wistar rats, obtained from the Animal Breeding Facility of the Gülhane Military Medical Academy (Ankara), were initially used for breeding. The animals' maintenance conditions during breeding and rearing were the same as in our previous studies (Dursun et al., 2006, Elibol-Can et al., 2014a-b). Rats were allowed to mate until a vaginal plug, an evidence of a successful fertilization, was observed. 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), and intact Control Group (C).

2.2. Ethanol Treatment

Starting from the GD7 throughout GD20, dams from A group were daily administered 6 g ethanol (96.5% v/v, Merck) per kg body weight by intragastric intubations using a stainless, curved feeding needle (18 ga, 3 in, Stoelting Co., Wood Dale, IL). The alcohol solution was prepared daily as a 25% (vol/vol) ethanol mixed with distilled water which was divided into two equal doses given to animals 1 h apart (Dursun et al., 2006; Elibol-Can et al., 2014a-b). Animals in IC group received the same volume of fluid with sucrose substituted isocalorically for ethanol. Animals in C group received *ad libitum* access to laboratory chow and water with no additional treatment. All animals were daily weighted and there were no significant differences between control and ethanol-treated dams in their body mass.

2.3. Pups

In order to exclude sex as an additional independent variable in the data analysis, in this study, we used only the male subjects (Dursun et al., 2006; Elibol-Can et al., 2014a-b). Male pups belonging to the mothers from each of treatment groups were randomly assigned to 4 age sub-groups sacrificed at birth (PD1) (n=87), at the end of the brain growth spurt period (PD10) (n=88), after weaning at the juvenile age (PD30) (n=90), and as young-adults (PD60) (n=90) with 7-8 pups per each treatment/age group.

To limit the effects attributable to contributions from individual litters the rats from each age group were intermixed between litters with one or maximum two pups from a single litter assigned to each of the age groups.

2.4. Tissue Preparation and Golgi Staining

Pups were first intracardially perfused with saline that was followed by a quick removal of the brains. The rapid Golgi staining was performed by using the FD Rapid GolgiStain™ kit. After impregnating and fixing the tissues for approximately 2 weeks, the brain specimens were cut into 100μm sections (Lawrence et al., 2012; Milatovic et al., 2010; Min et al., 2016) on Leica cryostat at -22^0C. Following drying, the sections were stained with appropriate solutions of the kit and counterstained with cresyl violet. After dehydrating in increasing concentrations of alcohol and clearing in xylene, the sections were cover-slipped with Permount mounting media and kept in a dark place.

2.5. Imaging and Morphometric Measures

Ten morphometric parameters of hippocampal principal neurons were evaluated in fetal-alcohol and control rats from each age group. Evaluated parameters included soma size, dendritic field area, total dendrite length (sum of all branch lengths), number of primary dendrites, dendrite diameter, number of dendritic branches, the highest branch order (the number of bifurcations from the cell body), mean branch angle (bifurcation angle), dendritic tortuosity (meandering estimated as the ratio of the distances from the soma to dendritic tips measured along the branches and in a straight line), and finally spine density (the number of spines per 1μm of dendrite length).

The stained cells from both left and right dorsal hippocampi were first traced with Neurolucida software under a light microscope at 100X magnification and then analyzed with Neuroexplorer software. The same criteria were used for the selection of the pyramidal neurons in CA1 and CA3 and granular neurons in DG of hippocampus (Ruan et al., 2006). According to these criteria: (1) the neurons should be densely stained so all the processes could be followed to the end at high magnification; (2) the clear image of dendritic spines on these processes is the indication of good staining; (3) no abrupt truncation of dendritic branches should be present; (4) only one neuron should be located within the viewing area to avoid possible mismatches of dendrites from other neurons.

Minimum 7 subjects were in each treatment/age group. From each hippocampal region (CA1, CA3, and DG) generally 5-8 neurons (total 1574 neurons) meeting the selection criteria were chosen for each animal. Most of the measurements were double checked by two researchers independently.

Statistical Analyses

Group means ± SEM were calculated from all measures. The morphological data showed normal distribution as assessed by Kolmogorov-Smirnov normality test. Under these conditions a two-way ANOVA (treatment x age) was used to evaluate the main effects of age and treatment as well as an age x treatment interaction. Additionally, the differences in the morphometric parameters between the treatment groups for each hippocampal region and each postnatal age, and between different ages for the same hippocampal region in each treatment group were analyzed by one-way ANOVA with subsequent post-hoc comparisons by Fisher's Least Significant Difference (LSD) test using SPSS 15 statistical package. The criterion of statistical significance was $p \leq 0.05$.

3. Results

3.1. Blood alcohol concentration (BAC)

At the GD20, the mean blood alcohol concentration in pregnant rats from A group estimated 3h after the second intubation was 246.6±40.9 mg/dl. Considering high rate of ethanol diffusion from the mother's placenta to the fetus blood, the mean fetal BAC should be close to this value.

3.2. Morphometric measures

The dendrites of pyramidal neurons were labelled as apical (AD) and basal (BD) according to their location in either stratum radiatum or stratum oriens. The developmental profiles as line drawings and the representative pictures of Golgi-impregnated hippocampal pyramidal and granular neurons are presented in Fig. 1. The following figures present treatment- and age-dependent changes in neurons' morphometric parameters over the protracted postnatal period.

INSERT Fig. 1.

3.2.1. Soma size

Figure 2 shows developmental changes in neurons' soma area in control and fetal alcohol pups. Two-way ANOVA (treatment x age) performed on these data for each hippocampal region independently yielded a significant main effect of age for the pyramidal neurons in both CA1 and CA3 regions ($F_{(3:74)}$=5.727, p=0.001, $F_{(3:74)}$=59.845 p<0.001, respectively) but not for the DG granule cells. As seen from this graphic, the greatest soma growth was noted in CA3 region during the brain growth spurt period between PD1-PD10 ($p \leq 0.001$) followed by some retraction in the soma size between PD10-PD30 ($p \leq 0.05$). In the DG region, the main effect of age was insignificant ($F_{(3:73)}$=2.378, p=0.078) with significant treatment x age interaction ($F_{(6:73)}$=2.995, p=0.011). On PD1, the soma of DG granular cells in A group was significantly smaller compared to control (p=0.018). At birth, the soma of CA3 pyramids was also smaller in the fetal-alcohol group compared to the intact control but the difference did not reach the accepted level of significance (p=0.065). Interestingly, in all three regions, during brain growth spurt period, the greatest increase in the soma size was observed in fetal alcohol groups. A one-way ANOVA with age as an independent factor confirmed significant increase in the soma size in CA1 pyramids and DG granule cells in A groups between PD1 and PD10 (p=0.009 and p=0.001, respectively).

INSERT Fig. 2.

3.2.2. Dendritic parameters.

The greatest developmental changes were noted in the dendritic arbor.

INSERT Fig. 3.

The dendritic parameters that underwent significant changes during the postnatal period studied are presented in the Fig.3 and Fig.4. A two-way ANOVA performed for the *dendritic field* confirmed a significant main effect of age for CA1, CA3, and DG regions ($F_{(3:74)}$=134.156, $p \leq 0.001$, $F_{(3:74)}$=224.511, $p \leq 0.001$, and $F_{(3:74)}$=88.243, $p \leq 0.001$, respectively) with a significant increase in the dendritic field area between PD1-PD30 in all three groups ($p<0.05$, Fig. 3A). In the intact control, the expansion of dendritic field of CA3 pyramids and DG granule cells still continued between PD30 and PD60 ($p \leq 0.05$). A one-way ANOVA yielded a significant treatment effect at PD1 only ($F_{(2:19)}$=8.893, p=0.002) with smaller dendritic field in A and IC groups compared to C group in CA pyramidal neurons ($p \leq 0.01$).

As estimated by a two-way ANOVA, in all groups, the main effect of age on the *total dendritic length* evaluated for AD and BD separately, was also highly significant in all three regions (CA1: $F_{(3:74)}$=124.386, $p \leq 0.001$ for AD and $F_{(3:74)}$=136.866, $p \leq 0.001$ for BD; CA3: $F_{(3:74)}$=122.992, $p \leq 0.001$ for AD, $F_{(3:74)}$=151.026, $p \leq 0.001$ for BD; DG: $F_{(3:72)}$=141.929, $p \leq 0.001$). A one-way ANOVA with treatment as an independent variable revealed significantly shorter ADs and BDs at PD1 in A and IC groups compared to C group ($p<0.05$) (Fig. 3B). Both CA1 pyramidal and granule cells' dendrites reached maximum length at PD30, while CA3 ADs still elongated during PD30-PD60 period (p=0.05) contributing to the further increase in the total dendritic field of CA3 pyramids. Interestingly, at PD60, in A and IC group, CA1 BDs manifested significant retraction compared to C group ($F_{(2:18)}$=4.989, p=0.019).

In all neurons, two-way ANOVA confirmed a significant main effect of age on the *total dendritic branch number* (CA1 ADs: $F_{(3:74)}$=70.991, $p \leq 0.001$; CA1 BDs: $F_{(3:74)}$=85.903, $p \leq 0.001$; CA3 ADs: $F_{(3:74)}$=77.085, $p \leq 0.001$, CA3 BDs: $F_{(3:74)}$=79.932, $p \leq 0.001$; DG; $F_{(3:73)}$=21.333, $p \leq 0.001$), with a significant increase in the number of branches between PD10-PD30 for AD and between PD1-PD30 for BD (Fig. 4A). At PD1, a one-way ANOVA with treatment as an independent variable confirmed a significantly lower total number of dendritic branches in the CA1 and CA3 pyramidal

neurons, in both intubated groups compared to C group ($p \leq 0.05$). Interestingly, at PD10, CA1 ADs of intubated rats had significantly more branches than ADs in the C group, and, at PD30, CA1 BDs from A group had more branches compared to both controls ($p \leq 0.05$). At PD60, a significant retraction of dendritic branches of CA1 BDs took place in A and IC groups ($p \leq 0.01$).

INSERT Fig.4.

A significant main effect of age effect was also revealed by a two-way ANOVA for *the highest dendritic branch order* in all studied types of neurons (CA1 ADs: $F_{(3:74)}=64.097$, $p \leq 0.001$; CA1 BDs: $F_{(3:74)}=118.644$, $p \leq 0.001$; CA3 ADs: $F_{(3:74)}=68.490$, $p \leq 0.001$; CA3 BDs: $F_{(3:74)}=143.709$, $p \leq 0.001$; DG: $F_{(3:73)}=16.280$, $p \leq 0.001$). A one-way ANOVA confirmed a significant increase in the branch order between PD10-PD30 in ADs ($p \leq 0.01$) and between PD1-PD30 in BDs of pyramidal cells ($p \leq 0.001$) (Fig. 4B). The main effect of treatment was significant in CA3 BDs only ($F_{(2:74)}=3.223$, $p=0.045$), while a significant age x treatment interaction was found in CA3 ADs ($F_{(6:74)}=3.112$, $p=0.009$). At PD1, the highest branch order in both ADs and BDs of CA pyramids was generally lower in the intubated groups compared to the intact control, however, this difference reached the level of significance only in CA3 area ($F_{(2:19)=}13.823$, $p \leq 0.001$ for ADs and $F_{(2:19)}=4.615$, $p=0.023$ for BDs). These relations were reversed at PD10 with A group manifesting a significantly higher order of CA1 AD branches compared to C group ($F_{(2:21)}=4.109$, $p=0.033$).

In contrast to the previously presented dendritic parameters, the postnatal changes in the total number of the primary primary dendrites, dendrite diameter, dendrite tortuosity and the planar dendritic branch angle were relatively small with minor treatment effect if at all (Suppl. Fig.1).

A two-way ANOVA performed on the *number of primary dendrites* (apical dendrites of DG granule cells and basal dendrites of CA pyramidal cells since the latter neurons have a single AD) yielded a significant main effect of age in all three regions ($F_{(3:74)}=5.611$, $p=0.002$ for CA1; $F_{(3:74)}=4.436$, $p=0.006$, for CA3; $F_{(3:72)}=8.776$, $p \leq 0.001$, for DG) which mainly reflected an increase in the dendrite number during brain growth spurt period in A groups and dendritic retraction in DG granule dendrites between PD10-PD30 ($p \leq 0.01$) (Supp Fig. 1A). Interestingly, on PD1, in all three regions, the mean number of primary dendrites in A group was lower compared to C group although these differences did not reach the required level of significance. Nevertheless, during brain growth spurt period, a significant increase in the dendrite number was noted in A groups in all regions

($p \leq 0.05$ or less), ending up with a significantly higher dendrite number in A group compared to control in CA1 region at PD10 ($p \leq 0.05$).

Analysis of the *dendritic diameter* revealed a significant main effect of age for ADs in CA1 ($F_{(3:74)}=2.780$, $p=0.047$) and DG granule cells' dendrites ($F_{(3:73)}=6.884$, $p \leq 0.001$) with a general tendency for a significantly larger dendrite diameter in A and IC groups on PD1 as compared to control (Supp Fig. 1B). This difference reached an accepted significant level in CA3 apical dendrites ($p \leq 0.01$). At PD10, the mean diameter of CA1/3 and DG apical dendrites and CA1 basal dendrites in the intubated groups has been reduced ($p<0.01$). This regressive process was especially pronounced in A group. As a result, at PD10, the diameter of CA1 apical and basal dendrites, CA3 basal dendrites, and DG granule cells' dendrites was significantly smaller in fetal alcohol group than in the intact control ($p \leq 0.05$), however, it recovered its normal size at PD30.

Analysis of *tortuosity* of ADs and BDs revealed a significant main effect of age in CA1 neurons of control rats ($F_{(3:74)}=5.298$, $p=0.002$ and $F_{(3:74)}=4.575$, $p=0.005$, respectively) and in granule neurons' dendrites in all three groups ($F_{(3:73)}=15.160$, $p \leq 0.001$). The dendritic tortuosity decreased along with dendritic elongation during the first postnatal months ($p \leq 0.05$) (Suppl. Fig. 1C).

A two-way ANOVA analysis performed for the *planar dendritic branch angle* yielded a significant age effect in CA1 BDs ($F_{(3:74)}=15.993$, $p \leq 0.001$) with a significant decrease between PD1-PD10 in the control ($p<0.01$) and between PD10-PD30 in the intubated groups ($p<0.05$) ending up with a significantly smaller branch angle in A group compared to control at PD30 ($p \leq 0.05$) (Suppl. Fig. 1D). In DG dendrites the main age effect on the dendritic branch angle was also yielded significant ($F_{(3:73)}=20.516$, $p \leq 0.001$) reflecting a decrease in the branch angle in intubated groups between PD1-PD30 ($p<0.05$).

To further evaluate the complexity of dendritic tree, the Sholl analysis was applied, wherein numbers of dendrite intersections for concentric circles of gradually increasing radius (10 μm increments) centered at the centroid of the cell body were counted.

INSERT Fig.5.

Results of Sholl analysis were largely consistent with the previous data. Here too, treatment effect was mostly seen on PD1 where a two-way repeated measure ANOVA analysis (sholl ring x

treatment) yielded significant group effect in BDs of CA1 and CA3 neurons ($F_{(2:16)}$=7.576 p=0.005, and $F_{(2:19)}$=9.111, p=0.002, respectively) (Fig. 5). On PD1, in the A group, a one-way ANOVA revealed significantly lower numbers of intersections in CA1 BDs between 40 and 60 μm ($p \leq 0.05$), in CA3 BDs between 50 and 60 μm ($p \leq 0.05$), in CA1 AD at 220 μm (p=0.011), and in CA3 AD between 10-70 μm from soma ($p \leq 0.05$). On the contrary, at PD10, in the A group compared to the control, an increase in the number of intersection was noted in CA1 ADs at 220 μm but also in granule cell dendrites at 100 μm ($p < 0.05$). At PD30, further increase in the number of intersections was observed in the A group in CA1 BD between 20 and 50 μm ($p \leq 0.05$). However, in the A group, at PD60, a significant decrease in the complexity of CA1 ADs between 300 and 350 μm ($p \leq 0.05$) and in CA1 BDs at 120 μm (p=0.039) was observed.

3.3.3. Spines

The dendritic spine density was measured in the third branch of AD and the second branch of BD in CA pyramidal neurons, and in the second branch of apical dendrites of DG granule cells.

INSERT Fig.6.

A two-way ANOVA analysis performed on dendritic spine density showed a significant main effect of age for both, AD and BD in all three hippocampal regions (CA1: $F_{(3:66)}$=44.550, $p \leq 0.001$ for AD and $F_{(3:65)}$=34.457, $p \leq 0.001$ for BD; CA3: $F_{(3:69)}$= 37.098, $p \leq 0.001$, for AD and $F_{(3:67)}$=11.286, $p \leq 0.001$ for BD; DG: $F_{(3:68)}$=66.175, $p \leq 0.001$). As seen from Fig. 6, the most pronounced increase in the spine density took place between PD1-PD10 (CA3 & DG) and PD10-PD30 period (CA1 & DG). According to a one-way ANOVA, at PD1, the spine densities on apical and basal dendrites of pyramidal neurons were lower in A and IC groups compared to C group ($p \leq 0.05$), however, this spine deficit was compensated by PD10. At PD60, a significant pruning of DG spines was noted in the fetal alcohol group ($p < 0.001$).

INSERT Fig.7.

In this study, the densities of different spine types were also evaluated. The representative pictures are presented in Fig.7A, and the densities of major spine types (thin, mushroom, stubby, and branched) are shown in the Fig.7B. At PD1, stubby spines (spines with head widths equal to neck lengths) prevailed. With maturation, thin spines became the second most crowded spine type. The

overall frequency of branched spines was the lowest. At PD1, the density of predominating stubby spines in CA1 and DG regions was significantly higher ($p \leq 0.05$) in the C group compared to the intubated groups, A and IC. At PD10, the number of the stubby spines in the two intubated groups caught up or even overrode their number in the C group. At PD10, the density of stubby spines on CA1 AD, density of thin, mushroom and branched spines on CA3 AD, and the density of thin spines on the granule neuron dendrites were significantly higher in the alcohol group compared to the control ($p \leq 0.05$). At PD30, the density of mushroom spines on CA1 basal dendrites was also significantly higher ($p \leq 0.05$) in the A group compared to controls. However, at PD60, the density of branched spines on CA3 basal dendrites was shown to be significantly lower in the A and IC groups compared to the intact control ($p \leq 0.05$).

4. Discussion

4.1. Postnatal development of hippocampus in intact animals.

To our knowledge, up-to-date, this is the only study simultaneously examining the development of such a large number of morphometric parameters of the principle hippocampal neurons during a protracted 2-months postnatal period in the same rat population. Basing on our results, the studied parameters could be easily divided into two classes: those which postnatally underwent profound developmental changes and those which, after the birth, changed very little.

Among morphometric parameters showing none or very small postnatal changes were dendrite tortuosity and the planar branch angle. In the CA1 and DG regions, these two parameters showed a decrease tendency observed especially between PD10-30 and thus after the most intense synaptogenesis as indicated by the peak in the spine density occurring around PD10 (Fig.6). It has been suggested that tortuosity might be especially important at very early stages of development when outgrowing fibers search their destinations (Stepanyants et al., 2004). On the other hand, a decrease in the branch angle and straightening of dendritic segments is increasing the distance between the cell body and tips of dendrites which may affect neuron geometry and thus its electrical properties.

Two other neuronal parameters which were fairly stable throughout postnatal ontogenesis of CA pyramidal cells are dendritic diameter and the number of the primary dendrites emerging from the soma. This is consistent with early reports by other authors (Minkwitz, 1976; Pokorný and Yamamoto, 1981) stating that the number of dendrites is established first and then followed by growth and branching. In contrast to this and consistently with other recent reports (Wu et al., 2015*)*, the number of primary dendrites in DG granule cells was reduced with time (PD10-PD30).

In neurons, reaching the adult size of soma at the early stage of postnatal ontogenesis is consistent with a role the cell body plays in the production of regulatory and structural proteins indispensable for the fiber outgrowth and formation of synaptic terminals. In this study, at PD1, the soma size of principal neurons was similar in all three hippocampal regions. The postpartum soma size was preserved in DG granule neurons and changed very little in CA1 pyramids. In contrast, the soma size of CA3 pyramids underwent a significant increase during PD1-PD10 period making these neurons the largest ones within the hippocampal formation. Some recent studies showed that, in

different types of neurons, the changes in the soma size are controlled by a differential gene expression, i.e. 18s rRNA expression (Ransdell et al., 2010). Interestingly, during the brain growth spurt period (PD1-10), the DG granule cells preserving their initial soma size undergo very rapid neurogenesis, showing a threefold increase in the number as compared to the CA region (Elibol-Can et al., 2014a).

Among the morphometric parameters manifesting the most pronounced postnatal changes were those related to dendritic ramification and formation of synaptic contacts, namely, the dendritic field area, the mean total dendritic length, total number of dendritic branches, the highest branch order, and the spine density, all manifesting a significant increase in all hippocampal regions and all treatment groups. It is consistent with some previous studies reporting a strong positive correlation between these parameters in the mouse retinal ganglion cells where, however, all four parameters reached maximum values at PD8 (Qu and Myhr, 2011). In contrast to that, in our study, the changes in dendritic field area, total dendritic length, number of dendritic branches, and the highest branch order, in both CA1 and CA3 subregions, were not confined to so called brain growth spurt period, but continued through 1-30 postnatal days reaching adult values at the end of juvenile period. In the DG, the most pronounced changes in the dendritic ramification (number of dendritic branches and the highest branch order) were observed during the period between PD10-PD30. Also in other species i.e. monkey, the maturation of DG granule cells followed the period of a profound increase in the cell numbers in this region, and thus, was delayed comparing to the pyramidal neurons (Jabes et al. 2011). In rat, the peak values of the dendritic parameters overlap with increased locomotor and sensory activity after weaning which promotes hippocampus-dependent learning of physical environment and social interactions (Rubin et al., 2014). The density of bifurcations, branch points, and the path length are known to have an important impact on the integration, filtering, and propagation of synaptic signals and may be sculptured in the activity-dependent manner. It is not surprising then, that, establishing the final pattern of dendritic arborization has been observed in many types of neurons throughout the whole postnatal development (Brown et al., 2008). Dendrites are dynamic structures and remodeling of the dendritic arbor may take place even in the adult nervous system (McAllister, 2000). Even more dynamic then dendrites themselves are dendritic spines, which constitute the main locus of excitatory synapses. There are different types of spines such as thin (immature), stubby (short and thick), mushroom-like (narrow neck and wide head) and those with branched heads. Shapes of

spines affect their electrical resistance and thus signal integration at dendritic synapses. Postnatal ontogenic development of dendritic spines involves changes in both, number and shape. In this study, the overall increase in spine density took place between PD1-PD10 in CA3 and between PD1-PD30 in CA1 and DG regions. The proportions of different spine types in different hippocampal regions were similar, with stubby spines predominating at PD1, and substantial increase in the count of thin spines at PD30 when they became the second, most abundant spine type. A significant increase in the numbers of mushroom and branched spines (an indirect index of synaptogenesis) was also observed during an extended period between PD1-PD30 especially on AD in CA1 and DG. However, the overall frequency of branched spines, was the lowest compared to other spine types. The latter results are consistent with reports by Rochefort and Konnerth (2012) confirming the prevalence of stubby spines during early postnatal period (PD1-PD10) and that of thin and mushroom spines later during the development (PD10-PD30). On the other hand, our observation of the presence of branched spines already at PD10 is somewhat contradictory to an early report by Fiala and coworkers (1998) stating the lack of branched spines in rat hippocampus until PD15.

In summary, the presented data demonstrate that the profound developmental changes in the rat hippocampus extend much beyond the brain growth spurt period (3rd trimester equivalent) lasting until the end of juvenile age with temporal profiles of these changes being region specific.

4.2. Effects of the exposure to fetal alcohol and maternal stress on postnatal development of hippocampus.

In this study, the effects of gestational ethanol administration very often overlapped with the effects of the intragastric intubation *per se*. The ethanol/intubation effects were manifested at PD1 as significantly decreased mean dendritic field area, total dendritic length, total number of dendritic branches, the highest branch order, and the mean spine density in the pyramidal CA neurons. In our previous study run under the same experimental paradigm, the lower spine density found at PD1 in A and IC groups was accompanied by an overall trend toward lower levels of a presynaptic marker protein, synaptophysin (Elibol-Can et al, 2014a). Overlapping effects of developmental ethanol and shame intubation were also reported by other authors. Boschen, and colleagues (2015), reported an increase in the hippocampal expression of BDNF after ethanol administration as well as shame intubation applied during the 3rd semester equivalent in rats. The effects of intubation *per*

se, are generally linked to the intubation-induced stress (Kelly and Lawrence, 2008, Boschen, et al., 2015*).* In line with this notion, in our previous studies, the behavioral symptoms of stress such as significantly lower locomotor activity and higher anxiety-like responses in the open field and the plus maze test have been manifested by the pups of both A and IC groups, although the amount of stress on the pregnant dams could be greater in IC group compared to A group where it was attenuated by sedative and anesthetic effects of ethanol during the 2nd daily ethanol application (Elibol-Can et al, 2014b). On the other hand, similar changes to those observed after the gestational intragastric intubation were also observed after the prenatal exposure to a restrained stress which was reported to have a detrimental effect on the growth and differentiation of dendritic arbor in the hippocampal pyramidal neurons (Fujioka et al., 2006, Jia et al., 2010) and produce a decline in neuronal size of granule cells in the hippocampus of adult male rats (Hosseini-Sharifabad et al., 2012). According to the literature (Schneider et al., 2004), the cross-section of the effects of prenatal ethanol and prenatal stress is not surprising since both treatments seem to cause an impairment of the feedback control over the pituitary-adrenal activity, resulting in a pituitary-adrenal hyperactivity. On the other hand, there is an evidence of high sensitivity of the hippocampus to the adrenal glucocorticoids (McEwen, 2001).

However, the differential effects of fetal ethanol and maternal stress were also reported. For instance, in our previous studies, fetal ethanol but not intubation stress affected rats' locomotor activity in the open field and the rate of place learning in the Morris Water Maze (Dursun et al., 2006). Also, a significantly lower neuron count in DG at PD30, a significantly higher doublecortin immunoreactivity (an indirect index of neurogenesis) in the dendate subgranular zone at PD30, and a significantly stronger synaptophysin immunoreactivity (an indirect index of synaptogenesis) in stratum radiatum of CA and DG regions on PD10 were observed in A group compared to both IC and C groups (Elibol-Can et al., 2014a,b).

Similarly, some previously reported results demonstrated the unique effects of intubation procedure such as significantly higher neuron counts in CA region at PD1 and PD10 in IC group compared to both, A and C groups (Elibol-Can et al., 2014a). These diverse results most probably arise from the fact that the observed effects depend on the ethanol dose, amount of stress, and the temporal pattern of their applications. The relationship between dose of ethanol and its detrimental effects on the central nervous system are well known (Pierce and West, 1986; Goodlett and Johnson,1997;

Savage et al., 2002). It was also postulated that ethanol intoxication during the 3rd trimester equivalent (the brain growth sprout period) results in more severe changes than those observed after ethanol administration during the 2nd trimester equivalent (Thomas et al., 1996; Cronise et al., 2002, Livy et al., 2003; Tran et al., 2003). Interestingly, it has been also shown that the differential effects of the fetal ethanol and maternal stress may be observed depending on the insult severity. Aberg and colleagues (2005) reported that the exposure of pregnant dams to low ethanol doses (1 and 3g/kg/day) increased neonatal neurogenesis in hypothalamus of offspring. It has been also reported that when a long-lasting, severe stress impaired development of dendritic arbor in hippocampal neurons, a short-lasting, mild prenatal stress enhanced neonatal neurogenesis and had a positive effect on neurons' development and differentiation (Fujioka et al., 2006).

In the present study, ethanol *per se* showed a deteriorating effect on the soma size of granule cells at birth. At PD1, the soma of CA3 pyramidal cell in the A group was also marginally smaller as compared to both controls. In addition, a detailed Sholl analysis of dendritic arbor for PD1 revealed significantly lower number of intersections at 60, 90, and 100 μm from soma in apical CA3 dendrites, and at 60 μm from soma in basal CA1 dendrites in the A group compared to both controls. These findings are in accordance with the previous studies by other authors also reporting a significant reduction in the soma size and total dendritic field area in the developing retinal ganglion cells in mouse (Dursun et al., 2011), reduction of the soma size and dendritic fields of neurons in the rat oculomotor nucleus (Burrows et. al., 1995), Bergmann glial cells (Perez-Torrero et al., 1997), and cerebellar granule cells (Smith et al., 1986) and eventually dendritic atrophy in the hippocampal pyramidal neurons due to a perinatal alcohol exposure (Saunders et al., 1995 and González-Burgos et al., 2006). Also the treatment of the hippocampal cell cultures with ethanol was reported to cause a decrement in the number and length of dendrites (Yanni and Lindsley, 2000). The latter ethanol effects may be partially explained by the negative interaction of ethanol with the fibroblast growth factor (bFGF) which, in turn, affects the outgrowth of nerve fibers and their bifurcation (Luo and Miller, 1998). The adverse effect of ethanol on the neurite growth can also arise from its interaction with GABA receptors which, at the early stages of brain development, have excitatory properties (Rogers and Hunter, 1992) and elevate the intracellular Ca^{2+} (Prendergast et al., 2004), damping the dendrite growth and branching (Kostyuk, 1986, Mameli et al., 2005). Ethanol was also reported by other authors to reduce the spine density both in vivo (Ferrer et al., 1988, Kuge et al., 1993), and in vitro (Yanni and Lindsley, 2000) and cause changes

in the shape of dendritic spines in hippocampal neurons in the rat (Tarelo-Acuña et al. 2000). The fact that sometimes the effects of the developmental ethanol intoxication and stress exposure overlap and sometimes they are unique may arise from different sensitivity of different parameters to the potentially adverse factors across the postnatal ages.

The adverse effects of prenatal ethanol and stress exposure observed in this study were transient. All the changes in the hippocampal cells' morphology observed at PD1 mostly disappeared at PD10, with some parameters in A and IC groups over passing the values from C group. This compensatory rebound from fetal alcohol/stress effects was seen in the mean dendritic field area in CA3, total number of dendrites in CA1 pyramids, mean branch number, the highest branch order, and the number of stubby spines in apical CA1 dendrites. At PD30, the number of mushroom spines on the basal CA1 dendrites and, at PD60, the soma size of granule cells reached normal levels. The transient character of neuronal deficits in fetal-alcohol and intubation-stress subjects can suggest developmental delay induced by these factors (Davies and Smith, 1981; West et al., 1984, Miller, 1988). The subsequent progress in the development of some morphometric features could be mediated by upregulated levels of neurotrophins. According to some previous studies, after a moderate fetal alcohol exposure, the postnatal neurotrophin levels in the cortex and hippocampus were significantly higher compared to controls (Nakano et al., 1996; Angelucci et al., 1997). Our results are in line with the results previously reported by another research group studying effects of the gestational exposure to a liquid diet containing a low dose of ethanol (6% (vol/vol)) on the cell counts, apical dendrite length, and spine density in hippocampal CA1 and CA3 regions in rats at 8 -15 months of age (Cullen et al., 2014). Here too, no detrimental ethanol effects on hippocampus morphology were noted.

In summary, this study presented a comprehensive analysis of the morphological development of three hippocampal subregions, CA1, CA3, and DG, during first two months after the birth showing that, in rat, different morphological features of hippocampal principal neurons complete their maturation during different periods of perinatal development. Soma size of CA1 and DG neurons, number and diameter of primary dendrites, dendritic tortuosity, and branch angle manifested minimal postnatal changes while dendritic field area and arborization showed a significant increase during the first month of postnatal life, with the growth and differentiation of granule cells' dendrites lagging behind that in CA pyramids. Also the densities and frequencies of different spine

types demonstrated significant changes until the post-weaning age. We believe that this study contribute to the elucidation of critical developmental periods for different morphological parameters which is important for better understanding the teratological effects of perinatal insults to the nervous system.

The effects of prenatal exposure to moderate ethanol concentrations largely overlapped with those of prenatal intubation stress. Being revealed only at birth (PD1), they were confined to the soma size of granule DG neurons, the size and arborization of the dendritic fields and the spine densities in CA pyramidal neurons. Rapid compensatory growth and dendritic differentiation occurring in A and IC groups within PD1-PD10 period point towards prenatal alcohol/stress-induced developmental delay. However, this delay in the morphological development of hippocampus could not be responsible for behavioral deficits often reported in juvenile fetal-alcohol subjects since by this age all morphometric parameters already reached their normal values. The overlap of ethanol and intubation effects indicates that this way of ethanol administration should be used with precautions.

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Figure Legends

Figure 1. Line drawings of Golgi-impregnated cells of CA1, CA3 and DG region of intact hippocampus for all ages studied (PD1, PD10, PD30, and PD60) and representative pictures of Golgi-impregnated CA1, CA3 and DG neurons at PD60.

Figure 2. Morphometric estimates of the mean soma area made for each postnatal age (PD1, PD10, PD30, and PD60) and for each treatment group: Alcohol (A), Intubated Control (IC) and Intact Control (C), in the three hippocampal regions (CA1, CA3 and DG). The degree of significance is denoted as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Error bars denote SEM.

Figure 3. Morphometric estimates of the total dendritic field area (A**)** and total dendritic length (B**)** made for each postnatal age (PD1, PD10, PD30, and PD60) and for each treatment group: Alcohol (A), Intubated Control (IC) and Intact Control (C), in the three hippocampal regions (CA1, CA3 and DG). The degree of significance is denoted as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Error bars denote SEM.

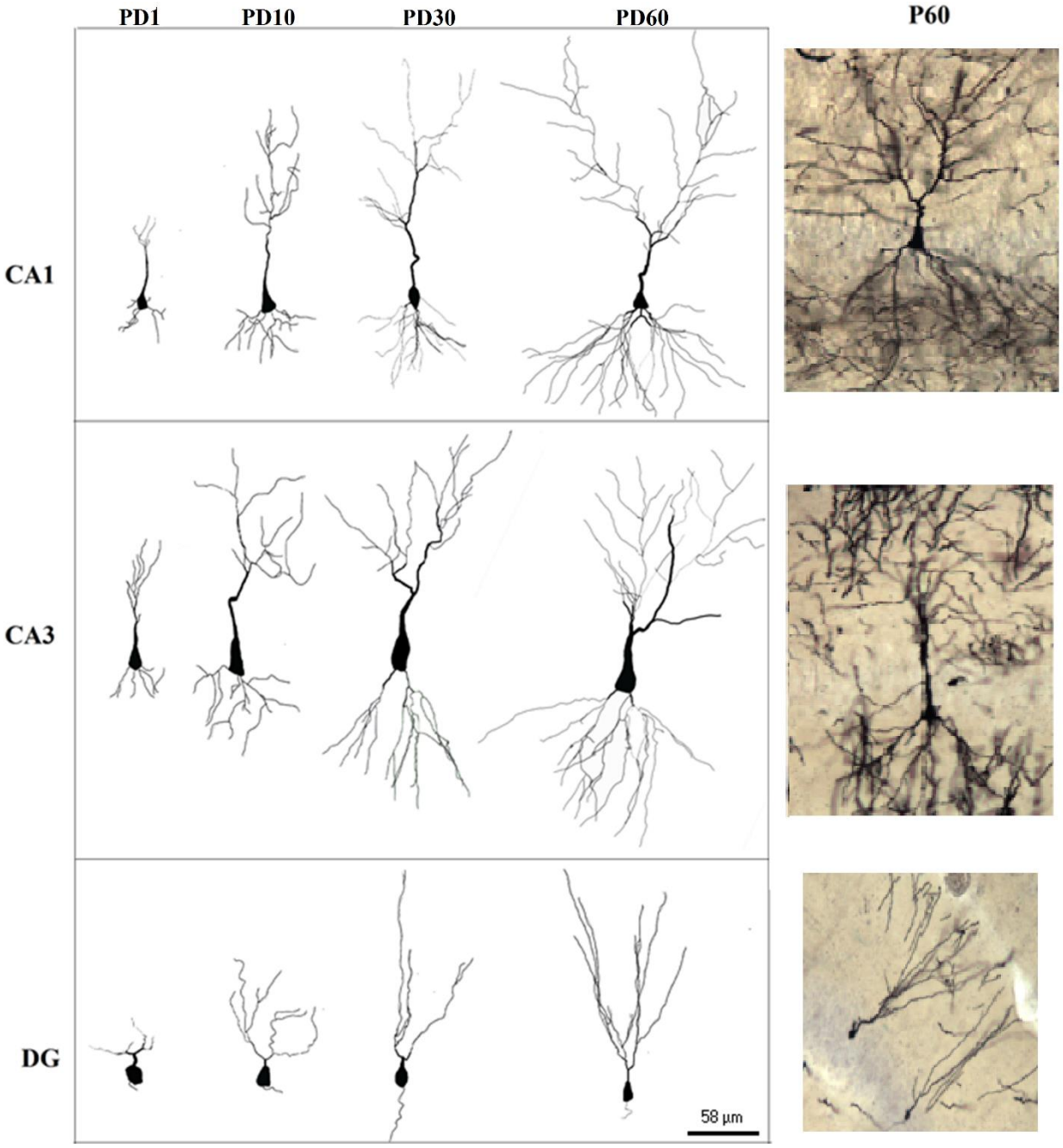
Figure 4. Morphometric estimates of the total number of dendritic branches (A), and mean highest branch order (B) made for each postnatal age (PD1, PD10, PD30, and PD60) and for each treatment group: Alcohol (A), Intubated Control (IC) and Intact Control (C), in the three hippocampal regions (CA1, CA3 and DG). The degree of significance is denoted as * $p \leq 0.05$ and ** $p \leq 0.01$. Error bars denote SEM.

Figure 5. Sholl analysis of number of intersections of dendritic branches measured for each group, each hippocampal region, and each postnatal age, independently. The degree of significance is denoted as $p \leq 0.05$*, and error bars denote SEM

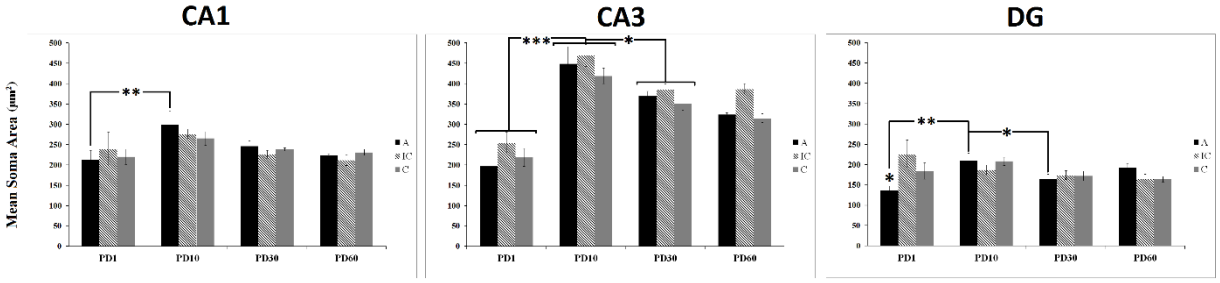
Figure 6. Morphometric estimates of the mean spine density made for each postnatal age (PD1, PD10, PD30, and PD60) and for each treatment group: Alcohol (A), Intubated Control (IC) and, Intact Control (C) in the three hippocampal regions (CA1, CA3 and DG). The degree of significance is denoted $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***. Error bars denote SEM.

Figure 7. A. Representative photomicrographs showing different types of spines in the neuropile of the hippocampal CA and DG region at PD60 under 100X magnification. (T: Thin spine, S: Stubby spine, M: Mushroom spine, B: Branched spine, F: Filopodia spine); **B.** Morphometric estimates of the density of thin, stubby, mushroom, and branched spines made for each postnatal age, each treatment group, and each hippocampal region, independently. Error bars denote SEM and the asterisks denote significance at $p \leq 0.05$ with respect to control.

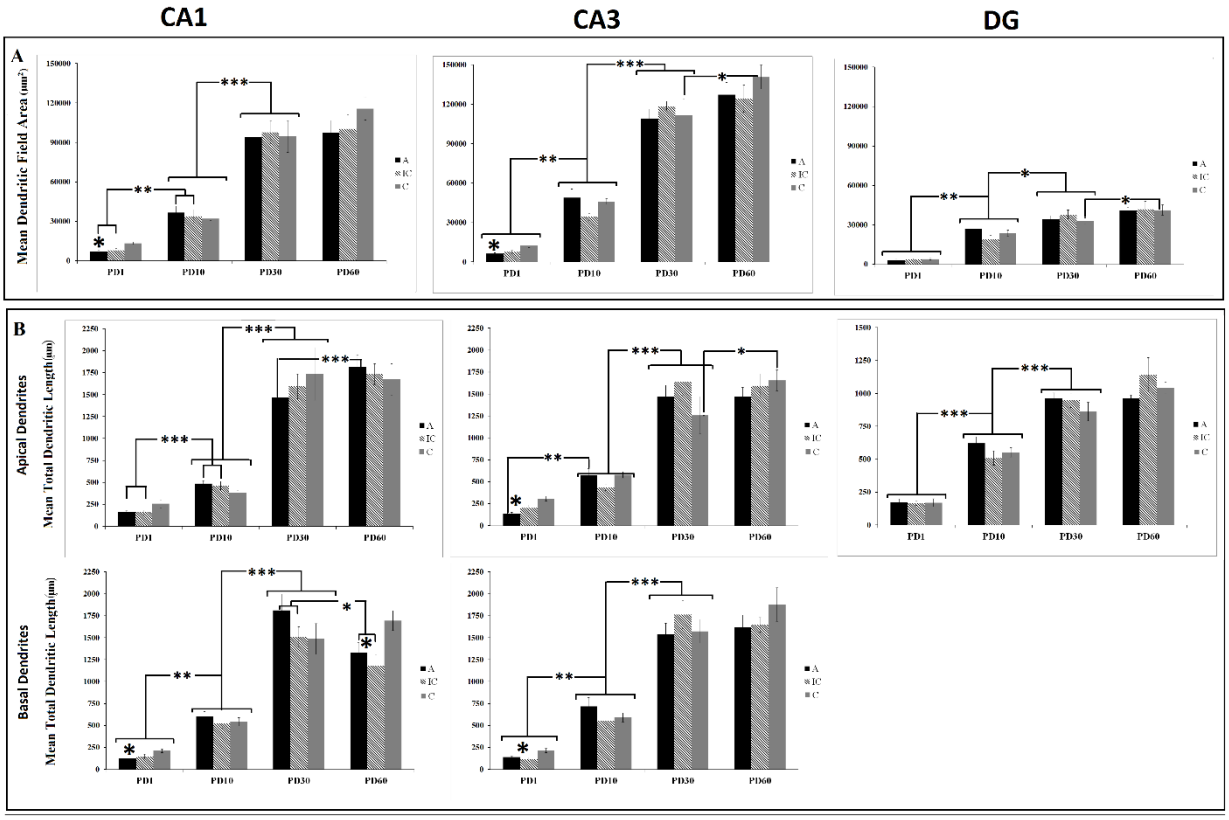
Figr-1



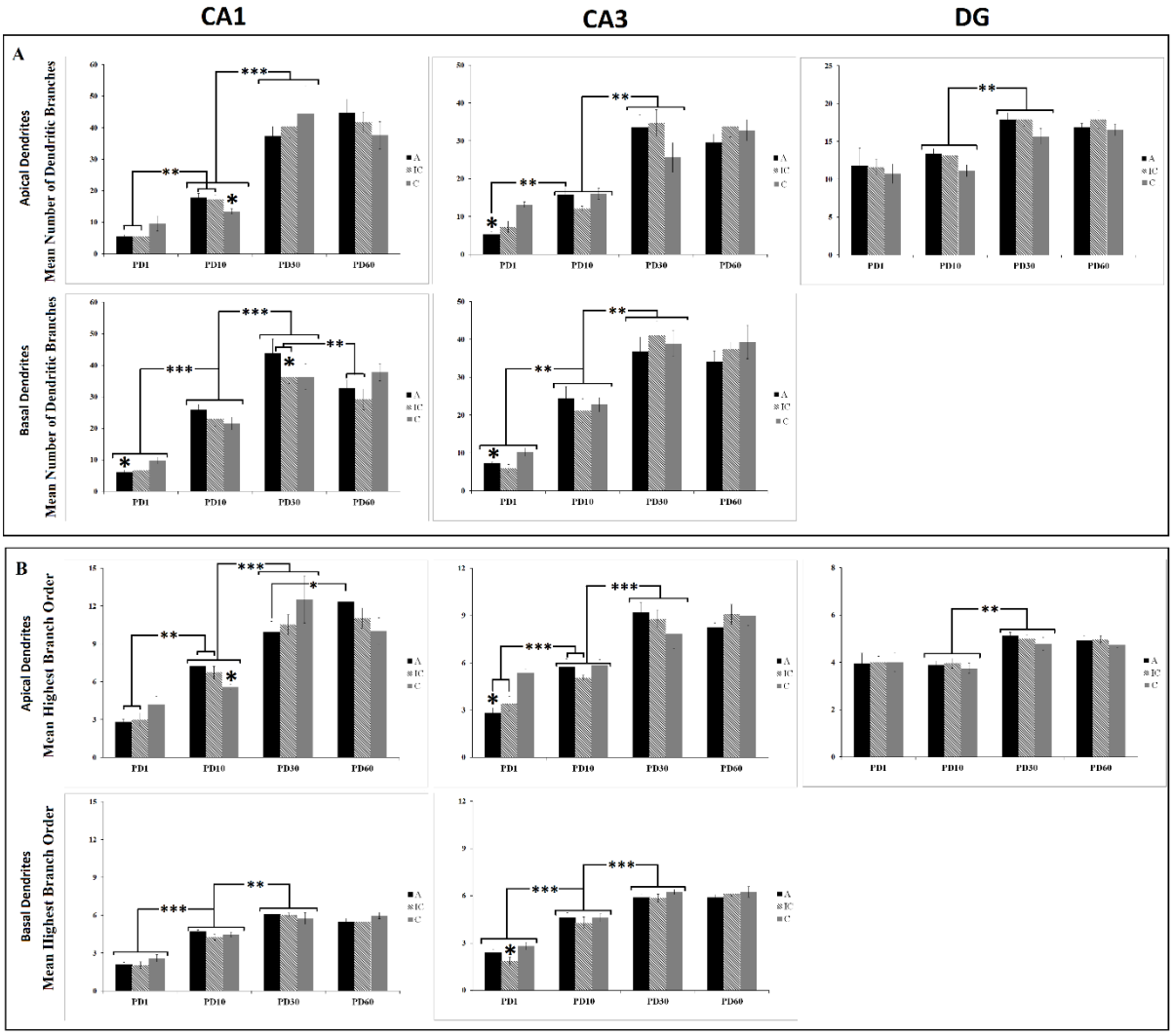
Figr-2

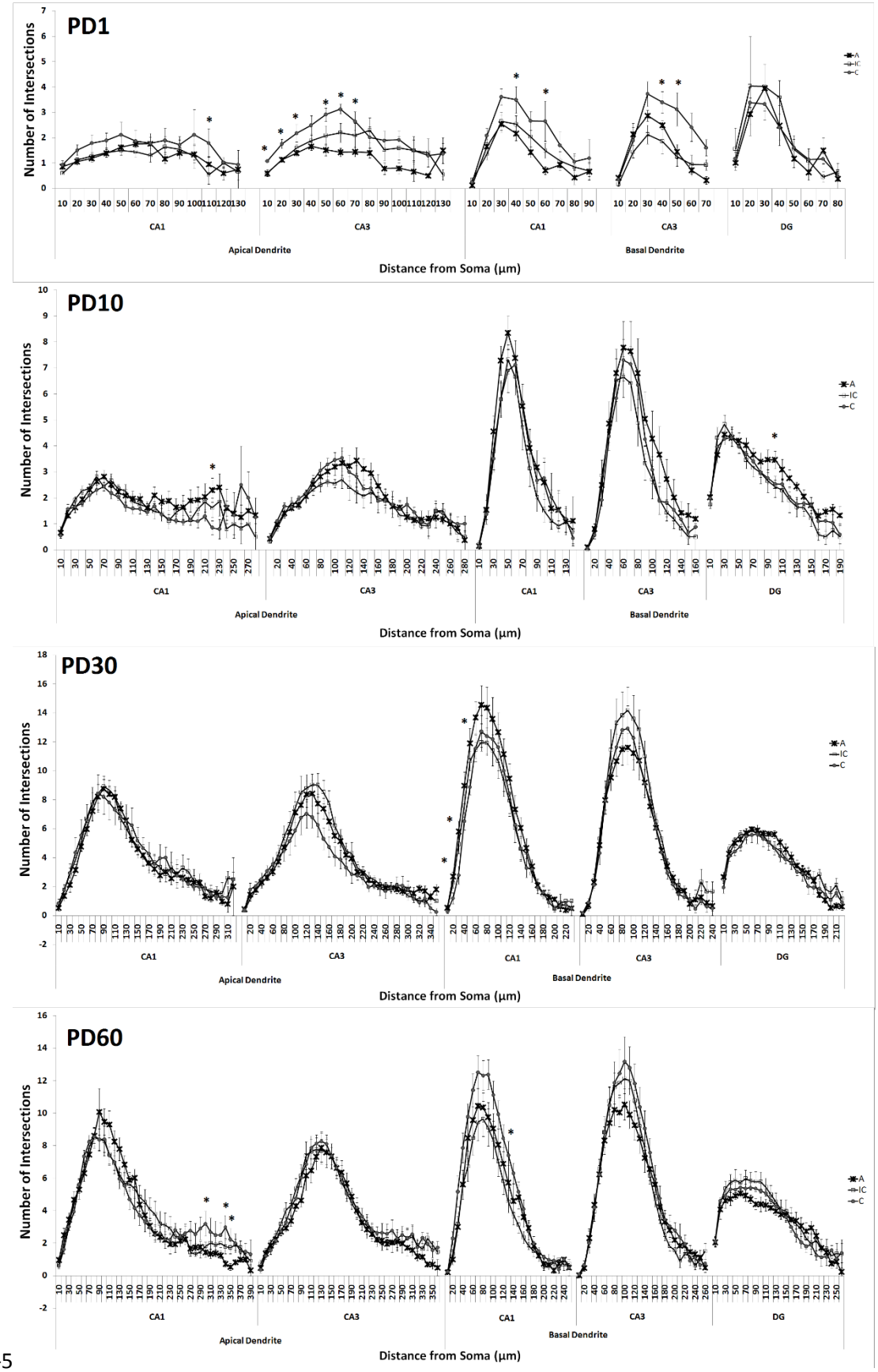


Figr-3



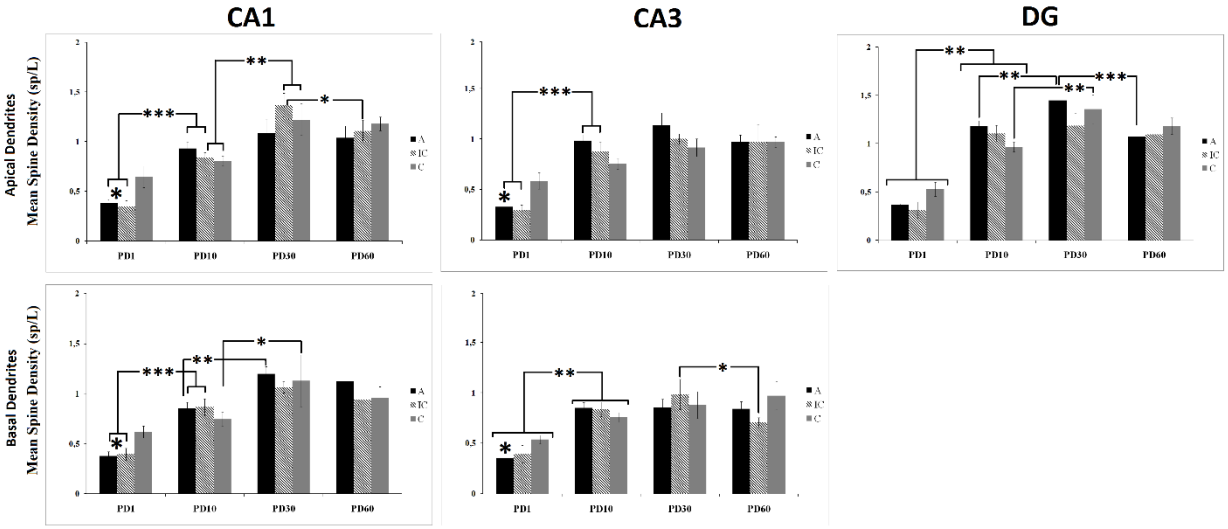
Figr-4





Figr-5

Figr-6



Figr-7