Maternal alcoholism affects cell lineage in adult hippocampal neurogenesis.

Nerina M. Villalba[1†], Catalina Madarnas[1†], Viviana Sanchez[2, 1], Alicia Brusco[2, 1*]

Corresponding author: hbrusco@fmed.uba.ar

[1]Universidad de Buenos Aires, CONICET, Instituto de Biología Celular y Neurociencia Prof. E. De Robertis (IBCN), Buenos Aires, Argentina,
[2]Universidad de Buenos Aires, Facultad de Medicina, 1° Unidad Académica del Departamento de Histologia, Biología Celular, Embriologia y Genética, Buenos Aires, Argentina

Abstract

Perinatal ethanol exposure (PEE) impacts the developing fetus, the central nervous system being particularly affected. These alterations can have clinical implications encompassed in Fetal Alcohol Spectrum Disorders (FASD), which include physical, mental, and behavioral deficits, with possible lifelong consequences.

The hippocampus is implicated in cognitive functions which are altered in adults with FASD.

The dentate gyrus (DG) of the hippocampus conserves the capacity to produce new neurons in adulthood. In this context, our study aimed to describe the effect of PEE on adult hippocampal neurogenesis using a murine model. Female CD1 mice were exposed to ethanol 6% v/v for 20 days prior to mating and along pregnancy and lactation. After weaning, pups had no further contact with ethanol. Characteristic cell types of the adult male DG were studied by immunofluorescence.

A lower percentage of type 1 and 4 cells and a higher percentage of type 2 cells were observed in PEE animals. This decrease in type 1 cells suggests that PEE reduces the population of remnant progenitors of the DG present in adulthood. The increase in type 2 cells and decrease in type 4 cells may indicate that the presence of ethanol during neurodevelopment alters the capacity of neuroblasts to become neurons in the adult neurogenic niche. These results suggest that pathways implicated in cell determination were affected by PEE and remained affect in adulthood. Since neurogenesis is associated to cognitive processes altered in FASD, these results may contribute to explaining one of the mechanisms involved in alcohol teratogenesis.

Keywords

Maternal alcoholism, Adult neurogenesis, Hippocampus, Dentate gyrus, Ethanol, Neuronal progenitor.

Abbreviations

PEE, Perinatal Ethanol Exposure; FASD, Fetal Alcohol Spectrum Disorders; DG, Dentate Gyrus; NPCs, Neuronal Progenitor Cells; SVZ, Subventricular Zone; SGZ, Subgranular Zone; GZ, Granular Zone; DCX, Doublecortin; CR, Calretinin; CB, Calbindin; C, control group; E, ethanol-exposed group; P, postnatal day; PB, Phosphate Buffer.

1. Introduction

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

Alcohol consumption during pregnancy can cause a spectrum of deleterious effects on offspring known as Fetal Alcohol Spectrum Disorders (FASD). These effects can be physical, mental or behavioral, with possible lifelong implications (Riley & McGee, 2005). The severity of such outcomes is linked to the amount and time window of alcohol consumption, among other factors (Maier & West, 2001).

During pregnancy, alcohol consumed by the mother can cross the placenta and reach the fetus, thus impacting fetal development. The central nervous system is one of the main targets of ethanol toxicity, undergoing morphological alterations that have been associated with neurobehavioral and cognitive deficits reported in individuals with FASD (Mattson et al., 2019).

Several neurobehavioral impairments registered in children with FASD –such as anxiety disorders and spatial learning and memory deficits– are connected to hippocampal function (Mattson et al., 2019). Moreover, FASD patients have shown a reduction in overall brain size and hippocampal volume (Autti-Rämö, 2002; Donald et al., 2016), an area especially affected by ethanol toxicity. These behavioral and neurologic alterations can persist into adulthood (Glass et al., 2014), although further research is needed to understand the lifelong effects of prenatal alcohol exposure.

Similar results are observed in rodent models of FASD, which have shown impairments in spatial learning and memory (Berman & Hannigan, 2000; Schambra et al., 2017) and an increase in anxiety-like behavior (Madarnas et al., 2020; Rouzer et al., 2017). As in humans, the rodent hippocampus is particularly affected by prenatal ethanol exposure. Alterations in hippocampal morphology have been registered in young rats perinatally exposed to ethanol (Evrard et al., 2003; Ramos et al., 2002), and a reduction in hippocampal volume has been reported in mouse fetuses after a single day of prenatal ethanol exposure (Godin et al., 2010). In addition, changes in hippocampal structural and functional plasticity have been observed in adolescent and adult rats chronically exposed to ethanol during development (Gil-Mohapel et al., 2011; Patten et al., 2013). These alterations could result from cell loss in the developing hippocampus (Livy et al., 2003), although the effects of ethanol may depend on the period of exposure and dosage, among other variables (Petrelli et al., 2018).

Neurons are particularly sensitive to ethanol exposure in periods of cell proliferation, migration and differentiation, cortical dysplasia being one of the most serious consequences (Aronne et al., 2011). Given that most of the neurobehavioral and neurologic impairments reported in children with FASD persist into adulthood and that newborn neurons are particularly sensitive to ethanol, perinatal ethanol exposure (PEE) may indeed affect neurogenesis in adulthood.

Adult neurogenesis is driven by neuronal progenitor cells (NPCs) located in neurogenic niches of the brain. The two neuroprogenitor regions in the adult brain of rodents that contain NPCs are: the subventricular zone (SVZ) along the walls of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus (Gonçalves et al., 2016). Neurogenesis is thought to be key to hippocampal function (Zhao et al., 2008).

While some authors have found a reduction in the number of proliferative cells and neurogenesis in adult rats and mice prenatally exposed to ethanol (Gil-Mohapel et al., 2014; Olateju et al., 2017; Redila et al., 2006), other studies have shown unaltered hippocampal cell proliferation after prenatal alcohol exposure (Choi et al., 2005; Gil-Mohapel et al., 2011).

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

Although Gil-Mohapel et al. (Gil-Mohapel et al., 2011) and Reid et al. (Reid et al., 2020) have extensively reviewed the effects of pre and perinatal ethanol exposure on adult neurogenesis in murine models, evidence is still controversial, as results in cell proliferation and neurogenesis may vary according to the time and period of exposure, dosage, route of administration, blood concentrations and animal age at the time of evaluation, among others.

In this context, the present study aimed to describe the effect of PEE on adult hippocampal neurogenesis using a murine model of chronic ethanol exposure throughout neurodevelopment. Furthermore, a wide range of markers was used to characterize the cell types present in neurogenic niches in the DG. The results presented here may help establish whether ethanol exposure during brain development has long-lasting effects on hippocampal neurogenic niches, altering cell survival, proliferation, migration, differentiation and maturation.

2. Material and methods

2.1. Animals

Twelve primiparous female CD1 mice (aged 45-50 days) and 6 adult male CD1 mice were randomly selected from different litters in the animal room at Instituto de Biología Celular y Neurociencia. Animals were housed in a temperature (21-22ºC) and photoperiod-controlled room (12 light/dark cycle; 8 am on, 8 pm off).
All procedures were in agreement with standards for the care of laboratory animals as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH). All procedures were administered under the auspices of CICUAL (Institutional Committee for the Care and Use of Laboratory Animals, Facultad de Medicina, Universidad de Buenos Aires, RES (CD) 2375/2017).

2.2. Ethanol exposure

Female mice were divided into two groups, a control group (C, 6 females) and an ethanol-exposed group (E, 6 females) and housed two per cage. Ethanol treatment was performed as previously described in detail (Madarnas et al., 2020).
Briefly, E females received a constant dilution of 6% v/v ethanol in water as the only beverage with standard food *ad libitum* as from 20 days previous to mating until pup weaning. C females and all male mice had free access to water and standard food. In both groups, a single male was put in a cage with two females for mating and pregnancy was determined by the detection of a vaginal plug (considered gestational day 0). Pregnant mice were separated for the rest of pregnancy and nursing. At postnatal day 1 **(**P1**)**, all the litters were reduced to no more than 10 pups, preferentially male. At P21, male offspring from both groups were weaned and housed 3-6 per cage from the same litter and received water and standard food *ad libitum,* with no further contact with ethanol. Pups from E mothers were defined as the perinatally exposed to ethanol group (PEE) and the pups from C mothers the Control group. C mothers and Control female pups were returned to complete their life as part of the colony. PEE and Control pups were submitted to morphological studies in adulthood (90-100 postnatal days).

2.3. Tissue processing

Twelve adult male pups per experimental group were randomly selected, two from each litter. Animals were deeply anesthetized with ketamine and xylazine in doses of 100 mg/kg and 10 mg/kg in physiological solution, respectively, and perfused through the left ventricle, initially with physiological solution added to 50 IU heparin, and subsequently with a fixative solution containing 4% (w/v) paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. Brains were removed and postfixed in the same cold fixative solution for 4 hours and then washed overnight

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

in 5% (w/v) sucrose in PB at 4 °C. Afterwards, brains were cryoprotected by immersion in 30% (w/v) sucrose in PB overnight at 4 ºC and stored at -80ºC. Coronal 50-µm-thick brain sections were cut using a cryostat (Leitz, Kryostat 1720 Digital), placed in glycerol 50% in phosphate buffer saline (PBS) and stored at -20ºC until used. Brain sections corresponding to -1 to -2.30 mm Bregma level for dorsal hippocampus (Franklin and Paxinos, 2008) were processed for the corresponding histological studies.

2.4. Immunofluorescence

Two coronal 50-µm-thick brain sections containing dorsal hippocampus were selected from each of the 5-6 mice per group from different litters. Slices were washed three times in PBS and immersed in a solution of 3% (v/v) normal equine serum plus 0.5% (v/v) Triton X-100 in PBS for 3 hours at 4 ºC under agitation to permeabilize and block nonspecific sites. Sections were then incubated with the following primary antibodies diluted in a solution of 1.5% (v/v) normal equine serum and 0.25% (v/v) Triton X-100 in PBS: mouse anti-NeuN (mouse anti-neuronal nuclei, monoclonal antibody, 1:750, Millipore, Cat# MAB377, RRID: AB_2298772), rabbit anti-GFAP (rabbit anti-gliofibrillary acidic protein, 1:3000, Sigma Aldrich, Cat# G9269, RRID: AB_477035), mouse anti-Nestin (monoclonal antibody, 1:1000, eBioscience, Cat# 14-5843-82, RRID: AB_1907435), rabbit anti-Ki67(polyclonal antibody, 1:3000, Abcam, Cat#ab15580, RRID: AB_443209), rabbit anti-CR (rabbit anti-calretinin, polyclonal antibody, 1:1000, Zymed, Cat# 180211), rabbit anti-DCX (rabbit anti-doublecortin, polyclonal antibody, 1:5000, Abcam, Cat#ab18723, RRID: AB_732011), rabbit anti-CB (rabbit anti-Calbindin D28k, polyclonal antibody, 1:7000, Swant Cat# CB38, RRID:AB_10000340). Slices were incubated at 4 ºC overnight under agitation. After three washes in PBS, sections were incubated for 1.5 hours in the dark with fluorescent secondary antibodies: goat anti-mouse IgG conjugated with Alexa FluorTM 568 (1:1000, Invitrogen, Cat# A11004, RRID: AB_143162) and goat anti-rabbit IgG conjugated with Alexa FluorTM 488 (1:1000, Invitrogen, Cat# A11008, RRID: AB_143165). Finally, slices were counterstained with Hoechst 33342 (1:1000, Sigma-Aldrich) to label nuclei, washed three times with PBS, mounted on gelatin-coated slides and coverslipped with 70% glycerol mounting medium. Negative controls were performed in each immunofluorescence, omitting the primary antibody to ensure technique specificity (see Supplementary Figure 1).
Photographs were taken on an inverted Olympus IX83 microscope with several objectives (10X, 20X, 60X) using an additional spinning disk unit for better resolution. Images were acquired using high-resolution digital monochromatic sCMOS *Orca* camera (Hamamatsu) and *CellSens Dimension CS-DI-V1* software.

2.5. Morphometric digital image analysis

Measurements were made on the photomicrographs at 20X primary magnification, analyzed by two blinded operators (Fig 12). The SGZ located near the crest of the hippocampal DG was the brain area selected for morphometric studies, and all measurements were made using ImageJ software (NIH, http://imagej.net) in the same region of the SGZ.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

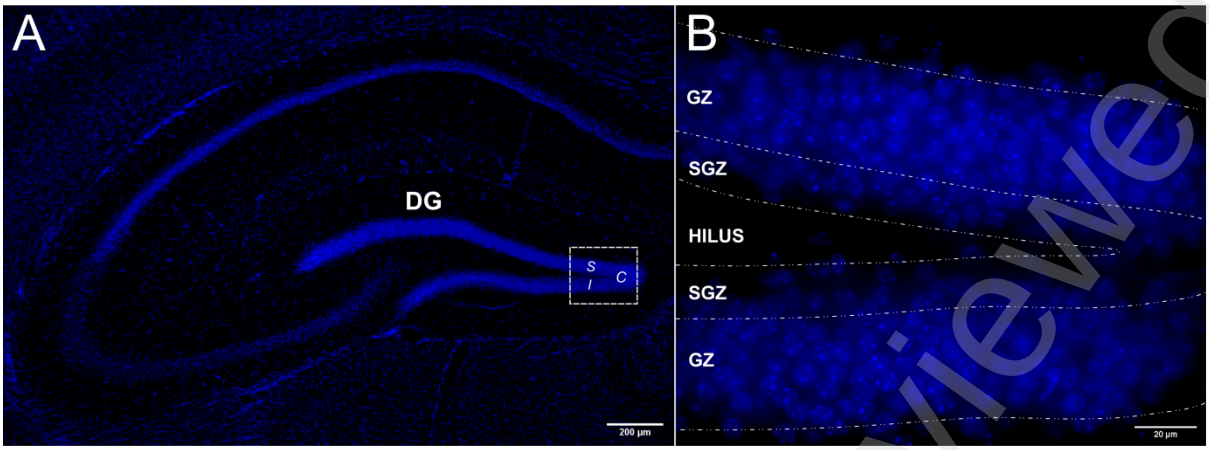

Figure 1. DG measuring area. Photomicrographs of coronal sections at the level of dorsal hippocampus stained with Hoechst. An overall view of the DG and hippocampus showing the crest (C) of the DG and its supracrest (S) and infracrest blades (I). Image at low magnification showing the area studied with a dotted box (A). Image at high magnification showing the detail of the three DG layers (GZ, SGZ and Hilus) delimited by dotted lines (B).

Double staining with different primary antibodies was performed to determine the proportion of cells at every stage of neurogenesis, relativizing to the total number of cells per unit of area. Ki67 immunofluorescence was used to assess proliferating cells. In order to study Ki67 expression in the SGZ, the Z axis was explored by Z projection in which Ki67+ cells and total nuclei were counted. The proportion of Ki67+ cells was then calculated regarding total nuclei.

Cell types were identified following the classification criteria stipulated by Kempermann (Kempermann et al., 2004). The total number of cells was determined in the same area by quantification of Hoechst-positive nuclei.

Marker / Cell Type	GFAP	Nestin	DCX	CR	CB	NeuN
1	+	+	-	-	-	-
2a	-	+	-	-	-	-
2b	-	+	+	-	-	-
3	-	-	+	-	-	-
4	-	-	+	+	-	+
5	-	-	-	-	+	+

Table 1. Markers used in the present work to identify each cell type involved in the neurogenic process.

Photomicrographs of double immunostaining were used to measure colocalization of two markers using ImageJ and the JACoP plugin. Thresholds of positive mark were set for each marker and the Manders' overlap coefficient was calculated (Bolte & Cordelières, 2006; Manders et al., 1996).

2.2.3 Data analysis

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

Statistical analysis was performed using GraphPad Prism v5.00 (GraphPad Software Inc.). Immunostaining quantifications (n = 3-5/treatment) were made from two slices per brain of each treatment and brain structure. Means and standard error of the mean (SEM) were obtained for all variables measured, the assumptions of normality and homoscedasticity were tested, and a two-tail Student's t test was performed to compare the two groups.

3. Results

Early precursor populations

In order to study the earliest stages of neurogenesis, Nestin and GFAP were used as specific markers to describe characteristic cell types –i.e., radial glial-like stem cells (type 1) and neuronal progenitors (type 2a)– in the adult Control and PEE hippocampal DG (Fig 2).

Type 1 cells were identified through GFAP+/Nestin+ immunolabeling (Fig 2 C and G). The cell body expresses both markers and is located in the SGZ, while neurites project through the granular zone (GZ). Of note, discontinuous GFAP+/Nestin+ immunostaining was observed in these processes (Fig 2 D and H). Type 2a cells were identified through GFAP-/Nestin+ immunolabeling (Fig 2 A and E). These cells present an oval-shaped soma located in the SGZ and lining the GZ, with no neuritic processes. In addition, astrocytic cells were identified through GFAP+/Nestin- immunostaining (Fig 2 B and F) and exhibited star-shaped morphology.

A smaller proportion of GFAP+/Nestin+ cells (Fig 2 I: Control 0.0612 ± 0,004, PEE 0.0447 ± 0.003; $p<0.05$) and a larger proportion of GFAP-/Nestin+ cells (Fig 2 J: Control 0.06443 ± 0.007, PEE 0.1181 ± 0.015; $p<0.05$) were observed in the SGZ of PEE animals. These results indicate that PEE animals present a lower percentage of type 1 cells and a higher percentage of type 2a cells in this neurogenic niche than Control animals.

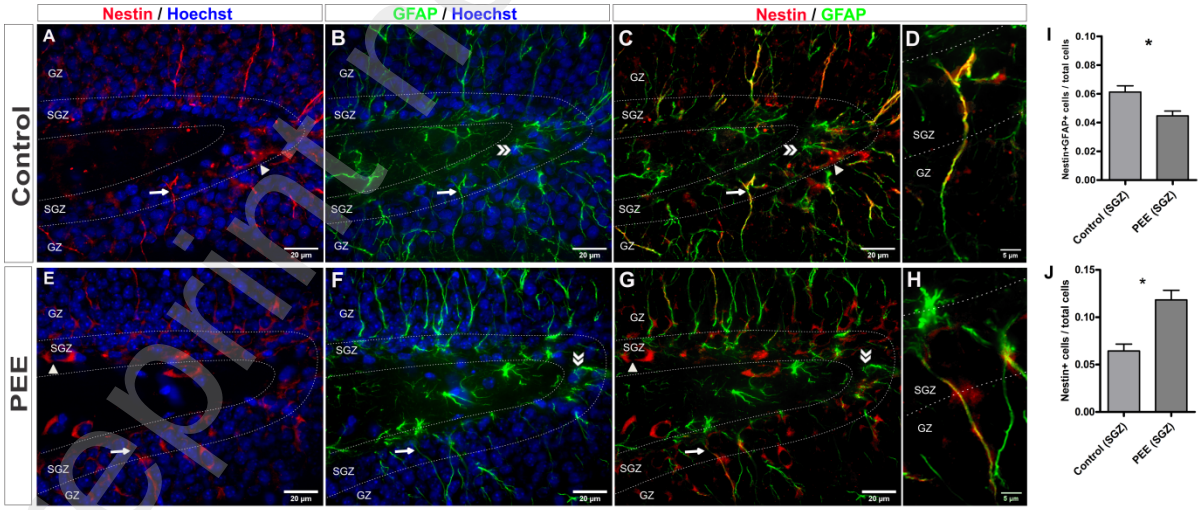

Figure 2. Cell types in early stages of neurogenesis in the adult mouse hippocampus. Radial glial-like stem cells (type 1 cells, GFAP+/Nestin+, arrows), neuronal progenitors (type 2a cells, GFAP-/Nestin+, arrowheads) and astrocytic cells (GFAP+/Nestin-, double arrows) in the SGZ of the DG of adult Control and PEE mice. Photomicrographs of coronal sections with Hoechst staining for cell nuclei (blue) and double immunofluorescence for Nestin (red) and GFAP (green). Sections at low

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

magnification (A-C and E-G) and merge at high magnification (D and H). Proportion of GFAP+/Nestin+ cells (I) and GFAP-/Nestin+ cells (J) in the SGZ. Data expressed as the mean ± SEM (Control n = 4, PEE n = 5); all parameters were analyzed through Student's t-test (*p < 0.05).

Given the larger proportion of GFAP-/Nestin+ cells and the fact that these cells retain the capacity to divide, assays were conducted on the number of proliferative cells. For this purpose, Nestin and Ki67 were used as specific markers to analyze whether PEE altered the number of proliferative type 2a cells in the adult DG (Fig and 4).

Contrary to expectations, no Nestin+/Ki67+ cells were found in the SGZ. Both experimental groups revealed Nestin-/Ki67+ cells in this area, arranged in clusters of four or more cells in the crest of the DG (Fig 3) and in clusters of two or three cells in the supra- and infra-crest regions of the DG (Fig 4), whereas no Nestin-/Ki67+ cells were found in the GZ or hilus. No significant differences were observed in the proportion of Nestin-/Ki67+ cells between Control and PEE animals in any of these areas (Fig 3 I, Control 0.0306 ± 0.003, PEE: 0.0267 ± 0.004; p = 0.504 and Fig 4 I, Control 0.0109 ± 0.0011, PEE: 0.0081 ± 0.0018; p = 0.199).

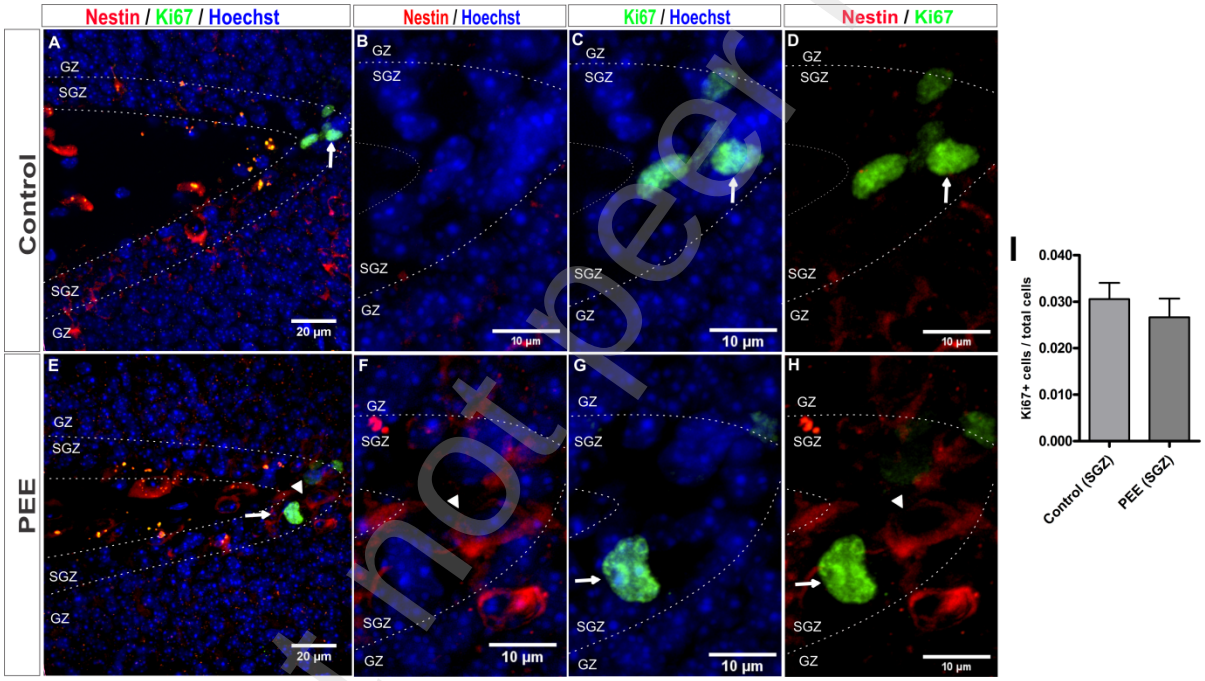

Figure 3. Distribution of proliferative cells in the crest of the DG in the adult mouse hippocampus. Proliferative cells (Nestin-/Ki67+, arrows) and neuronal progenitors (type 2 cells, arrowheads) in the SGZ of the DG of adult Control and PEE mice. Photomicrographs of coronal sections with Hoechst staining for cell nuclei (blue) and immunofluorescence for Ki67 (green) and Nestin (red). Sections at low magnification (A and E) and at high magnification (B-D and F-H). Proportion of Ki67+ cells in the SGZ in the crest of the DG (I). Data expressed as the mean ± SEM (Control n = 4, PEE n = 4); all parameters were analyzed through Student's t-test.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

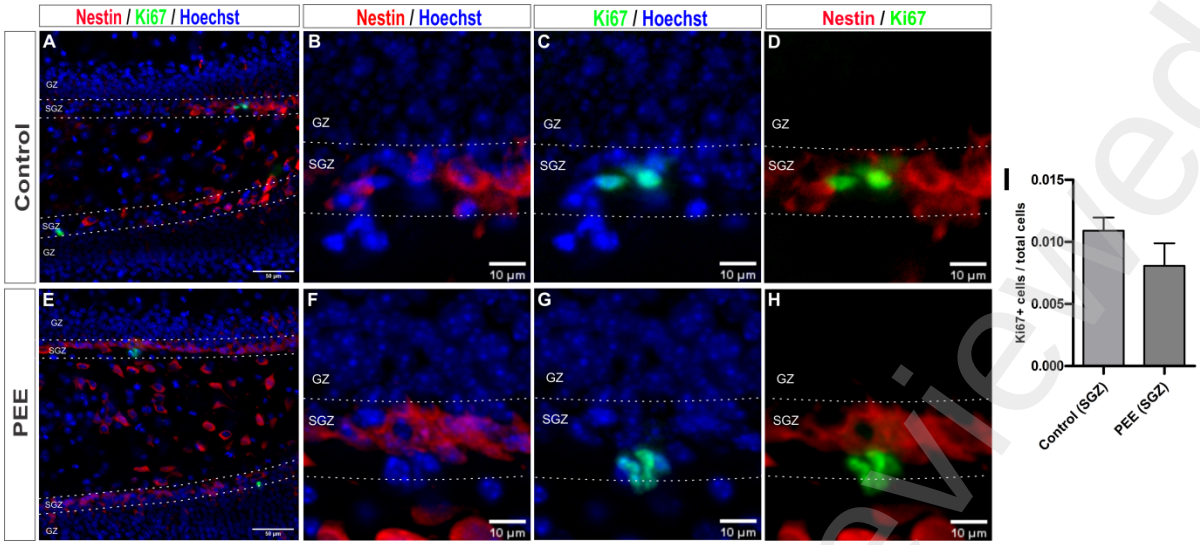

Figure 4. Distribution of proliferative cells in the supra- and infra-crest regions of the DG in the adult mouse hippocampus. Photomicrographs of coronal sections with Hoechst staining for cell nuclei (blue) and immunofluorescence for Ki67 (green) and Nestin (red). Sections at low magnification (A and E) and at high magnification (B-D and F-H). Proportion of Ki67+ in SGZ in the supra- and infra-crest regions of the DG (I). Data expressed as the mean ± SEM (Control n = 4, PEE n = 4); all parameters were analyzed through Student's t-test.

With the view to analyzing a later stage in the neurogenic process, studies were carried out using Nestin and doublecortin (DCX) as specific markers (Fig 5). Interestingly, no Nestin+/DCX+ (type 2b cells) were found in the SGZ of the DG in either experimental group (Fig 5 C-D and G-H). This result may indicate a transient co-expression of these markers, reflecting a short time window in which type 2b cells can be found. Nestin-/DCX+ (type 3 cells) –which are at a more advanced stage in the neurogenic process than type 2b cells– were detected in the SGZ of both Control and PEE animals, with round-shaped or slightly triangular nuclei (Fig 5 B and F).

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

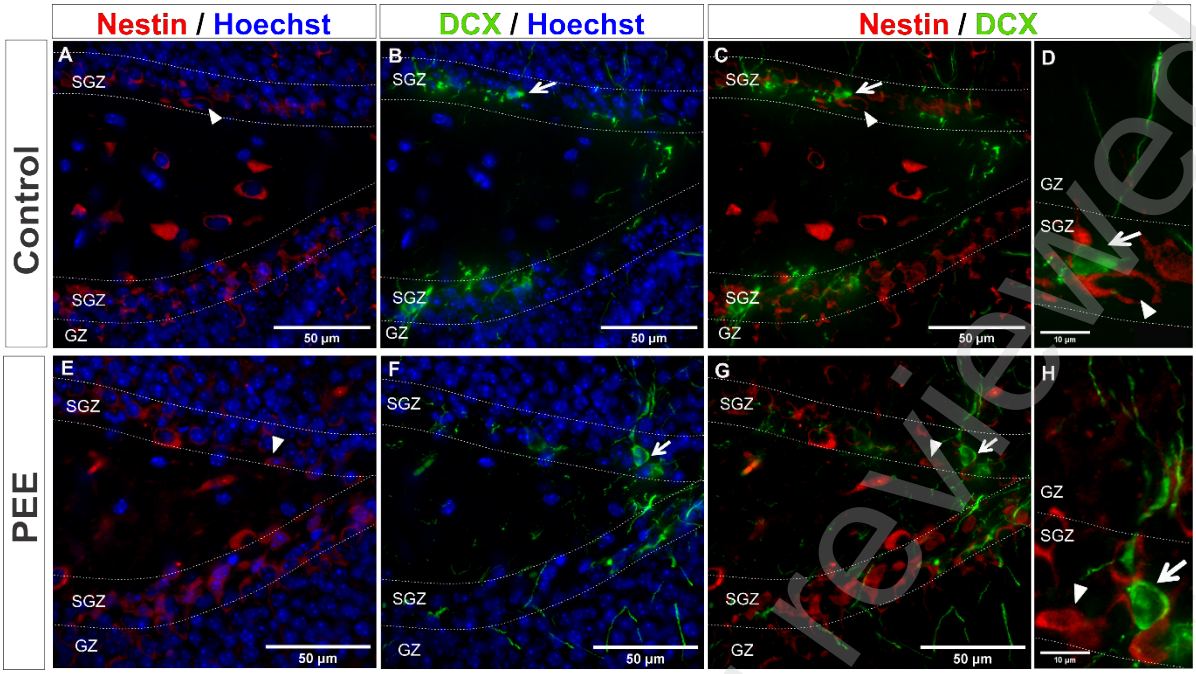

Figure 5. Neuronal progenitors and migratory cells in the adult mouse hippocampus. Neuronal progenitors (type 2a, Nestin+/DCX-, arrowheads) and migratory (type 3 cells, Nestin-/DCX+, arrows) in the SGZ of the DG of adult Control and PEE mice. Photomicrographs of coronal sections with Hoechst staining for cell nuclei (blue) and double immunofluorescence for Nestin (red) and DCX (green). Sections at low magnification (A-C and E-G) and at high magnification (D and H).

Interestingly, the exploration of the rostro-caudal axis unveiled a particular expression pattern for DCX and Nestin immunolabeling: fewer DCX+ than Nestin+ cells were found -1.46 to -1.82 from Bregma (Fig 6 A and C), whereas more DCX+ than Nestin+ cells were observed -1.94 to -2.30 from Bregma (Fig 6 B and D).

The expression pattern of these two markers was further analyzed in the SGZ and GZ along the rostro-caudal axis (Fig 6 E and G, rostral level; Fig 6 F and H, caudal level). Results showed a significantly larger number of Nestin+/DCX- type 2a cells in the PEE group in both areas but only in rostral slices (Fig 6 E: Control 0.1501 ± 0.013, PEE 0.3178 ± 0.037; $p<0.01$ / F: Control 0.0279 ± 0.003, PEE 0.01816 ± 0.004; $p>0.05$), while no differences were found in the number of Nestin-/DCX+ cells (Fig 6 G: Control 0.174 ± 0.006, PEE 0.1574 ± 0.013; $p>0.05$ / H: Control 0.199 ± 0.010, PEE 0.186 ± 0.020; $p>0.05$).

These results hint at an expression pattern for Nestin+/DCX- neuronal progenitors and Nestin-/DCX+ migratory cells along the rostro-caudal axis and suggest that PEE disrupts the localization pattern of neuronal progenitors in the SGZ and GZ.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

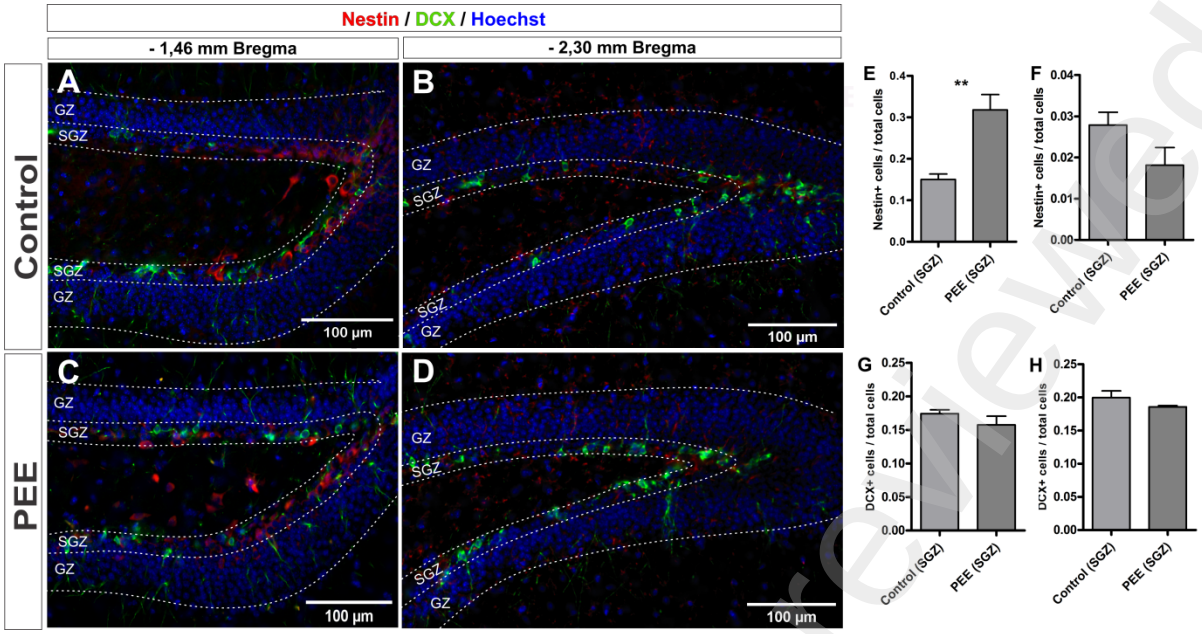

Figure 6. Rostro-caudal distribution of neuronal progenitors and migratory cells in the adult mouse hippocampus. Rostral (A and C) and caudal (B and D) sections of dorsal DG of Control and PEE adult mice. Neuronal progenitors (type 2a, Nestin+/DCX-) and migratory cells (type 3 cells, Nestin-/DCX+). Photomicrographs of coronal sections at low magnification with Hoechst staining for cell nuclei (blue) and double immunofluorescence for Nestin (red) and DCX (green). Proportion of Nestin+ cells at rostral (E) and caudal (F) level and DCX+ cells at rostral (G) and caudal (H) level in the SGZ. Data expressed as the mean ± SEM (Control n = 3-4, PEE n = 3-4); all parameters were analyzed through Student's t-test (**$p < 0.01$).

Migratory cells

When DCX+ cells were analyzed, it could be noticed how they started to migrate from the SGZ to the GZ, as their orientation changed from tangential to radial and their morphology became more polarized. Their neurites projected to the GZ and increased in number and complexity as the cells matured (Fig 7). Moreover, DCX+ cells presented different morphologies and localizations and, as the time window for DCX expression is considerably wide, immature granule cells (type 4) were also found to express it: Early type 3 cells exhibited tangential orientation of their soma, with the major cellular axis parallel to the layers distribution of the DG, late type 3 cells had radial orientation and were located in SGZ, while type 4 cells were located in the edge between the GZ and SGZ, with their neurites/dendrites extending to the first third of the molecular layer. Morphology and localization were conserved in PEE animals regarding Control (Fig 7 A-D), as was the proportion of DCX+ cells in the SGZ (Fig 6 G and H).

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

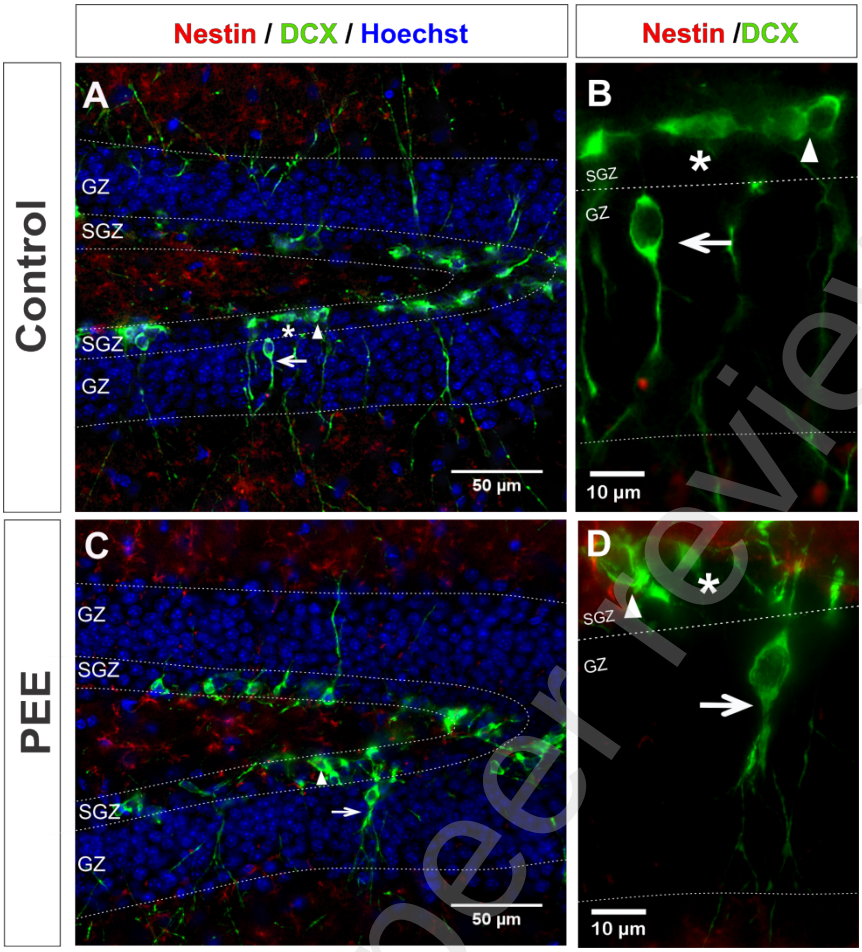

Figure 7. Migratory cells in the adult mouse hippocampus. Early type 3 cells (Nestin-/DCX+, asterisks), late type 3 cells (Nestin-/DCX+, arrowheads) in SGZ and early type 4 cells (Nestin-/DCX+, arrows) in the GZ of the DG of adult Control and PEE mice. Photomicrographs of coronal sections with Hoechst staining for cell nuclei (blue) and double immunofluorescence for Nestin (red) and DCX (green). Sections at low magnification (A and C) and at high magnification (B and D).

As mentioned above, DXC+ cells were found in the GZ as in SGZ (Fig 8). A smaller proportion of DXC+ cells were observed in PEE than in Control in GZ (Fig 8 E: Control 0.00415 ± 0.00017, PEE: 0.00288 ± 0.00018; $p = 0.0042$). Therefore, early exposure to ethanol during development may be hypothesized to affect either the differentiation or migration of neuroblasts, causing a delay in their transit toward the granular layer.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

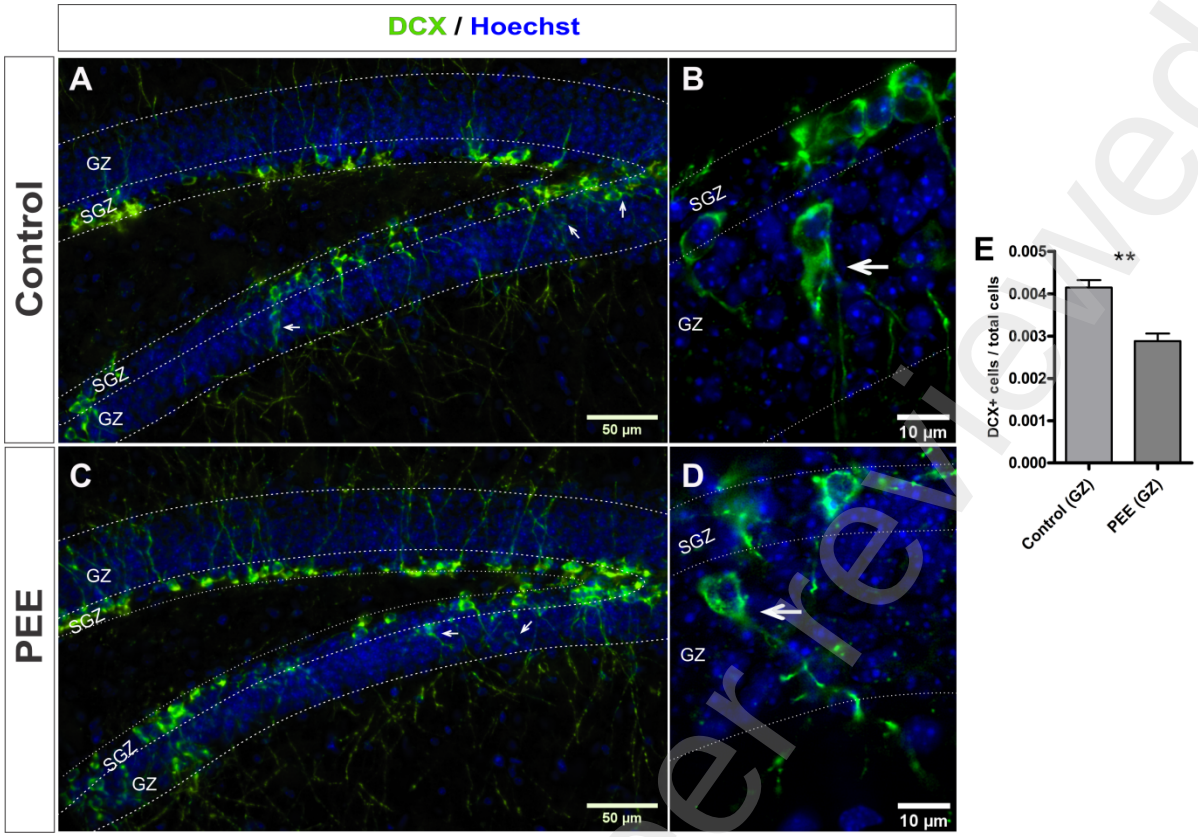

Figure 8. Migratory and immature granule cells in the adult mouse hippocampus. Type 3 cells (DCX+) in the SGZ and early type 4 cells (DCX+, arrows) in the GZ of the DG of adult Control and PEE mice. Photomicrographs of coronal sections with Hoechst staining for cell nuclei (blue) and immunofluorescence for DCX (green). Sections at low magnification (A and C) and at high magnification (B and D). Proportion of DCX+ cells (E) in the GZ. Data expressed as the mean ± SEM (Control n = 4, PEE n = 4); all parameters were analyzed through Student's t-test (**$p < 0.01$).

Double NeuN –a specific marker of mature neurons– and DCX immunostaining was further used to study more advanced stages in the neurogenic process (Fig 9). Type 3 cells may have differentiated from more mature neurons for being DCX+/NeuN- (cellular type described in Fig 7 and 8), with their somas located in the SGZ (Fig 9 B-C and F-G) and their neurites entering the granular zone.

Postmitotic populations

Type 4 cells continue expressing DCX and begin to express NeuN. These cells were thus identified as DCX+/NeuN+, and their somas were located both in the SGZ and GZ, which indicates that migrating neurons began transit from the SGZ to their definite location in the GZ (Fig 9 B, D, F and H). In contrast, mature granule cells (type 5) no longer express DCX and were hence identified as DCX-/NeuN+ (Fig 9 B-D and F-H). The proportion of type 4 cells showed no significant differences between experimental groups in the SGZ (Fig 9 I: Control 0.0163 ± 0.0025, PEE: 0.0171 ± 0.0027; $p = 0.783$) but a smaller value for PEE animals in the GZ (Fig 9 J: Control 0.00415 ± 0.00017, PEE: 0.00288 ± 0.00018; $p = 0.0042$). In addition, the proportion of mature granular cells showed no significant differences between conditions

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

in either zone (Fig 9 K: Control 0.821 ± 0.013, PEE: 0.812 ± 0.027; p = 0.781 / L: Control: 0.9634 ± 0.0049, PEE: 0.9478 ± 0.0078; p = 0.1441). These results suggest that the differentiation process in adulthood is not affected by PEE.

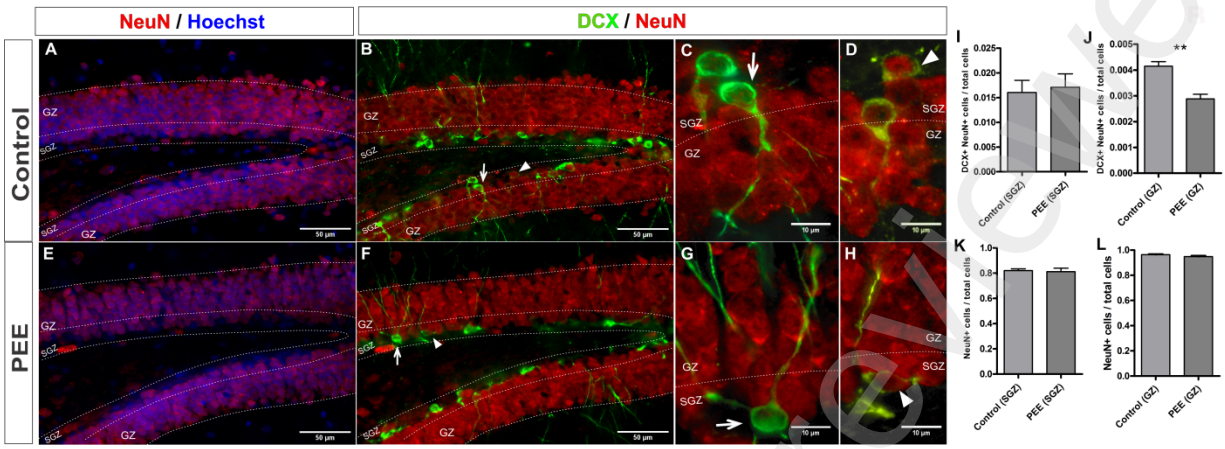

Figure 9. Migratory and granule cells in the adult mouse hippocampus. Migratory cells (type 3 cells, NeuN-/DCX+, arrows), immature granule cells (type 4 cells, NeuN+/DCX+, arrowheads) and granule cells (type 5, NeuN+/DCX-) in the SGZ and GZ of the DG of adult Control and PEE mice. Photomicrographs of coronal sections with Hoechst staining for cell nuclei (blue) and double immunofluorescence for NeuN (red) and DCX (green). Sections at low magnification (A-B and E-F) and at high magnification (C-D and G-H). Proportion of NeuN+/DCX+ cells in the SGZ (I) and GZ (J) and NeuN+/DCX- cells in SGZ (K) and GZ (L). Data expressed as the mean ± SEM (Control n = 4, PEE n = 4); all parameters were analyzed through Student's t-test (**p< 0.01).

Given that postmitotic stages can be identified through the expression of different types of Ca2+ binding proteins, Calretinin (CR) and Calbindin (CB), calcium-binding and buffering proteins, were used as specific markers for type 4 and type 5 cells, respectively. Furthermore, the transition from migratory type 3 to immature granule type 4 cells was analyzed using double DCX/CR (Fig 10) and NeuN/CR (Fig 11) immunostaining.

Once again, type 3 cells only expressed DCX, while type 4 cells expressed both DCX and CR with their somas located in the SGZ (Fig 10 C-F and J-M). The proportion of DCX+/CR+ cells was smaller in PEE than in Control animals (Fig 10 N: Control 0.07424 ± 0.0067, PEE: 0.04984 ± 0.0041; p = 0.037).

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

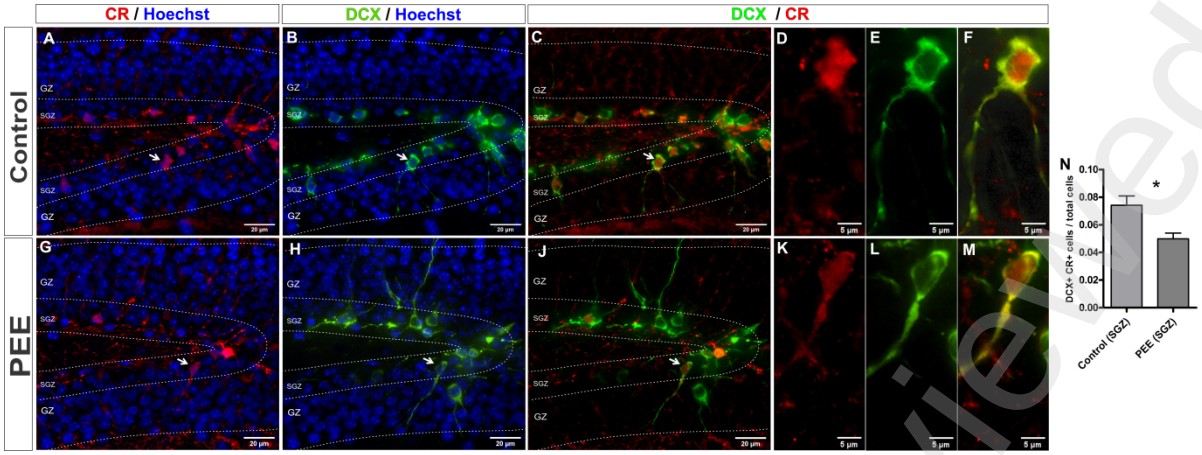

Figure 10. Migratory and immature granule cells in the adult mouse hippocampus. Migratory cells (type 3 cells, CR-/DCX+), immature granule cells (type 4 cells, CR+/DCX+, arrows) in the SGZ of the DG of adult Control and PEE mice. Photomicrographs of coronal sections with Hoechst staining for cell nuclei (blue) and double immunofluorescence for CR (red) and DCX (green). Sections at low magnification (A-C and G-J) and at high magnification (D-F and K-M). Proportion of CR+/DCX+ cells in the SGZ (N). Data expressed as the mean ± SEM (Control n = 4, PEE n = 4,); all parameters were analyzed through Student's t-test (*p < 0.05).

In agreement with the results shown above, the PEE group presented a smaller proportion of NeuN-/CR+ cells than the Control group (Fig 11 S: Control: 0.06112 ± 0.004 y PEE: 0.03863 ± 0.005; p = 0,008), while no significant differences were found in the proportion of NeuN+/CR+ cells between experimental groups (Fig 11 T: Control: 0.02124 ± 0.0019, PEE: 0.02475 ± 0.0033; p = 0.3702).

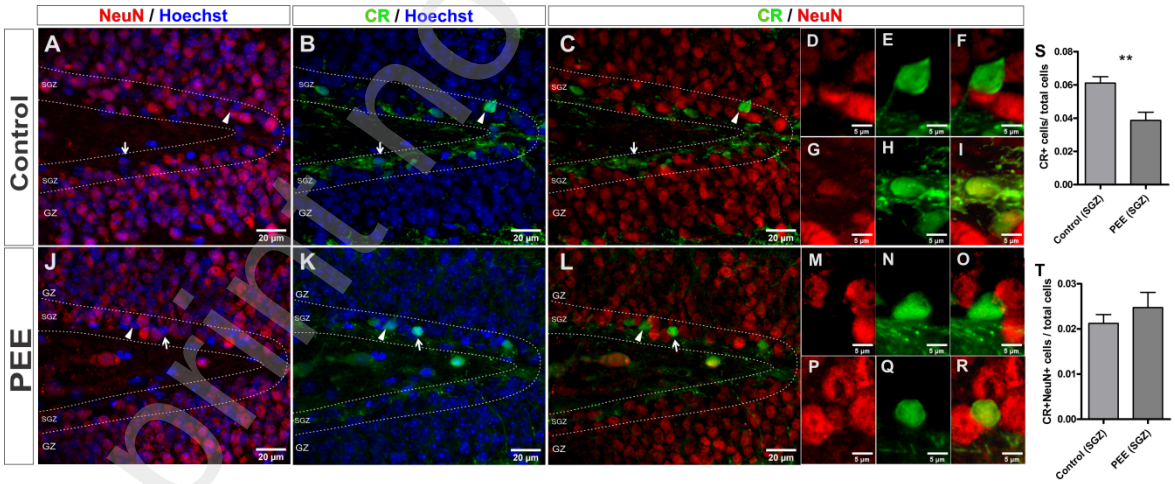

Figure 11. Immature granule cells in the adult mouse hippocampus. Early type 4 cells (NeuN-/CR+, arrowheads) and late type 4 cells (NeuN+/CR+, arrows) in the SGZ of the DG of adult Control and PEE mice. Photomicrographs of coronal sections with Hoechst staining for cell nuclei (blue) and double immunofluorescence for NeuN (red) and CR (green). Sections at low magnification (A-C and J-L) and at high magnification (D-F and M-O, NeuN-/CR+ cells; G-I and P-R, NeuN+/CR+ cells). Proportion

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

of NeuN-/CR+ (S) and NeuN+/CR+ (T) cells in the SGZ. Data expressed as the mean ± SEM (Control n =5, PEE n = 4,); all parameters were analyzed through Student's t-test (**p < 0.01).

Finally, mature granule cells were studied employing CB and NeuN as specific markers. NeuN+/CB+ cells were detected in both the GZ and SGZ (Fig 12 A-F and G-L), and their proportions did not vary between experimental groups in either area (Fig 12 M: Control: 0.03885 ± 0.0052, PEE: 0.03852 ± 0.0046, p = 0.647; N: Control: 0.7976 ± 0.0016, PEE: 0.8071 ± 0.011; p = 0.646).

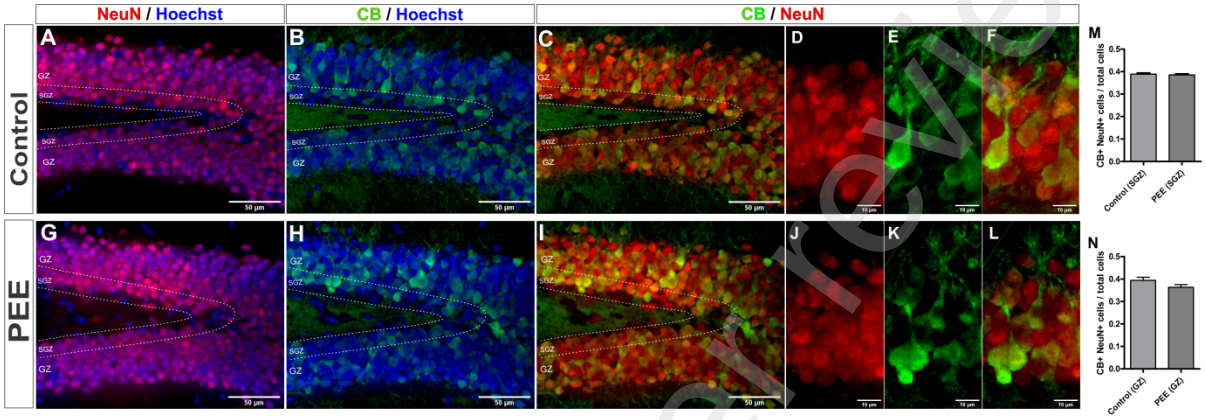

Figure 12. Mature granule cells in the adult mouse hippocampus. Mature granule cells (NeuN+/CB+) in the SGZ and the GZ of the DG of adult Control and PEE mice Photomicrographs of coronal sections with Hoechst staining for cell nuclei (blue) and double immunofluorescence for NeuN (red) and CB (green). Sections at low magnification (A-C and G-I) and at high magnification (D-E and J-L). Proportion of NeuN+/CB+ cells in the SGZ (M) and GZ (N). Data expressed as the mean ± SEM (Control n = 4, PEE n = 4); all parameters were analyzed through Student's t-test.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

4. Discussion

Accumulating evidence from studies in humans and animal models demonstrates the deleterious effects of alcohol exposure during development on the central nervous system and particularly on the hippocampus, which are linked to many of the neurobehavioral and cognitive disorders reported in FASD.

The present work focused on the effects of PEE on the adult hippocampal structure and, in particular, the DG, where neurogenesis persists throughout life. This process includes six stages or cellular types from a presumable bipotential radial glial-like stem cell (type 1), to a transiently amplifying lineage determined by progenitor cells (type 2) and migratory cells (type 3) to early postmitotic and mature neurons (type 4 and 5) (Kempermann et al., 2004; Nicola et al., 2015). As each of these stages presents specific molecular and morphological features, injury such as ethanol exposure during embryonic and early postnatal life is expected to affect them in different ways.

The first cell type involved in adult neurogenesis (type 1 cells) showed a smaller number in adult PEE animals, even though ethanol exposure was interrupted after weaning. Given that ethanol exposure in the embryonic period has been shown to reduce the pool of radial glial-like progenitors (Rubert et al., 2006), the neurotoxic effect of ethanol during the entire neurodevelopmental period (both embryonic and early postnatal life) may be thought to reduce the pool of stem cells in PEE pups. Therefore, PEE may bring about the decrease in type 1 cells while ethanol is present in blood, with damage persisting into adulthood after ethanol removal from the diet.

It has been demonstrated that cell differentiation varies along the rostro-caudal axis in the adult DG, being higher in the rostral region (Snyder et al., 2009). Consistently with these findings, the analysis of neuronal precursor populations revealed a larger number of GFAP-/Nestin+ progenitor cells (types 2) in the rostral than in the caudal region of the SGZ. Therefore, early precursors populations were analyzed in the rostral zone.

Type 2 cells were more abundant in the DG of adult PEE than in Control animals. It has been reported in different experimental models that exposure to ethanol during early development can affect neuronal differentiation (Gil-Mohapel et al., 2011; Singh et al., 2009; Tateno et al., 2005). Although the molecular mechanism underlying this alteration is still unclear, it can be speculated that PEE may dysregulate the neurogenic process in adulthood, delaying the differentiation of type 2 cells into type 3.

Most of the proliferative activity in the adult SGZ corresponds to type 2 cells, also referred to as transient amplification progenitor cells (Steiner et al., 2006). Interestingly, double-immunostaining assays on late type 2 cells (type 2b) rendered no Nestin/DCX colocalization, which may be due to the transient expression of these markers (Harris et al., 2017; Seri et al., 2004). In addition, the analysis of proliferative capacity yielded no significant differences between experimental groups. In line with findings reported by Olateju et al. (Olateju et al., 2017), our results suggest that PEE has a mild effect on the proliferative process in the adult hippocampus. Alternatively, the naturally low rate of cell proliferation in adulthood may mask the possible effects of PEE.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

Early type 3 cells in the SGZ have a horizontal arrangement with very short processes, while more mature cells acquire a radial arrangement in which the extension ascends to the granular layer (Knoth et al., 2010). Our studies revealed no differences in horizontal or radial type 3 cells in the SGZ between PEE and Control animals. In contrast, fewer type 3 cells were found in the GZ of PEE animals. Taken together, these results suggest that PEE had a negative effect on the migration of neuroblasts from the SGZ to the GZ, generating a delay in the process.

In agreement with these results, PEE animals presented a lower number of type 4 cells than Control in the GZ, while type 5 cells (granular neurons) did not vary between groups. This is probably due to the low number of new mature granular cells generated in adult hippocampal neurogenesis (Ciric et al., 2019; Kerloch et al., 2019), which may have made it difficult to detect differences induced by perinatal treatment with the methods used.

Various studies have shown no effects of PEE on hippocampal neurogenesis in adolescent/young adult animals (Joana Gil-Mohapel et al., 2011; Klintsova et al., 2007), and some reports have even shown a slight increase in neuronal differentiation (Boehme et al., 2011). However, these data do not conflict with our findings, as young animals present a different hippocampal neurogenesis rate, and compensatory mechanisms may be at play. Indeed, as neurogenesis declines with age, comparisons between adolescence/youth and adulthood can hardly be made (Hamilton et al., 2016; Kuhn et al., 2018; Lugert et al., 2010; Smith et al., 2018). Furthermore, our work finds support in previous studies using different experimental designs which have reported a reduction in neurogenesis in adults perinatally exposed to ethanol (Choi et al., 2005; Gil-Mohapel et al., 2014; Ieraci & Herrera, 2007; Klintsova et al., 2007).

In addition to age, several other factors may influence the effects of alcohol exposure during development. Alcohol intake during gestation produces central nervous system damage in both humans and animal experimental models, with alterations depending on the developmental time point, duration and dose of exposure and epigenetic features, among others (Reid et al., 2020). Scientific research has long addressed the influence of these factors and the cell mechanisms involved in neuronal damage using different animal models and experimental designs such as exposure to ethanol vapors, forced ethanol intake, intraperitoneal ethanol injections, intragastric ethanol administration, intermittent and continuous exposure (Gaztañaga et al., 2020). Moreover, murine models have revealed differences in PEE effects across mouse strains (Amiri et al., 2019).

Our group has previously used a model of maternal exposure to low doses of ethanol consisting in 6% (v/v) ethanol intake before mating and along pregnancy and lactation, with no other stressors for mothers or pups. Results showed a reduction in the cerebral cortex thickness , probably due to neuronal death and alterations in neuronal migration during corticogenesis (Aronne et al., 2011). Other previous studies by our group have further shown alterations in the morphological organization of limbic system areas which correlate with anxious behavior in adult CD1 mice perinatally exposed to ethanol (Madarnas et al., 2020). These results are in agreement with reports using similar experimental models of maternal alcoholism (Abbott et al., 2016; El Shawa et al., 2013; Madden et al., 2020), but differ from those using stressors other than ethanol exposure (Gil-Mohapel et al., 2014).

One of the processes where the neuroplasticity of the brain becomes evident throughout life is neurogenesis, with the hippocampus being one of the few brain areas where neurogenesis persists in adulthood. Several groups have aimed to characterize the cell types present in the adult DG of the hippocampus and their histological organization (Kempermann et al, 2004;

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

Alenina and Klempin 2014; Aguilar-Arredondo et al, 2015; Harris et al, 2017). Due to the neurotoxicity of ethanol along neuronal development and, more precisely, its effect on immature neurons and the entire neurogenic process, the aim of the present work was to analyze the cellular composition of the adult DG in mice only perinatally exposed to moderate but continuous dose of ethanol.

It is well known that the developing central nervous system is mostly affected by maternal alcoholism and that behavioral and cognitive alterations in early life –for instance, fetal alcohol syndrome– can persist in adulthood even in the absence of alcohol intake (Alati et al, 2006; Day et al, 2013; Rangmar et al, 2015; Goldschmidt et al, 2019). As several disorders are associated with maternal alcoholism (Comasco et al., 2018; Mattson et al., 2019; Willford et al., 2004), it is necessary to establish a connection between perinatal exposure and alterations observed in adulthood. FASD encompass a wide range of clinical expressions, and some of them may result from alterations in adult neurogenesis, as this mechanism works as a plastic response of the brain to adapt to a changing environment (Lledo et al., 2006).

Campaigns to discourage alcohol consumption during pregnancy and lactation should raise awareness of its negative impact and prevent FASD, which is often difficult to detect. Likewise, preclinical studies should be carried out to test potential neuroprotectors and transfer them to properly designed clinical studies, since few groups have achieved translation from animal models to humans (Ernst et al., 2022).

5. Conclusion

The results shown in this study demonstrate that exposure to low ethanol concentrations throughout the neurodevelopmental period alters the hippocampal neurogenic process in adulthood, as PEE animals showed fewer type 1 cells, more type 2 cells and then again fewer type 4 cells in the DG.

It is clear that the generation of neuronal precursors is affected by ethanol exposure during neurodevelopment, and that this impact is not offset in the absence of ethanol after weaning and persists into adulthood, together with an impairment in neuronal differentiation.

Given the plasticity of the hippocampus and particularly the DG, alterations remain in adulthood and may account for the typical neurobehavioral and cognitive features observed in FASD.

Funding

This work was supported by grants from Universidad de Buenos Aires (UBACYT 20020170100371BA) and FONCYT (PICT2017-0610).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

CRediT authorship contribution statement

Nerina Villalba and Catalina Madarnas: contributed equally to the research work. Investigation, Methodology, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Viviana Sánchez**: Project design, Conceptualization, Visualization, Supervision, Writing - review & editing. **Alicia Brusco**: Project design, Conceptualization, Visualization, Supervision, Formal analysis, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

Authors declare no conflict of interest.

Acknowledgements

We appreciate the technical assistance in the care and maintenance of animals of Lic. Andrea Pecile.

References

Abbott, C. W., Kozanian, O. O., Kanaan, J., Wendel, K. M., & Huffman, K. J. (2016). The Impact of Prenatal Ethanol Exposure on Neuroanatomical and Behavioral Development in Mice. *Alcoholism: Clinical and Experimental Research*, *40*(1), 122–133. https://doi.org/10.1111/ACER.12936

Aguilar-Arredondo A., Arias C., Zepeda A. (2015) Evaluating the functional state of adult-born neurons in the adult dentate gyrus of the hippocampus: from birth to functional integration. Rev Neurosci. 26(3):269-79. https://doi: 10.1515/revneuro-2014-0071.

Alati R., Al Mamun A., Williams G.M., O'Callaghan M., Najman J.M., Bor W. (2006) In utero alcohol exposure and prediction of alcohol disorders in early adulthood: a birth cohort study. Arch Gen Psychiatry. 63(9):1009-16. https://doi: 10.1001/archpsyc.63.9.1009.

Alenina N., Klempin F. (2014) The role of serotonin in adult hippocampal neurogenesis. Behav Brain Res. 277:49-57. https://doi: 10.1016/j.bbr.2014.07.038.

Amiri, S., Davie, J. R., & Rastegar, M. (2019). Chronic Ethanol Exposure Alters DNA Methylation in Neural Stem Cells: Role of Mouse Strain and Sex. *Molecular Neurobiology 2019 57:2*, *57*(2), 650–667. https://doi.org/10.1007/S12035-019-01728-0

Aronne, M. P., Guadagnoli, T., Fontanet, P., Evrard, S. G., & Brusco, A. (2011). Effects of prenatal ethanol exposure on rat brain radial glia and neuroblast migration. *Experimental Neurology*, *229*(2), 364–371. https://doi.org/10.1016/j.expneurol.2011.03.002

Autti-Rämö, I. (2002). Foetal alcohol syndrome – a multifaceted condition. Developmental Medicine and Child Neurology, 44(2), 141–144. https://doi.org/10.1017/S0012162201001839

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

Berman, R. F., & Hannigan, J. H. (2000). Effects of Prenatal Alcohol Exposure on the Hippocampus: Spatial Behavior, Electrophysiology, and Neuroanatomy. Hippocampus, 10, 94–110. https://doi.org/10.1002/(SICI)1098-1063(2000)10:1

Boehme, F., Gil-Mohapel, J., Cox, A., Patten, A., Giles, E., Brocardo, P. S., & Christie, B. R. (2011). Voluntary exercise induces adult hippocampal neurogenesis and BDNF expression in a rodent model of fetal alcohol spectrum disorders. *The European Journal of Neuroscience*, *33*(10), 1799–1811. https://doi.org/10.1111/J.1460-9568.2011.07676.X

Bolte, S., & Cordelières, F. P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. Journal of Microscopy, 224(3), 213–232. https://doi.org/10.1111/J.1365-2818.2006.01706.X

Choi, I. Y., Allan, A. M., & Cunningham, L. A. (2005). Moderate fetal alcohol exposure impairs the neurogenic response to an enriched environment in adult mice. *Alcoholism, Clinical and Experimental Research*, *29*(11), 2053–2062. https://doi.org/10.1097/01.ALC.0000187037.02670.59

Ciric, T., Cahill, S. P., & Snyder, J. S. (2019). Dentate gyrus neurons that are born at the peak of development, but not before or after, die in adulthood. *Brain and Behavior*, *9*(10). https://doi.org/10.1002/BRB3.1435

Comasco, E., Rangmar, J., Eriksson, U. J., & Oreland, L. (2018). Neurological and neuropsychological effects of low and moderate prenatal alcohol exposure. *Acta Physiologica*, *222*(1), e12892. https://doi.org/10.1111/APHA.12892

Day N.L., Helsel A., Sonon K., Goldschmidt L. (2013). The association between prenatal alcohol exposure and behavior at 22 years of age. Alcohol Clin Exp Res. 2013 Jul;37(7):1171-8. https://doi: 10.1111/acer.12073.

Donald, K. A., Fouche, J. P., Roos, A., Koen, N., Howells, F. M., Riley, E. P., Woods, R. P., Zar, H. J., Narr, K. L., & Stein, D. J. (2016). Alcohol exposure in utero is associated with decreased gray matter volume in neonates. Metabolic Brain Disease, 31(1), 81–91. https://doi.org/10.1007/s11011-015-9771-0

El Shawa, H., Abbott, C. W., & Huffman, K. J. (2013). Prenatal Ethanol Exposure Disrupts Intraneocortical Circuitry, Cortical Gene Expression, and Behavior in a Mouse Model of FASD. *Journal of Neuroscience*, *33*(48), 18893–18905. https://doi.org/10.1523/JNEUROSCI.3721-13.2013

Ernst, A. M., Gimbel, B. A., de Water, E., Eckerle, J. K., Radke, J. P., Georgieff, M. K., & Wozniak, J. R. (2022). Prenatal and Postnatal Choline Supplementation in Fetal Alcohol Spectrum Disorder. *Nutrients 2022, Vol. 14, Page 688*, *14*(3), 688. https://doi.org/10.3390/NU14030688

Evrard, S. G., Vega, M. D., Ramos, A. J., Tagliaferro, P., & Brusco, A. (2003). Altered neuron-glia interactions in a low, chronic prenatal ethanol exposure. Developmental Brain Research, 147(1–2), 119–133. https://doi.org/10.1016/j.devbrainres.2003.09.004

Gaztañaga, M., Angulo-Alcalde, A., & Chotro, M. G. (2020). Prenatal Alcohol Exposure as a Case of Involuntary Early Onset of Alcohol Use: Consequences and Proposed

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

Mechanisms From Animal Studies. *Frontiers in Behavioral Neuroscience*, *14*, 26. https://doi.org/10.3389/FNBEH.2020.00026

Gil-Mohapel, J., Titterness, A. K., Patten, A. R., Taylor, S., Ratzlaff, A., Ratzlaff, T., Helfer, J., & Christie, B. R. (2014). Prenatal ethanol exposure differentially affects hippocampal neurogenesis in the adolescent and aged brain. *Neuroscience*, *273*, 174–188. https://doi.org/10.1016/J.NEUROSCIENCE.2014.05.012

Gil-Mohapel, Joana, Boehme, F., Patten, A., Cox, A., Kainer, L., Giles, E., Brocardo, P. S., & Christie, B. R. (2011). Altered adult hippocampal neuronal maturation in a rat model of fetal alcohol syndrome. *Brain Research*, *1384*, 29–41. https://doi.org/10.1016/J.BRAINRES.2011.01.116

Glass, L., Ware, A. L., & Mattson, S. N. (2014). Neurobehavioral, neurologic, and neuroimaging characteristics of fetal alcohol spectrum disorders. Handbook of Clinical Neurology, 125, 435–462. https://doi.org/10.1016/B978-0-444-62619-6.00025-2

Godin E.A., O'Leary-Moore S.K., Khan A.A., Parnell S.E., Ament J.J., Dehart D.B., Johnson B.W., Allan Johnson G., Styner M.A., Sulik K.K. (2010). Magnetic resonance microscopy defines ethanol-induced brain abnormalities in prenatal mice: effects of acute insult on gestational day 7. Alcohol Clin Exp Res., 34(1):98-111. https://doi.org/ 10.1111/j.1530-0277.2009.01071.x.

Goldschmidt, L., Richardson, G. A., De Genna, N. M., Cornelius, M. D., and Day, N. L. (2019). Prenatal alcohol exposure and offspring alcohol use and misuse at 22 years of age: a prospective longitudinal study. Neurotoxicol.Teratol. 71, 1–5. https://doi: 10.1016/j.ntt.2018.11.001

Gonçalves, J. T., Schafer, S. T., & Gage, F. H. (2016). Adult Neurogenesis in the Hippocampus: From Stem Cells to Behavior. Cell, 167(4), 897–914. https://doi.org/10.1016/J.CELL.2016.10.021

Hamilton, G. F., Bucko, P. J., Miller, D. S., DeAngelis, R. S., Krebs, C. P., & Rhodes, J. S. (2016). Behavioral deficits induced by third-trimester equivalent alcohol exposure in male C57BL/6J mice are not associated with reduced adult hippocampal neurogenesis but are still rescued with voluntary exercise. *Behavioural Brain Research*, *314*, 96–105. https://doi.org/10.1016/J.BBR.2016.07.052

Harris, L., Zalucki, O., Oishi, S., Burne, T. H., Jhaveri, D. J., & Piper, M. (2017). A morphology independent approach for identifying dividing adult neural stem cells in the mouse hippocampus. *Developmental Dynamics*, *247*(1), 194–200. https://doi.org/10.1002/DVDY.24545

Ieraci, A., & Herrera, D. G. (2007). Single alcohol exposure in early life damages hippocampal stem/progenitor cells and reduces adult neurogenesis. *Neurobiology of Disease*, *26*(3), 597–605. https://doi.org/10.1016/J.NBD.2007.02.011

Kempermann, G., Jessberger, S., Steiner, B., & Kronenberg, G. (2004). Milestones of neuronal development in the adult hippocampus. *Trends in Neurosciences*, *27*(8), 447–452. https://doi.org/10.1016/J.TINS.2004.05.013

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

Kerloch, T., Clavreul, S., Goron, A., Abrous, D. N., & Pacary, E. (2019). Dentate Granule Neurons Generated During Perinatal Life Display Distinct Morphological Features Compared With Later-Born Neurons in the Mouse Hippocampus. *Cerebral Cortex*, *29*(8), 3527–3539. https://doi.org/10.1093/CERCOR/BHY224

Klintsova, A. Y., Helfer, J. L., Calizo, L. H., Dong, W. K., Goodlett, C. R., & Greenough, W. T. (2007). Persistent impairment of hippocampal neurogenesis in young adult rats following early postnatal alcohol exposure. *Alcoholism, Clinical and Experimental Research*, *31*(12), 2073–2082. https://doi.org/10.1111/J.1530-0277.2007.00528.X

Knoth, R., Singec, I., Ditter, M., Pantazis, G., Capetian, P., Meyer, R. P., Horvat, V., Volk, B., & Kempermann, G. (2010). Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. PloS one, 5(1), e8809. https://doi.org/10.1371/journal.pone.0008809

Kuhn, H. G., Toda, T., & Gage, F. H. (2018). Adult Hippocampal Neurogenesis: A Coming-of-Age Story. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *38*(49), 10401–10410. https://doi.org/10.1523/JNEUROSCI.2144-18.2018

Livy, D. J., Miller, E. K., Maier, S. E., & West, J. R. (2003). Fetal alcohol exposure and temporal vulnerability: Effects of binge-like alcohol exposure on the developing rat hippocampus. Neurotoxicology and Teratology, 25(4), 447–458. https://doi.org/10.1016/S0892-0362(03)00030-8

Lledo, P. M., Alonso, M., & Grubb, M. S. (2006). Adult neurogenesis and functional plasticity in neuronal circuits. *Nature Reviews Neuroscience 2006 7:3*, *7*(3), 179–193. https://doi.org/10.1038/nrn1867

Lugert, S., Basak, O., Knuckles, P., Haussler, U., Fabel, K., Götz, M., Haas, C. A., Kempermann, G., Taylor, V., & Giachino, C. (2010). Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. *Cell Stem Cell*, *6*(5), 445–456. https://doi.org/10.1016/J.STEM.2010.03.017

Madarnas, C., Villalba, N. M., Soriano, D., & Brusco, A. (2020). Anxious Behavior of Adult CD1 Mice Perinatally Exposed to Low Concentrations of Ethanol Correlates With Morphological Changes in Cingulate Cortex and Amygdala. *Frontiers in Behavioral Neuroscience*, *0*, 92. https://doi.org/10.3389/FNBEH.2020.00092

Madden, J. T., Thompson, S. M., Magcalas, C. M., Wagner, J. L., Hamilton, D. A., Savage, D. D., Clark, B. J., & Pentkowski, N. S. (2020). Moderate prenatal alcohol exposure reduces parvalbumin expressing GABAergic interneurons in the dorsal hippocampus of adult male and female rat offspring. *Neuroscience Letters*, *718*. https://doi.org/10.1016/J.NEULET.2019.134700

Maier, S. E., & West, J. R. (2001). Drinking patterns and alcohol-related birth defects. Alcohol Research and Health, 25(3), 168–174. https://europepmc.org/articles/PMC6707176

Manders, E. M. M., Stap, J., Strackee, J., Van Driel, R., & Aten, J. A. (1996). Dynamic behavior of DNA replication domains. Experimental Cell Research, 226(2), 328–335. https://doi.org/10.1006/EXCR.1996.0233

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

Mattson, S. N., Bernes, G. A., & Doyle, L. R. (2019). Fetal Alcohol Spectrum Disorders: A Review of the Neurobehavioral Deficits Associated With Prenatal Alcohol Exposure. *Alcoholism, Clinical and Experimental Research*, *43*(6), 1046–1062. https://doi.org/10.1111/acer.14040

Nicola, Z., Fabel, K., & Kempermann, G. (2015). Development of the adult neurogenic niche in the hippocampus of mice. *Frontiers in Neuroanatomy*, *9*(MAY), 53. https://doi.org/10.3389/FNANA.2015.00053

Olateju, O. I., Spocter, M. A., Patzke, N., Ihunwo, A. O., & Manger, P. R. (2017). Hippocampal neurogenesis in the C57BL/6J mice at early adulthood following prenatal alcohol exposure. Metabolic Brain Disease 2017 33:2, 33(2), 397–410. https://doi.org/10.1007/S11011-017-0156-4

Patten, A. R., Gil-Mohapel, J., Wortman, R. C., Noonan, A., Brocardo, P. S., & Christie, B. R. (2013). Effects of Ethanol Exposure during Distinct Periods of Brain Development on Hippocampal Synaptic Plasticity. Brain sciences, 3(3), 1076–1094. https://doi.org/10.3390/brainsci3031076

Petrelli, B., Weinberg, J., & Hicks, G. G. (2018). Effects of prenatal alcohol exposure (PAE): Insights into FASD using mouse models of PAE. Biochemistry and Cell Biology, 96(2), 131–147. https://doi.org/10.1139/BCB-2017-0280

Ramos, A. J., Evrard, S. G., Tagliaferro, P., Tricárico, M. V., & Brusco, A. (2002). Effects of chronic maternal ethanol exposure on hippocampal and striatal morphology in offspring. Annals of the New York Academy of Sciences, 965, 343–353. https://doi.org/10.1111/j.1749-6632.2002.tb04176.x

Rangmar J., Sandberg A.D., Aronson M., Fahlke C. (2015). Cognitive and executive functions, social cognition and sense of coherence in adults with fetal alcohol syndrome. Nord J Psychiatry.69(6):472-8. https://doi: 10.3109/08039488.2015.1009487.

Redila, V. A., Olson, A. K., Swann, S. E., Mohades, G., Webber, A. J., Weinberg, J., & Christie, B. R. (2006). Hippocampal cell proliferation is reduced following prenatal ethanol exposure but can be rescued with voluntary exercise. Hippocampus, 16(3), 305–311. https://doi.org/10.1002/HIPO.20164

Reid, H. M. O., Lysenko-Martin, M. R., Snowden, T. M., Thomas, J. D., & Christie, B. R. (2020). A Systematic Review of the Effects of Perinatal Alcohol Exposure and Perinatal Marijuana Exposure on Adult Neurogenesis in the Dentate Gyrus. *Alcoholism: Clinical and Experimental Research*, *44*(6), 1164–1174. https://doi.org/10.1111/ACER.14332

Riley, E. P., & McGee, C. L. (2005). Fetal alcohol spectrum disorders: An overview with emphasis on changes in brain and behavior. Experimental Biology and Medicine, 230(6), 357–365. https://doi.org/10.1177/15353702-0323006-03

Rouzer, S. K., Cole, J. M., Johnson, J. M., Varlinskaya, E. I., & Diaz, M. R. (2017). Moderate Maternal Alcohol Exposure on Gestational Day 12 Impacts Anxiety-Like Behavior in Offspring. Frontiers in Behavioral Neuroscience, 0, 183. https://doi.org/10.3389/FNBEH.2017.00183

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487

Rubert, G., Miñana, R., Pascual, M., & Guerri, C. (2006). Ethanol exposure during embryogenesis decreases the radial glial progenitorpool and affects the generation of neurons and astrocytes. *Journal of Neuroscience Research*, *84*(3), 483–496. https://doi.org/10.1002/JNR.20963

Schambra, U. B., Lewis, C. N., & Harrison, T. A. (2017). Deficits in spatial learning and memory in adult mice following acute, low or moderate levels of prenatal ethanol exposure during gastrulation or neurulation. Neurotoxicology and Teratology, 62, 42–54. https://doi.org/10.1016/J.NTT.2017.05.001

Seri, B., García-Verdugo, J. M., Collado-Morente, L., McEwen, B. S., & Alvarez-Buylla, A. (2004). Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. *The Journal of Comparative Neurology*, *478*(4), 359–378. https://doi.org/10.1002/CNE.20288

Singh, A. K., Gupta, S., Jiang, Y., Younus, M., & Ramzan, M. (2009). In vitro neurogenesis from neural progenitor cells isolated from the hippocampus region of the brain of adult rats exposed to ethanol during early development through their alcohol-drinking mothers. *Alcohol and Alcoholism (Oxford, Oxfordshire)*, *44*(2), 185–198. https://doi.org/10.1093/ALCALC/AGN109

Smith, L. K., White, C. W., & Villeda, S. A. (2018). The systemic environment: at the interface of aging and adult neurogenesis. *Cell and Tissue Research*, *371*(1), 105–113. https://doi.org/10.1007/S00441-017-2715-8

Snyder, J. S., Radik, R., Wojtowicz, J. M., & Cameron, H. A. (2009). Anatomical gradients of adult neurogenesis and activity: Young neurons in the ventral dentate gyrus are activated by water maze training. *Hippocampus*, *19*(4), 360–370. https://doi.org/10.1002/HIPO.20525

Steiner, B., Klempin, F., Wang, L., Kott, M., Kettenmann, H., & Kempermann, G. (2006). Type-2 cells as link between glial and neuronal lineage in adult hippocampal neurogenesis. Glia, 54(8), 805–814. https://doi.org/10.1002/glia.20407

Tateno, M., Ukai, W., Yamamoto, M., Hashimoto, E., Ikeda, H., & Saito, T. (2005). The effect of ethanol on cell fate determination of neural stem cells. *Alcoholism: Clinical and Experimental Research*, *29* (12 SUPPL.). https://doi.org/10.1097/01.ALC.0000190658.56149.D4

Willford, J. A., Richardson, G. A., Leech, S. L., & Day, N. L. (2004). Verbal and Visuospatial Learning and Memory Function in Children With Moderate Prenatal Alcohol Exposure. *Alcoholism: Clinical and Experimental Research*, *28*(3), 497–507. https://doi.org/10.1097/01.ALC.0000117868.97486.2D

Zhao, C., Deng, W., & Gage, F. H. (2008). Mechanisms and Functional Implications of Adult Neurogenesis. Cell, 132(4), 645–660. https://doi.org/10.1016/J.CELL.2008.01.033

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4195487