The Effects of Voluntary Exercise on Adult Hippocampal Neurogenesis and BDNF Levels in a Rodent Model of Fetal Alcohol Spectrum Disorders

by

Fanny Boehme
B.Sc., University of Applied Sciences Lausitz, 2008

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of the Requirements for the Degree of

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in the Department of Biology

Supervisory Committee

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Abstract

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Alcohol consumption during pregnancy is detrimental to the developing nervous system of the unborn offspring. The hippocampus, one of the two brain regions where neurogenesis persists into adulthood, is particularly sensitive to the teratogenic effects of alcohol. The present study examined the effects of alcohol exposure throughout all three trimester equivalents on the stages of adult neurogenesis. Prenatal and early postnatal alcohol exposure (PPAE) altered cell proliferation in adult but not adolescent animals and increased early neuronal differentiation without affecting cell survival in both age groups. The levels of brain-derived neurotrophic factor (BDNF) were not affected by PPAE in the dentate gyrus but were significantly decreased in the Cornu ammonis region of the hippocampus. These results might explain the functional deficits seen in this hippocampal sub-region. This study identified that voluntary wheel running increased cell proliferation, differentiation and survival as well as BDNF expression in both PPAE and control animals.

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ACTH	adrenocorticotrophic hormone
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ANOVA	analysis of variance
ARBD	alcohol related birth defects
ARND	alcohol related neurological disorders
BAC	blood alcohol content
BAD	Bcl-2 associated death promoter
Bcl-2	B-cell leukemia-2
BDNF	brain-derived neurotrophic factor
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CA	cornu's ammon (Ammon's horn) region
Ca^{2+}	Calcium ion
CaMKII	calcium/calmodulin dependent protein kinase II
Casp	caspase
Cdc42	cell division control protein 42 homolog
CNS	central nervous system
CORT	corticosterone
CRE	cyclic AMP response element
CREB	CRE- binding protein
DCX	doublecortin
DAB	2,2-diaminobenzedine
DAG	diacyl glycerol
DNA	deoxyribonucleic acid
DG	dentate gyrus
EC	entorhinal cortex
ECL	enhanced chemiluminescence

EGTA	ethylene glycol bis (2-aminoethyl ether)- *N,N,N′N′*- tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extra-cellular signal-regulated kinase
FAS	fetal alcohol syndrome
FASD	fetal alcohol spectrum disorder
GABA	γ-aminobutyric acid
GCL	granule cell layer
GCs	glucocoriticoids
GND	gestational day
HCl	hydrochloric acid
HPA	hypothalamus-pituitary-adrenal axis
HRP	horseradish peroxidase
Ig	Immunoglobin
i.p.	intraperitoneal
IP_3	inositol-1,4,5-triphosphate
JNK	Jun kinase
LTP	long-term potentiation
MAP	mitogen-associated protein
MAPK	mitogen-activated protein kinase
mBDNF	mature BDNF
MEK	mitogen-activated protein kinase kinase
Msk	mitogen and stress-activated protein kinase
mGluR	metabotrobic glutamate receptor
NaCl	sodium chloride
NFκB	nuclear factor kappa-light-chain- enhancer of activated B-cells
NGF	nerve growth factor
NPC	neuronal progenitor cells
NR	non-runner
NSC	neuronal stem cells
NT	neurotrophin

NMDA	*N*-methyl-D-aspartate
PBS	phosphate-buffered saline
PBST	PBS with Tween-20
PFA	paraformaldehyde
PI_3K	phosphatidylinositol-3 kinase
PND	postnatal day
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PPAE	pre and postnatal alcohol exposure
PSA-NCAM	polysialiated form of the neural cell adhesion molecule
PVDF	polyvinylidene fluoride
R	runner
Rsk	ribosomal S6 kinase
RT	room temperature
SC	Schaffer collateral
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SGZ	sub-granular zone
SVZ	sub-ventricular zone
TBS	tris-buffered saline
TBST	TBS with Triton X-100
TNF	tumor necrosis factor
TRAF	tumour necrosis factor receptor associated factor
Trk	tropomyosin-receptor-kinase

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1. Introduction

1.1. Fetal Alcohol Spectrum Disorders

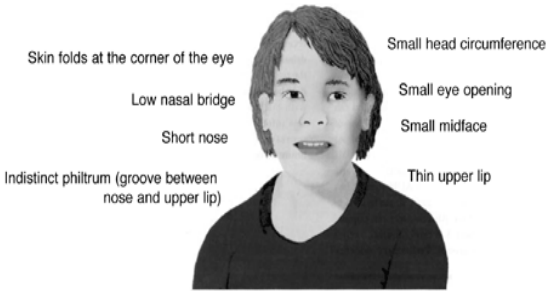

Figure 1. Facial abnormalities in infants born with Fetal Alcohol Syndrome. **(**http://www.niaaa.nih.gov/Resources/GraphicsGallery/FetalAlcoholSyndrome)

Alcohol exposure during the period of brain development can influence cell proliferation, differentiation, and survival in the central nervous system (CNS) inducing a constellation of symptoms grouped under the term: Fetal Alcohol Spectrum Disorders (FASD) (Abel, 2006; Mancinelli et al., 2007; Niccols, 2007). The spectrum of disorders includes Alcohol Related Neurological Disorders (ARND), Alcohol Related Birth Defects (ARBD), and Fetal Alcohol Syndrome (FAS). In particular, a diagnosis of FAS is made when a child presents particular cranio-facial dysmorphologies (Figure 1) (such as small head circumference, small and widely spaced eyes, flat midface, short and upturned nose, smooth and wide philtrum, and a thin upper lip), growth retardation, CNS impairments, and confirmed prenatal alcohol exposure (Stratton and Howe, 1996). Recent estimates have suggested that the incidence of FASD may be as high as 2-5 % in the most industrialized countries making it a serious health issue (May et al., 2009). Indeed, exposure to alcohol *in utero* has been cited as the leading cause of mental retardation and preventable birth defects (May et al., 2009; Oesterheld et al., 1998; Stratton and Howe, 1996).

In humans, alcohol exposure *in utero* is linked to a myriad of cognitive (in particular learning and memory), behavioural, emotional and social deficits. Behavioural deficits observed in these children include spatial and memory deficits that are indicative of learning and memory impairment, and are typically associated with hippocampal dysfunction (Coles et al., 1991; Streissguth et al., 1989; Uecker and Nadel, 1996). Moreover, neuroimaging studies have shown that children with FASD have significantly smaller brains, due to volume reduction in the cerebral cortex, amygdaloid body, basal ganglia, corpus callosum, cerebellum, and the hippocampal formation. This reduction in brain volume is thought to be the result of alcohol-induced apoptotic cell loss and reduced cell proliferation in the developing CNS (Archibald et al., 2001; Autti-Ramo, 2002; Ikonomidou et al., 2000; Klintsova et al., 2007; Roebuck et al., 1998).

Exposure of the developing fetus to alcohol may also affect biological mediators, including the synthesis and release of growth factors by cells of the CNS (Goodlett et al., 2005). If alcohol is administered during pregnancy, it can easily cross the placental barrier and may irreversibly impair multiple neurotrophin signalling pathways (Moore et al., 2004). Neurotrophins are a critical mediator for cell survival and have been shown to influence adult hippocampal neurogenesis as well as hippocampal-dependent learning and memory (for review see Lee and Son, 2009). Thus, this dysregulation of neurotrophic signalling together with a decrease in hippocampal neurogenesis may account, at least in part, for the learning and memory deficits seen in children affected with FASD and in rodent models of pre- and early postnatal alcohol exposure (PPAE).

1.1.1. Animal Models of Pre- and Postnatal Alcohol Exposure

Animal models are effective and valid tools used in experimental studies designed to evaluate the teratogenic effects of alcohol on the developing brain and have made valuable contributions to our understanding of FASD. While the study of human subjects is invaluable, epidemiological studies are limited by ethical constraints and a multitude of extraneous and confounding variables including; multi-substance abuse, maternal health, social or socioeconomic variables, experiential variability, and limitations or discrepancies in self-reporting. Animal models of FASD eliminate these obvious confounds associated with human subject studies and further allow for control over experimental design and magnitude, as well as allowing an appropriate control of stress, nutrition, and variation in alcohol consumption patterns (Abel and Hannigan, 1995). Importantly, each of the major characteristics of human FAS, (facial feature dysmorphology, CNS abnormalities, neurodevelopmental effects and growth deficiency or restriction) have been identified in one or more animal models of FASD (Goodlett and Horn, 2001). That said, no single animal model has been shown to exhibit all diagnostic criteria for FAS (Cudd, 2005; Hannigan, 1996) and there is no single ‘best’ model of FASD.

In rats, the physiological responses to alcohol are similar to that of humans (Hannigan, 1996) and behavioural outcomes of prenatal alcohol exposure have been fairly consistent with clinical and behavioural outcomes in human studies (Driscoll et al., 1990; Hannigan, 1996). For example, rodent models have demonstrated pre and postnatal growth restrictions (Abel, 1980), physical malformation (Abel and Dintcheff, 1978; Chernoff, 1977; Leichter and Lee, 1979; Randall et al., 1977; Tajuddin and Druse, 1996; Weinberg

and Gallo, 1982; Weinberg, 1993) physiological abnormalities (Sliwowska et al., 2006; Weinberg and Bezio, 1987; Zhang et al., 2005) as well as CNS dysfunction. In rats, CNS dysfunction includes impairments in basic adaptive functioning, as well as reductions in neural plasticity and poor performance in learning and memory tests (reviewed by Berman and Hannigan, 2000).

The timing of alcohol exposure is an additional consideration when working with rodent models. In both rats and mice, a brain growth spurt that corresponds to the human third trimester occurs during the first 10 days of postnatal life (Cudd, 2005). Thus, in these rodent models, alcohol must be administrated postnatally to examine the effect of alcohol in a time frame that is viewed as the equivalent to the third trimester in humans. The current models of alcohol administration during the period of brain development can be evaluated in terms of how well each model accommodates these considerations. There are four major modes of administration of alcohol: **1)** ethanol in water or liquid diet **2)** vapor inhalation, **3)** artificial rearing, and **4)** oral/intragastric intubation or gavage (for reviews see Abel, 1980; Gil-Mohapel et al., 2010; Kelly and Lawrence, 2008; Riley and Meyer, 1984). Alternative, less common methods, which will not be discussed here, include subcutaneous (s.c.) or intraperitoneal (i.p.) injections of alcohol solutions (Pal and Alkana, 1997; Rose et al., 1981).

1.1.1.1.Alcohol via Liquid Diet

Voluntary consumption of alcohol in rodents does not typically produce a blood alcohol content (BAC) higher than 150 mg/dl (reviewed by Abel and Hannigan, 1995). By using this method, rats can consume on average 12 g of ethanol/kg/day (or up to ~18 g of ethanol/kg/day). In some studies alcohol is introduced prior to pregnancy, starting

with a low dose dissolved in a saccharine solution and over a series of days the alcohol concentration increases in order to get rats/mice accustomed to it (Allan et al., 2003; Choi et al., 2005). This technique is advantageous in that it is less time consuming and labour intensive than other methods, reduces the potential handling stress of more invasive techniques, and is generally safer (i.e., results in fewer animal fatalities). This method has been shown to be reliable at producing low stable BAC levels in pregnant dams. However, this self-administration method does not allow for the control of precise dosage or timing of consumption and cannot be used to mimic binge drinking behaviour, as BACs do not reach a binge-like level (> 200 mg/dl). Lastly, this form of administration can only be used for pregnant dams and not breast-feeding pups and therefore cannot be used in third trimester equivalent models of FASD.

1.1.1.2. Inhalation Method

In the vapour inhalation method, pregnant dams or neonates are placed in an inhalation chamber filled with ethanol vapour for several hours (Karanian et al., 1986; Miki et al., 2008b; Rogers et al., 1979; Ryabinin et al., 1995). This technique is characterized by a rapid rise in BACs and has been shown to produce reliable and consistent high BACs (Miki et al., 2008b; Nelson et al., 1990). In many ways, the effects obtained with this technique are comparable with the ones obtained with gavage feeding (see below) with the advantage of requiring less labor and involving less handling of the animals (i.e., pups). This is also an excellent model to use with smaller rodents like mice (Kang et al., 2004). On the downside, this method does not mimic the routes of intake in humans and therefore may not accurately replicate several important aspects of human prenatal ethanol exposure. Moreover, in some studies, pups must be removed from their

mothers for extended periods of time (up to 3 hours a day) (Miki et al., 2008b), and there is no effective control group to account for the loss in nutrition of the ethanol-exposed pups. Alternatively neonatal pups can be placed inside the vapour chamber together with their dam in order to minimize the stress associated with the separation from the mother.

1.1.1.3.Artificial Rearing

In the artificial rearing or ‘pup-in-the-cup' method, neonate pups are exposed to alcohol in a way that is functionally similar to the third trimester of human pregnancy (Dobbing and Sands, 1979). Pups are typically maintained in a plastic cup supplied with nesting materials, floating in warm water, designed to mimic warm nesting and maternal interaction (Kelly et al., 1988; Kelly et al., 1991; Samson and Diaz, 1981; West, 1993). The process involves surgically implanting an intragastric tube or gastric cannula into the pups’ stomach (Hall, 1975) and ethanol is administered using a programmable pump that can administer alcohol chronically or periodically. While this model attempts to mimic the third trimester of human pregnancy and is reliable in producing consistent BAC levels, the procedure is extremely invasive, and requires the separation of pups from their mother (and consequently from the mother’s milk) as well as from their litter-mates during this important period of brain development. Furthermore, the procedure is expensive, labor intensive, and involves many potential health complications for the neonates.

1.1.1.4.Intragastric Intubation

In the intragastric intugation method ethanol is delivered directly to the stomach using a gavage (or force-feeding) strategy (Cronise et al., 2001; Kelly and Tran, 1997; Light et al., 1998; Tran et al., 2000). This procedure employs the use of a syringe attached to a curved steel gavage needle or plastic tubing that is inserted through the esophagus down to the entrance of the stomach. The ethanol is diluted either in water, in a vehicle solution (e.g. saline), or in a nutritional formula (e.g. milk). A nutrition and stress control group can be treated with an iso-caloric and iso-volumic substitute (e.g. maltose-dextrin solution) in replacement of ethanol and the consumption of standard chow can be restricted to that of the ethanol group's consumption. Alternatively, a stress/handling control group can receive a sham intubation. The doses of ethanol typically range between 2-6 g/kg/day in this model (Berman and Hannigan, 2000). There are several advantages to this method. Firstly, this model allows for precise control over the dose administered, and hence accurate control of the peak BAC reached. In this way, the model can be used to mimic binge-like alcohol consumption more accurately. As well, a modified version of the intragastric intubation technique (typically using plastic tubing instead of steel gavage needles) can be used in the treatment of neonate pups, and thus inclusion of the third-trimester equivalent is possible. However, there are several potential stress effects involved in this invasive procedure leading to an increased risk of animal death (e.g. due to an accidental perforation of the esophagus during the procedure).

In conclusion, there is current no "ideal" rodent model to mimic FASD, as all the methods described above are associated with potential advantages and disadvantages.

Given these considerations, in the present study we used a model of intragastic intubation throughout all three trimester equivalents, as this is the only model that mimics ethanol exposure through all three trimester equivalents (pre and postnatally) with consistent and reliable high BAC levels.

1.2. The Mechanisms of Alcohol Induced Damage

The chemical properties of ethanol allow for the rapid crossing from the maternal blood stream through the placental barrier into the developing fetus (Guerri and Sanchis, 1985). The placenta is involved in the transfer of essential nutrients between mother and fetus, so any impairment of placental cell function by ethanol will adversely affect fetal development and contribute to the pathophysiology of FASD (Burd et al., 2007).

In detail, ethanol impairs placental and umbilical cord blood flow by constricting blood vessels resulting in hypoxia and malnutrition of the fetus (Fisher et al., 1982; Mukherjee and Hodgen, 1982). This malnutrition can additionally increase the toxic effects of ethanol in the fetus (Shankar et al., 2007). Interestingly, ethanol metabolism is increased in pregnant rats (Badger et al., 2005), indicating a complex relationship between nutritional status and ethanol toxicity which can hardly be separated.

Besides its effects on nutrition and oxygen supply, ethanol also has a direct impact on brain development. Alcohol exposure during pregnancy has deleterious effects on the developing nervous system (reviewed by Goodlett and Horn, 2001). In short, ethanol interferes with neuronal migration and gliogenesis (Gressens et al., 1992; Hirai et al., 1999; Miller, 1986), disrupts cell adhesion molecules (Hirai et al., 1999; Hoffman et al., 2008), decreases myelination (Shetty and Phillips, 1992), alters development and

function of several neurotransmitter systems (including γ-aminobutyric acid (GABA) and glutamate signalling) (Olney, 2004), changes developmental gene regulation (Lee et al., 2008), reduces transport and uptake of glucose (Yao and Gregoire Nyomba, 2007), and disrupts mitochondrial function (leading to increased oxidative stress and cell death) (Henderson et al., 1999).

The work presented in this thesis will focus on the effects of ethanol on cell proliferation and survival in the hippocampus and its regulation by growth factors such as brain-derived neurotrophic factor (BDNF) (Heaton et al., 2000).

1.3. The Hippocampal Formation

The hippocampal formation is perhaps one of the most widely studied regions of the brain. The hippocampus is part of the limbic system and is a bilateral structure embedded within the medial temporal lobes of the cerebrum. Its name derives from its curved shape that can be observed in coronal brain sections, which resembles a seahorse. The hippocampus has three subdivisions: CA1, CA2, CA3 (CA comes from the Latin word *cornu ammonis*) (Lorente de Nó, 1934). The dentate gyrus (DG), the subiculum, presubiculum, the parasubiculum and the entorhinal cortex (EC) are part of the hippocampal formation. These regions differ in their connectivity patterns and expression of certain genes. The EC, DG and CA constitute the essential trisynaptic core circuit of this region (Andersen et al., 1969) (Figure 2).

The hippocampal formation is known to be involved in the control of several learning and memory behaviours, including spatial learning (reviewed by Gorchetchnikov and Grossberg, 2007; Schmajuk, 1990; Treves and Rolls, 1994). In addition, it is also one of

the regions of the brain that appears to be particularly vulnerable to the effects of ethanol exposure during early life (Barnes and Walker, 1981; West et al., 1986). Based on this observation, several researchers speculated that ethanol-induced hippocampal damage may be one of the causes of alterations in learning ability commonly seen in humans and rodents with FASD (reviewed by Berman and Hannigan, 2000).

1.3.1. Developmental Neurogenesis in the Hippocampus

In rodents, hippocampal neurogenesis starts prenatally and nears completion shortly after birth, with differentiation progressing through a CA1 to CA3 gradient and a dorsal to ventral gradient (Altman and Das, 1966; Bayer, 1980b). The formation of the granule cell layer (GCL) of the DG can be divided into two stages: during the first stage, progenitor cells proliferate within the periventricular zone of the medial part of the embryonic cerebral cortex, followed by the migration of the neural progenitor cells and neuroblasts to the prospective DG sub-region during the perinatal period (Altman and Bayer, 1990a; Eckenhoff and Rakic, 1984; Rickmann et al., 1987; Sievers et al., 1992). In rats, the first granule neurons in the DG are born during late embryogenesis (i.e. at around gestational day (GD) 17, one day after the first pyramidal neurons appear), these cells first move to the outer shell of the GCL, which is finally formed by postnatal day (PND) 5. Thus, only 15% of the granular cells are generated before birth (Bayer and Altman, 1975). Therefore, the time span of granule cell generation is approximately three times longer than pyramidal cell development and continues into adulthood. On the day of birth, the oldest granule neurons are visible in the suprapyramidal blade and exhibit rudimentary dendrites extending into the molecular layer (Rahimi and Claiborne, 2007). These neurons develop primarily during the first 3 weeks after birth (Bayer, 1980a), and

get incorporated into the hippocampal circuit as early as the end of the first week (Rahimi and Claiborne, 2007).

In the second stage, newborn cells are added to the inner thicker shell of the GCL, which is formed during infant and juvenile periods. Between PNDs 20-30 the proliferative cells become largely confined to the SGZ at the base of the GCL, which is the region where adult neurogenesis occurs (Altman and Bayer, 1990b). Neurogenesis produces a gradient of cell ages and dendritic morphologies across the GCL of the DG, while new neurons integrate with a spatial temporal gradient into the existing GCL.

Older cells occupying the outer edge of the GCL near the molecular layer, and younger cells being more prevalent in the inner most layer (Altman and Bayer, 1990b; Crespo et al., 1986; Kuhn et al., 1996; Wang et al., 2000). As it is known that the developing hippocampus is particularly vulnerable during the period of brain growth spurt (PNDs 4-10), therefore it is likely that ethanol exposure during this critical period will have drastic effects on the structure and function of this region.

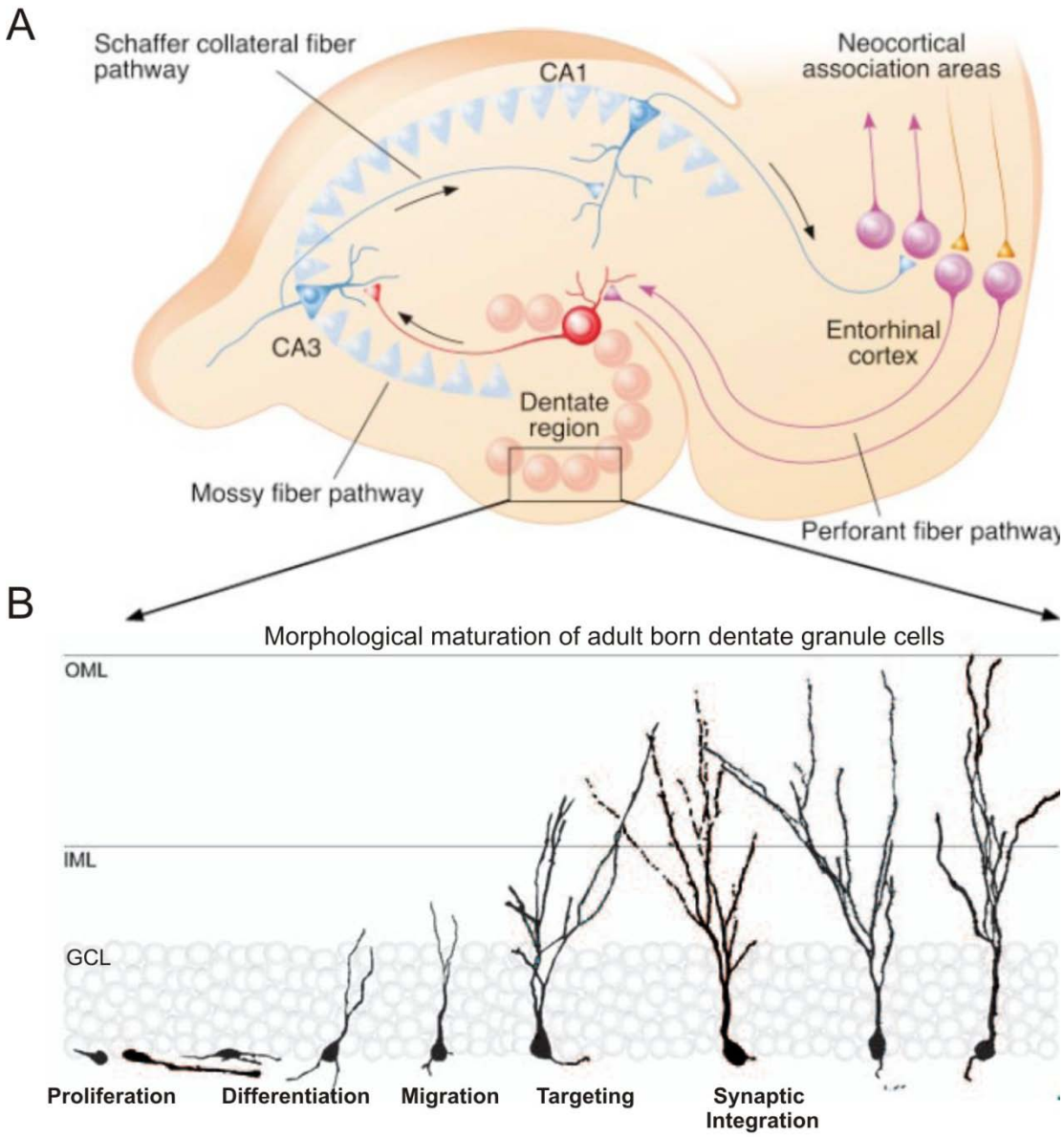

Figure 2. Neuronal circuitry and developmental stages of the hippocampus.
A, The hippocampus forms a principal unidirectional network, receiving inputs from the entorhinal cortex that form connections with the DG via the perforant path. CA3 neurons also receive inputs from the DG via mossy fibres. They send axons to CA1 via the schaffer collateral fiber pathway. These neurons send the main hippocampal output back to the EC, to form a loop (adapted from Lie et al., 2004). B, Neuronal development in the DG proceeds through a series of stages from proliferation to differentiation, migration and final integration into the trisynaptic circuitry (for detail see section 1.3.2) (adapted from Piatti et al., 2006).

1.3.2. Adult Hippocampal Neurogenesis

The initial belief that neurogenesis only occurs during embryonic development has changed dramatically over the past 20 years. In fact, proliferation and differentiation of new neurons are now known to occur in selective regions of the adult mammalian brain, primarily in the subventricular zone (SVZ) adjacent to the lateral ventricles and in the subgranular zone (SGZ) of the hippocampal DG (Altman and Das, 1965; Cameron et al., 1993; Kaplan and Hinds, 1977; Kuhn et al., 1996).

In the hippocampus, newborn neurons migrate just a short distance from the SGZ of the DG to the granule zone where they incorporate into the existing circuitry (Zhao et al., 2007). Dividing progenitor cells gives rise to daughter cells which differentiate, migrate, and integrate by extending dendrites towards the molecular layer and axons towards the CA3 region of the hippocampus (Kempermann et al., 2004) (Figure 2).

In young adult rats, where the cell cycle length is calculated to be ~25h (Cameron and McKay, 2001), about 9000 new cells are generated each day in the hippocampus, thus there are hundreds of thousands of cells created each month; the equivalent of about 6% of the total granule neuronal population (Cameron and McKay, 2001). These new cells define their neuronal phenotype within the first few days and are subject to a selection process, during which they are either recruited into function or eliminated (Biebl et al., 2000). About 50 % of newly generated granule cells die by apoptosis (Dayer et al., 2003) and out of the surviving cells, 80 – 90 % become neurons (Cameron and McKay, 2001). The surviving neurons mature over a period of 4 to 5 weeks of mitosis and become functionally indistinguishable from older granule cells within 7 weeks (van Praag et al., 2002).

1.3.2.1.Stages of Adult Hippocampal Neurogenesis

Adult hippocampal precursor cells have been found to be multipotent, giving rise to neurons, astrocytes and oligodentrocytes *in vitro* (Palmer et al., 1995; Palmer et al., 1999). These radial glial-like precursor cells are putative neuronal stem cells (NSC), providing scaffolding, which is necessary for normal development of the DG (Forster et al., 2002). These so called type-1 cells usually extend a strong apical process into the molecular layer of the DG and may establish contacts with blood vessels (Filippov et al., 2003).

These putative NSC give rise to fast proliferating intermediate precursors (type-2 cells), which are morphologically distinct from the stem cells: their processes are short and horizontally oriented. They express glial and neuronal markers, such as brain lipid binding protein, Nestin, doublecortin (DCX), NeuroD, and Prox1. Morphologically, type-2 cells have a small soma, an irregularly shaped nucleus and lack a strong apical process. Type-3 cells invariably express markers of the neuronal linage (DCX, NeuroD, and Prox1) and lack glial markers. Type-3 cell processes vary considerably in length, complexity and orientation. About 3 days after the initial division, the maturing granule cells become postmitotic. At this stage, the cells retain the vertical morphology of (late) type-3 cells, with a rounded or slightly triangular nucleus, clearly visible apical dendrites, and perhaps, early signs of protrusion of an axon (Figure 2 B).

Axonal contact in the target CA3 region has been found as early as 3 to 5 days after division (Hastings and Gould, 1999). The cells now express the post-mitotic neuronal marker neuronal nuclei (NeuN), the most widely used indicator for ‘mature neurons’. Even one day after a single injection of 5-bromo-2’-deoxyuridine (BrdU), a thymidine

analogue which in cooperates into the DNA during S-phase of the cell cycle, one can find a considerable number of cells labelled, which reaches their maximum 3 days later (Steiner et al., 2004). The majority of these neurons are subject to a selection process, during which they are either recruited or eliminated (Biebl et al., 2000).

During the late phase of maturation, which takes about 4 to 7 weeks, new cells become functionally indistinguishable from older granule cells (van Praag et al., 2002). After structural integration into the existing network, the new cells switch their intracellular calcium binding protein from calretinin to calbindin (Brandt et al., 2003). At this time the new neurons must find their place in the hippocampal circuitry, establish their connections in the local network (Ambrogini et al., 2004; van Praag et al., 2002).

1.3.2.2. Regulation of Adult Hippocampal Neurogenesis

Each phase of adult neurogenesis is tightly regulated and can be influenced by many factors. While being partially regulated by genetics, adult neurogenesis is also regulated by physiological, pathological, and behavioural factors that influence the proliferation, differentiation, and survival of new neurons. For example, stress (Gould et al., 1998), glucocorticoids (GCs) (Gould et al., 1992), inflammation (Ekdahl et al., 2003), alcohol (Nixon and Crews, 2002), opiates (Eisch et al., 2000), and the process of aging (Altman and Das, 1965; Kuhn et al., 1996) can all down-regulate adult neurogenesis. Indeed, neurogenesis naturally decreases with age (Altman and Das, 1965), with a very strong decline early in life follwed by a presistent reduction that levels off at a dramatically reduced rate in later life. This decline seems to result from a reduction in the number of precursor cells, mainly transient amplifying (type-2 and type-3) cells, which leads to a decrease in proliferation (Seki, 2002). Conversely, estrogens (Brannvall et al., 2002;

Perez-Martin et al., 2003), antidepressant drugs (Malberg et al., 2000; Manev et al., 2001), electroconvulsive therapy (Madsen et al., 2000), growth factors such as BDNF (Zigova et al., 1998) and insulin growth factor 1 (IGF-1) (Aberg et al., 2000), learning (Gould et al., 1999), physical exercise (van Praag et al., 1999a; van Praag et al., 1999b), and environmental enrichment (Kempermann et al., 1997) can up-regulate the capacity for neurogenesis in the adult mammalian brain.

Newly generated neurons have been linked to the functioning of the hippocampus, a brain region that is critical for learning and the formation of new explicit memories (Deng et al., 2010). However, in order to better understand the link between cognitive impairment and deficits in neurogenesis, it is crucial to elucidate both the regulatory mechanisms of adult hippocampal neurogenesis and how changes in these mechanisms can impact behaviour and hippocampal functioning. As mentioned above, prenatal ethanol exposure has been shown to cause learning and memory deficits, and might also alter adult neurogenesis in individuals affected with FASD.

Initial PPAE studies observed reductions in the size and number of cells in several CNS structures (e.g. cerebellum, cerebral cortex and hippocampus) (Bauer-Moffett and Altman, 1977; Miller, 1995; Nathaniel et al., 1986; Phillips and Cragg, 1982; West, 1986; Wigal and Amsel, 1990). Only recently have researchers started to investigate the differential effects of either pre- or early postnatal alcohol exposure on adult hippocampal neurogenesis (Table 1). The magnitude of alterations on hippocampal neurogenesis appears to depend on the dosage, timing and method of alcohol administration. Alcohol exposure during the first two trimester equivalents did not alter adult neurogensis (Choi et al., 2005; Redila et al., 2006), whereas alcohol during the third trimester equivalent

leaded to alterations in some studies (Helfer et al., 2009; Ieraci and Herrera, 2007; Klintsova et al., 2007; Wozniak et al., 2004).

Nevertheless, it should be noticed that none of these studies investigated the effect of alcohol exposure during all three trimester equivalents or analysed the neurogenic deficits in an age dependent manner.

Table 1. Summary of the published literature on the effects of prenatal or early postnatal alcohol exposure on adult hippocampal cell proliferation and/or cell survival/neurogenesis.

Period of exposure	Method	BAC mg/dl	Changes in cell prolif.	Age of animals (prolif.)	Changes in cell survival /neurogenesis	Age of animals (survival)	Reference
GDs 1-20	Liquid Diet	121	BrdU: ↑ F (ns) ↓ M (ns)	PND 95	BrdU: ↑ F (ns) ↓ M (ns)	PND 123	(Choi et al., 2005)
	Liquid Diet	184	BrdU↓ (ns)	PND 57 (M)	BrdU ↔	PND 85 (M)	(Redila et al., 2006)
PNDs 4-9	Gavage	315	Ki67 ↔	PND 50 (M)	BrdU ↓ BrdU/NeuN ↓	PND 80 (M)	(Klintsova et al., 2007)
	Gavage	330	BrdU ↔	PND 42 (M)	BrdU/DCX ↔ BrdU/NeuN ↔	PND 72 (M)	(Helfer et al., 2009)
PND7	1x s.c. 5g/kg	nd	PCNA ↓ Sox/GFAP↓	PND 147	BrdU ↓	PND 147	(Ieraci and Herrera, 2007)
	2x s.c. 2.5g/kg	510	-	-	BrdU/NeuN ↔	PND 54	(Wozniak et al., 2004)

BAC, blood alcohol level; F, female; GD, gestational day; M, male; n.d. not determined, ns, non significant trend; PND postnatal day; prolif, proliferation; s.c., subcutaneous; ↔ no change; ↑, increase in cell number; ↓ decrease in cell number.

1.4. Neurotrophins

Neurotrophins are a family of growth factors that have a critical role in many aspects of neuronal development and function. Although originally identified as neuronal survival factors, neurotrophins exert many regulatory roles ranging from proliferation to synapse formation and axonal pathway finding (Patapoutian and Reichardt, 2001).

The four neurotrophins expressed in the mammalian CNS are BDNF, nerve-growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), and they all share homologies in sequence and structure and have a similar genomic segment organisation. They are initially synthesized as precursors (proneurotrophins) and are proteolytically cleaved to originate mature, biologically active neurotrophins (Edwards et al., 1988). Interestingly, recent work has demonstrated that regulation of their maturation is an important post-transcriptional control point that limits and adds specificity to their actions (Lee et al., 2001).

Neurotrophins activate two different receptor classes, the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases and the p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor (TNF) receptor superfamily. The neurotrophin proforms preferentially activate $p75^{NTR}$ to mediate apoptosis whereas the mature forms activate Trk receptors to promote survival (Lee et al., 2001; Nykjaer et al., 2005). The four neurotrophins exhibit specificity in their interactions with the three members of the Trk receptor family, NGF activating TrkA, BDNF and NT-4 activating TrkB, and NT-3 activating TrkC.

1.4.1. Brain-Derived Neurotrophic Factor (BDNF)

BDNF, originally purified from pig brain as a survival factor for several neuronal populations not responsive to NGF, was the second neurotrophin to be characterized by Barde and his colleges (Barde et al., 1982). BDNF is synthesized as a precursor pro-BDNF protein, which is cleaved in the endoplasmatic reticulum into 32 kDa pro-BDNF. Pro-BDNF is either proteolytically cleaved inside the cell by furin or protein convertases and subsequently secreted as the 14 kDa mature BDNF, or secreted as pro-BDNF and then cleaved by extracellular proteases, such as metallo-proteases or plasmin (reviewed by Lessmann et al., 2003) (Figure 3).

BDNF is primarily expressed in the granule cells of DG, in excitatory pyramidal neurons of the hippocampus and cerebral cortex, and to a lesser extent, in the cerebellum, striatum and amygdala (Dugich-Djordjevic et al., 1995; Kawamoto et al., 1996; West, 2008; Wetmore et al., 1993). All of these regions are directly or indirectly involved in cognitive function, indicating a critical role of BDNF in cognition, specifically during learning and memory formation (Barot et al., 2008; Kalmbach et al., 2009; Squire and Zola, 1996). Furthermore, BDNF has been shown previously to play a diverse role in modulating the structure and function of the brain. BDNF regulates dendritic and axonal morphology and affects synaptogenesis and synaptic transmission (Thoenen, 1995; Levine et al., 1995). In addition, BDNF plays a major role in the differentiation and survival of neuronal progenitor cells (Barnabe-Heider and Miller, 2003; Lee et al., 2002). In the hippocampus, BDNF signalling is required for the long-term survival of newborn neurons (Sairanen et al., 2005).

The pro-survival and pro-differentiation effects of BDNF result from the activation of two key signalling pathways. First, binding of BDNF to TrkB receptor induces the activity of the phosphatidylinositol-3-kinase (PI3K) leading to protein kinase B (PKB/Akt) activation, which, in turn, phosphorylates and deactivates pro-apoptotic targets, including the transcription factors Forkhead and BAD, playing a cricial role in development and apoptosis (Brunet et al., 2001; Datta et al., 1997) (Figure 3). Second, binding of BDNF to TrkB also induces an activation of extracellular signal-regulated protein kinase (Erk) (either through phospholipase C (PLC)-induced intracellular calcium (Ca^{2+}) release or through protein kinase C (PKC) activation). This results in an increase in the activities of both ribosomal S6 kinase (Rsk2) and mitogen and stress activated protein (Msk) kinases, which, in turn, enhances the phosphorylation of cyclic-AMP response element-binding protein (CREB) (Arthur et al., 2004; Bonni et al., 1999). Once activated by phosphorylation, CREB induces the transcription of a variety of genes (e.g. polysialylated neural cell adhesion molecule, PSA-NCAM; and Bcl-2) that facilitate neuronal differentiation and survival (Figure 3).

On the other hand, when pro-BDNF is not processed into mature BDNF inside the cell, it can be released into the extracellular milieu where it binds to $p75^{NTR}$. This binding leads to the activation of pro-apoptotic signalling pathways, through Jun kinase cascade signalling and subsequent activation of caspases -3,-6, and -9 (Nykjaer et al., 2005). However, this pathway can also modulate neuronal survival through activation of NFκB (nuclear factor kappa-light-chain-enhancer of activated B-cells) via the TNF receptor associated factor (TRAF) (reviewed by Chao, 2003) (Figure 3).

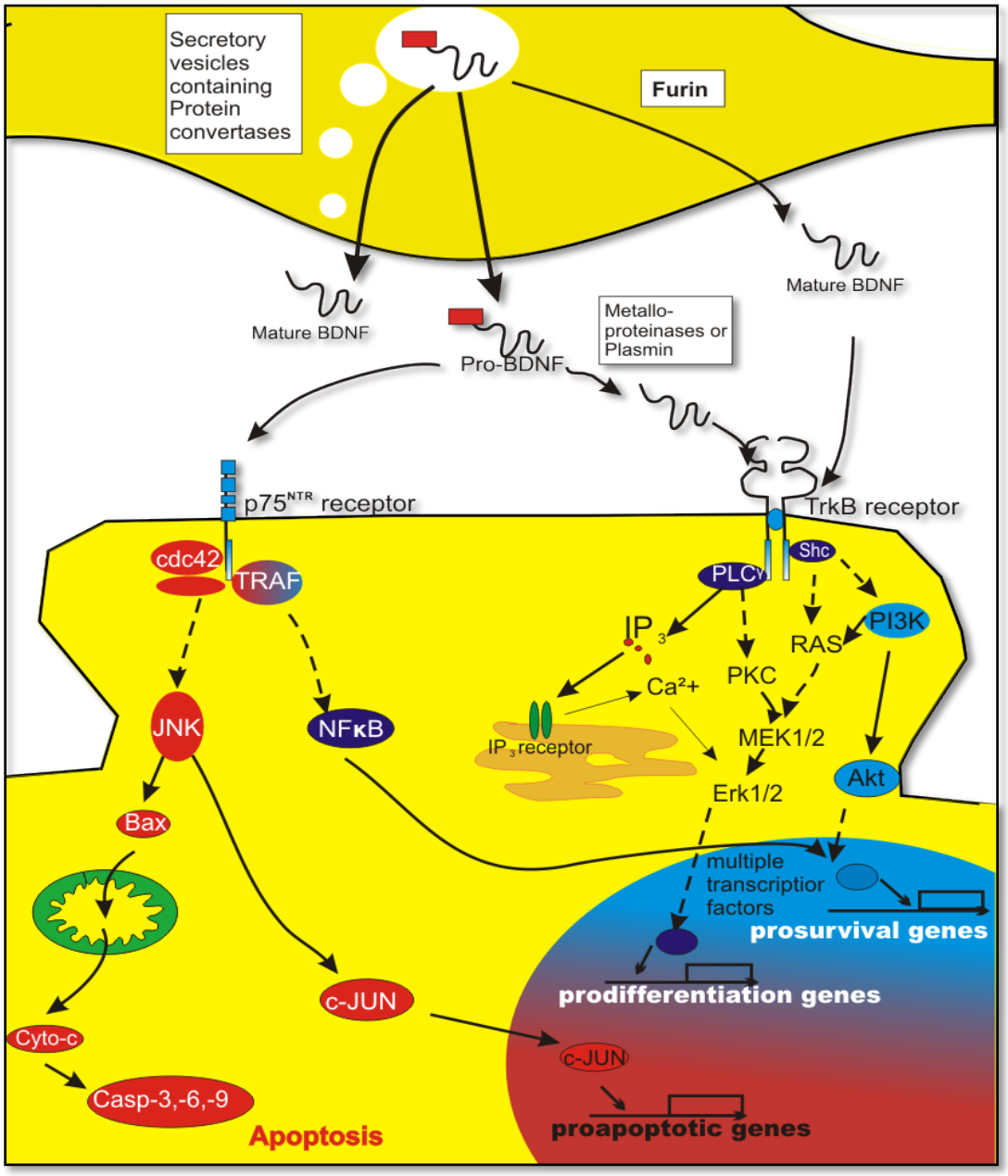

Figure 3. The processing of Pro-BDNF into BDNF and its involvement in signalling cascades leading to cell survival or apoptosis.

BDNF is synthesized as a precursor pro-BDNF protein and is subsequently cleaved into pro-BDNF. Pro-BDNF is either proteolytically cleaved inside the cell by furin or protein convertases and secreted as mature BDNF, or secreted as pro-BDNF and subsequently cleaved by extracellular proteases, such as metallo-proteases or plasmin into BDNF. Mature BDNF binds with high affinity to theTrkB receptor, activating PLC or RAS through binding of Shc, which results in Erk activation and an increased transcription of several pro-differentiation genes. On the other hand, TrkB activation can induce PI3K activity resulting in activation of the PKB/Akt pathway, which increases the transcription of pro-survival genes. Pro-BDNF, however, has a high affinity for p75 ^{NTR}, which subsequently results in the activation of the JNK signalling cascade, andof caspases (Casp) -3,-6, and -9 as well as the transcription of several pro-apoptotic genes. Activation of p75^{NTR} has also been shown to have pro-survival effects through the activation of NFκB (for details and abbreviations see text).

1.4.1.1.Expression Pattern of BDNF, NGF and NT-3

Neurotrophic factors play key roles during development particularly in the modulation of neuronal survival, morphology, and plasticity (Roback et al., 1992; Thoenen, 1991). In the CNS, NGF, BDNF and NT-3 mRNAs are highly enriched during postnatal cortical development, displaying distinct spatiotemporal patterns of expression (Das et al., 2001). Interestingly, the levels of expression of all three neurotrophins are highest in the adult hippocampus when compared to other regions, such as the neocortex and the cerebellum (Ernfors et al., 1990; Maisonpierre et al., 1990). Thus, in this brain region mRNA and protein levels of NGF, BDNF, NT-3 increase during the brain growth spurt in an age-depend manner reaching a plateau at PND21, PND14, and PND14 respectively (Das et al., 2001).

Importantly, the spatiotemporal expression of these neurotrophins (particularly BDNF) is tightly regulated throughout life and an imbalance in this regulation (caused by external factors such as alcohol) can have a considerable impact on neuronal development as well as neuronal integration and survival.

1.4.1.1. Regulation of BDNF Expression upon PPAE

The development of the brain is critically dependent on growth factors, in particular BDNF. As fetal exposure to alcohol induces profound neuronal disorganization and brain damage, it was hypothesized that alcohol interferes with the expression of growth factors through an unknown mechanism.

Table 2. Summary of the published literature on the effects of prenatal and early postnatal alcohol exposure on BDNF mRNA and protein levels in the hippocampus.

	Ethanol exposure	Species	Peak BAC (mg/dl)	BDNF-mRNA levels (Animal age)	BDNF-protein levels (Animal age)	Reference
Prenatal	Ethanol liquid diet (14 days before gestation and until birth)	mouse	Avg. 78 (GD 14)	↓ 60 % (PNDs 60-90)*	↔ (PNDs 60-90)	(Caldwell et al., 2008)
	Ethanol liquid diet (60 days before gestation until weaning)	mouse	133 (GD 15), 11 (PND 7)		↓ 60 % (PND 7) ↓ 60 % (PND 30)	(Fiore et al., 2009)
	Ethanol liquid diet during gestation	rat	161 (GD 18)		↔ (PND 1)	(Heaton et al., 2000)
	Intragastic intubation (GDs 5- 20)	rat	n.d. (exposed to 1g/kg)** n.d. (exposed to 3g/kg)**	↓30% (PNDs 7-8)	↔ (PNDs 7-8) ↓ 35 % (PNDs 7-8)	(Feng et al., 2005)
	Ethanol liquid diet (28 days before gestation until weaning)	rat	n.d. (exposed to 10% v/v)**	↑ 150 % (PND 60) ↔(PND 14, 30, 90)		(Barbier et al., 2008)
Postnatal	Vapor inhalation method (PNDs 4-10)	rat	307(PND 10)		↑ 40 % (PND 10)	(Heaton et al., 2000)
	vapor inhalation method (PNDs 10-15)	rat	336 (PNDs 10-15)	↑ 50 % (PND 15) ↑ 60 % (PND 20) ↔(PND 30) ↓ 30 % (PDN 60)		(Miki et al., 2008a)
	Intragastic intubation (PNDs 5-8)	rat	310 (PND 8)	↓ 50 % (PND 8)	↓ 30 % (PND 8)	(Tsuji et al., 2008)

*Only for BDNF splice variants containing Exon III, IV and VI. ** BAC levels were not determined, but the ethanol dose is stated in brackets. BAC, blood alcohol content; GD, gestational day; PND, postnatal day; n.d. not determined, ↔ no change; ↑, increase in mRNA or protein levels; ↓ decrease in mRNA or protein levels.

Particularly in the hippocampus, BDNF is thought to regulate cell proliferation and survival and to further act as a signalling molecule contributing to hippocampal-dependent learning (reviewed by Cunha et al., 2009; Lee and Son, 2009).

Thus, several research groups have investigated the impact of pre or early postnatal alcohol exposure on the expression of BDNF in this brain region. The reported effects on protein and mRNA levels of BDNF have been somewhat contradictory and it is possible

that BDNF expression can be differentially modulated depending on the animal model used, the BAC achieved, as well as the age of the offspring at the time of analysis (Table 2).

Additional factors, such as different levels of stress during critical developmental stages may have also contributed to the discrepancies among these studies. This is a confound that needs to be carefully considered as stress has been shown to impact BDNF expression (Burton et al., 2007; Koo et al., 2003; Zuena et al., 2008).

1.5. Exercise as a Therapeutic Strategy for FASD

It is well established that voluntary exercise has a positive impact on hippocampal structure and function and can particularly enhance learning and memory (Adlard et al., 2005; van Praag et al., 1999a). Rats that are given free access to a running wheel are incredibly active and can run up to 5 km/night (Farmer et al., 2004). In a 7-12 day period, this can result in generating up to three times more newborn cells in the SGZ than observed in sedentary controls (Farmer et al., 2004; Kronenberg et al., 2003; van Praag et al., 1999a). Other studies have also shown that voluntary exercise can increase blood flow to the DG (Pereira et al., 2007) and cause morphological changes in the hippocampal formation including: an increase in dendritic arborization and spine density (Eadie et al., 2005; Redila and Christie, 2006). In addition, exercise reduces oxidative stress and improves neuroendocrine autoregulation which has been shown to counteract damages from stress- and age-related neuronal degeneration (reviewed by Kiraly and Kiraly, 2005). Thus, there are a multitude of factors that contribute to the exercise induced changes in the structure and function of both new and existing neurons.

Although the exact molecular mechanisms underlying the running-induced up-regulation of hippocampal neurogenesis are still under debate, the levels of many trophic factors, including BDNF, were shown to be increased after physical exercise (Adlard and Cotman, 2004; Adlard et al., 2005; Farmer et al., 2004; Johnson and Mitchell, 2003; Neeper et al., 1995; Rasmussen et al., 2009). Importantly, since alcohol exposure during the third trimester equivalent has been shown to differentially affect the MAPK pathway, leading to a decreased activation of Erk and MAPK (Tsuji et al., 2008). Exercise on the other hand side activates the MAPK pathway through BDNF signalling (Ma, 2008), and might therefore be a beneficial therapeutic approach for FASD affected individuals. Thus, it is crucial to further investigate the beneficial effects of exercise in FASD models and to better understand the underlying regulatory mechanisms. Furthermore, increasing the understanding of the impact of ethanol on growth factor regulation might be relevant for the design of potential therapeutic interventions for these disorders.

1.6. Aims of This Study

Several different models have been used to study the effects onto adult hippocampal neurogensis and BDNF expression, but none of these looked into alcohol exposure throughout all three trimester equivalents. Alcohol exposure throughout all three trimester equivalents might identify different alterations than alcohol exposure during a specific period of fetal development. Our approach will be to generate and characterize a new model of moderate alcohol exposure in rats throughout all three trimester equivalents. This model will mimic a binge-like pattern of alcohol consumption through the entire period of human pregnancy. Furthermore, it will allow us to compare the

effects of ethanol exposure throughout gestation and early postnatal life with previous studies that have only analyzed either one of these periods alone.

We hypothesize that PPAE will alter hippocampal neurogenesis in adolescent and adult rats and will thus analyse cell proliferation, differentiation and survival in the adolescent and adult hippocampus of PPAE animals.

In addition we want to test, if alcohol consumption during all three trimester equivalents produces long lasting deficits in BDNF expression in the adult hippocampus and these these changes are in correlation to the neurogenesis results.

Lastly, we hypothesize that PPAE will impact the capacity of voluntary exercise to enhance adult hippocampal neurogensis and BDNF expression. Therefore we will immunohistochemically analyse animals after 12 days of voluntary wheel running and in addition perform biochemical analysis to measure BDNF levels in the different sub-regions of the hippocampus.

2. Methods

2.1. Subjects

80 female virgin Sprague-Dawley rats (Charles River, Quebec, Canada) and 8 proven male breeders were utilized for the study. All animal procedures were conducted in accordance with the Canadian Council on Animal Care and the University of Victoria Animal Care Committee (for details on the number of animals used for each experiment see Appendix B, Table B 1).

2.1.1. Acclimatization and Breeding

Upon arrival at the University of Victoria animal care facility, dams were given a 1 week acclimatization period. The colony room was maintained at a constant temperature of 21 °C with a 12h dark-light cycle. Dams were housed in pairs and males housed singly during acclimatization in clear polycarbonate cages (46 X 24 X 20 cm) with Carefresh contact bedding (Absorption Corp., Bellingham, WA, USA). Standard rat chow (no. 5, 012, Jamiesons Pet Food Distributors, Delta, BC, Canada) was used for feeding throughout the study. Following the acclimatization period, the ethanol-exposed and pair-fed animals were handled by experimenters for 5 days prior to the start of the experimental procedure. In the first day of training, animals were handled and wrapped in a towel in a restraint position. On the second day of training, animals were restrained and gavage needles were inserted through their esophagus and held in place for 30 seconds. Over the final 3 days of training, animals received an intragrastric intubation of increasing volumes of tap water varying from 1 ml to 5 ml in order to familiarize them to the gavage procedure.

Once the handling period was completed, each dam was paired with one male breeder in the evening. A plastic enrichment tube was included in the housing cage to create an environment conducive to breeding. A vaginal swab with 0.9 % NaCl solution was performed each morning. The swab was then visually examined on a microscope slide with an Olympus microscope with a 10x objective (Olympus CX21, Center Valley, PA, USA) for the presence of sperm (see Figure 4). The presence of sperm was taken to indicate pregnancy, and this time point was assigned as GD 1. A minimum of eight pregnant dams were assigned for each condition (ethanol-exposed, pair-fed, *ad libitum*) (Figure 5).

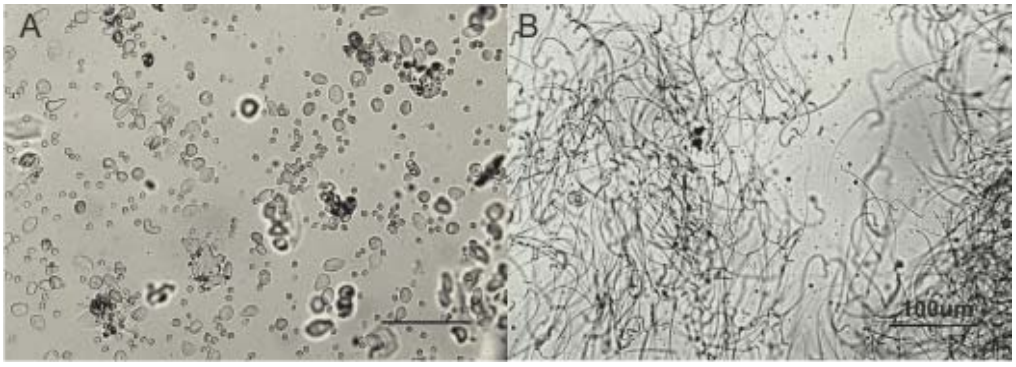

Figure 4. Images of vaginal swabs taken in the early morning for pregnancy detection.
(A) This swab was taken from a non-pregnant female in di-estrus phase of the oestrous cycle, which is characterized by a large number of leucocytes (very small, round cells) and a small number of non-nucleated epithelia cells (large, oval shaped cells). (B) This swab was taken from a pregnant female and shows a large number of sperm cells and only a few leucocytes.

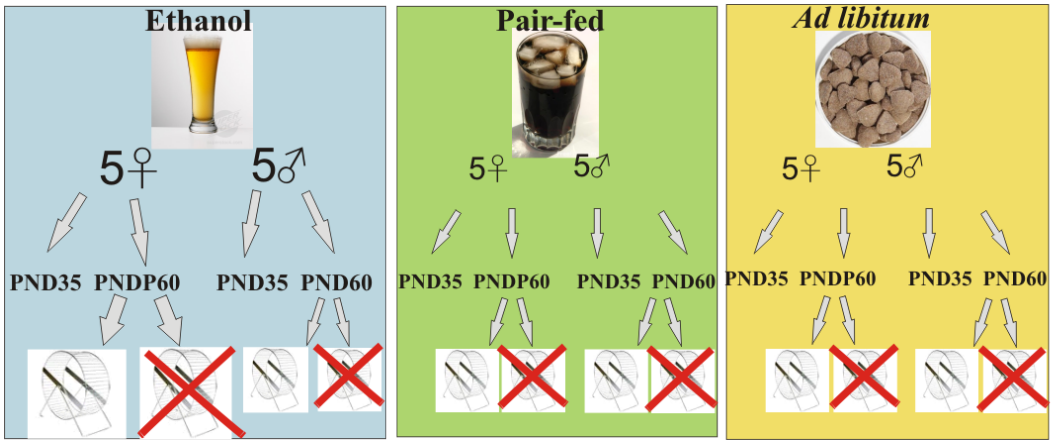

Figure 5. The pregnant dams were assigned to either *ad libitum*, pair-fed or ethanol treatment. Each litter was culled to five females and five males at postnatal day (PND) 2. At PND 23 litters were seperated and animals were further divided into two age groups: PND35 (adolescent stage) and PND 60 (adult stage).Of each treatment condition, one female and one male were assigned for PND35 and two females as well as two males were assigned for PND 60. Out of the PND 60 animals one female and one male were assigned to voluntary exercise (i.e. were housed in cages with free access to a running wheel). The remaining animals were kept under standard housing conditions.

2.1.2. Treatment

Intragastric intubation of pregnant dams (GDs 1-22): The first females to get pregnant were assigned to the ethanol condition to determine their daily food consumption during gestation. Rats that were deemed pregnant after the ethanol-exposed group was filled were assigned to the pair-fed or the *ad libitum* conditions.

All pregnant dams were singly housed. Ethanol-exposed, pair-fed and *ad libitum* dams were weighed every morning and their food consumption was recorded. *Ad libitum* and ethanol dams had free access to food and water. The food supply for the pair-fed group was restricted to the average amount of food consumed by ethanol dams for that particular day of gestation. For the pair-fed and ethanol-exposed dams, all food was removed from the cages 2 hours prior to the first gavage to ensure the stomach of the rats were sufficiently empty to accommodate the volume of solution intubated. All ethanol and pair-fed pregnant rats were intubated starting on GD 1. Pregnant ethanol-exposed dams received a dose of 4.3 g/kg of 36% ethanol (1.98 kcal/ml) from GD 1 to GD 22,

resulting in an applied volume of 15.27ml/kg body weight. Pair-fed dams received a dose of an iso-caloric and iso-volumic maltose-dextrin solution for the same period. Appropriate dose was calculated for each animal based on body weight measurements from that day. A 20 gage curved gavage feeding needle (7.62 cm, 2.25 mm ball; Popper & Sons, New Hyde Park, NY) was attached to 3 or 5 ml syringes for the delivery of the solution. The solution was administered in two separate intubations spaced 15-20 minutes apart in order to accommodate for volume reabsorption.

Intragastric intubation of pups (PDs 4-10): The day of birth (usually GD 23) was assigned as PND 1. Litters averaged fifteen pups per dam and were culled to ten pups (five males and five females, whenever possible) on PND 2. The litters were unhandled until PND 4 when the gavage procedure commenced. On PND 4, pups were permanently

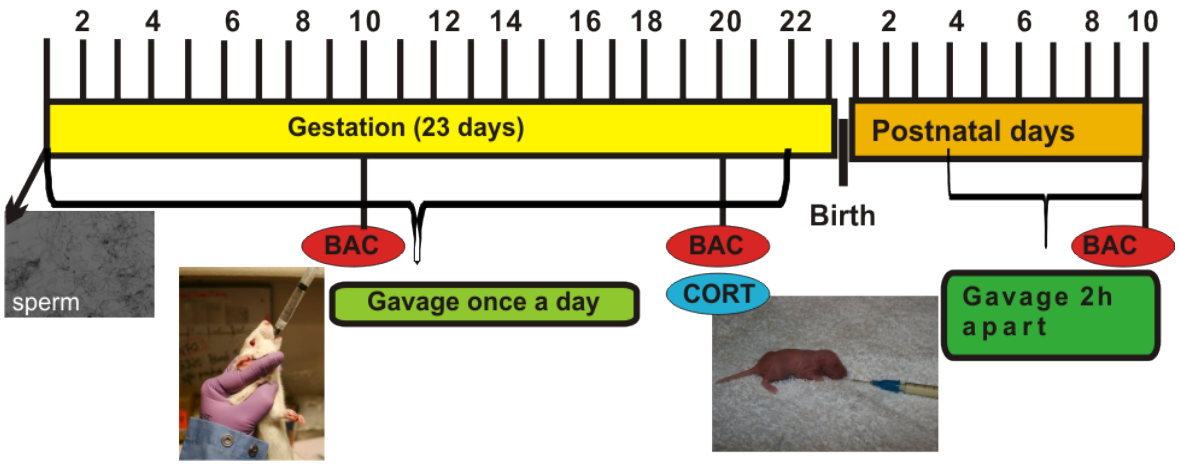

Figure 6. Time line of treatment paradigm for ethanol-exposed and pair-fed dams and litters. The detection of sperm determined GD 1. At this time females were assigned to their treatment condition. Ethanol and pair-fed damns were gavaged daily from GDs 1-22. Litters were left untouched for the first 3 days and were subsequently gavaged from PNDs4-10. Blood samples were taken 3 hours after the procedure at GD 10 and GD 20 from all pregnant dams as well as at PND 10 from all litters. CORT blood samples were taken 30 min after the procedure at GD 20. BAC, blood alcohol content; CORT, corticosterone.

paw-marked with India ink for identification purposes (Geller and Geller, 1966). India ink was s.c. injected using a Spaulding special electric tattoo marker (Spaulding & Rogers, Voorheesville, NY, USA). Each morning (from PNDs 4-10) all ethanol-exposed and pair-fed pups (males/females separately) were weighed and an average pup weight was determined. Pups that were significantly smaller than the others were weighed individually and were euthanized if they weigh less than half the mass of all others that litter. The ethanol dose of 4g/kg of 12% ethanol was calculated from the average pup weight, resulted in an applied ethanol milk solution of 0.02085ml/g pup weight. The ethanol or maltose-dextrin was dissolved in a nutritional milk solution, similar in composition to rat milk (West et al., 1984), and supplemented with a specially formulated vitamin mix (Bio-Serv; Frenchtown, NJ, USA) (see Appendix A for detailed information). The solution was administered in separate intubations 2 hours apart using a modified rat tail vein catheter attached to a 1 ml syringe as depicted in Figure 6. The catheter was lubricated in corn oil to aid in the swallowing of the tubing. An additional feeding of pure milk was supplied to ethanol-exposed pups in the evening. This measure was deemed necessary due to inadequate nutrition, low birth weight, and high mortality rate of ethanol-exposed pups. The pair-fed pups were mock-intubated during the third feeding; tubing was inserted for 30 seconds with no solution being injected. Previous research has indicated that the extra feeding of the pair-fed pups can create excess growth in these animals. The sham-intubation was therefore deemed more suitable (Gil-Mohapel et al., 2010; Kelly and Lawrence, 2008).

2.1.3. BAC and CORT Assay

To assess BAC, blood tail samples were taken from the dams at GD 10 and GD 20, 3 hours following intubation (determined in a pilot study as the peak of BAC, data not shown). 500 µl of blood were taken via tail nick sampling. Pup blood tail samples were taken on PD 10, 2 hours following the last ethanol intubation. The tail was nicked and 20 µl of blood were collected in heparinized capillary tubes. Samples were stored at 4 °C over night and then centrifuged for 30 minutes at 3000 *g*. Serum was extracted from the samples and stored at -20 °C until processing. Alcohol levels were assessed using an ANALOX machine (Analox Instruments, Lunenburg, MA, USA).

Blood samples for analysis of corticosterone (CORT) levels were taken on GD 20 and processed like described above. CORT analysis was performed according to the manufacturer instructions with the enzyme immunoassay kit (900-097, Assay Designs, Ann Habor, MI, USA). Briefly, the provided donkey anti-sheep IgG-coated 96 well plate was loaded with a CORT standard (in the range of 0-20,000 pg/ml) as well as serum samples (diluted in assay buffer containing a steroid displacement reagent and run in duplicates). An alkaline phosphatase conjugated to CORT as well as the polyclonal antibody against CORT was added to the wells and the plate was shaken for 2 hours at room temperature (RT). The CORT present in the samples and standards competes with the conjugate over the binding sites of the antibody. Following 3 washes, wells were aspirated and p-nitrophenyl phosphate substrate solution was added and incubated for 1 hour at RT to start the reaction of the alkaline phosphatase. The reaction was stopped by adding stop solution containing trisodium phosphate and CORT levels were determined at 405nm with a VersaMax microplate reader (Molecular Devieces, Sunnyvale, CA,

USA) and analysed with the SoftMax Pro 5.2 software (Molecular Devieces). CORT levels were calculated from the standard curve prepared for each plate and were expressed as ng/ml serum.

2.1.4. Voluntary Exercise

Animals were weaned and assigned to the different experimental conditions at PND 23. From PND 23 until sacrificed animals were group-housed (2-3) according to their sex and condition. Animals assigned for voluntary exercise, had free access to a running wheel from PNDs 48-60 (for details see Figure 7). The running wheels were connected to a computer and the running distance was constantly recorded (in intervals of 1 min) using the Vitalview software (Mini Mitter, Bend, OR, USA). The total distance run during the 12 days period was calculated and expressed in kilometres (km).

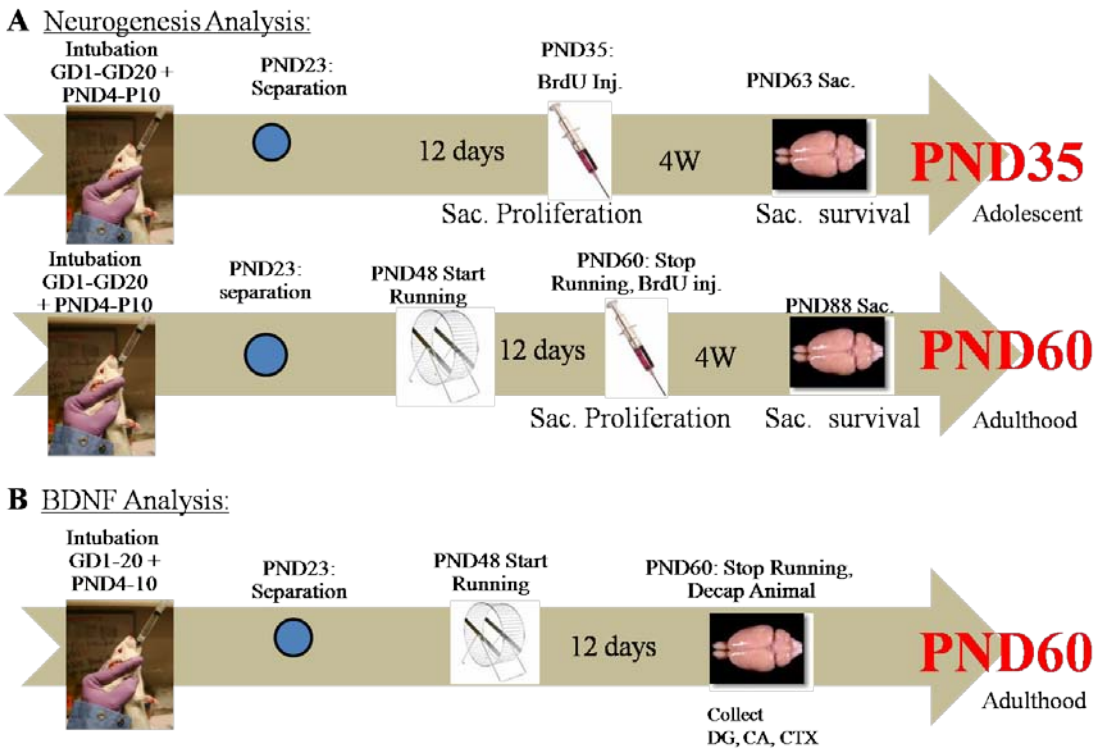

Figure 7. Time line of experimental design for neurogenesis (A) and BDNF (B) experiments
All pups were separated according to their experimental condition at PND 23. (A) Animals assigned for the analysis of neurogenesis at PND35 received an i.p. injection of BrdU at PND35 and were either sacrificed 2 hours later (for analysis of cell proliferation) or 4 weeks later (for analysis of cell survival). Animals assigned for analysis of neurogenesis at PND 60 had either access to a running wheel from PNDs 48-60 or where kept under standard housing conditions. This set of animals received an i.p. injection of BrdU at PND60 and were either sacrificed 2 hours later (for analysis of cell proliferation) or 4 weeks later (for analysis of cell survival). (B) Animals assigned for BDNF analysis where handled in the same way as animals assigned for neurogenesis experiment. At PND 60 animals were quickly decapitated and fresh tissue samples from the detage gyrus (DG), cornu amonis (CA) and cortex (CTX) were collected.

2.2. Histological Analysis

2.2.1. BrdU Administration

To label cells undergoing cell division, we used BrdU immunohistochemistry (Miller and Nowakowski, 1988). This technique is based on the administration of BrdU, a thymidine analogue which incorporates into the DNA of cells during the S-phase of the cell cycle. This exogenous marker can be subsequent detected with immunohistochemistry using an antibody against BrdU. It should be noted that a single injection of BrdU labels the DNA in the nuclei of cells that are in the S-phase, but not the proliferating cells that are in other phases of the cell cycle (Cameron and McKay, 2001). BrdU (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9 % NaCl (10 mg/ml) and delivered via i.p. injections to rats in a saturating dose (200 mg/kg). The animals assigned for neuronal proliferation were given a single i.p. injection of BrdU in the early morning (~9:00 am) and were sacrificed 2 hour later. For neuronal survival studies, rats were sacrificed 4 weeks following the BrdU injection.

2.2.2. Tissue Preparation

Animals were deeply anesthetised with urethane (250 mg/ml in water, i.p. injection 1.5 g/kg of body weight) and transcardially perfused with 0.9 % NaCl followed by 4 % paraformaldehyde (PFA) to fix the brains prior to removal. The brains were removed and left in 4 % PFA overnight at 4 ºC and then transferred to 30 % sucrose. Saturation on sucrose alters the water content of the cells and prevents freezing artefacts during tissue sectioning. Following saturation in sucrose, serial coronal sections were obtained on a freezing microtome (Model 860, American Optical Corporation, Buffalo, NY, USA) at

30 µm thickness. Sections were collected into a 1/6 section sampling fraction and stored in Walters anti-freeze cryoprotectant solution (0.04 M Tris-buffered saline (TBS), 30 % ethylene glycerol, 30 % glycerol) at 4 ºC.

2.2.3. Immunohistochemistry

The information on primary and secondary antibodies used in this study is summarized in Tables 3 and 4. For immunohistochemical analysis brain slices were transferred from the anti-freeze solution into mesh net inserts and rinsed 10 minutes for three times in 0.1M TBS buffer (84 mM Tris-HCl, 16 mM Tris, 0.9 % NaCl, pH = 7.4) at RT with gentle agitation on an orbital shaker (Belly Dancer; Stovall, Greensboro NC, USA).

Every sixth 30 µm brain section was used for quantitative detection of cells that incorporated BrdU. Free-floating sections were placed in a 2 N HCl solution for 30 minutes at 65°C in order to break the hydrogen bonds and denature the DNA double-helix. After rinsing three times in 0.1 M TBS for 10 minutes at RT, sections were incubated in blocking solution (0.1 M TBS with 5 % normal horse serum and 0.25 % Triton X-100) for 1 hour at RT. After blocking, slices were incubated in primary antibody in 5 % blocking solution for 48 hours at 4 °C. After three rinses in TBS/Triton X-100 wash buffer (0.1 M TBS with 0.25 % Triton X-100), sections were incubated with secondary antibody in 5% blocking solution at RT for 2 hours. After rinsing, brain sections were incubated with the avidin-biotin-peroxidase complex (Vectastain ABC Elite Kit PK4000, Vector Laboratories, Burlingame, CA, USA) for 1 hour and washed three times with 0.01 M TBS. As substrate for the peroxidase reaction, 2,2-

diaminobenzedine (DAB; DAB Kit SK 4100, Vector Laboratories) was applied for 10-15 minutes at a concentration of 0.25 mg/ml in TBS with 0.01 % hydrogen peroxide. Sections were thoroughly rinsed in TBS, mounted onto 2 % gelatine-coated microscope slides, and dehydrated through a series of ethanol solutions of increasing concentrations (50, 70, and 90 %) followed by a 5 minutes incubation with a xylene substitute (CitriSolv, Fisher Scientific, Pittsburgh, PA, USA). The slides were then coverslipped with Permount medium (Fisher Scientific) using 24 x 60 mm cover glass (thickness #1, Fisher Scientific).

Table 3. Summary of all primary antibodies used in this study.

Primary Antibody	**Immunogen**	**Host**	**Dilution**	**Marker for:**	**Source**
BrdU monoclonal	Purified BrdU	Mouse	1:60	proliferation and survival	M0744, Dako, Glostrup, Denmark
Ki67 polyclonal	recombinant fusion protein corresponding to a cDNA fragment containing the Ki67 motif	Rabbit	1:500	proliferation	VP-K451, Vector Laboratories, Burlingame, CA, USA
NeuroD polyclonal	peptide mapping the N-terminus of NeuroD	Goat	1:200	differentiation	SC-1084, Santa Cruz Biotechnology, Santa Cruz, CA, USA

Table 4. Summary of all secondary antibodies used in this study.

All antibodies were purchased from Vector Laboratories, Burlingame,CA, USA.

Secondary Antibody	**Dilution**	**Ref. №**
biotin-conjugated horse anti-mouse IgG	1:200	BA-2001
biotin-conjugated goat anti-rabbit IgG	1:200	BA-1000
biotin-conjugated horse anti-goat IgG	1:200	BA-9500

For evaluation of cell proliferation, immunohistochemistry against the endogenous Ki67 protein (also known as MK167) was also performed. Ki67 is a mitotic marker that is expressed during all active phases of the cell cycle (G1, S, G2 and M) (Scholzen and Gerdes, 2000). A separate series of brain sections were incubated twice in 10 mM citric acid (dissolved in 0.1 M TBS) for 5 minutes at 95 °C in order to completely unmask the antigens. After three washes in 0.1M TBS, the sections were transferred into a 3 % hydrogen peroxide quenching solution (0.1M TBS with 3 % hydrogen peroxide and 10 % methanol) for 10 minutes at RT. Tissue was then blocked with blocking solution (0.1 M TBS with 5 % normal goat serum and 0.25 % Triton X-100), incubated with the primary antibody for 48 hours at 4 °C and finally detected with a secondary antibody as described above.

For evaluation of early neuronal differentiation, NeuroD immunodetection was performed. NeuroD is a helix-loop-helix transcription factor that is expressed during the early phases of neuronal differentiation (Brunet and Ghysen, 1999; Miyata et al., 1999). A separate series of brain sections was incubated in 3 % hydrogen peroxide quenching solution for 15 minutes at RT followed by three washes with 0.1 M TBS. Tissue was then blocked with blocking solution (0.1 M TBS with 5 % normal horse serum and 0.25 % Triton X-100), incubated with the primary antibody for 48 hours at 4°C and finally detected with the secondary antibody as described above.

2.2.4. Cell Quantification

Cell quantification was performed in coded slides (to ensure the experimenter was blinded to the group identity) using conventional light microscopy with an Olympus BX51 microscope equipped with 10x, 40x, and 100x objectives. Image Pro-Plus software

(version 5.0 for Windows^{TM}, Media Cybernetic, Inc., Silver Spring, MD, USA) and a Cool Snap HQ camera (Photometrics, Tucson, AZ, USA) were used to capture images for analysis. The area of each individual DG section was determined by tracing the GCL (for details see Figure 8) using the Image Pro Plus measurement tools. Volumes were calculated by multiplying the area by the thickness of the slice (30 μm). The number of BrdU-, Ki67-, and NeuroD-immunopositive cells present in the SGZ of the DG was quantified by manually counting all DAB-positive cells present within 30 microns of the GCL in 1/6 brain sections that contained identifiable portions of the DG formation (i.e. from 2.56 posterior to Bregma to 6.04 anterior to Bregma (Paxinos and Watson, 1982)). The cell density in SGZ for each animal was calculated from the number of cells per 30 μm DG cross-section and the volume of the GCL (mm^3), in order to control for changes in hippocampal size to the alcohol exposure.

Images were processed with Adobe Photoshop 4.0 (Adobe Systems Mountain View, CA, USA). Only contrast enhancements and colour level adjustments were made; otherwise images were not digitally manipulated.

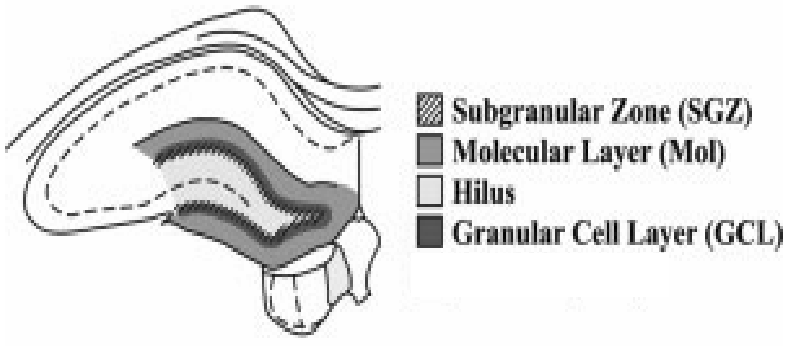

Figure 8. Localisation of the SGZ and the GCL within the DG.
Adult Neurogenesis occurs in the SGZ. The number of BrdU+ cells present in this region was quantified and the entire GCL was used as a reference volume. The dense cell bodies that form the GCL make it clearly visible under the microscope. (Adapted from Mandyam et al., 2007)

2.3. Biochemical BDNF Analyses

2.3.1. Sample Preparation

Animals assigned to BDNF analysis were anesthetized at PND 60 with isoflurane (Abbott Laborities, North Chicago, IL, USA) and quickly decapitated. The brains were removed on ice; the hippocampi and the cortex from both hemispheres were dissected. The hippocampi were further dissected into their DG and CA sub-regions. Tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C. Samples were sonicated four times for 5 seconds in 5 ml of lysis buffer per g of tissue. The composition of this buffer was as follows: 20 mM Tris (pH = 8), 137 mM NaCl, 1% NP-40, 10% glycerol, 2 mM ethylene glycol bis (2-aminoethyl ether)- N,N,N′N′- tetraacetic acid (EGTA), and 1x Halt ^{TM} Proteinase Inhibitor Cocktail (Thermoscience, Rockford, IL, USA). Lysates were then centrifuged at 2000 *g* for 15 minutes at 4 °C and supernatant was used for sample preparation. The total protein content of the samples was determined by the bicinchoninic acid (BCA) method using a BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA).

2.3.2. ELISA Assay

BDNF protein levels were measured by enzyme-linked immunosorbent assay (ELISA) using a BDNF Emax Immuno Assay System kit (Promega, Madison, WI, USA) according to the protocol provided by the manufacturer. Briefly, 96-well microplates were treated with a monoclonal anti-BDNF antibody in carbonate buffer (pH = 9.7) and incubated overnight at 4 ºC. The plates were washed with PBST (8mM sodium phosphate diabesic heptahydrate, 32mM sodium phosphate monobasic monohydrate, 240mM NaCl,

0.05% Tween-20), and blocked for 1 hour at RT. A standard curve was prepared with purified BDNF. Tissue samples were diluted in blocking buffer (Promega) and subsequently added to the remaining wells (100 μl/well). The plates were incubated with gentle agitation for 2 hours at RT and washed five times with PBST. A polyclonal anti-human BDNF was then added to the wells and incubated for 2 hours at RT. After five washes with PBST, a horseradish peroxidase (HRP)-conjugated anti-IgY antibody was added to the wells and the plates were incubated for 1 hour at RT. After five more washes with PBST, a peroxidasesubstrate was added for 15 minutes. The reaction was stopped by adding 1 N HCl, and absorbance read in a microplate reader at 450 nm (VersaMax, Molecular Devieces). All samples were analyzed in duplicates with the SoftMax Pro Software (Molecular Devieces). BDNF levels were calculated from the standard curve prepared for each plate and were expressed as pg/mg protein.

2.3.3. Western-Blotting

For detection of BDNF by western-blotting, 15 μg of protein were separated on 17 % sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Perklin Elmer, Boston, MA, USA) at 40 V in transfer buffer (25 mM Tris, 192 mM glycine, 20 % (v/v) methanol) overnight at 4ºC. Membranes were blocked for 1 hour at RT with 5 % (w/v) Difco^{TM} – skim milk powder (Beckon, Dickson and Company, Sparks, MD, USA) and then incubated with the primary antibody polyclonal rabbit anti-BDNF N-20 (1:1000; sc-546, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% bovine serum albumine (BSA) in PBST at 4 °C overnight. Following three 5 minutes washes with PBST, membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:5000; Millipore, Temecula, CA, USA) for 1 hour at

RT. Bands were visualized by enhanced chemiluminescence (ECL Plus, GE Healtcare, Buckinghamshire,UK) and exposed to X-OMAT^{TM}-film (Kodak, Rochester, NY, USA). Pro- and mature BDNF forms were analyzed on the same blots. Blots were incubated for 30 minutes at 50ºC in stripping buffer (62.5 mM Tris/HCl (pH = 6.7), 2 % SDS, 100mM β-mercaptoethanol), washed three times for 5 minutes in PBST, blocked for 1 hour at RT and re-probed with mouse monoclonal anti β-actin (1:20,000; AC-15, Sigma-Aldrich) in 5% BSA overnight at 4ºC. Blots were then washed three times for 5 minutes in PBST and incubated with HRP-conjugated goat anti-mouse IgG (1:10,000; KPL, Gaitherburg, MD, USA) in PBST for 1h at RT prior to developing with ECL. Densitometric scanning of the films was performed under linear exposure conditions with the Quantity One software (BioRad Laboratories, Hercules, CA) and a Gel Doc XR camera (BioRad Laboratories).

2.4. Statistical Analysis

Statistical analysis was performed using the Statistica 7.1 analytical software (StatSoft Inc., Tulsa, OK, USA). The body weight of the pregnant dams and pups as well as the dams' food consumption data were analyzed with repeated measures analysis of variance (ANOVA). The BAC, CORT, BDNF levels and cell proliferation/neurogenesis data were analyzed with a factorial ANOVA. Post-hoc analyses were conducted using Fishers least significant difference. Data are presented in figures as means ± standard error of the mean (SEM). A p value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Characterization of the Rat Intragastric Intubation Model

3.1.1. Effect of Ethanol Administration on Body Weight and Food Consumption

Food consumption was recorded on a daily basis, in order to control for a potential ethanol-induced reduction in caloric intake. The pair-fed group had their daily food allotments matched to the amount of food consumed by the ethanol-exposed group in each particular day of gestation; as expected, repeated measures ANOVA detected an effect of *ethanol treatment* [F (2, 50) = 142.325, $p < 0.001$; with *ad libitum* > pair-fed $p < 0.001$; *ad libitum* > ethanol-exposed, $p > 0.001$] (Figure 9 A).

The same effect was observed when weight gain throughout gestation was analysed [repeated measures ANOVA, F (2, 67) = 30.754, $p < 0.001$; with *ad libitum* > pair-fed, $p < 0.001$; *ad libitum* > ethanol-exposed, $p < 0.001$]. This result indicated that the pair-fed group served as an effective control for any weight-loss related to a potential ethanol-induced reduction in caloric intake (Figure 9 B).

Following birth, pup weights were recorded until PND 22. No effect of *ethanol treatment* [repeated measures ANOVA, F (2, 38) = 0.401, $p = 0.673$] or *sex* [repeated measures ANOVA, F (2, 38) = 3.299, $p = 0.077$], on offspring body weight was observed suggesting that the additional milk intubation administered to the ethanol-exposed pups was sufficient to counteract any malnourishment that may have resulted from ethanol exposure (Figure 9 C).

3.1.2. Intoxication and Stress Levels of Alcohol-Exposed Animals

Peak BAC levels measured 3 hours after ethanol exposure at the last day of each trimester equivalent are presented in Figure 9 D. The mean BAC levels in mg/dl were 155 ± 8 at GD 10, 195 ± 11 at GD 20 and 228 ± 9 at PND 10. The highest BAC was reached at PND 10 which is not surprising, since at this early age the metabolic pathways of ethanol breakdown (which involve the enzyme alcohol dehydrogenase) are not fully developed (Raiha et al., 1967).

CORT levels were used as an indicator of the stress (Odio and Brodish, 1990; Reul and de Kloet, 1985; Titterness and Christie, 2008), experienced by the animals as a consequence of the daily handling and gavage procedure. Significantly elevated CORT levels were detected 30 minutes after the intragastric intubation procedure in both ethanol-exposed and pair-fed dams at GD 20 [one-way ANOVA, $F(2, 58) = 25.250$, $p < 0.001$ with significant differences between all three treatment conditions: ethanol > pair-fed, $p = 0.005$; ethanol and pair-fed > *ad libitum*, $p < 0.001$] (Figure 9 E). CORT levels were further elevated in ethanol-exposed animals since ethanol acts as a maternal stressor, causing activation of the hypothalamus-pituitary adrenal (HPA) axis and leading to a further elevation of CORT response (Rivier et al., 1984; Weinberg and Gallo, 1982).

3.2. Effects of Sex and Ethanol Treatment on Running Distances

In order to verify that animals that were assigned to the voluntary physical exercise condition were running an appreciable distance, the total distance run/cage (i.e., two animals) was recorded. It is however important to emphasize that the two animals

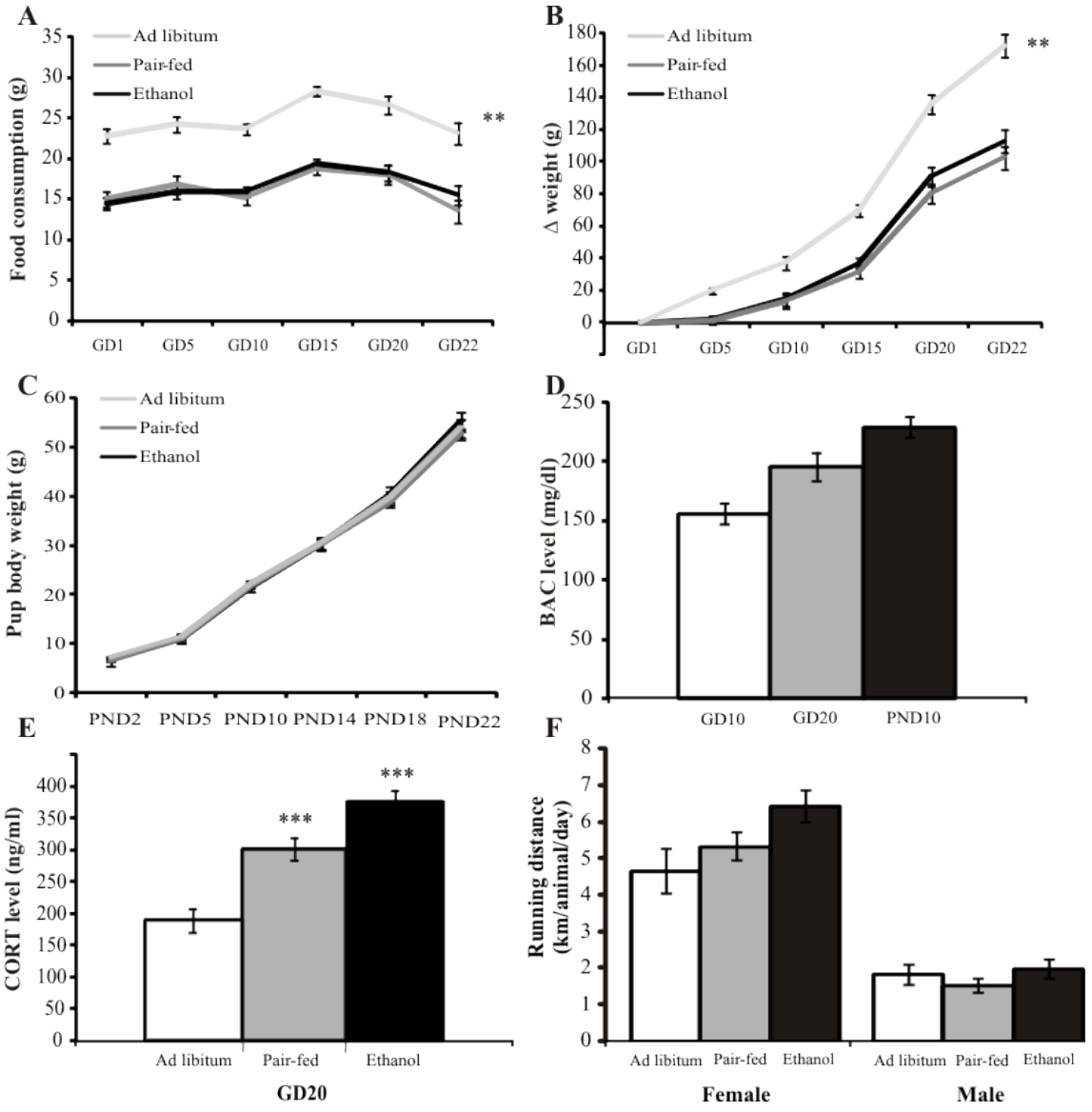

Figure 9. Comparison of body weight, food consumption, intoxication, CORT levels, and running distance of ethanol-exposed dams and litters and their pair-fed and *ad libitum* controls.
(A) The food consumption of the pair-fed dams was restricted to the amount of food consumed by the ethanol group on that particular day of gestation. Thus, a significant difference in food consumption was only detected between the ethanol/pair-fed groups and *ad libitum* dams but not between the ethanol and pair-fed animals. (B) Due to the reduction in food consumption, ethanol and pair-fed dams gained significantly less weight throughout gestation. (C) The weight gain of the offspring, however, was not affected by the pre- and postnatal gavage. (D) Blood alcohol content (BAC) of the ethanol exposed animals determined at the end of each trimester equivalent. The highest level of intoxication was detected at PND 10. (E) Corticosterone (CORT) levels of ethanol and pair-fed animals were significantly elevated in comparison to *ad libitum* controls. (F) Differences in running distance of female and male animals in each treatment condition. Data are represented as means ± SEM. (** indicates $p < 0.01$, *** indicates $p < 0.005$).

assigned to an exercise cage might have contributed unequally to the total running distance, and therefore the values presented (running distance/animal/day) are an approximation.

Statistical analysis of the running distance per animal/day revealed significant effects of *ethanol treatment* [two-way ANOVA, F (2, 59) = 5.506, p = 0.047 with ethanol-exposed > pair-fed , p = 0.011; ethanol > *ad libitum*, p = 0.023] and *sex* [two-way ANOVA, F (1, 59) = 128.485, $p < 0.001$, with female > male, $p < 0.001$], but no significant interaction between *ethanol treatment* and *sex* [two-way ANOVA, F (2, 59) = 2.094, p = 0.132] (Figure 9 F). Females ran three times more than males in all conditions, with an range of average running distance between 4.5-6.4 km per day for females and 1.5-2.0 km per day for males. On the other hand, the effect of treatment is relatively minor, as ethanol-exposed animals run on average 4.3 km/day whereas pair-fed and *ad libitum* rats run 3.3 and 3.4 km/day respectively.

3.3. Effect of PPAE on Adult Hippocampal Neurogenesis

In order to ascertain whether PPAE affects cell proliferation and ultimately adult neurogenesis in the DG of the hippocampus, two different time points were analysed, an adolescent stage (PND 35) and an early adult stage (PND 60). All animals received one injection of BrdU and were perfused either 2 hours (for proliferation analysis) or 4 weeks (for cell survival analysis) after the BrdU injection. In addition, the endogenous proliferation marker Ki67 was used to label cells in all phases of the cell cycle whereas the immature neuronal marker NeuroD was employed to evaluate early neuronal differentiation.

3.3.1. Effect of PPAE on Cell Proliferation in the DG

At the adolescent stage (PND 35), BrdU immunohistochemistry revealed no significant main effect of *ethanol treatment* [two-way ANOVA, F (2, 39) = 0.987, p = 0.523) nor a significant interaction between *ethanol treatmen*t and *sex* [ANOVA, F (2, 39) = 1.842, p = 0.172] on the density of BrdU+ cells in the SGZ of DG (Figure 10 A-C). These results indicate that cell proliferation 2 hours after injection of BrdU is not altered by PPAE in adolescent animals.

Immunohistochemistry for the endogenous cell cycle marker Ki67 confirmed the BrdU results and revealed no significant main effect of *ethanol treatment* [two-way ANOVA, F (2, 38) = 0.265; p = 0.768] nor a significant interaction between *ethanol treatment* and *sex* [two-way ANOVA, F (2, 38) = 0.478, p = 0.623] on the density of Ki67+ cells in the SGZ of the DG (Figure 10 D-F). Collectively, the Ki67 and BrdU results suggest that PPAE does not affect cell proliferation in the DG rats at PND 35.

Similarly, when cell proliferation was examined at an early adult stage (PND 60), no significant main effect of *ethanol treatment* on the density of BrdU+ cells in the DG was observed [two-way ANOVA, F (2, 39) = 2.022, p = 0.146]. Further, there was no significant interaction between *ethanol treatment* and *sex* [two-way ANOVA, F (2, 39) = 0.782, p = 0.465] (Figure 11 A-C).

However, unlike the results obtained with BrdU, immunohistochemistry for the endogenous marker Ki67 revealed a significant main effect of *ethanol treatment* [two-way ANOVA, F (2, 47) = 3.214, p = 0.049, with ethanol > pair-fed, p = 0.029; and ethanol > *ad libitum*, p = 0.022] but no significant interaction between *sex* and *ethanol treatment* [two-way ANOVA, F (2, 47) = 1.399, p = 0.256] was observed (Figure 11 D-

H). In conformity with these results, it is worth mentioning that a significant ethanol-induced increase in the number of Ki67+ cells was also observed at PND 90 (i.e., at a later stage of adulthood) [two-way ANOVA, F (2, 39) = 6.735, $p = 0.003$; with ethanol > pair-fed, $p = 0.001$ and ethanol > *ad libitum*, $p = 0.006$] (data not shown).

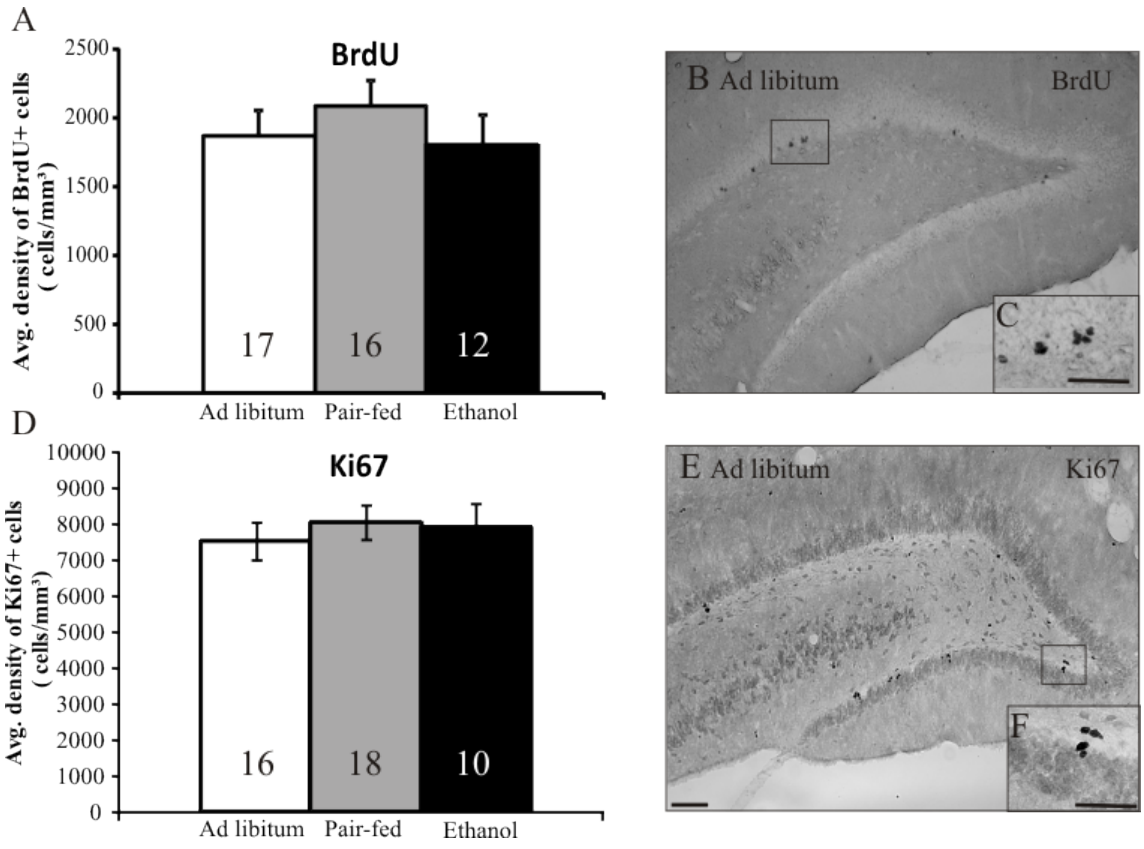

Figure 10. PPAE does not alter DG cell proliferation at PND 35 (adolescence).
(A) Cell proliferation as assessed by BrdU immunohistochemistry (2 hours after BrdU injection) was not significantly altered in ethanol-exposed animals when compared to both pair-fed and *ad libitum* controls. (B, C) Representative micrographs of DG sections processed for BrdU immunohistochemistry taken with a 10x (B) and 40x (C) objectives respectively. (D) Cell proliferation as assessed with the endogenous cell cycle marker Ki67 confirmed that ethanol did not affect DG cell proliferation at PND 35. (E, F) Representative micrographs of DG sections processed for Ki67 immunohistochemistry taken with a 10x (E) and 40x (F) objective respectively. The number of animals per treatment group is indicated inside the respective bar. Data are presented as means ± SEM. Scale bar = 100 μm.

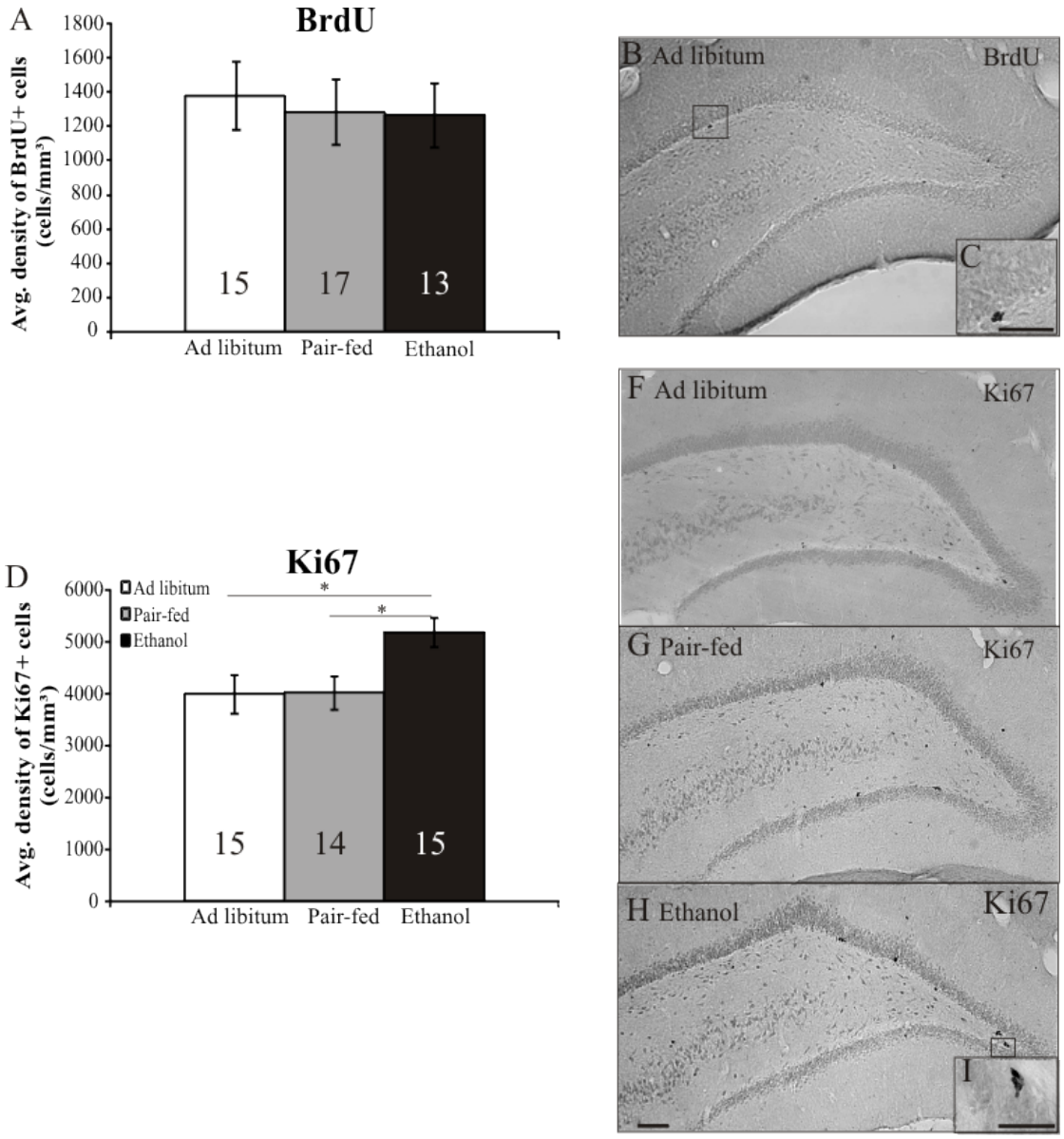

Figure 11. PPAE increases the density of Ki67-positive cellsbut not BrdU+ cells at PND 60 (adulthood).

(A) Cell Proliferation as assessed by BrdU immunohistochemistry (2 hours after BrdU injection) was not significantly altered in ethanol-exposed animals when compared to both pair-fed and *ad libitum* controls. (B, C) Representative micrographs of DG sections processed for BrdU immunohistochemistry taken with a 10x (B) and 40 x (C) objectives respectively. (D) However, cell proliferation as assessed with the endogenous cell cycle marker Ki67 was significantly increased in ethanol-exposed animals (E-I). Representative micrographs of DG sections processed for Ki67 immunohistochemistry from an *ad libitum* control (F), a pair-fed (G) and an ethanol-exposed (H, I) animal. Micrographs were obtained with either a 10x (F-H) or a 40x (I) objective. The number of animals per treatment group is indicated inside the respective bar. Data are presented as means ± SEM. * indicates $p < 0.05$; ** indicates $p < 0.01$. Scale bar = 100 μm.

3.3.2. Effect of PPAE on Neuronal Differentiation in the DG

Analysis of the immature neuronal marker NeuroD at the adolescent stage (PND 35) revealed a significant main effect of *ethanol treatment* [two-way ANOVA, F (2, 36) = 5.856; p= 0.006, with ethanol > pair-fed, p=0.006; ethanol > *ad libitum*, p= 0.003] (Figure 12).

A similar effect was observed at the adult stage (PND 60), since *ethanol treatment* significantly altered the density of NeuroD+ cells in DG of ethanol-exposed animals [two-way ANOVA, F (2, 33) = 4.440; p = 0.019 with ethanol > pair-fed, p = 0.007, trend towards ethanol > *ad libitum*, p = 0.061] (Figure 12). Collectively, these results indicate that PPAE alters early neuronal differentiation both in adolescent and adult rats.

3.3.3. Effect of PPAE on Cell Survival in the DG

Exposure to ethanol *in utero* and throughout the early postnatal brain growth spurt did not alter cell survival as analysed by BrdU immunohistochemistry 4 weeks post injection. Statistical analysis revealed no significant main effect of *treatment* [two-way ANOVA, F (2, 31) = 1.641, p = 0.210], and no significant interaction between *treatment* and *sex* [two-way ANOVA, F (2, 31) = 2.029, p = 0.148] on the density of BrdU+ cells in PND 35 animals that were sacrificed 4 weeks later (i.e. at PND 63) (Figure 13 A).

Similar results were obtained at PND 88 (i.e., in animals that received a single injection of BrdU at PND 60 and were sacrificed 4 weeks later). Thus, no significant main effect of *ethanol treatment* [two-way ANOVA, F (2, 40) = 1.148, p = 0.327] and no significant interaction between *ethanol treatment* and *sex* [two-way ANOVA, F (2, 40) = 0.006, p = 0.994] were found in these animals (Figure 13 B).

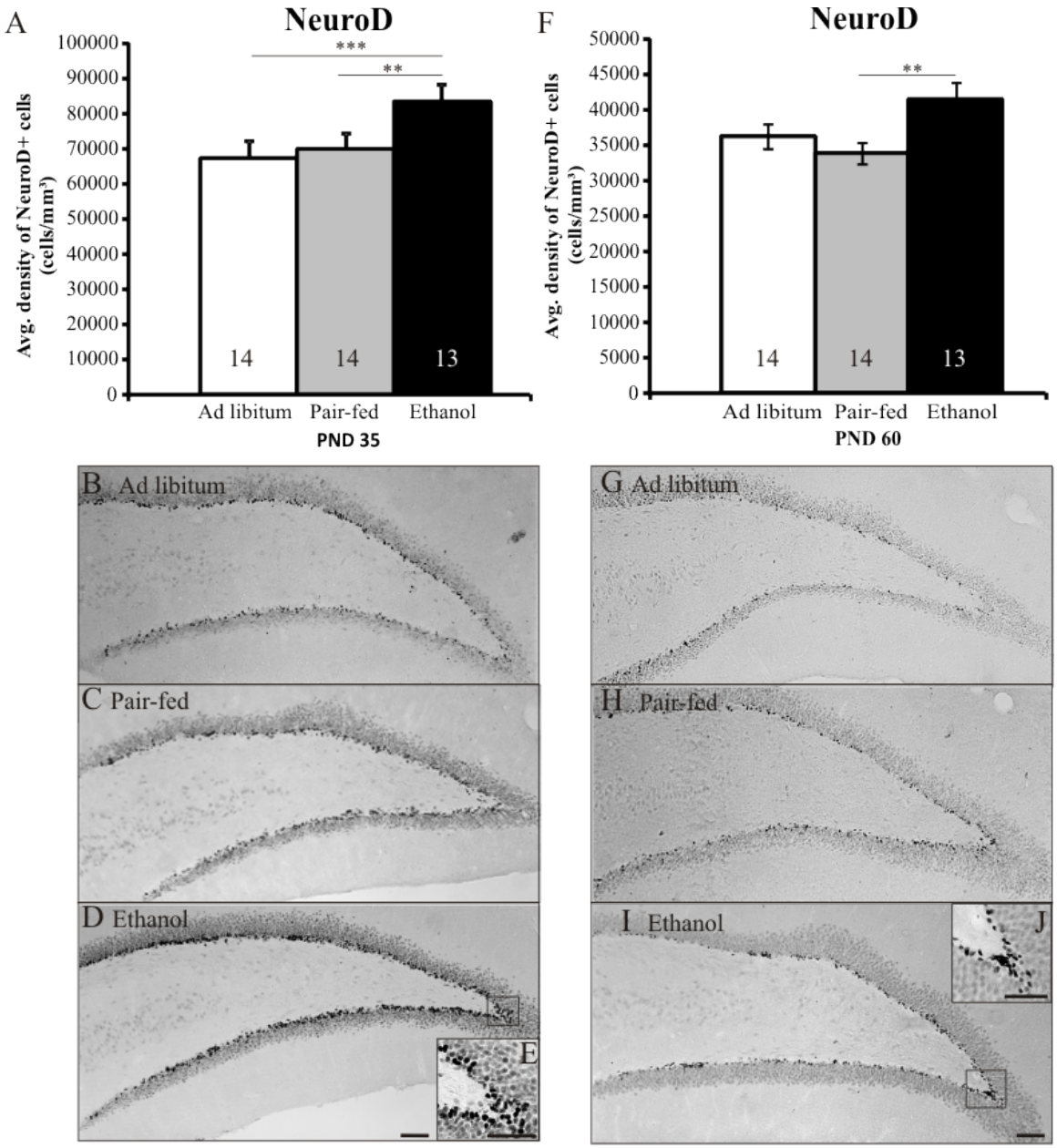

Figure 12. PPAE enhances NeuroD expression in the DG at PNDs 35 and 60.
PPAE significantly alters the expression of the early neuronal marker NeuroD in both adolesecent (PND 35, A) and adult (PND 60, F) animals. Representative micrographs of DG sections processed for NeuroD immunohistochemistry from *ad libitum* (B, G), pair-fed (C, H), and ethanol-exposed (D, E, I, J) animals. Both *ad libitum* and pair-fed rats show a lower density of NeuroD+ cells in the DG when compared to ethanol-exposed (D, I) animals. The number of animals per treatment group is indicated inside the respective bar. Data are presented as means ± SEM. ** indicates $p < 0.01$, *** indicates $p < 0.005$. Scale bar = 100 μm.

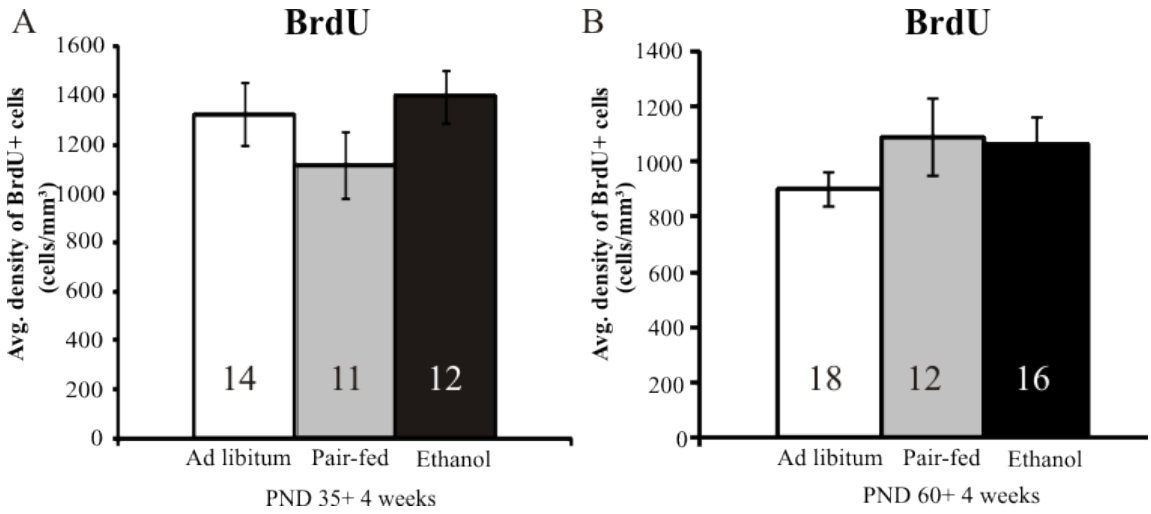

Figure 13. PPAE does not affect DG cell survival - 4 weeks after a single BrdU injection at PNDs 35 and 60.
Cell survival was not significantly altered by PPAE. (A) After a single injection of BrdU at PND 35 animals remained under standard housing conditions and were sacrificed 4 weeks later at PND63. (B) After a single injection of BrdU at PND 60 animals were kept under standard housing conditions and were sacrificed 4 weeks later at PND 88. Brains were processed for BrdU immunohistochemistry and the density of BrdU+ cells in the DG was calculated. The number of animals per treatment group is indicated inside the respective bar. Data are represented as means ± SEM.

3.4. Effects of Voluntary Exercise on Proliferation, Differentiation and Survival of New Cells in the DG of Adult PPAE Rats

The potential beneficial effects of a regime of 12 days of voluntary physical exercise on hippocampal neurogenesis in adult PPAE animals were also investigated. Statistical analysis of the density of BrdU+ cells revealed a significant main effect of *sex* [three-way ANOVA, $F(1, 77) = 7.761$, $p = 0.007$] and *running* [three-way ANOVA, $F(1, 77) = 48.93$, $p < 0.001$] as well as a significant interaction between *sex* and *running* [three-way ANOVA, $F(1, 77) = 5.120$; $p = 0.026$, with female runners (R) > female non-runners (NR), $p > 0.001$; male R > male NR, $p = 0.001$; and female R > male R, $p = 0.004$]. Interestingly, running doubled the density of BrdU+ cells in the DG of females but not males (Figure 14). No significant interaction between *ethanol treatment* and *running* was observed [three-way ANOVA, $F(2, 77) = 0.212$, $p = 0.809$].

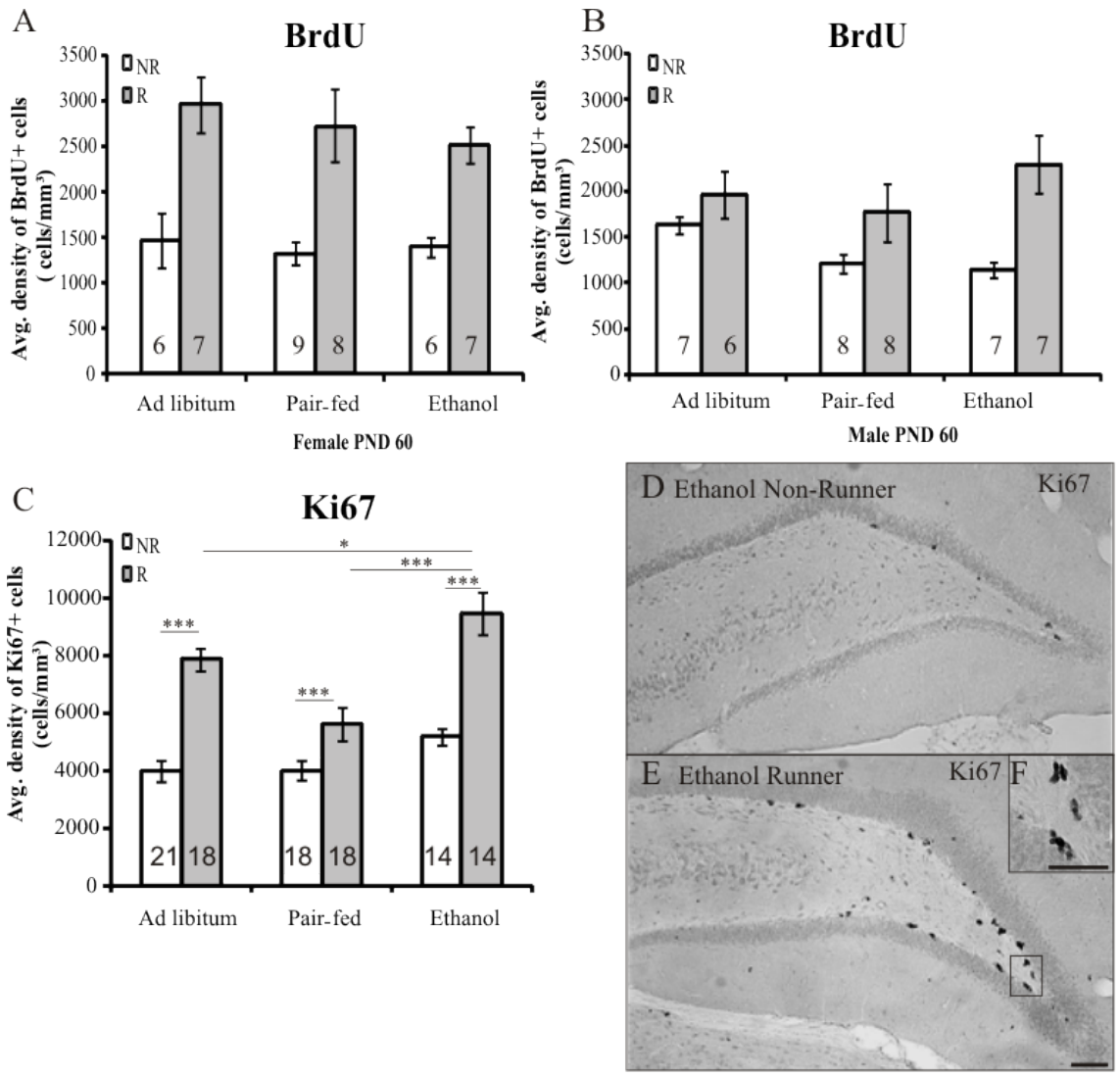

Figure 14. Voluntary exercise enhances DG cell proliferation in PPAE adult rats (PND 60).

(A, B) Exercise significantly increased cell proliferation, as assessed by BrdU immunohistochemistry, in all treatment groups equally. However, a sex versus running interaction was observed and therefore the results from both sexes are presented separately (for details see text).(C) The density of Ki67+ cells is significantly increased in exercising animals as compared with their respective sedentary controls (non-runners, NR). In addition, Ethanol-exposed runners (R) have higher levels of cell proliferation than *ad libitum* R and pair-fed R. (D) Representative micrographs of DG sections processed for NeuroD immunohistochemistry from an ethanol-exposed non-runners (NR) (D) and an ethanol-exposed R (E-F). Images were taken with a 10x (D-E) and a 40x (F) objective respectively. The number of animals per treatment group is indicated inside the respective bar. Data are presented as means ± SEM.* indicates $p < 0.05$; *** indicates $p < 0.005$. Scale bar = 100 μm.

Statistical analysis of the DG density of Ki67+ cells revealed a significant interaction between *treatment* and *running* [three-way ANOVA, F (2, 91) = 4.833; p = 0.010, with *ad libitum* R > NR, $p < 0.001$; pair-fed R > pair-fed NR, $p = 0.012$; ethanol R > ethanol NR, $p < 0.001$; ethanol R > *ad libitum* R, $p < 0.001$; and ethanol R > pair-fed R, $p < 0.018$]. Further, no interaction between *sex* and *running* was detected [three-way ANOVA, F (1, 91) = 2.557, $p = 0.113$] but significant main effects of *sex* [three-way ANOVA, F (1, 91) = 4.784, $p = 0.031$], *ethanol treatment* [three-way ANOVA, F (2, 91)=14.112, $p < 0.001$] and *running* [three-way ANOVA, F (1, 91) = 73.618, $p < 0.001$] were observed. The density of proliferating Ki67+ cells in ethanol-exposed R was more than double the density of Ki67+ observed in both *ad libitum* and pair-fed R (see Figure 14).

The expression of the early neuronal marker NeuroD was also significantly altered by *ethanol treatment* [three-way ANOVA, F (2, 71) = 5.038, $p = 0.009$, with ethanol > pair-fed, $p = 0.002$], *sex* [three-way ANOVA, F (1, 71) = 7.861, $p = 0.006$], and *running* [three-way ANOVA, F (1, 71) = 32.484, $p < 0.001$]. Additionally, a significant interaction between *sex* and *running* [three-way ANOVA, F (1, 71) = 10.601, $p = 0.002$, with female R > female NR, $p < 0.001$; and female R > male R, $p < 0.001$] was found (Figure 15).

Analysis of cell survival 4 weeks post BrdU injection (i.e. at PND 88), revealed a significant main effect of *running* [three-way ANOVA, F (1, 79) = 35.617, $p < 0.001$]. Furthermore, significant interactions between *ethanol treatment* and *running* [three-way ANOVA, F (2, 79) = 4.609, $p = 0.013$], *sex* and *ethanol treatment* [three-way ANOVA, F (2, 79) = 3.587, $p = 0.032$], and *ethanol treatment*, *sex* and *running* [three-way ANOVA,

F (2, 79) = 3.757; $p = 0.027$, with *ad libitum* female R > *ad libitum* NR, $p < 0.001$; *ad libitum* male R >*ad libitum* male NR, $p = 0.016$; ethanol female R > ethanol female NR, $p < 0.001$; ethanol male R > ethanol male NR, $p = 0.018$] were also detected (Figure 16). It is important to note that neither male nor female pair-fed animals showed a running-induced increase in hippocampal cell survival. However, both *ad libitum* and ethanol-exposed female R show a two-fold increase in cell survival when compared with both *ad libitum* and ethanol-exposed male R.

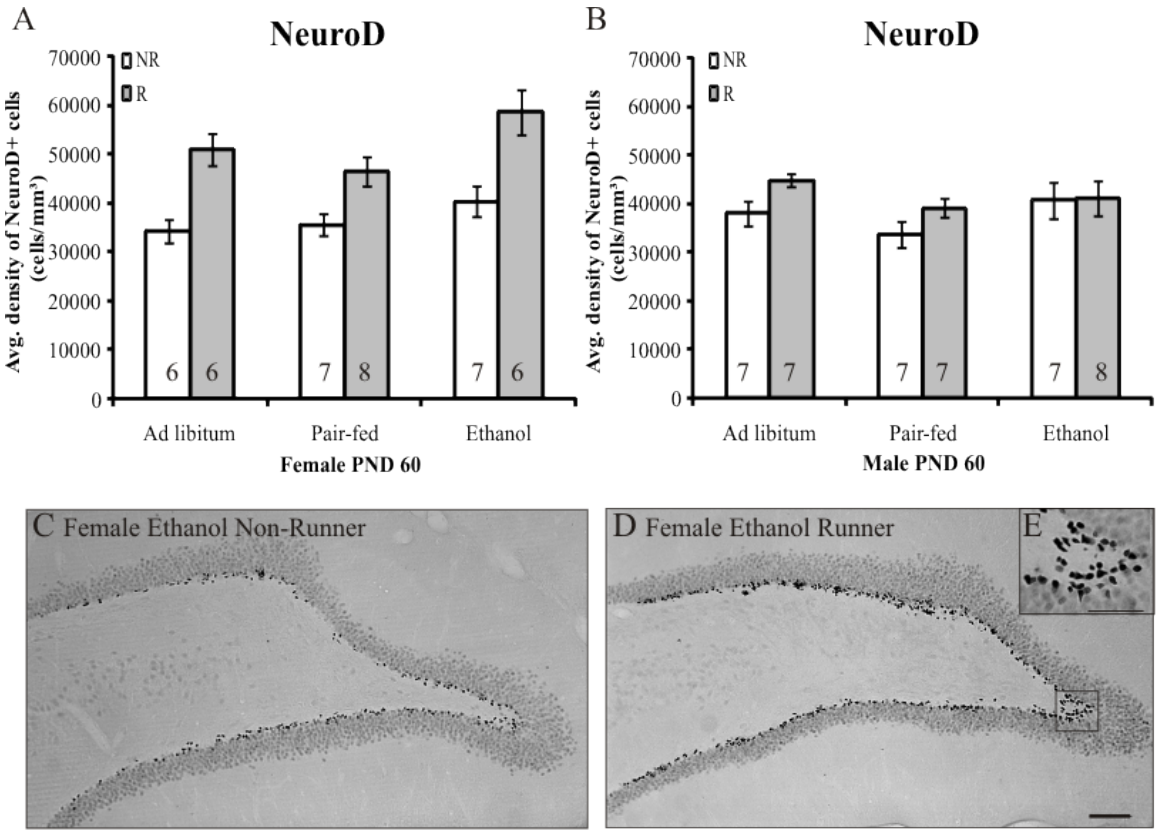

Figure 15. Voluntary exercise preferentially enhances NeuroD expression in PND 60 female rats. (A, B) Voluntary exercise significantly increases NeuroD expression independently from the treatment condition. However, sexes are affected differently with females R (A) showing a higher increase in the density of NeuroD+ cells when compared with male runner (R) (B). (C, D) Representative micrographs of DG sections processed for NeuroD immunohistochemistry from an ethanol-exposed female non runner (NR) (C) and an ethanol-exposed female R (D, E). Images were taken with a 10x (C-D) and 40x (E) objective respectively. The number of animals per treatment group is indicated inside the respective bar. For statistical significances, see details in the text. Data are presented as means ± SEM. Scale bar = 100 μm.

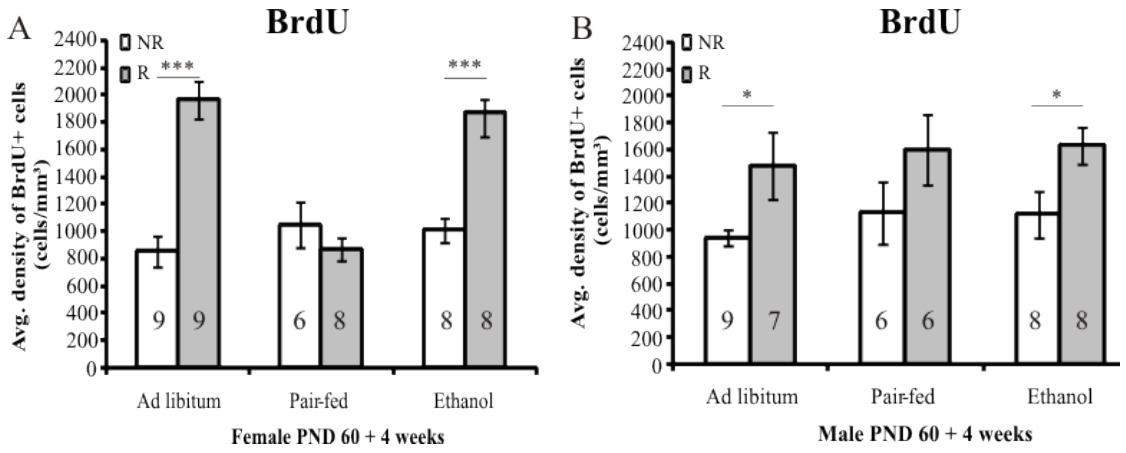

Figure 16. Voluntary exercise enhances cell survival in female and male PPAE adult rats.
(A, B) Animals received a single injection of BrdU at PND 60 and were sacrificed 4 weeks later (i.e., at PND 88). 12 days of voluntary exercise significantly increased the density of BrdU+ cells in the DG of both *ad libitum* and ethanol-exposed animals. The running-induced increase in DG cell survival was significantly larger in females (A) than in males (B) (for details see text). NR, Non-Runner; R, Runner. The number of animals per treatment group is indicated inside the respective bar. Data are represented as means ± SEM. * indicates $p < 0.05$; *** indicates $p < 0.001$.

3.5. Effect of PPAE and Voluntary Exercise on BDNF Expression

BDNF is a critical neurotrophic factor that has been shown to regulate cell survival and differentiation in the adult hippocampus (Barnabe-Heider and Miller, 2003; Lee et al., 2002). Several lines of evidence indicate that the beneficial effects of voluntary wheel running might be mediated, at least in part, by an increase in BDNF expression (Adlard and Cotman, 2004; Adlard et al., 2005; Johnson and Mitchell, 2003; Rasmussen et al., 2009). In order to analyze possible ethanol- and/or exercise-induced alterations in BDNF expression levels in the DG and CA sub-regions of the hippocampal formation, tissue lysates of PND 60 animals were obtained and BDNF levels were analyzed with both ELISA and western-blotting techniques. Importantly, while ELISA quantifies the total BDNF levels present in the lysate, the western-blotting technique allows for the specific detection of the mature form of BDNF. Thus, by using both methods, it was possible to

detect whether changes in total BDNF levels were due to alterations in the mature isoform of this neurotrophic factor.

Statistical analysis of the ELISA results revealed a significant main effect of *running* on total BDNF levels in the DG [three-way ANOVA, F (1, 86) =7.47, p = 0.007, with R > NR, p = 0.011]. However, no significant main effects of *ethanol treatment* [three-way ANOVA, F (2, 87) = 0.683, p=0.507] or *sex* [three-way ANOVA, F (1, 86) = 0.233, p = 0.630] were observed. Nevertheless, we decided to present the results regarding the total BDNF levels in the DG of females and males independently (Figure 17 C-D), as sex-specific alterations in the levels of this neurotrophin might explain, at least in part, the differences in cell survival that were observed between females and males. In fact, the observed sex specific differences in total BDNF levels (Figure 17) mimic the cell survival differences that were detected in PND 60 animals (Figure 16). We also examined the expression of mature BDNF, which can be detected as a 14 kDa band by western-blotting. Since the results from females were expressed as a percentage of change in comparison to *ad libitum* female NR, whereas the results from males were expressed as a percentage of change in comparison with *ad libitum* male NR, comparisons were only possible within each sex but not across sexes. In agreement with the results obtained with ELISA, we detected a main significant effect of *running* in female animals [two-way ANOVA, F (1, 42) = 4.279, p = 0.045] but no significant effect of *ethanol treatment* [two-way ANOVA, F (2, 42) = 0.349, p = 0.707] (Figure 17 A). In males, no significant effects of *running* [two-way ANOVA, F (1, 42) = 0.203, p = 0.654] or *treatment* [ANOVA, F (2, 42) = 1.405, p = 0.257] were detected (Figure 17 B).

Due to the fact that PPAE has been repeatedly shown to induce cell death in the CA1 sub-region of the hippocampus (Barnes and Walker, 1981; Bonthius and West, 1991; Wigal and Amsel, 1990), we also prepared CA lysates in order to analyze BDNF levels in this hippocampal subfield. A significant interaction between *treatment* and *running* was detected [three-way ANOVA, $F(2, 86) = 4.074$, $p = 0.020$; with ethanol R > ethanol NR, $p = 0.0016$; and ethanol NR < pair-fed NR, $p = 0.448$) but no main effect of *ethanol treatment* [three-way ANOVA, $F(2, 86) = 0.025$, $p = 0.975$] or *sex* [three-way ANOVA, $F(2, 86) = 0.016$, $p = 0.900$] was obtained (Figure 17 C).

In the cortex (which served as a control region) no significant main effects of *treatment* [three-way ANOVA, $F(2, 83) = 2.246$, $p = 0.112$] or *running* [ANOVA, $F(1, 83) = 0.001$ $p = 0.995$] were observed (see Figure 17 D).

Finally, it should be mentioned that the DG is highly enriched in BDNF protein (112.6 ± 3 ng/mg of protein) in comparison to both the CA region (51.0 ± 1.7 ng/mg of protein) and the cortex (10.6 ng/mg of protein) [one-way ANOVA $F(3,384) = 577.490$, $p < 0.001$; with DG > CA, $p < 0.001$; DG > cortex, $p < 0.001$; and CA > cortex $p < 0.001$], which suggests its important regulatory role of this sub-region.

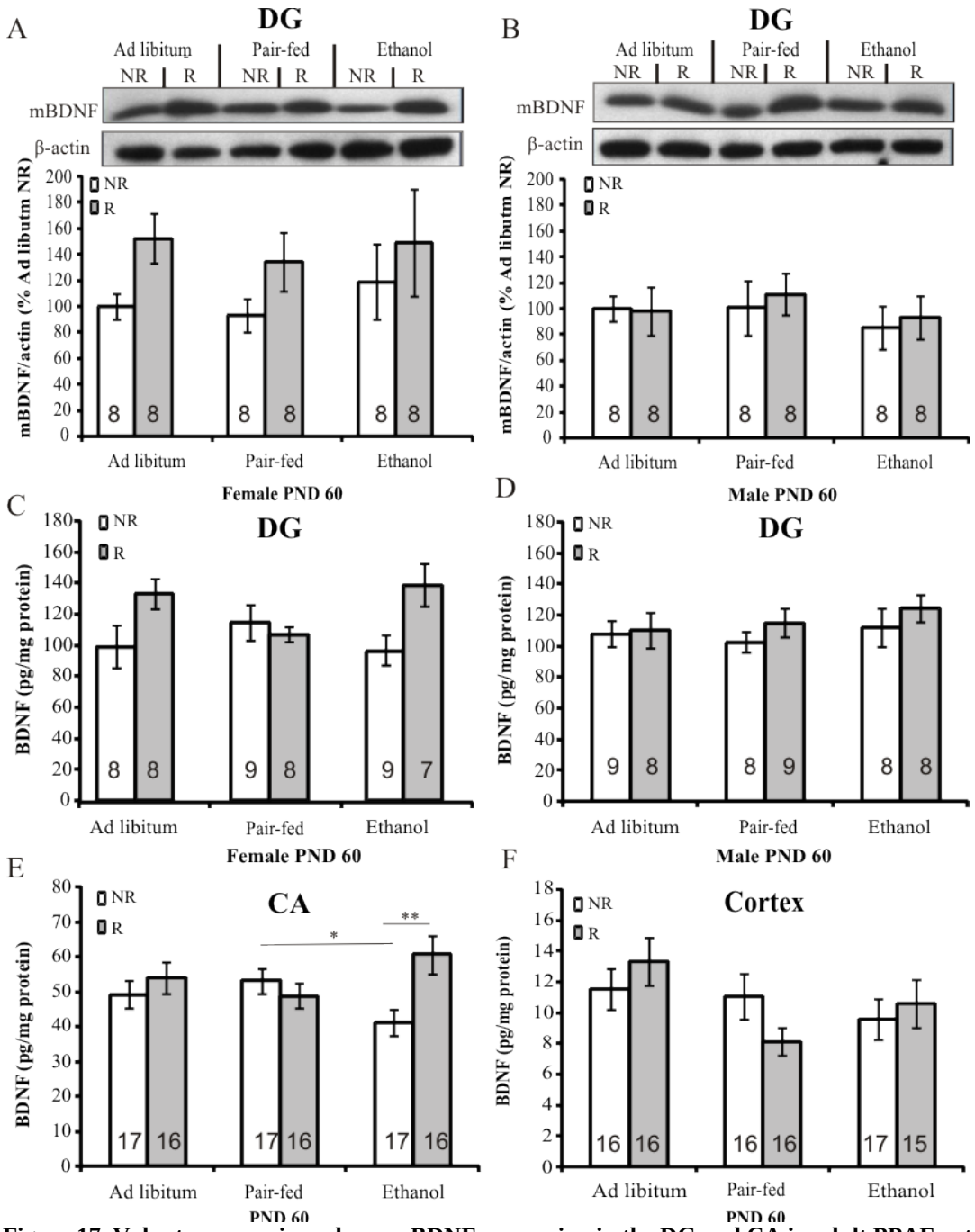

Figure 17. Voluntary exercise enhances BDNF expression in the DG and CA in adult PPAE rats. Detection of BDNF expression in lysates from the DG (A-D) and CA (E) of the hippocampus as well as the cortex (F) by either western-blotting (A-B) or ELISA (C-F). Exercise increased the expression of the 14kDa mature BDNF (mBDNF) in the female DG (A) but not in the male DG (B) as analyzed by western blotting (representative blots are shown above the graphs). Expression of mBDNF, normalized to β-actin, is graphed as % change in comparison to *ad libitum* NR (A, B). ELISA assay revealed a similar effect of running on BDNF levels independent of treatment and sex (C, D).The increase in total BDNF levels upon exercise is more pronounced in females (C) than in males (D). In the CA sub-region (E), running only increased BDNF levels in PPAE animals. In addition, a decrease in total BDNF levels in PPAE NRs in comparison with pair-fed NRs was detected. (F) In the cortex, no alterations due to treatment or exercise were observed. NR, Non-Runner; R, Runner. The number of animals per treatment group is indicated inside the respective bar. Data are presented as means ± SEM.* indicate $p < 0.05$, **indicate $p < 0.01$.

4. Discussion

The aims of this study were to establish and characterize a highly controlled model of FASD (Section 4.1); and determine possible long-term effects of alcohol- induced alteration in cell proliferation, differentiation and cell survival in the DG of adolescent and adult rats (Section 4.2). Finally, investigate if alcohol impacts the capacity for voluntary physical exercise to enhance adult neurogenesis (Section 4.3) and BDNF expression (Section 4.4) in this FASD model.

4.1. Characterization of the Intragastric Intubation Model of PPAE

The severity of FASD can be affected by factors such as timing and pattern of ethanol exposure, BAC levels reached, as well as the maternal and procedural stress, and nutrition status (Ethen et al., 2009; Gil-Mohapel et al., 2010). By using a highly controlled model of FASD, in which alcohol is administered during the entire gestation as well as the early postnatal period, we tried to control for the majority of these influencing factors. Furthermore, the intragastric intubation technique allowed for a weight-calibrated ethanol administration that simulated binge-like drinking throughout the entire human pregnancy.

Moderate to high levels of alcohol intoxication (BAC 155 to 225mg/dl), during all three trimester equivalents, were reached. The used of a pair-fed group allowed us to account for factors that we could not control for, such as procedural stress as well as ethanol-induced reduction in caloric intake and weight loss. The effectiveness with which the pair-fed group controlled for a reduction in caloric intake is reflected in the non-significant differences between pair-fed and ethanol-exposed animals with respect to food

consumption and weight gain of dams and pups. These results indicate that the pair-fed group addressed dietary confounds associated with ethanol ingestion.

Another noteworthy aspect of this model is the procedural stress. The pair-fed animals underwent the same intubation procedure and handling as the ethanol-treated rats, thus CORT levels in both conditions (pair-fed and ethanol) were found to be elevated. Ethanol can also act as an additional maternal stressor, causing the activation of the HPA axis and leading to a further increase in CORT response (Rivier et al., 1984; Weinberg and Gallo, 1982). In agreement, a significant increase in CORT levels was detected in ethanol-exposed dams when compared to those of pair-fed controls. Thus, when attempting to separate the effects of ethanol from those of stress it is vital to have an effective pair-fed control group that undergoes the exact same procedures as the ethanol-treated group.

However, it is important to point out that prenatal stress induced by the intubation procedure can result in a reprogramming of the HPA axis (reviewed by Weinberg et al., 2008; Weinstock, 2007). Indeed, maternal stress can increase CORT response in the offspring. Moreover, alcohol can additionally affect the development of the HPA axis (i.e. by differentially regulating ovarian steroids, which can result in increased HPA sensitivity to estradiol) (Weinberg et al., 2008), and thus potential confounding effects may arise which are important when considering the limitations of the pair-fed control group. Therefore the pair-fed condition can be even seen as a treatment group rather than a control condition, since prenatal stress through procedure and food restriction might differentially effect the development of the HPA axis in comparison to ethanol treatment and procedural stress.

Ultimately, our model successfully addressed a number of the important factors that are believed to influence the manifestation of FASD. However, the used model does have its drawbacks, as stated above; the intragastric intubation procedure can be more stressful than other models, such as the use of an ethanol-liquid diet. Furthermore, this is a very labour intensive model since ethanol has to be administered by the investigator thus requiring commitment 7 days a week for the duration of the gestational period and through the first 10 postnatal days. Therefore, this PPAE FASD model is most effective when the timing of ethanol exposure (e.g. only during a specific period of gestation) or when the modeling of binge-like drinking is of interest.

4.2. Influence of PPAE on Adult Hippocampal Neurogenesis

The effects of prenatal alcohol exposure on adult hippocampal neurogenesis are just now beginning to be explored. Only a few research groups have investigated the effects of ethanol on neuronal linage in the adult hippocampus upon prenatal or early postnatal ethanol exposure (reviewed by Gil-Mohapel et al., 2010). These effects seem to be related to dosage, timing, and method of alcohol administration. In this respect, rodent studies have used one of two different time frames of alcohol exposure: either the first and second trimester equivalents together (approximately GDs 1-23), or the third trimester equivalent alone (approximately PNDs 4-10) (Table 1). In the current study, however, ethanol was administered during both time frames (i.e., prenatal and early postnatal) in order to mimic a pattern of drinking throughout the entire human pregnancy. Since this paradigm has not been used previously, caution needs to be taken when comparing the effects of ethanol exposure on adult hippocampal neurogenesis and BDNF expression obtained in the present study to previous work.

Our goal was to study the age-dependent effects of PPAE on adult neurogenesis. Therefore, we chose to analyze two distinct time-points: PND 35, an adolescent stage (since cognitive deficits in children affected with FASD mainly become apparent during the educational years), and PND 60, a young adult stage (a time point which has been mostly used in previous studies; see Table 1). A third time-point at PND 90 is currently being analyzed (data not shown).

4.2.1. Alteration in the Phases of the Cell Cycle but not in Cell Proliferation Rate upon PPAE

To address possible alterations in cell cycle kinetics upon PPAE, two cell cycle markers were analyzed: the exogenous marker BrdU and the endogenous protein Ki67.

A single BrdU injection approximately labels only 2-10 % of NSCs (Filippov et al., 2003). Since the cell cycle in adult rats takes about 25 hours and the S-phase accounts for 9.5 hours, a single BrdU injection will only provide a “snap-shot” of the cells that were entering the S-phase at the time of injection. Ki67 however, was shown to label 80% of the neuronal progenitor cells but only 25 % of NSCs, since these transient amplifying progenitor cells continue to divide for 3-5 days (Tozuka et al., 2005). These considerations have to be taken into account when interpreting the results obtained with both BrdU and Ki67 labelling.

In adolescent rats (PND 35), PPAE had no significant effect on overall cell proliferation or the phases of the cell cycle. However, at PND 60 a significant increase in the density of Ki67+ cells but not BrdU+ cells in the SGZ of the hippocampus was detected, indicating a potential alteration in cell cycle kinetics (Figure 18).

Alterations in the cell cycle have long been suggested as an effect of alcohol exposure on fetal brain development (Bauer-Moffett and Altman, 1977; Jacobs, 2001; Luo and Miller, 1998; Miller, 1989; Miller, 1995; Miller and Nowakowski, 1991). Miller and Nowakowski (1991) first reported that prenatal ethanol exposure increases the cell cycle length without altering the duration of the S-phase. This statement provides a possible explanation for the differences observed with BrdU and Ki67 after PPAE. On the other hand, Ki67 is an endogenous protein that is expressed during all active stages of the cell cycle. Therefore, ethanol-induced increases in cell cycle length likely resulted in more dividing cells per unit of time and hence a higher expression of Ki67 in the DG of ethanol-exposed animals.

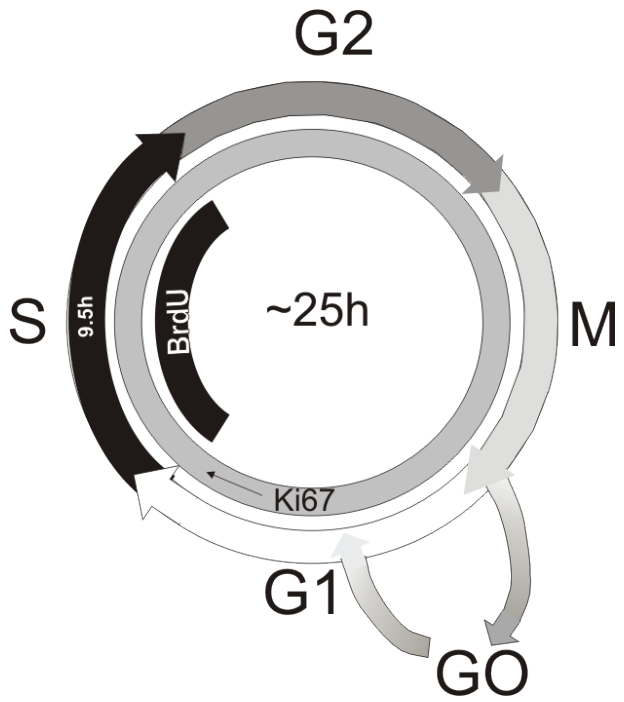

Figure 18. Illustration the cell cycle phases in rats

The cell cycle consists of a series of 4 phases leading to the duplication of the DNA and division of the cell. The cell is ready to enter the cell cycle when it has passed the G1/S checkpoint (which is controlled by cyclin-dependent kinases). In the S-phase (calculated to be 9.5h in young rats), the DNA is synthesised and BrdU can be in cooperated. During G2 the cell grows and prepares for mitosis. In the mitotic phase (M) the chromosomes are drawn apart. In G1 the cell senses growth factors that start the process of cell division. In this phase the cell might either exit the cell cycle to G0 (resting phase) and stop dividing or proceed to the S-phase initiating a new cycle of division.

Following from the above mentioned hypothesis one might conclude that the overall proliferation rate of NSCs and progenitor cells is not significantly altered by PPAE, since the increase in the density of Ki67+ cells was not accompanied by a similar increase in the density of BrdU+ cells. Interestingly, deficits in cell proliferation due to PPAE (especially when animals were exposed to ethanol during the third trimester equivalent) were only observed when animals were examined at an older age (Ieraci and Herrera, 2007) with no significant differences have been detected during adolescence or early adulthood (Helfer et al., 2009; Klintsova et al., 2007). Moreover, in our model of moderate alcohol intoxication the BAC levels were always below 300 mg/dl and previous studies with similar intoxication levels were also not able to detect PPAE-induced changes in adult neurogenesis (Choi et al., 2005; Redila et al., 2006). Taken together, this model of pre- and early postnatal ethanol exposure result in change in the cell cycle length which persist into late adulthood, since a similar increase in Ki67 labelling was observed in PND 90 PPEA animals (preliminary data not shown).To further address this question a different BrdU injection paradigm need to be used to exactly measure the length of the S-phase and cell cycle (for details see Hayes and Nowakowski, 2002)

4.2.2. PPAE Alters Neuronal Differentiation

In addition to the above-mentioned changes in the cell cycle, alterations in neuronal differentiation were observed as assessed by immunohistochemistry for the immature neuronal marker NeuroD. An increase in the density of NeuroD+ cells was detected at both examined ages. To the best of our knowledge, this is the first study that has analyzed NeuroD expression upon prenatal or early postnatal ethanol exposure. However, Helfer

and colleagues (2009) have previously reported no alterations in DCX expression at PND42 after ethanol exposure during the third trimester equivalent (PNDs 4-9). Importantly, DCX is a cytoskeletal protein that has the same expression profile as NeuroD and commonly used as an immature neuronal marker (Brown et al., 2003; Steiner et al., 2006).

A possible underlying mechanism to explain these results might be related with a lengthening of the cell differentiation stages by ethanol, leading to a slower maturation of neurons and thus resulting in an increased NeuroD expression. Interestingly, similar effects due to prenatal and early postnatal ethanol exposure have been observed in oligodendrocytes. Alcohol exposure throughout gestation and the first 10 PNDs caused a delay in oligodendrocyte maturation but appeared to have no long-term effects on the glial cell population of the optic nerve in 15-20 day-old rats. These results indicate that the number of mature astrocytes is unaffected by alcohol despite the observed delay in their differentiation rate (Phillips and Krueger, 1992). The mechanisms of neuronal and oligodentrocyte differentiation/maturation might be regulated by similar triggers, which can explain, at least in part, the results observed in this study.

Further support to this idea comes from an *in vitro* study by Singh and colleagues (2009), who detected a delay in neuronal maturation in hippocampal cultures of neuronal progenitor cells that were harvested at PND 70 (pregnant dams were exposed to ethanol liquid diet throughout gestation and nursing (~PND 21). Besides the delayed maturation of neuronal progenitor cells, a decreased expression of synapsin III was also observed (Singh et al., 2009). Interestingly, synapsin III was previously shown to regulate the commitment of NSCs to the neuronal linage (Kao et al., 2008). Whether synapsin III

expression is also decreased in the present model of PPAE is a hypothesis that derives further investigation.

Another noteworthy explanation for these results might be related with alterations in cell signalling. GABA, a major inhibitory neurotransmitter in the adult brain, activates synaptic and extrasynaptic $GABA_A$ receptors, causing the hyperpolarization of mature neurons. However, in the embryonic nervous system as well as in neuronal progenitor cells of the SGZ, GABA has an excitatory action leading to depolarization of the cell. Since GABAergic inputs are a necessary trigger for the initiation of NeuroD expression in progenitor cells (Tozuka et al., 2005), alterations in GABAergic excitatory inputs might have resulted in a prolonged NeuroD expression. In fact, GABAergic signalling has been implicated in dictating the "tempo" with which cells progress through the various stages of the neurogenic process (Ge et al., 2007). In agreement with this hypothesis, previous studies have shown alterations in GABA and glutamate signalling due to alcohol exposure during fetal development (Allan et al., 1998; Galindo et al., 2005; Ledig et al., 1988). It is believed that this ethanol-induced potentiation of GABAergic activity is the result of increased GABA release at interneuronal synapses (Galindo et al., 2005). Singh et al. (2009) detected amplified stimulatory effects of GABA and attenuated stimulatory effects of glutamate in 29 day-old NPCs harvested from the hippocampus of PND 90 prenatal ethanol-exposed rats. In addition, an increased expression of the β 2/3-subunit of the $GABA_A$ receptor was observed in the hippocampal formation of young adult prenatal ethanol-exposed guinea pigs (Iqbal et al., 2004), indicating that alcohol might not only lead to increased GABA release but also cause attenuated expression of the $GABA_A$ receptor. In conclusion, PPAE might influence the

duration of each neurogenic phase through alterations in GABAergic signalling, leading to an increased expression of NeuroD.

4.2.3. Cell Survival is Not Effected by PPAE

Previous studies have shown that 4 weeks after BrdU injection an abrupt drop in the number of BrdU+ cells can be observed as a result of cell death (Gage, 2000). 80 to 90% of the surviving cells become functional integrated neurons within the next 2 to 4 weeks (Cameron and McKay, 2001). Our data reveals that PPAE does not affect the density of BrdU+ surviving cells in the DG of both adolescent and adult animals 4 weeks after a single injection of this exogenous marker. These results are in agreement with previous reports on the effects of alcohol exposure (through prenatal liquid diet) on the survival of newly born hippocampal cells (Choi et al., 2005; Redila et al., 2006). Early postnatal gavage (Klintsova et al., 2007) and an acute i.p. injection of ethanol at PND7 (Wozniak et al., 2004) also revealed no alterations in cell survival when animals were analyzed at an younger age. However, when BrdU was injected over a period of 14 consecutive days at an early adult stage or for at least 7 consecutive days in aged animals, a 25 % reduction in BrdU+ and BrdU/NeuN co-labelled cells was observed (Ieraci and Herrera, 2007; Klintsova et al., 2007). This indicates that in order to detect significant differences in cell survival/neurogenesis, a larger progenitor pool needs to be labelled, and therefore, one single BrdU injection might not be enough to observe alterations in cell survival. It is also important to note that the levels of intoxication reached in these studies were in the 300-500 mg/dl range (Helfer et al., 2009; Ieraci and Herrera, 2007; Klintsova et al., 2007; Wozniak et al., 2004) and therefore these results need to be evaluated with caution, since

alcohol levels in humans are estimated to reach a peak BAC of ~190 mg/dl in third-trimester drinkers (0.19 %) (May et al., 2009).

In addition, when alcohol exposure is restricted to the third trimester equivalent (Ikonomidou et al., 2000; Klintsova et al., 2007; Mooney et al., 1996; Tran and Kelly, 2003; West et al., 2001) different aspects of adult hippocampal neurogenesis may be affected. For example, it is possible that prenatal alcohol exposure (as well as the effects of reduced nutrition and stress) might "habituate" the SGZ stem cell pool. In fact, developmental proliferation of neuronal precursors in this neurogenic region takes place between GDs 5-11 and the first granule neurons in the DG are born during late embryogenesis (GD 17) in rats. Thus, alcohol exposure during the early PNDs or throughout all three trimester equivalents might differently affect progenitor cells. *In vitro* studies show that if ethanol is administrated in a progressive way (i.e. when the dose is increased gradually over the course of the experiment) and over a longer period of time no deficits in cell signalling (e.g. long term potentiation (LTP)) can be observed. However, if ethanol is acutely administrated, LTP is inhibited (Tokuda et al., 2007). This might explain why more robust effects on adult neurogenesis have been observed in models of a single or short-term exposure to alcohol during the period of brain growth than in models of alcohol exposure during the entire gestational period. Thus, drawing comparisons between this study and previous reports might be difficult in light of the different exposure paradigms used.

In conclusion, the present results indicate that PPAE does not induce drastic deficits in adult hippocampal neurogenesis. However, it is still possible that the physiological

properties as well as the maturation of these newly born neurons might be affected by PPAE.

This hypothesis is supported by various studies that have reported impairments in synaptic plasticity and/or deficits in hippocampal-dependent learning and memory tasks upon prenatal alcohol exposure (Christie et al., 2005; Kelly et al., 1988), even when no alterations in adult hippocampal neurogenesis were observed (Redila et al., 2006).

4.2.4. Age-Related Decline in Neurogenesis is Not Effected by PPAE

Neurogenesis declines noticeably with age, resulting in a decrease of up to 80 % within 40 days after birth (Altman and Das, 1965). To the best of our knowledge, this is the first study that investigated the effects of age-dependent alterations in adult hippocampal neurogenesis in pre- and/or postnatal ethanol-exposed animals. No alterations in the age-induced decline in adult neurogenesis were observed between PND 35 and PND 60. A decrease of 23–30 % in the density of BrdU+ cells and a reduction of 40-50 % in the density of NeuroD+ cells were observed between the two age groups regardless of the treatment condition. However, the age-induced reduction in the density of Ki67+ cells was more pronounced in both control groups (50 %) when compared with ethanol-exposed rats (30 %). Although the exact reasons for this difference are not known, it is possible that the effects of alcohol on the onset of puberty, and in the case of females, on vaginal opening, might contribute to this finding. The onset of puberty is reported to be at an age of 34-35 days in control animals and at 38-39 days in prenatal alcohol-exposed animals (McGivern and Yellon, 1992). Especially in females, ovarian hormones (i.e. estrogen) have been shown to increase cell proliferation (Tanapat et al., 1999). Thus a delayed increase in estrogen levels in ethanol-exposed females might have

counteracted the alterations in cell cycle kinetics and thus resulted in a less robust age-induced decline in cell proliferation. In agreement with this idea, the difference in age-induced decline of Ki67+ cells between control and ethanol-exposed animals was only observed between PND 35 and PND 60 but not between PND 60 and PND 90 (at which time point a consistent decrease of ~30% in the density of Ki67+ cells was observed across all treatment groups; data not shown).

4.3. Effect of Voluntary Wheel Running on Adult Hippocampal Neurogenesis in Adult Rats after PPAE

Exercise is an important facet of behaviour that enhances brain health and function. Further, voluntary wheel running has been shown to increase adult hippocampal cell proliferation and neurogenesis as well as enhance learning and memory (Adlard and Cotman, 2004; Eadie et al., 2005; van Praag et al., 1999a; van Praag et al., 1999b). In agreement, a recent study has reported beneficial effects of exercise on hippocampal-dependent spatial learning tasks in rats that were exposed to ethanol during the third trimester equivalent (Thomas et al., 2008), indicating that exercise during adulthood might be a potent therapeutic strategy to improve hippocampal function in FASD.

With respect to the effects of physical exercise on hippocampal neurogenesis, our laboratory was the first to investigate the potential benefits of this non-invasive therapeutic strategy on animals that were exposed to ethanol during the first and second trimester equivalents (i.e., dams had access to a liquid diet containing ethanol throughout gestation) (Redila et al., 2006). In this initial study a decrease in cell proliferation (as measured by BrdU labelling at PND 50) was observed in ethanol-exposed subjects as compared to non-handled controls although no significant differences between ethanol-

exposed and pair-fed treatment groups were detected. Furthermore, no differences were found in neuronal differentiation between all three conditions. After voluntary wheel running cell proliferation and cell survival (4 weeks after BrdU injection) were found to be significantly increased in all treatment conditions in the study by Redila et al, (2006). The present study also found no significant alterations in exercise–induced up-regulation of adult hippocampal neurogenesis due to PPAE. Helfer et al.(2009) have also recently analysed the effect of 12 days of voluntary running on rats that were exposed to ethanol during the third trimester equivalent (PNDs 4-9). Although the authors observed no significant effect of ethanol exposure on cell proliferation, the long-term survival of BrdU+ cells was impaired in ethanol and sham-intubated animals (assessed at PND 72), and this effect could not be rescued by physical exercise. However, since this impairment was seen in both ethanol and sham-intubated animals, stress rather than ethanol might have caused this alteration.

Despite the fact that in the present study adult hippocampal neurogenesis was not significantly altered in ethanol-exposed animals, Ki67 immunohistochemistry revealed that the exercise-induced up-regulation of neurogenesis was significantly higher in ethanol-exposed animals than in pair-fed and *ad libitum* controls. Furthermore, BrdU immunohistochemistry also revealed that this exercise-induced up-regulation of the neurogenic process was significantly higher in ethanol-exposed females than in ethanol-exposed males. These results might be explained by the higher exercising rate that was observed in PPAE females, which is in agreement with the hyperactive phenotype that is characteristic of both rodents and humans with FASD (Hellemans et al., 2008; Melcer et al., 1994; Thomas et al., 2008). In addition, the fact that males run less than age-matched

females might be a result of competitive territorial behaviour (since in our experimental conditions two males had to share a cage and its respective running wheel) and increased body mass and overall length of male rats (Cortright et al., 1997). Further, sex-specific hormonal response (e.g. testosterone in males and estrogen in females), energy metabolism and protein and fat mass between males and females (Cortright et al., 1997) might also account for these results. For this study we could not perform any correlation analysis to test if the exercise rate is responsible for increases obtained in cell proliferation, since animals were group housed and running data was only recorded per cage but not per specific animal.

A decrease in cell survival in female pair-fed runners was also detected in this study; this deficit might be a result of the combined effects of both prenatal stress (due to constant handling and the gavage procedure) and prenatal food restriction. Prenatal stress leads to increased basal CORT levels, especially in females offspring (Szuran et al., 2000; Takahashi et al., 1998), as well as reduced expression of glucocorticoide receptors (GR) (Takahashi, 1998). Stress and physical exercise have opposite effects on adult hippocampal neurogenesis through the regulation of pathways involving growth factors such as IGF-1, VEGF and BDNF (Heine et al., 2005; Kuhn et al., 1997; Ma, 2008; Trejo et al., 2001). Therefore, it is possible that the compounding effects of prenatal stress and food restriction might have induced impairment in the regulation of growth factor expression in adult pair-fed females. This hypothesis is supported by an impairment of the DG to increase BDNF levels upon voluntary exercise in female pair-fed runners (see below).

4.4. Alteration in BDNF Expression upon PPAE

BDNF has an essential regulatory role in the long-term survival as well as integration of new neurons into the existing circuitry (Lee et al., 2002; Sairanen et al., 2005). Several lines of evidence indicate that PPAE impacts the expression of BDNF in the hippocampal formation (Barbier et al., 2008; Caldwell et al., 2008; Fiore et al., 2009; Heaton et al., 2000; Miki et al., 2008a; Tsuji et al., 2008). Alterations of BDNF in this brain region might contribute to impairments in hippocampal-dependent learning as well as cell proliferation and survival (reviewed by Cunha et al., 2009; Lee and Son, 2009).

In the present study we detected an exercise-induced significant increase in the levels of BDNF in the hippocampus, which is in agreement with previous reports (Adlard and Cotman, 2004; Adlard et al., 2005; Farmer et al., 2004; Johnson and Mitchell, 2003; Rasmussen et al., 2009). Since total BDNF (analysed by ELISA) as well as mature BDNF (analysed by western-blotting) were increased in a similar manner, it can be hypothesized that BDNF activated the PKB/Akt and ERK signalling cascades leading to an increased expression of pro-differentiation and pro-survival genes (Barnabe-Heider and Miller, 2003; Sairanen et al., 2005).

A recent study by Berchthold and colleagues (2010) analyzed the temporal expression pattern of BDNF after 3 weeks of exercise and detected that BDNF levels remain elevated for a period of 2 weeks after the removal of animals from the running wheels. Therefore, it is likly that the levels of BDNF measured in this study not only affected proliferation and differentiation of the newly born cells but also their survival. For further addressing a this correlation an assay measuring the activation of the Erk and Akt pathway needs to be performed as well as an analysis of the mRNA expression of

survival and differentiation genes (like BAD, Forkhead, PSA-NCAM). The linkage to the obtained BDNF levels is particularly important when interpreting the cell survival results, since animals were removed from their running wheels, injected with BrdU, and sacrificed 4 weeks later. Within this scenario, it is relevant to note that the observed changes in DG BDNF levels follow a pattern identical to the differences observed in BrdU cell survival, strongly suggesting that cell survival is indeed mediated, at least in part, by BDNF expression. Since females run twice as much as males, greater alterations in BDNF expression levels due to exercise were observed in females. Thus, greater exercising levels resulted in higher BDNF protein levels, and a consequent greater increase in cell proliferation, differentiation and survival in females when compared to males.

With regards to the different treatment conditions, no alterations in BDNF expression levels were observed in ethanol-exposed animals in comparison to *ad libitum* controls, indicating that PPAE does not affect BDNF levels in adulthood. These results are supported by several other studies. Caldwell and colleagues (2008) failed to detect any changes in BDNF expression in adult (PNDs 60-90) PPAE animals, indicating that a moderate ethanol exposure during the first two trimester equivalents might not significantly influence BDNF levels in adulthood. The remaining studies have only analyzed PPAE-induced changes in the levels of this neurotrophin during development or early adolescence (Feng et al., 2005; Heaton et al., 2000; Tsuji et al., 2008) (for details on these studies see Table 2).

In addition, a deficit in running-induced increase in BDNF expression was found in pair-fed animals. Interestingly, the same deficit was also detected in cell survival,

indicating that the expression of BDNF might account for the decrease in cell survival observed 4 weeks after BrdU injection in PND60 pair-fed controls.

4.4.1. PPAE-Induced Deficits in BDNF Expression in the CA Region

BDNF also plays a critical role in activity-dependent synaptic plasticity (reviewed by Waterhouse and Xu, 2009). LTP in the CA1 subfield of the hippocampus is a commonly used measure of hippocampal synaptic plasticity. LTP deficits observed in this subfield after PPAE (Savage et al., 1998) have been proposed as a mechanism for the learning impairment seen in humans with FASD (Christie et al., 2005; Richardson et al., 2002). Thus, evaluating BDNF levels in the CA subfield of the hippocampus might elucidate potential deficits that may be associated with this impairment in synaptic plasticity.

In agreement with this hypothesis, a significant decrease in BDNF expression due to PPAE in the CA subfield of the hippocampal formation was detected. To the best of our knowledge, this is the first study reporting PPAE-induced alterations in the expression levels of BDNF that are specific to the CA sub-region of the hippocampal formation. Thus, the results reported in previous studies regarding PPAE-induced changes in BDNF hippocampal levels might correspond to specific alterations in the CA subfield, since this sub-region accounts for the larger portion of the hippocampal formation (in PND 60 rats: weight of CA sub-region is ~40 mg whereas weight of DG sub-region is ~20 mg).

This specific deficit in CA BDNF levels might also explain why cell loss (i.e., a decrease in cell number and/or cell density) upon PPAE is mainly found in the CA1 sub-region of the hippocampus (Bonthius and West, 1990; Bonthius and West, 1991; Livy et al., 2003; Wigal et al., 1990). Decreases in cell density in the CA1 subfield were

observed in both adolescent and adult animals in different modes of ethanol exposure during the period of brain development. Interestingly, Livy et al. (2003) identified that ethanol exposure during the third trimester equivalent produced more robust changes in cell loss than prenatal ethanol exposure in this hippocampal sub-region. In agreement, Tsuji and colleagues (2008) recognized that ethanol exposure during this same period not only decreases the levels of BDNF but also the levels of phospho-MAPK and phospho-PKB/Akt in the hippocampus. This decrease in Akt phosphorylation can potentially lead to a reduction in the expression of pro-survival genes, which may then account for the increased cell loss found in the CA1 sub-region.

Finally, BDNF also mediates the stimulation of *de novo* protein synthesis, which is crucial for the maintenance of late-phase LTP (Kang et al., 1996). Richardson and colleagues (2002) failed to induce LTP in the CA1 subfield of the hippocampus in PPAE animals (Richardson et al., 2002). Thus it is possible that a decrease in BDNF expression in the CA sub-field can account, at least in part, for the cellular and physiological deficits leading to the impairment in hippocampal function and the deficits in learning and memory seen in FASD models.

4.5. Conclusions

In summary, the present study demonstrated that PPAE does not significantly impair cell proliferation and cell survival in the hippocampus of adolescent and adult rats while increased neuronal differentiation at both time points was found. Since PPAE-induced alterations in the expression of the endogenous cell cycle marker Ki67, but did not change the levels of BrdU incorporation, it can be hypothesized that alcohol exposure

during thorough all three trimester equivalents lengthens the cell cycle without altering the length of the S-phase.

In this study, newly born cells in the adult DG of PPAE animals display normal proliferation and survival but altered neuronal differentiation and maturation, a process observed with normal aging (Rao et al., 2005). In order to address these particular deficits, further quantification of the stages of neuronal differentiation upon PPAE is warranted.

Voluntary exercise up-regulated all analyzed stages of adult neurogenesis (i.e. cell proliferation, differentiation and survival) in PPAE and control animals indicating that the capacity of voluntary exercise to enhance adult hippocampal neurogenesis and BDNF expression was not effected ethanol exposure during all three trimester equivalents. Further, no PPAE-induced alterations in the levels of total BDNF and mature BDNF were found in the DG. However, BDNF deficits in the CA sub-region of the hippocampus were observed, and these might explain some of the deficits seen in FASD animal models including a decrease in cell density in the CA1 subfield, impairments in synaptic plasticity and deficits in hippocampal-dependent learning and memory. Future studies are necessary in order to determine whether physical exercise can rescue these deficits seen in animal models of FASD.

4.6. Future Directions

Based on the results presented in this study, it is of interest to examine adult neurogenesis in the DG of aged animals in order to determine whether the alterations observed in this study are maintained or even exacerbated with age. Indeed, it is possible that PPAE might interfere with the age-depend decrease in adult hippocampal

neurogenesis. Therefore we are currently analyzing how cell proliferation and differentiation is affected in PND 90 PPAE animals. Since we also detected alterations in early neuronal differentiation with the immature neuronal marker NeuroD, it would also be of interest to further characterize the phenotype of the BrdU+ cells that survived the 4 week period. To analyse the late stages of neuronal maturation, triple-labelling for BrdU, calbindin, and calretinin would be necessary. These proteins are used to distinguish the two final stages of neuronal maturation since newly generated neurons switch their main calcium-binding protein from calretinin to calbindin once they are fully functional and integrated into the existing circuitry (Brandt et al., 2003). This analysis would give an indication on whether the maturation stage of these new cells and their integration into the existing network is affected by PPAE.

Previous work from our laboratory has demonstrated that alterations in synaptic function upon prenatal alcohol exposure can be detected (Christie et al., 2005) even when only minor alterations in adult neurogenesis were observed (Redila et al., 2006). The present BDNF results suggest that the CA sub-region of the hippocampus might be particularly affected in PPAE animals. Thus, further analysis of the intra- and extracellular BDNF signalling pathways in this subfield might be of further interest.

The neurotransmitter GABA controls not only synaptic signalling but also early neuronal differentiation, which was found to be increased in this model. Since prenatal alcohol exposure has been reported to increase GABAergic signalling (Ikonomidou et al., 2000), it might be also relevant to investigate the regulation of GABA in PPAE animals. Thus, determining the expression levels of glutamate decarboxylase (GAD), which

converts glutamate into GABA, might elucidate potential underlying mechanisms for these alterations.

Finally, our laboratory has recently observed reductions in the activities of enzymes involved in the glutathione redox cycle in the DG of PND 60 PPAE rats (Brocardo et al., unpublished results). The glutathione system is a key component for removal and neutralization of reactive oxygen species (ROS). Based on these promising preliminary results, we are currently examining the levels of other oxidative stress markers (such as the activities of the ROS-scavenging enzymes, catalase and superoxide dismutase as well as the levels of protein oxidation and lipid peroxidation) in the DG and other CNS regions of PPAE animals.

Moreover, we also examining whether PPAE increases depression- and anxiety-like behaviours in PND 60 animals using a battery of behavioural tests that include the elevated plus maze, the activity in the open field, and the forced swim test. Finally, given the potential beneficial effects of physical exercise, we are also interested in evaluating whether this non-invasive therapeutic strategy can ameliorate the above-mentioned deficits in the pathways that regulate oxidative stress as well as the development of the depressive- and anxiety-like phenotypes induced by PPAE.

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Appendices

Appendix A - Milk Solution Composition

Table A 1. Milk solution composition

Material	Amount required per 1l	Concentration
evaporated milk	704 ml	
mineral mix	23.5 ml	
dH_2O	211.3 ml	
corn oil	61 ml	
methionine	0.94g	6.29mM
tryptophan	0.469 g	4.93mM
vitamin mix (Bio-serv)	4.69g	
calcium phospate diabasic	0.094g	54 μM
deoxycholic	0.094g	23 μM

Table A 2. Mineral mix composition

material	Concentration(mM)
$ZnSO_4$	14.8
$CuSO_4$	15
$FeSO_4$	8.6
$MgCl_2$	420
KCL	536.5

Table A 3. Vitamin mix composition (Bio-serv, Frenchtown, NJ, USA)

Vitamin	g/kg
vitamin A (500,000 IU/g)	0.845
vitamin D3 (400,000 IU/g)	0.106
vitamin E acetate	3.622
ascorbic Acid	63.990
inostitol	374.280
choline chloride	495.015
menadione sodium bisulfite	12.074
Para-amino benzoic acid	0.121
niacin	26.562
riboflavin	10.866
pyridoxine HCl	0.918
thiamin	1.328
calcium pantothenate	7.244
biotin	0.009
folic acid	0.003
vitamin B-12 (1%)	3.018

Appendix B - Summary of the Subjects Used in this Study

Table B 1. Summary of the subjects used in the present study.

Condition	Dams	Pups	BrdU	Ki67	NeuroD
PND 35 proliferation	15(AL) 20(PF) 20(E)	8(AL-M) 11(AL-F) 9(PF-M) 10(PF-F) 8(E-M) 7(E-F)	17(AL) 16(PF) 12(E)	19(AL) 19(PF) 13(E)	14(AL) 15(PF) 13(E)
PND 63 BrdU survival		9(AL-M) 8(AL-F) 8(PF-M) 8(PF-F) 9(E-M) 8 (E-F)	14(AL) 11(PF) 12(E)	–	–
PND 60 proliferation		8(AL-M-NR 8(AL-M-R) 8(AL-F-NR) 10(AL-F-R) 8(PF-M-NR) 9(PF-M-R) 8(PF-F-NR) 9(PF-F-R) 6(E-M-NR) 8(E-M-R) 7(E-F-NR) 6(E-F-R)	15(AL-NR) 14(AL-R) 16(PF-NR) 18(PF-R) 13(E-NR) 13(E-R)	21 (AL-NR) 18(AL-R) 18(PF-NR) 18(PF-R) 14(E-NR) 14(E-R)	7(AL-M-NR 7(AL-M-R) 7(AL-F-NR) 7(AL-F-R) 7(PF-M-NR) 7(PF-M-R) 7(PF-F-NR) 7(PF-F-R) 6(E-M-NR) 8(E-M-R) 7(E-F-NR) 6(E-F-R)
PND 88 BrdU survival		10(AL-M-NR 8(AL-M-R) 9(AL-F-NR) 8(AL-F-R) 8 (PF-M-NR) 8(PF-M-R) 8(PF-F-NR) 8(PF-F-R) 9(E-M-NR) 9(E-M-R) 8(E-F-NR) 8(E-F-R)	9(AL-M-NR 7(AL-M-R) 9(AL-F-NR) 7(AL-F-R) 6(PF-M-NR) 6(PF-M-R) 6(PF-F-NR) 8(PF-F-R) 8(E-M-NR) 9(E-M-R) 8(E-F-NR) 8(E-F-R)	–	–
PND 60 BDNF study	8(AL) 8(PF) 9 (E)	9 (AL-M-NR 8 (AL-M-R) 8(AL-F-NR) 8(AL-F-R) 8(PF-M-NR) 9(PF-M-R) 9(PF-F-NR) 8(PF-F-R) 8(E-M-NR) 8(E-M-R) 9(E-F-NR) 8(E-F-R)	–	–	–
Total:	**55** for neurogenesis **25** for BDNF	**422** for neurogenesis **100** for BDNF			

The number of animals is presented per experimental conditions. E, Ethanol; PF, Pair-fed; AL, *Ad libitum*; M, Male; F, Female; NR, Non-runner; R, Runner