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Review

Hippocampal cell loss and neurogenesis after fetal alcohol exposure: Insights from different rodent models

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ABSTRACT

Prenatal ethanol exposure is invariably detrimental to the developing central nervous system and the hippocampus is particularly sensitive to the teratogenic effects of ethanol. Prenatal ethanol exposure has been shown to result in hippocampal cell loss, altered neuronal morphology and impaired performance on hippocampal-dependent learning and memory tasks in rodents. The dentate gyrus (DG) of the hippocampus is one of the few brain regions where neurogenesis continues into adulthood. This process appears to have functional significance and these newly generated neurons are believed to play important functions in learning and memory. Recently, several groups have shown that adult hippocampal neurogenesis is compromised in animal models of fetal alcohol spectrum disorders (FASD). The direction and magnitude of any changes in neurogenesis, however, appear to depend on a variety of factors that include: the rodent model used; the blood alcohol concentration achieved; the developmental time point when alcohol was administered; and the frequency of ethanol exposure. In this review we will provide an overview of the different rodent models of FASD that are commonly used in this research, emphasizing each of their strengths and limitations. We will also present an up-to-date summary on the effects of prenatal/neonatal ethanol exposure on adult hippocampal neurogenesis and cell loss, highlighting some of the possible molecular mechanisms that might be involved.

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Abbreviations: ARBD, Alcohol Related Birth Defects; ARND, Alcohol Related Neurological Disorders; BAC, blood alcohol content; BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; CNS, central nervous system; DG, dentate gyrus; EDC, ethanol derived calories; FASD, fetal alcohol spectrum disorders; FAS, Fetal Alcohol Syndrome; FGF, fibroblast growth factor; GABA, gamma-aminobutiric acid; GD, gestational day; GDNF, glial cell-derived neurotrophic factor; IGF-1, insulin growth factor 1; i.p., intraperitoneal; LTP, long-term potentiation; NeuN, neuronal nuclei; NMDA, N-methyl-D-aspartate; PKB/Akt, phospho-protein kinase B; pMAPK, phospho-mitogen-activated protein kinase; PND, postnatal day; s.c., subcutaneous; SGZ, subgranular zone; SVZ, subventricular zone

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1. Introduction

The consumption of alcohol during pregnancy can lead to a variety of developmental problems that include: deficits in the formation of the central nervous system (CNS); restricted physical growth; and impaired cognitive abilities. The appearance of these symptoms can vary widely and often is dependent upon the developmental period during which alcohol was consumed (Autti-Ramo and Granstrom, 1991). The term Fetal Alcohol Spectrum Disorders (FASD) was coined to encompass the vast array of teratogenic effects that can arise when alcohol is consumed during different periods of the pregnancy. The spectrum of disorders can include Alcohol Related Neurological Disorders (ARND), Alcohol Related Birth Defects (ARBD), and Fetal Alcohol Syndrome (FAS). In particular, a diagnosis of FAS is done when a child presents stereotypic cranio-facial dysmorphologies that result from alcohol-induced damage during the formation of the facial bones (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 impairment, and confirmed prenatal alcohol exposure (for review see Hill et al., 1989; Moore et al., 2001; Robin and Zackai, 1994). Unfortunately, this is usually a disconcerting situation for the mother, and often the symptoms of FASD are not apparent until the educational years, so it is hard to obtain an accurate estimate of how many individuals are afflicted with FASD. Recent estimates suggest that the incidence of FASD may be as high as 2–5 % making it a serious health issue. Indeed, exposure to alcohol *in utero* is the leading cause of preventable mental retardation and birth defects in the United States (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, behavioral, emotional and social deficits. Behavioral deficits observed in these children include impaired spatial memory performance that is typically associated with hippocampal dysfunction (Coles et al., 1991; Streissguth et al., 1989; Uecker and Nadel, 1996; Hamilton et al., 2003). Similarly, rodent models of FASD also show impairments in hippocampal-dependent behaviors: including spatial learning and memory and contextual fear-conditioning tasks (Allan et al., 2003; Berman and Hannigan, 2000; Choi et al., 2005; Kelly et al., 1988). In support of this work, neuroimaging studies have shown that children with FASD have significantly smaller brains, with reduced hippocampal volume (Autti-Ramo, 2002), in addition to reductions in the size of the cerebral cortex, amygdaloid body, basal ganglia, corpus callosum, and cerebellum (Archibald et al., 2001; Autti-Ramo, 2002). Similarly, in a recent imaging study, significant reductions in the volume of several brain structures including the hippocampus have been observed in mouse fetuses following a single day of prenatal alcohol exposure (Parnell et al., 2009). This reduction in brain volume is likely to reflect reductions in cell size, dendritic arborization, and synaptic connections (Abel et al., 1983; Davies and Smith, 1981; Diaz Perez et al., 1991; Ferrer et al., 1988), as well as apoptotic cell loss in conjunction with a decrease in 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).

It has long been known that the hippocampus is a brain area that is particularly sensitive to the effects of ethanol during its development. Some of the earliest studies on the effects of alcohol during gestation noted that ethanol could cross the placental barrier, readily reaching all brain areas including the hippocampal formation (Ho et al., 1972). More recently, studies have quantified how cell numbers change in the hippocampus after prenatal exposure to alcohol (Livy et al., 2003; Section 3). The type and extent of neuronal loss in this brain structure and related cognitive impairments seems to depend on the

developmental timing as well as the dose and extent of prenatal ethanol exposure. Specifically, ethanol exposure during the rapid growth spurt that occurs in the third trimester equivalent induces significant cell loss in this brain region (Ikonomidou et al., 2000; Kelley et al., 2003; Klintsova et al., 2007; Mooney et al., 1996; West et al., 1986). In rats, the third trimester equivalent period occurs postnatal (see Section 2.1.2), and animals exposed to ethanol for only a few days during this period can show deleterious effects on hippocampal structure and function (Bonthius and West, 1988; Dobbing and Sands, 1979; Guerri, 1998; Miki et al., 2000, 2003, 2004). The pattern of ethanol exposure also plays a significant role and the extent of damage can be particularly severe with binge-like drinking [which typically achieves a blood alcohol content (BAC) over 200 mg/dl] (Bonthius and West, 1990, 1991; Kelly and Lawrence, 2008).

While it is now widely accepted that the hippocampus is one of the few regions in the mammalian brain where neurogenesis (i.e., the generation of new neurons) continues to occur throughout adulthood (see Section 4.1), the effects of ethanol on this process have only been recently explored. Interestingly, and perhaps not surprisingly, the effects of ethanol on hippocampal neurogenesis seem to parallel those observed on hippocampal cell numbers, and deficits in this process are also related to the dosage, timing, pattern, and method of alcohol administration during prenatal or early postnatal life. Because the model of exposure used can dramatically impact these findings, in the following section we will review the different FASD rodent models that are currently used in research.

2. Animal models of FASD

Animal models can be effective and reliable tools for the experimental study of the teratogenic effects of alcohol on the developing brain and they have made numerous 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 uncontrollable and confounding variables that include; multi-substance abuse, diet, maternal health, genetic background, social or socioeconomic variables, experiential variability, and limitations or discrepancies in self-reporting. Animal models of FASD eliminate many of the obvious confounds associated with human subject studies. Furthermore, properly designed animal studies allow the experimenter to control for stress and nutrition variables, in addition to variations in alcohol consumption patterns (Kelly and Lawrence, 2008). Moreover, animal models allow for investigation of specific patterns of alcohol exposure (i.e. chronic versus binge-like drinking), manipulation of the peak BAC reached, the developmental timing and duration of exposure, as well as the investigation of specific brain regions or cells types (Maier et al., 1996; West et al., 1994).

Animal models also mimic the human condition fairly well, and 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 prenatal alcohol exposure (e.g., chicks, mice, rats, guinea pigs, or nonhuman primates) (Goodlett and Horn, 2001). However, similar to the variability observed in humans, there is no single animal model that mimics all of the classical features of FAS and FASD (Cudd, 2005; Hannigan, 1996). Nevertheless, different models do mimic different facets of this disorder, and the experimental question is paramount to determining what model to use.

Although in the present review we will focus on rodent models of FASD, it is worth mentioning that nonhuman primate models of this disorder have also been established (for review see Cudd, 2005; Schneider et al., 2001, 2007). The use of these models may surpass several limitations associated with rodent models (see Section 2.1.2), as gestation in nonhuman primates closely mimics human pregnancy. Moreover, the use of nonhuman primates is useful when studying complex behaviors and validating basic science findings in an animal that is more like the human. However, rodent models are useful in exploring basic science questions that relate to molecular biology and genetics and that cannot yet be explored in higher-order animals. Indeed, rodents are the most commonly used animal species in FASD research, and there is an extensive body of literature on rodent physiology, anatomy, behavior, reproduction, development and teratology (Cudd, 2005). The physiological responses of rodents to alcohol are also similar to that of humans (Hannigan, 1996) and neurobehavioral outcomes of prenatal alcohol exposure have been fairly consistent with clinical and behavioral outcomes in human studies (Driscoll et al., 1990; Hannigan, 1996). For example, rodent models have demonstrated pre- and postnatal growth restrictions, 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 rodents, CNS dysfunction involves impairment in basic adaptive functioning, reduced neural plasticity and poor learning in tests of learning and memory. In fact, there are several hippocampal dependent learning and memory tasks (i.e., eye-blink conditioning and Morris water maze tests) that can be used for both rodents and humans; this allows for the inclusion of measures of hippocampal functioning, making rodents a prime candidate for such studies.

There are several factors to consider when choosing a rodent model of FASD. These include: the peak BAC level achieved; the timing of exposure to ethanol during the period of brain development; the use of appropriate control groups; and the mode or route of ethanol administration. These factors will be discussed in the following sections.

2.1. *Factors to consider when choosing a model of FASD*

2.1.1. *Blood alcohol content (BAC)*

The peak BAC depends on both dose and pattern of exposure. Indeed, lower doses of ethanol can produce higher peak BACs when administered in a binge-like fashion (Bonthius and West, 1990, 1991), further emphasizing the importance of these two factors on the BAC achieved. In epidemiological studies, estimation of the BAC levels may also depend on additional factors such as metabolic rate, food consumption, tolerance, and genetics (Abel, 1996; Hannigan, 1996). In rodent

studies, the BAC measurement is recorded as the amount of alcohol per unit of blood (usually mg/dl) and this measure is typically taken between 2 and 4 h after the exposure, when the alcohol concentration reaches peak levels in the circulating blood (Serbus et al., 1986). Repeated binge-like exposure (BAC over 200 mg/dl) is usually associated with increased neurotoxicity (Olson et al., 1998), although a single episode of binge-like drinking has also been shown to have deleterious effects (Ieraci and Herrera, 2007; Parnell et al., 2009; see Sections 3 and 4). Furthermore, neuronal damage has also been observed in models characterized by low BAC levels (Choi et al., 2005; Miller, 1995; Redila et al., 2006; Wigal and Amsel, 1990; see Sections 3 and 4). Therefore, although critical, the peak BAC is not the only factor that determines the magnitude of the neuronal damage that is observed in rodent models of FASD.

2.1.2. *Developmental timing of alcohol exposure*

The type and extent of ethanol-induced neurotoxicity are not only determined by the alcohol dose and pattern of administration (i.e., the BAC achieved), but also by the timing of exposure during the period of brain development. Within this scenario, it is critical to recognize that gestation and development in rodents differ from humans, and that this has to be taken into account when designing studies. The human gestation period is characterized by three trimesters, all of which occur prenatally. A brain growth spurt occurs between weeks 25 and 38 of gestation in the third trimester, a time in which marked growth and differentiation take place. This period of rapid brain growth begins at the end of the second trimester, peaks at birth, and then tapers off in early years (Dobbing and Sands, 1979; West, 1987). The rat and mouse gestational period is significantly shorter than humans (rats: 21–22 days; mice: 18–21 days), and the offspring continue to undergo substantial brain development postnatal (Cronise et al., 2001; Tran et al., 2000; Wigal and Amsel, 1990). As with humans, the mouse and rat development period is usually divided into three trimester equivalents, however these rodents do not perfectly mimic the developmental phases of human gestation. The first trimester equivalent corresponds to gestational days (GDs) 1–10. The second trimester equivalent corresponds to GDs 11–21/22 (mice usually give birth on GD21 and rats on GD22). The third trimester equivalent, however, actually corresponds to postnatal days (PNDs) 1–10. Guinea pigs, on the other hand, have a longer gestational period than rats and mice (68 days versus 21/22 days) and have been used to study the effects of alcohol on the developing brain when the growth spurt occurs *in utero*. Consistently with the results that have been observed in rats, guinea pigs that have been exposed to ethanol *in utero* also show evidence of hippocampal dysfunction, including impairments in hippocampal-dependent tasks (Byrnes et al., 2004; Iqbal et al., 2004, 2006; McAdam et al., 2008; Richardson et al., 2002), reduced hippocampal long-term potentiation (LTP) (Byrnes et al., 2004; Richardson et al., 2002), and increased hippocampal cell loss (Gibson et al., 2000; Green et al., 2005; McGoey et al., 2003). While offering the advantage of being a true *in utero* exposure model, the longer gestational period and smaller litter size can be a disadvantage for some experimental designs.

In addition, the sensitivity of the CNS to the effects of alcohol varies throughout the perinatal period, with specific cell types being more sensitive at certain stages or even on certain days (Brodie and Vernadakis, 1990; Cartwright and Smith, 1995; Coles et al., 1992; Coles, 1994; Davis et al., 1990; Rahman et al., 1994; West et al., 1990). In the rat brain, the first critical period of neuronal development occurs between GDs 5 and 11, when organogenesis occurs, the neural tube is formed, and the proliferation of neuronal precursors takes place in zones adjacent to the neural tube (for review see Guerri, 1998). Exposure of rodents to high levels of ethanol during this period can cause major neural tube defects and lead to facial dysmorphologies similar to the ones observed in children affected with FAS (Sulik et al., 1981). The second critical developmental stage occurs between GDs 11 and 21. At this period, most of the areas of the nervous system, except the cerebellum, are differentiating. During this time, neuronal generation and migration also occur in some areas of the brain (including the cerebral cortex and the hippocampus). However, even within a given brain region, the developmental stage of the various cell populations might differ. For example, in the hippocampus, pyramidal cells are generated during gestation, whereas dentate granule cells are not produced until the postnatal period (for review see Guerri, 1998). Ethanol exposure during this stage can affect the proliferation, generation, and migration of neurons from the neocortex, hippocampus, and the main sensory nucleus (Miller, 1992). The last critical period of development (equivalent to the brain growth spurt in humans) occurs from GD18 to PND9. During this period there is a marked increase in brain weight due in part to the proliferation of astroglial and oligodendroglial cells, as well as synaptogenesis, and dendritic arborization. At the same time, neurogenesis (i.e., the generation of new neurons) continues to occur in the cerebellum (for review see Guerri, 1998) and the dentate gyrus (DG) of the hippocampus (Bayer, 1980, 1982; Schlessinger et al., 1975). Ethanol exposure during this period may induce severe neuronal loss (Bonthius and West, 1990, 1991), alterations in dendritic arborization and synaptogenesis (Guerri, 1987), reactive gliosis (Goodlett et al., 1993), delayed myelination (Lancaster et al., 1984), as well as damage in the hippocampal (West et al., 1984) and cerebellar (Borges and Lewis, 1983) regions (the two main brain areas undergoing rapid development at this period). Importantly, certain structures may be more vulnerable to the deleterious effects of alcohol during the period of intensive cell genesis (Vorhees and Fernandez, 1986), while others (e.g., the Purkinje cells in the cerebellum) might be more susceptible during the phase of cell differentiation (Marcussen et al., 1994). Thus, prenatal/neonatal exposure will affect different brain areas depending on the proliferation, differentiation and migration patterns of neurons within these specific regions.

Given the above considerations, it is important to consider regional and temporal windows of neuronal vulnerability when studying FASD. In particular, the inclusion of the third trimester equivalent must be considered when developing an effective animal model. This is especially true in that the third human trimester appears to be a particularly sensitive period to the deleterious effects of ethanol (Bonthius and West, 1990, 1991; Goodlett et al., 1990; Maier et al., 1997; Napper and West, 1995; Phillips and Cragg, 1982). For example, cessation of

alcohol consumption by a pregnant woman during this period lessens microencephaly and limits the severity of physical and cognitive deficits (Coles et al., 1985, 1991). However, it must be emphasized that the third trimester equivalent in rats and mice is fundamentally different from the third trimester of human pregnancy, as it occurs outside the confines of the placental barrier. Any third trimester equivalent model that uses these species must therefore consider contributing factors such as parturition and the absence of the placental barrier when evaluating the effects of alcohol on the developing brain. For example, when ethanol is metabolized by the maternal liver alone, the dam and pup BAC levels are known to be the same throughout gestation (Miki et al., 2008). Alcohol dehydrogenase can be first detected in the pup liver at GD18 at which time it functions at approximately 25% of adult capacity, whereas adult activity levels are not reached until after parturition at PND18 (Raiha et al., 1967). Because of this, it is commonly reported that higher pup BAC levels can be achieved with lower alcohol doses than the doses used for the pregnant dams (Livy et al., 2003; Tran and Kelly, 2003).

Very few studies have looked at the effects of alcohol exposure across all three trimester equivalents of rodent pregnancy, likely due to the time and technical difficulties of such a paradigm. Furthermore, as there are numerous potential stressors associated with the treatment of pregnant dams and neonate rodents, *in vivo* FASD studies require a careful experimental design.

2.1.3. *Nutrition, stress and control groups*

Animal studies have illuminated the fact that nutritional effects that accompany alcohol consumption are difficult to separate from the teratogenic effects of alcohol alone (Dreosti, 1993; Fisher, 1988; Redila et al., 2006; Schenker et al., 1990). Reduced maternal diet associated with alcohol consumption has been known to exacerbate the effects of alcohol exposure, (Leibel et al., 1993) and ameliorating the nutritional deficits that accompany alcohol consumption may in turn limit the extent of alcohol teratogenesis (Dreosti, 1993; Hannigan, 1996).

Given the fact that poor nutrition associated with alcohol consumption can profoundly affect the developing fetus (Abel and Hannigan, 1995) and since ethanol-treated animals have a reduced dietary intake, appropriate pair-fed controls are important to control for the effects of dietary restrictions. One way this can be achieved is by introducing a calorie-matched control group. Typically a carbohydrate supplement is used (such as maltose-dextrin) in replacement of ethanol, and any consumption of standard rodent chow should be restricted to that of the ethanol condition. Furthermore, a pair-fed control can also act as a control for the stress related to any procedures involved in the administration of alcohol, especially since interactions between prenatal stress and alcohol exposure have been previously reported (McGivern, 1989; Ward and Wainwright, 1991). While control for nutrition/stress is important, pair-feeding may be viewed as an imperfect control (i.e., it has been repeatedly observed that these control groups differ in several biological and behavioral measures, such as body weight) (Redila et al., 2006). Furthermore, the pattern of food consumption of the pair-fed group will likely differ from that of the ethanol-treated group, as these control animals will typically consume their allotted daily food as soon as it is available, and so may experience additional stress from food restriction during the remaining period of the day. As well, pair-fed animals may not control for secondary nutritional effects, such as the effect ethanol may have on absorption of nutrients. In this sense, this form of 'control' may be viewed as a treatment group in itself and therefore a 'base-line' (non-handled, *ad libitum*, or sham) control group is always appropriate (Glavas et al., 2007; Hofmann et al., 1999; Redila et al., 2006; Weinberg, 1984).

2.2. *Mode of administration*

There are several different modes for the administration of ethanol during pregnancy. In the following sections we will provide an overview of the four methods of administration that have been most commonly used in FASD research: (1) via the drinking water or a specific liquid diet; (2) via oral/intragastric intubation or gavage; (3) via vapor inhalation, or (4) using an artificial rearing paradigm (for additional reviews see Abel, 1980; Kelly and Lawrence, 2008; Riley and Meyer, 1984) (Table 1).

It is also worth mentioning that ethanol can also be administered as either a subcutaneous (s.c.) (see for example, Ikonomidou et al., 2000; Izumi et al., 2005; Wozniak et al., 2004), or an intraperitoneal (i.p.) injection (see for example, de Licona et al., 2009; Dunty et al., 2001; Strahlendorf and Strahlendorf, 1983; Ukita et al., 1993; Webster et al., 1983). These routes of administration have been consistently used in acute mouse models of prenatal/early postnatal ethanol exposure (rats are usually preferred for chronic models), allowing for a rapid increased in BAC with limited handling-induced stress. However, these methods are far less reliable for long-term/chronic exposure paradigms and the extremely high BACs achieved might not reflect the typical values obtained upon ethanol ingestion. Furthermore, they do not mimic the routes of intake in humans and may not accurately replicate several important aspects of human prenatal ethanol exposure. In fact, it has been shown that administration of ethanol i.p. to pregnant mice during the organogenic period results in a significantly higher incidence of malformations when compared with the same ethanol dose delivered via intubation (Webster et al., 1983). Furthermore, Clarke and collaborators (1985) have also observed that i.p. administration of ethanol to pregnant guinea pigs results in the distribution of ethanol from the peritoneal space across the uterus and chorioamniotic membranes into the amniotic fluid in addition to absorption into the maternal blood circulation and subsequent placental transfer into the fetus. As a consequence, the fetus is exposed to a very high ethanol concentration in the amniotic fluid immediately after ethanol administration, which strongly indicates that the i.p. route does not accurately mimic ingestion of ethanol by pregnant rodents (Clarke et al., 1985).

2.2.1. *Ethanol in liquid diet*

Adding ethanol to a flavored liquid diet has proven to be a very effective method for self-administration of ethanol in rodents (Lieber and DeCarli, 1982; Redila et al., 2006; Sherwin et al., 1979; Weinberg, 1984). In most self-administration models,

Table 1 – Advantages and disadvantages of the four most commonly used rodent models of ethanol administration during the period of brain development.

Method	Advantages	Disadvantages
Liquid diet	• Time and labor efficient • Low handling/procedural stress • Low stable BAC • Nutritional control • Used prenatally	• Cannot control dose/timing • Does not mimic binge drinking • Cannot achieve high BAC • Cannot be used postnatally
Gastric intubation	• Mimics binge drinking • Precise control over dose/timing • Reliable high BAC • Nutritional/stress control • Used both pre and postnatally	• Potentially stressful and invasive • Handling of neonates • Can lead to loss of animals • Nutritional controls show discrepancies
Inhalation	• High stable BAC • Low handling stress • Time and labor efficient • Used both pre and postnatally	• Neonates away from mother for extended period • No nutritional control • Requires special equipment
Artificial rearing	• Postnatal • Control dose/timing of exposure • Mimics human third trimester	• Postnatal use only • Mother and litter deprivation • Extremely invasive • Stressful • Requires special equipment

ethanol is added to a commercially available nutritional formula. Common formulas include Liqui-diet, (Bio-Serve, Frenchtown, NJ) or Sustacal (Mead Johnson, Evansville, IN). Using this method rats can consume on average 12 g of ethanol/kg/day (or up to 18 g of ethanol/kg/day) (Fig. 1C). To achieve low BACs, rats require approximately 18% ethanol derived calories (EDC), while higher BACs require approximately 35% EDC (Berman and Hannigan, 2000). 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 is increased in order to get animals accustomed to it (Allan et al., 2003; Choi et al., 2005). In these models, pair-fed diets can use an iso-volumic and iso-caloric replacement (such as maltose-dextrin) for the ethanol calories and food can be restricted to that of the ethanol group's consumption (Berman and Hannigan, 2000).

This technique is advantageous in that it is less time consuming and labor intensive than other methods, reduces the potential handling stress of more invasive techniques, and is generally safer (i.e., results in fewer animal fatalities). Furthermore, it is shown to be reliable at producing low stable BAC levels in pregnant dams. However, this method does not allow for the control of precise dosage or timing of consumption. For example, Mankes et al. (1992) found that the ethanol consumption of a group of 221 rats that were fed with a liquid diet containing 6.1% ethanol, ranged anywhere from 4 to 18 g of ethanol/kg/day depending on the rat. As well, this technique cannot be used to mimic binge-like drinking behavior, as BACs do not reach a binge-like level (>200 mg/dl). Lastly, although this form of administration could potentially be used for breast-feeding pups by continuing the dam on an ethanol-containing diet, the actual dose of ethanol that would be consumed by the pups could not be properly controlled. Moreover, intoxicated mothers could potentially fail to engage in appropriate maternal behaviors, and their pups could incur both social and nutritional stress. Thus, this mode of administration is not preferred when specifically using third trimester equivalent (PNDs 1–10) mouse or rat models of FASD.

2.2.2. *Intragastric intubation*

In this method, ethanol is delivered directly to the stomach using a gavage (or force-feeding) strategy (Cronise et al., 2001; Kelly and Tran, 1997; Serbus et al., 1986; Tran et al., 2000). The procedure uses a syringe attached to a curved steel gavage needle or plastic tubing that is inserted down the esophagus to the entrance of the stomach. The ethanol is diluted in water, in vehicle solution (e.g., saline), or in a nutritional formula (Fig. 1A). A nutritional 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 rodent chow can be restricted to that of the ethanol group's consumption. Alternatively, a stress/handling control group can receive a sham intubation. Doses of ethanol typically range between 2 and 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) can be used in the treatment of neonate pups (Fig. 1B), and thus inclusion of the third-trimester equivalent is possible. However, when intragastric intubation is used postnatally, it is common to observe body weight differences between alcohol-exposed and control pups, in large part because high levels of alcohol will inhibit suckling behavior. In addition, if an iso-caloric diet is given to the control subjects, they will often get heavier than normal controls (Goodlett and Johnson, 1997; Goodlett et al., 1998). A possible

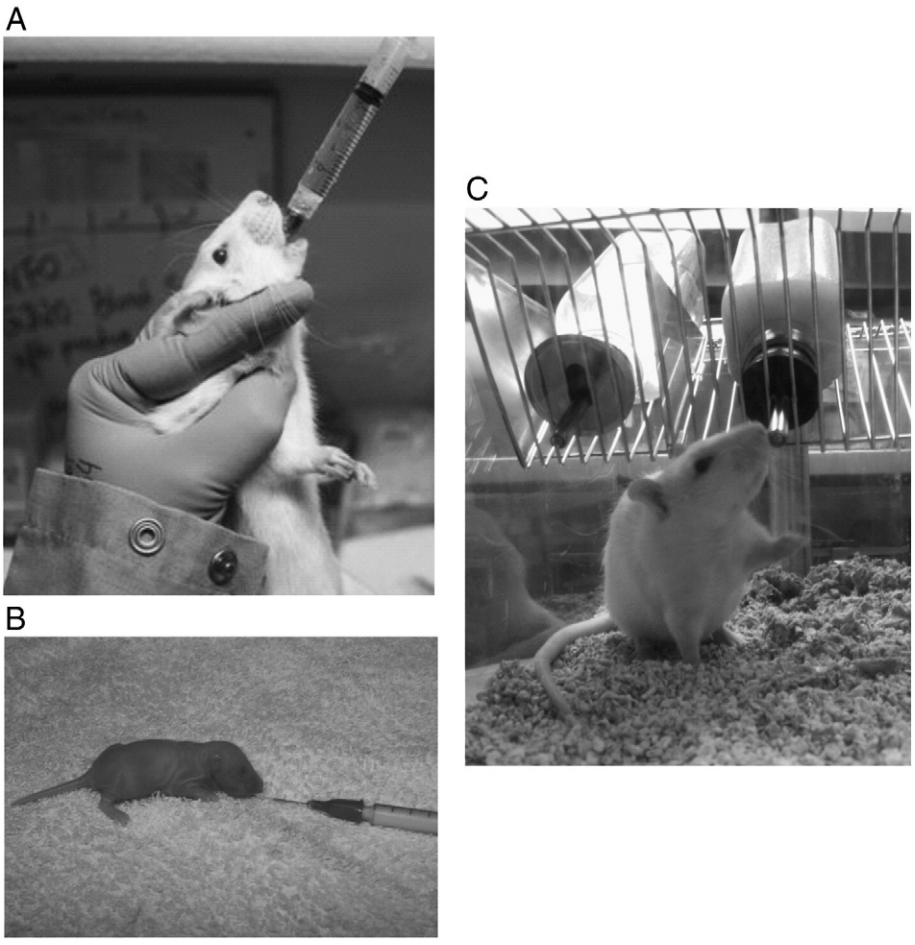

Fig. 1 – The most widely used rat models of FASD. (A, B) The intragastric intubation (gavage) model. In this model, ethanol is delivered directly to the stomach of pregnant dams using a syringe attached to a curved steel gavage needle or plastic tubing that is inserted down the esophagus to the entrance of the stomach (A). This procedure (typically using plastic tubing attached to a syringe) can also be employed in the treatment of neonate pups (B). In this model of binge-like drinking, doses of ethanol typically range between 2 and 6 g/kg. (C) The liquid diet model. In this self-administration model, ethanol is added to a commercially available nutritional formula and no standard rodent chow is provided. Pregnant dams will voluntarily consume on average 12 g of ethanol/kg/day.

alternative will be to supplement the diet of the ethanol-treated pups with an extra-feeding of milk solution (with no ethanol) while performing a sham intubation on the control pups (Gil-Mohapel et al., unpublished observations). Importantly, there are also several potential stress effects involved in this invasive procedure. Researchers need to be specially trained to perform the technique safely and effectively to minimize stress in pregnant dams and neonate pups, and even with adequate training fatalities in these animals are not uncommon.

2.2.3. *Inhalation method*

In this method, pregnant dams or neonates are placed in an inhalation chamber filled with ethanol vapor for several hours (Karanian et al., 1986; Miki et al., 2008; Pal and Alkana, 1997; 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., 2008; Nelson et al., 1990). In many ways, the effects obtained with this technique are comparable with the ones obtained with gavage feeding with the advantage of requiring less labor and involving less handling of the 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 h a day) (Miki et al., 2008), and there is no effective control group to account for the loss in nutrition of the ethanol-exposed pups. Alternatively, neonatal pups can be exposed to alcohol without being separated from the dam. Because of differences in metabolism (see Section 2.1.2), the dam can remain in the vapor inhalation chamber but experience blood alcohol levels that are much lower than that of the pups.

2.2.4. *Artificial rearing*

In this "pup-in-the-cup" method, neonate pups are exposed to alcohol in a way that is functionally similar to that of the third human trimester of 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 (Bonthius and West, 1990; Kelly et al., 1988, 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 (or a control solution) is administered using a programmable pump that can administer alcohol chronically or periodically. Additionally, a non-treated control group is required in order to properly distinguish the effects of ethanol administration from those of the procedure. Therefore, this model mimics the third trimester of human pregnancy, is reliable in producing consistent BAC levels, and allows for a pair-fed control that receives the exact same amount of food as the ethanol-exposed group (a problem with neonatal gavage; Section 2.2.2). Thus, although this method cannot account for any ethanol-related differences in nutrient absorption, at least food intake can be properly controlled. However, the procedure is costly, labor intensive, and extremely invasive, involving potential health complications for the neonates. Furthermore, it requires pups to be removed from their mother (and consequently from the mother's milk) as well as from their litter-mates during this important period of brain development. Nevertheless, it is surprising that many studies do not report major differences between artificially reared and non-treated control groups, likely because the pups, although separated from the mother, receive stimulation from the researchers, which can potentially mimic maternal care.

In conclusion, there is currently no "ideal" rodent model to mimic FASD, as all the methods described above are associated with different potential advantages and disadvantages (Table 1). These have to be carefully taken into consideration when planning FASD studies. Furthermore, caution is needed when drawing comparisons between studies that used different FASD rodent models, as the different characteristics of each model might have influenced the results.

3. Effects of prenatal/neonatal ethanol exposure on hippocampal cell populations

It is now well established that prenatal ethanol exposure induces a decrease in brain volume, which results from apoptotic cell loss. The affected brain structures include the cerebral cortex, amygdaloid body, basal ganglia, corpus callosum, cerebellum, and the hippocampus (Archibald et al., 2001; Autti-Ramo, 2002; Byrnes et al., 2004; Ikonomidou et al., 2000; Klintsova et al., 2007; Roebuck et al., 1998). The observed cellular damage and its persistency depend on the dose, time, and frequency of ethanol exposure during development (Rosett et al., 1983).

Furthermore, it is likely that withdrawal symptoms can also occur during fetal development and that alcohol withdrawal by a pregnant woman might exacerbate the adverse effects of alcohol on the developing fetus (for review see Thomas and Riley, 1998). Indeed, several rodent studies have shown that ethanol withdrawal may lead to excitotoxic neuronal cell death [caused by an over-activation of glutamate *N*-methyl-D-aspartate (NMDA) receptors] (for review see Hoffman and Tabakoff, 1994). Moreover, these alterations in NMDA receptor activation were shown to contribute to the behavioral deficits associated with developmental alcohol exposure, as these could be significantly attenuated in rats treated with the NMDA receptor antagonist MK-801 during ethanol withdrawal (Thomas et al., 1997, 2001, 2002). It is important to point that the contribution of alcohol withdrawal to the neuropathology (i.e., neuronal cell death) and behavioral deficits that result from prenatal ethanol exposure is especially relevant when interpreting the results obtained using rodent models of binge-like drinking (e.g., gastric intubation), when acute exposure to high doses of ethanol is experienced throughout gestation and/or the first 10 days of postnatal life.

In addition, different populations of neurons might be more or less susceptible to the deleterious effects of alcohol depending on their location within the developing brain. In particular, the effects of prenatal alcohol exposure on the cell populations of the hippocampus have been inconsistent (Table 2).

3.1. *Effects of prenatal/neonatal ethanol exposure on the number of hippocampal cells*

The liquid diet model and artificial rearing have been commonly used alone or in combination to evaluate the effects of ethanol exposure during critical periods of brain development (i.e., first and second trimester equivalents and/or third trimester equivalent, respectively) on hippocampal cell density. In an initial study, Barnes and Walker (1981) have examined the effects of prenatal ethanol exposure on the density of dorsal hippocampal pyramidal and DG granule cells of the offspring using the liquid diet model throughout gestation (i.e., first and second trimester equivalents). Interestingly, they found 20% fewer dorsal hippocampal pyramidal cells at PND60 (i.e., when animals reached adulthood) in the brains of the rats that were exposed to ethanol during gestation when compared with those of control animals. Prenatal ethanol exposure, however, did not affect the density of DG granule cells (Barnes and Walker, 1981). In a subsequent study, West and colleagues (1986) used an artificial rearing procedure from PNDs 4–10 to evaluate the effects of ethanol exposure on hippocampal cell populations. They found that a BAC of approximately 380 mg/dl induced a 20% reduction in the brain weight to body weight ratio in PND10 ethanol-exposed rats. However, at this time point, ethanol exposure did not produce deficits in the densities of CA1 or CA3 neurons but induced a 10% increase in the density of DG granule cell layer neurons (West et al., 1986). In agreement, rat pups that were reared artificially from PNDs 4–10 and received a daily ethanol dose of 4.5 g/kg condensed into two or four feedings (BACs of 361.6 and 190.7 mg/dl, respectively) showed significant microencephaly and cell loss, relative to controls (Bonthius and West, 1990). With respect to the hippocampus however, CA1 neuronal density was significantly reduced only by the most condensed alcohol treatment, while CA3, CA4, and the DG populations were not affected by any paradigm (Bonthius and West, 1990). Interestingly, the same findings were observed when the offspring was allowed to live up to adulthood (PND90), indicating that early postnatal ethanol exposure causes the permanent loss of neurons specifically in the CA1 region of the hippocampus

Table 2 – Summary of the published literature on the effects of prenatal and early postnatal ethanol exposure on cell numbers or density of the various hippocampal cell populations.

Species and period of exposure	Method	BAC mg/dl	Changes in cell numbers or density	Age of animals	Reference
Rat GD7	25% ethanol, i.p.	nd	↓ all hippocampal sub-regions	PND66	(Diaz Perez et al., 1991)
Rat GDs 1–21	Liquid diet	nd	↓ 20% dorsal hippocampal pyramidal cells ↔ DG granule cells	PND60	(Barnes and Walker, 1981)
Rat GDs 6–21	Liquid diet	144	↓ CA1 neurons ↔ DG granule cells	PNDs 30–35	(Miller, 1995)
PNDs 4–10	Artificial Rearing	132 339	↑ DG granule cells ↓ DG granule cells ↔ CA1 neurons	PNDs 30–35	
Rat GDs 1–20	Gavage	314	↔ CA1, CA3 and DG neurons	PND10	(Livy et al., 2003)
PNDs 4–10	Gavage	359	↓ CA1, CA3 and DG neurons	PND10	
GDs1–20 and PNDs 4–10	Gavage	335	↓ CA1, CA3 and DG neurons	PND10	
Rat GDs 1–20	Gavage	136–422	↔ CA1, CA3 and DG neurons	PND10	(Maier and West, 2001)
Rat GDs 1–21 and PNDs 4–12	Liquid diet and artificial rearing	82.6	↓ 12% CA1 pyramidal cells ↓ 11% mature DG granule cells	PND21	(Wigal and Amsel, 1990)
Rat GDs 1–22 and PNDs 2–10	Gavage	298–398	↓ CA1 neurons ↔ CA3 and DG neurons	PNDs 112–174	(Tran and Kelly, 2003)
Rat PNDs 4–10	Artificial rearing	380	↔ CA1 and CA3 neurons ↑ 10% DG granule cells	PND10	(West et al., 1986)
Rat PNDs 4–10	Artificial rearing	361.6	↓ CA1 neurons ↔ CA3, CA4 and DG neurons	PND10	(Bonthius and West, 1990)
Rat PNDs 4–10	Artificial rearing	361.6	↓ CA1 neurons ↔ CA3, CA4 and DG neurons	PND90	(Bonthius and West, 1991)
Rat PNDs 10–15	Ethanol inhalation	430	↔ DG granule cells ↓ Hilus neurons	PND16 PND30	(Miki et al., 2003)
Mouse PND7	2× s.c. 2.5 g/kg	510	↑ apoptosis in diencephalic structures that form an hippocampal circuit	PND54	(Wozniak et al., 2004)
Guinea pig GD2–67	Gavage 4 g/kg	246	↓ 25–30% CA1 pyramidal cells	PND12	(Gibson et al., 2000)
Guinea pig GD2–67	Gavage 4 g/kg	220–230	↓ 25–30% CA1 pyramidal cells	GD62 PNDs 1, 5, 12	(McGoey et al., 2003)
Guinea pig GD2–67	Gavage 4 g/kg	281	↑ hippocampal apoptosis	GD65 PND1	(Green et al., 2005)
Guinea pig GDs 44–62	Gavage 2–4 g/kg	340	↔ CA1, CA3 and DG neurons	GD63 PND10	(Byrnes et al., 2004)

BAC, blood alcohol level; GD, gestational day; nd, not determined; PND postnatal day; i.p., intraperitoneal; ↑ significant increase; ↓ significant decrease; ↔ no change.

(Bonthius and West, 1991). Differences in the BACs achieved might account for the discrepant results observed between the study by West et al. (1986) (i.e., increased density of DG granule neurons) and those by Bonthius and West (1990, 1991) (i.e., a reduction in the density of CA1 neurons).

In a different study, Wigal and Amsel (1990) used the liquid diet model throughout gestation (GDs 1–21) and artificial rearing during the third trimester equivalent (PNDs 4–12). They found that exposure to 3% ethanol (i.e., low BAC of 82.6 mg/dl) was enough to induce alterations in hippocampal neuroanatomy that were detectable at PND21. More precisely, hippocampal cell density showed a 12% reduction in CA1 pyramidal cells and an 11% reduction in mature granule cells in ethanol exposed rats when compared with their age-matched controls (Wigal and Amsel, 1990). In agreement, Miller (1995) reported that ethanol exposure through most of gestation (using the liquid diet model from GDs 6–21; BAC: 144 mg/dl) induced a decrease in the total number of neurons (assessed by a modified stereological method) in the CA1 segment of the hippocampus that was detected at PNDs 30–35

and had little impact on neuronal numbers in the DG. However, when ethanol was administered postnatally by artificial rearing, the effects on the number of DG granule cells were highly dependent on the BAC achieved, with moderate levels (132 mg/dl) inducing an increase in the number of granule cells, and higher levels (339 mg/dl) causing a decrease in DG cell numbers. Postnatal ethanol treatment had no effect on the number of CA1 neurons (Miller, 1995).

The results obtained with the intragastric intubation model have been somewhat different. Using stereology, Livy and colleagues (2003) found a reduction in cell numbers in the CA1, CA3 and DG at PND10 following third trimester equivalent binge-like exposure (i.e., using the intragastric intubation procedure; BAC: 359 mg/dl). Similar results were observed after binge-like ethanol exposure throughout gestation and the third-trimester equivalent (BAC: 335 mg/dl). However, when administered throughout gestation alone (i.e., no exposure during the early postnatal period; BAC: 314 mg/dl), alcohol exposure did not significantly reduce cell counts in these sub-regions of the hippocampus (Livy et al., 2003). Importantly, this finding is in agreement with a previous study by Maier and West (2001), who showed that binge-like exposure to ethanol throughout gestation alone (via intragastric intubation) had no effect on the total number of pyramidal cells in the CA1 and CA3 sub-regions as well as on the total number of granule neurons in the DG at PND10 (Maier and West, 2001). These results suggest, that at least in this model, hippocampal cell loss is the result of alcohol exposure during the early postnatal period (PNDs 4–10) (Livy et al., 2003; Maier and West, 2001). On the other hand, Tran and Kelly (2003) used the gavage technique throughout gestation and during the first 10 days of life (producing BAC levels from 298 mg/dl at GD10 to 398 mg/dl at PND10) to estimate the total number of pyramidal and granule cells within hippocampal regions CA1, CA3 and DG when rats reached adulthood (PNDs 112–174). They concluded that the CA1 region is highly susceptible to ethanol exposure over the early neonatal period or all three trimester equivalents, while areas CA3 and DG were more resistant to ethanol (Marino et al., 2004; Tran and Kelly, 2003). The age of the offspring at the time of analysis may contribute to differences between these studies, and a reduction in CA3 and DG cell populations can be initially detected at PND10 when animals were exposed to ethanol during the three trimester equivalents (Livy et al., 2003) but not later on, when animals reach adulthood (Tran and Kelly, 2003). Thus, it is possible that compensatory mechanisms might have underly a recovery in these hippocampal sub-regions.

Interestingly, rats exposed to a high daily dose of ethanol vapor from PNDs 10–15 also do not seem to show DG cell loss. Indeed, placement in an ethanol vapor chamber for 3 h a day to produce high BACs (430 mg/dl) did not produce significant differences in the total number of DG granule cells between ethanol-treated rats and their age-matched controls at either PND16 or PND30. In contrast, ethanol-treated rats had significantly fewer neurons in the hilus sub-region of the DG than control animals at PND16, but this effect was not present at PND30 (Miki et al., 2003). This study suggest that a short period of ethanol vapor exposure during early life can affect the numbers of neurons in some DG cell populations, but not all, and that the decrease can be compensated over time, likely through the process of adult neurogenesis. However, Diaz Perez and colleagues (1991) found that a single exposure to ethanol (one i.p. injection of 25% ethanol at GD7), was enough to induce a decrease in the density of neuronal bodies in all sub-regions of the rat hippocampus that was detectable at PND66 (Diaz Perez et al., 1991).

Studies with guinea pigs have also confirmed the common trend of an increased susceptibility of CA1 neurons to the effects of prenatal ethanol exposure that has been observed with the rat models. Indeed, Gibson and colleagues (2000) have reported that chronic prenatal ethanol exposure (i.e., intragastric administration of 4 g of ethanol/kg throughout gestation) produced a decrease in brain and hippocampal weights with no change in body weight (Gibson et al., 2000), and induced a reduction in the density of hippocampal CA1 pyramidal cells by 25–30% that can be detected as early as PND1 (McGoey et al., 2003) and is still observed at PND12 (Gibson et al., 2000; McGoey et al., 2003). Importantly, this reduction appears to be due to mitochondrial apoptotic cell loss (i.e., cytochrome *c* release and subsequent caspase activation) (Green et al., 2005). However, when pregnant guinea pigs were orally intubated with a daily dose of 2 g ethanol/kg on GD43 and/or GD44 and then 4 g ethanol/kg from GDs 45–62 to mimic ethanol consumption during the period of brain growth spurt (i.e., the third trimester equivalent), the results were somewhat different (Byrnes et al., 2004). Indeed, ethanol consumption during this period did not affect the density of hippocampal CA1 and CA3 pyramidal cells and DG granule cells either at GD63 (near-term fetus) and PND10.

Overall, these results indicate that certain hippocampal cell populations might be particularly susceptible to the teratogenic effects of ethanol exposure during different gestational periods. However, a direct comparison between these studies is somewhat problematic due to the different modes of alcohol administration used, the timing of administration, the dosage, the age of the offspring at the time of analysis (see Table 2), as well as the method of cell quantification used. Indeed, while the initial studies reported their results in terms of cell densities (Barnes and Walker, 1981; Bonthius and West, 1990, 1991; Diaz Perez et al., 1991; Wigal and Amsel, 1990), a stereological analysis of the total number of cells is currently the preferred method (Livy et al., 2003; Maier and West, 2001; Marino et al., 2004; Miki et al., 2008; Miller, 1995; Tran and Kelly, 2003). Thus, the sensitivity and accuracy of cell number estimates might vary considerably depending on the method used for the estimations. Importantly, differences in cell densities do not necessarily indicate changes in total cell numbers. For example, density measures with frozen tissue assume that shrinkage is equal among subjects. On the other hand, these potential differences can be accounted for with unbiased stereology [by applying the Cavalieri's principle (Gundersen et al., 1988) and the optical dissector method (West and Gundersen, 1990)]. Together, these experimental differences might explain why some authors find that prenatal, but not postnatal (or third trimester equivalent), ethanol exposure has no effect on DG cell numbers (Livy et al., 2003; Miller, 1995), while others have shown that both prenatal and postnatal ethanol exposure combined have a greater impact on DG granule cell density (Livy et al., 2003; Wigal and Amsel, 1990). Furthermore, while

the severity of deficits in the DG is strongly related to the duration of exposure, it may be that administration of ethanol at different time points can influence when hippocampal cell loss will be most pronounced. Thus, juvenile rats show greater cell loss when ethanol is administered in the third trimester equivalent, than when it is administered prenatal (Livy et al., 2003). However, the same trend has not been observed in PND10 guinea pigs, where ethanol exposure limited to the period of brain growth spurt had no effect on hippocampal cell numbers (Byrnes et al., 2004). On the other hand, while prenatal ethanol exposure appears to have no effect on hippocampal cell numbers when the animals are examined early on (i.e., at PND10) (Livy et al., 2003; Maier and West, 2001), cell loss can be detected later on in life (i.e., when animals are allowed to reach adolescence or adulthood) (Barnes and Walker, 1981; Diaz Perez et al., 1991; Miller, 1995). Nevertheless, a common feature in most studies is that the CA1 hippocampal sub-region is always more affected than the DG (Miller, 1995; Tran and Kelly, 2003).

3.2. *Effects of prenatal/neonatal ethanol exposure on the ultrastructural features of hippocampal cells*

Besides a decrease in neuronal numbers in the various sub-regions of the hippocampus, various studies have also indicated that prenatal/early postnatal exposure to ethanol influences cellular maturation, dendritic architecture, and synapse density of the existing cells in the hippocampal formation.

Particularly in the DG, neurogenesis (see Section 4.1) produces a gradient of cell ages and dendritic morphologies across the dentate granule cell layer, with older cells occupying the outer edge of the granule cell strata near the molecular layer, and younger cells being more prevalent in the inner most layer (Altman and Bayer, 1990; Crespo et al., 1986; Kuhn et al., 1996; Wang et al., 2000). Morphologically, the younger granule cells in the inner layer have smaller and more narrow dendritic processes than the granule cells in the outer layers that extend numerous dendritic processes in a large fan-shaped pattern (Redila et al., 2006; Wang et al., 2000). Both narrow and fan-like cell types can be synaptically activated and they extend axons into the CA3 region to establish contacts with pyramidal neurons (Wang et al., 2000). Importantly, ethanol has been shown to inhibit the development of dendrites in cultures (Lindsley et al., 2002; Yanni and Lindsley, 2000; Yanni et al., 2002), and several groups have reported reductions in the numbers of spines on hippocampal dendrites following prenatal ethanol exposure (Abel et al., 1983; Davies and Smith, 1981; Diaz Perez et al., 1991; Ferrer et al., 1988).

Furthermore, rats that were exposed to ethanol throughout their gestation were found to have abnormally distributed mossy fibers in temporal regions of the hippocampus, causing alterations in neuronal circuitry that persist to maturity (West et al., 1981). Moreover, electron microscopy studies have shown that chronic ethanol exposure during gestation and the third trimester equivalent significantly decreased synapse densities in the strata radiatum and lacunosum-moleculare of the CA1 region of the hippocampus. These changes could be detected as early as PND2 and persisted through the juvenile period (PNDs 14–21) and adulthood (PND70) (Kuge et al., 1993). Importantly, a single dose of ethanol administered at GD7 (25%, administered by i.p. injection) has also been shown to be enough to decrease the number of apical and basal dendritic spines in hippocampal pyramidal neurons of the offspring (detected at PNDs 21, 36, and 66) (Diaz Perez et al., 1991).

Together, these results strongly indicate that prenatal/early postnatal ethanol exposure can have dramatic ultrastructural effects on the various cell populations that comprise the hippocampal formation and that these effects can persist into adulthood. Importantly, a reduction both in the number of cells and in dendritic arborization and spine density is likely to have major functional implications and to contribute, at least in part, to the hippocampal-dependent learning and memory deficits observed in animals that were exposed to ethanol during the period of brain development.

4. Effects of prenatal/neonatal ethanol exposure on adult hippocampal neurogenesis

4.1. *Adult hippocampal neurogenesis*

One of the central dogmas of neuroscience has been the belief that neurogenesis (i.e., the generation of new neurons) only occurs during embryonic development. However, mounting evidence over the past 40 years has challenged that notion (Altman and Das, 1965; Cameron et al., 1993; Kaplan and Hinds, 1977; Kuhn et al., 1996). In fact, proliferation and differentiation of new neurons are now known to occur in selective regions of the adult mammalian brain, primarily the subventricular zone (SVZ) adjacent to the lateral ventricles and the subgranular zone (SGZ) of the DG of the hippocampus.

In the hippocampus, newborn neurons migrate just a short distance from the SGZ of the DG to the granule zone where they integrate into the existing circuitry (Zhao et al., 2007). A dividing progenitor cell gives rise to daughter cells which differentiate, migrate, and integrate extending dendrites towards the molecular layer and an axon towards the CA3 region of the hippocampus (Kempermann et al., 2004). New neurons are fully mature within 4 weeks of mitosis. About 9000 new cells are generated each day in the rodent hippocampus (hundreds of thousands of cells each month, accounting for 6% of the total granule neuronal population) of which about 80–90% differentiate into neurons (Cameron and McKay, 2001). This entire process and each one of its phases are tightly regulated and can be influenced by many factors. While being partially regulated by genetics, adult neurogenesis is also regulated by physiological, pathological, and behavioral factors that regulate the proliferation, differentiation, and the ultimate survival of new neurons. For example, stress (Gould et al., 1998), glucocorticoids (Gould et al., 1992), inflammation (Ekdahl et al., 2003), ethanol (Nixon and Crews, 2002; Section 4.2), opiates (Eisch et al., 2000), and the process of aging (Kuhn et al., 1996) can all down-regulate adult neurogenesis. Conversely, estrogen (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 brain-derived neurotrophic factor (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,b), and environmental enrichment (Kempermann et al., 1997) can up-regulate the capacity for neurogenesis in the adult mammalian brain.

Importantly, the hippocampus is critical for certain aspects of cognition such as learning and memory and newly generated neurons are linked to the functioning of the hippocampus. Furthermore, correlative studies have shown that hippocampal neurogenesis can be modulated by learning and behavioural experience, and that loss of hippocampal neurogenic function can have consequences on memory formation (for review see Bruel-Jungerman et al., 2007). Thus, although still debatable, it is possible that adult hippocampal neurogenesis might contribute to these cognitive processes. Since, as mentioned above, ethanol exposure has been shown to cause learning and memory deficits, adult hippocampal neurogenesis might also be impaired in individuals affected with FASD.

4.2. *Adult hippocampal neurogenesis in models of FASD*

Pioneering studies by Miller have clearly shown that prenatal exposure to ethanol significantly affects the proliferation and migration of cortical neurons in the offspring of female rats that were fed with an ethanol-containing diet during gestation (Miller, 1986, 1987, 1988, 1993). Such abnormalities may cause a desynchronization of cortical development, making it impossible for cortical neurons to form normal circuits. Furthermore, the discovery that prenatal ethanol exposure alters neurogenesis during development has raised the question of whether such insults that are suffered early on in life can have long-lasting effects and compromise the generation of new neurons later on (i.e., during adulthood).

Within this scenario, the effects of prenatal and early postnatal alcohol exposure on adult hippocampal neurogenesis are just now beginning to be explored and a few research groups have investigated various ethanol treatment paradigms to evaluate the effects of ethanol on neuronal linage in the adult hippocampus. These effects seem to be also related to dosage, timing, and method of alcohol administration. In this respect, most rodent studies have used one of two different timeframes of fetal ethanol exposure: either the first and second trimester equivalents together (approximately GDs 1–23), or the third trimester equivalent alone (approximately PNDs 4–10) (Table 3).

Redila and colleagues (2006) administered alcohol to pregnant Sprague–Dawley rats, during the first and second trimester equivalents, in an ethanol liquid diet (35.5% ethanol-derived calories). A pair-fed control was provided an isocaloric liquid diet (maltose-dextrin substitute) and a non-handled control had *ad libitum* access to food and water. The ethanol-exposed rats had free access to the ethanol-diet and the food intake of the pair-fed control group was restricted to that of the ethanol group. Their results showed decreases in cell proliferation, as measured by bromodeoxyuridine (BrdU, an exogenous thymidine analogue that incorporates into the DNA during the S-phase of the cell cycle) labeling at PND50, in ethanol-exposed subjects as compared to non-handled controls but no significant differences between ethanol-exposed and pair-fed conditions. Furthermore, no differences were found in neuronal differentiation between all three conditions. Several factors might have influenced these results. BAC levels peaked at only a moderate dose level of 184 mg/dl. Moreover, stress additionally reduced food consumption in pair-fed controls by approximately 25% of their normal daily intake and a period of fasting that was not accounted for in the ethanol condition. Most importantly, no alcohol was administered during the third trimester equivalent. As previous research has indicated, the hippocampus is particularly sensitive during this period (Ikonomidou et al., 2000; Klintsova et al., 2007; Mooney et al., 1996; Tran and Kelly, 2003; West

Table 3 – Summary of the published literature on the effects of prenatal and early postnatal ethanol exposure on adult hippocampal cell proliferation and/or cell differentiation/neurogenesis.

Species and period of exposure	Method	BAC mg/dl	Changes in cell prolif.	Age prolif.	Changes in cell survival/neurogenesis	Age survival	Reference
Mouse GDs 1–20	Liquid diet	121	BrdU: F ↑ (ns trend) M ↓ (ns trend)	PND95	BrdU: F ↑ (ns trend) M ↓ (ns trend)	PND123	Choi et al. (2005)
Rat GDs 1–20	Liquid diet	184	BrdU↓ (ns trend)	PND57 (M)	BrdU ↔	PND85 (M)	Redila et al. (2006)
Rat PNDs 4–9	Gavage	315	Ki67 ↔	PND50 (M)	BrdU ↓ BrdU/NeuN ↓	PND80 (M)	Klintsova et al. (2007)
Rat PNDs 4–9	Gavage	330	BrdU ↔	PND42 (M)	BrdU/NeuN ↔	PND72 (M)	Helfer et al. (2009)
Mouse PND7	1× s.c. 5 g/kg	nd	PCNA ↓ Sox/GFAP ↓	PND147	BrdU ↓	PND147	Ieraci and Herrera (2007)
Mouse PND7	2× s.c. 2.5 g/kg	510	nd	nd	BrdU/NeuN ↔	PND54	Wozniak et al., 2004

BAC, blood alcohol level; BrdU, bromodeoxyuridine; F, female; GD, gestational day; M, male; nd, not determined; NeuN, neuronal nuclei; PND postnatal day; prolif, proliferation; s.c., subcutaneous; ↑ (ns trend), non-significant trend towards an increase; ↓ (ns trend), non-significant trend towards a decrease; ↑ significant increase; ↓ significant decrease; ↔ no change.

et al., 2001) and leaving out this critical window of development may have influenced the results. Due to the non-significant differences between pair-fed and ethanol-conditions in terms of proliferation and differentiation rates, the effects of handling-induced stress, food restriction, and nutritional factors cannot be accurately parceled out.

Choi and colleagues (2005) used a mouse model of fetal alcohol exposure based on a voluntary drinking paradigm in which mice were trained to drink 10% ethanol throughout pregnancy. Voluntary consumption of 10% alcohol (peak BAC 121 mg/dl) resulted in no changes in the size of the progenitor pool (as assessed by a 12-day BrdU injection regime) and no differences in cell proliferation (as assessed by immunohistochemistry for the endogenous cell cycle marker Ki-67) or neurogenesis (as evaluated by double-labeling for BrdU and neuronal nuclei, NeuN, a commonly used marker for mature neurons). However, exposure to moderate levels of alcohol resulted in a long lasting impaired neurogenic response to an enriched environment, suggesting that moderate levels of alcohol exposure are enough to induce a long-term, persistent defect in the neurogenic responses to behavioral challenge.

In a different study, Klintsova and colleagues (2007) have examined the effects of binge-like exposure to ethanol during the third trimester equivalent alone. Rat pups were intubated with ethanol in milk solution (11.9%, 5.25 g/kg) via intragastric gavage from PNDs 4–10. In this study, no pair-fed control was used, but a sham intubation control was introduced to account for any potential stress effects involved in the intubation procedure. The use of a sham intubation, however, does not adequately account for possible nutritional or calorie restriction-related stress effects of ethanol intubation. With this model, a high BAC (approximately 315 mg/dl) was achieved, causing a significant reduction in the number of mature neurons in the adult hippocampal DG both at PND50 and PND80, without altering cumulative cytogenesis at PND50, indicating that early postnatal (i.e., the third trimester equivalent) binge alcohol exposure results in long-term deficits on adult hippocampal neurogenesis.

In agreement with this study, Ieraci and Herrera (2007) have also shown that even a single exposure of mouse pups to high levels of alcohol at PND7 (by s.c. injection of 5.5 g/kg of ethanol) was enough to decrease the pool of hippocampal progenitor cells and to reduce cell survival in the adult hippocampus. Similarly, Wozniak and colleagues (2004) have shown that binge-like exposure (by two s.c. injections of 2.5 g/kg of ethanol) of infant mice to ethanol on a single day (PND7) induced neuronal apoptosis in specific brain regions that are integral components of an extended hippocampal-diencephalic circuit (that includes the hippocampus, the anterior thalamic nuclei, the mammillary bodies, and the retrosplenial cortex) that is thought to mediate learning and memory. As a result, these mice showed profound impairments in spatial learning and memory at 1 month of age. This impairment was significantly attenuated during subsequent development, indicating recovery of function. However, this recovery was not associated with an increase in adult hippocampal neurogenesis, suggesting that it was a plastic reorganization of the existing neuronal networks that compensated for the early neuronal loss. Importantly, these last two studies indicate that a single ethanol exposure during the early postnatal period can have long-lasting deleterious effects both on the structure and function of the hippocampus. However, it is important to note that in both cases s.c. injections were used as the route of ethanol administration, and as discussed above (see Section 2.2), this method does not reflect the routes of intake in humans and therefore may not accurately replicate several important aspects of human prenatal ethanol exposure.

To summarize, alterations on adult hippocampal neurogenesis have been reported in different models of FASD and the magnitude of the changes observed depends on the model used as well as the BAC achieved (Table 3). Furthermore, differences in the protocols of BrdU administration (namely dose of BrdU, one versus multiple administrations, interval between administrations, and time of sacrifice after the last administration) are also important factors to consider when comparing the outcomes of the studies that used this exogenous marker as a measure of cell proliferation. Nevertheless, a few studies have indicated that even when only a low to moderate BAC is reached (e.g., with a liquid diet that provides 35.5–36.5% ethanol-derived calories and is administered throughout gestation) and no significant reduction in neurogenesis is detected (Redila et al., 2006), significant impairments in hippocampal LTP and hippocampal dependent learning and memory can still be observed (Christie et al., 2005). These studies suggest that at least in the liquid diet model, the dose of ethanol that is required to observe significant changes in hippocampal neurogenesis is higher than the one needed to induce functional alterations in the hippocampal circuitry. Nevertheless, we cannot rule out the hypothesis that subtle alterations in adult hippocampal neurogenesis still occur with low doses of ethanol but cannot be detected due to limitations of the experimental design.

Finally, it is important to point out that the effects of ethanol exposure during the period of brain development on the second neurogenic zone in the adult mammalian brain (the SVZ) have not been thoroughly investigated. Nevertheless, there is at least one study showing a decrease in cell proliferation in this region in rats that were exposed to ethanol during adolescence (Crews et al., 2006), which demonstrates that this region is also sensitive to the effects of ethanol. Whether prenatal and early postnatal ethanol exposure will also have long-lasting effects on the neurogenic function of the SVZ is a topic that deserves further investigation.

4.3. *Possible mechanisms for the ethanol-induced impairment of adult hippocampal neurogenesis*

The reasons for the observed reductions in adult hippocampal neurogenesis upon ethanol exposure during the period of brain development are not entirely clear. *In vitro* studies have shown that ethanol slows cell proliferation, and increases the length of the cell cycle, as well as the rate of cell death (Jacobs and Miller, 2001). Ethanol has also been shown to inhibit neuronal differentiation (Singh et al., 2009; Tateno et al., 2005) and delay neuronal maturation (Singh et al., 2009). In fact, neural progenitor cells isolated from the hippocampus of adult rats that were exposed to ethanol during the period of brain development are characterized by amplified stimulatory effects in response to gamma-aminobutiric acid (GABA), attenuated

stimulatory effects in response to glutamate, an increase in the expression of Wnt proteins, and an increase in beta-catenin-probe binding, characteristics that are associated with a delay in neuronal maturation and synapse formation (Singh et al., 2009).

On the other hand, Klintsova and colleagues (2007) have recently proposed that ethanol may affect the fate of progenitors by causing a dysfunction in astrocytic regulatory activity. Astrocytes release factors that promote differentiation of neural progenitors, such as neurogenesin I, thus being considered a major component of the neurogenic niche. While the effect of alcohol on the release of neurogenesin I is unknown, prenatal alcohol exposure has been shown to reduce levels of other proliferative agents such as fibroblast growth factor (FGF) (Bartlett et al., 1994; Rubert et al., 2006).

An alternative hypothesis is related with the effects of ethanol on hippocampal synaptic circuitry. Interestingly, there has been some controversy regarding the effects of ethanol on the developing hippocampus, partly due to its effects on GABA synapses and the fact that these can have either excitatory or inhibitory actions depending on the developmental stage. Thus, some studies have shown that ethanol can potently increase the frequency of giant depolarizing potentials (which, during development, are generated by the excitatory actions of GABA and glutamate) that occur during the period of growth spurt in the CA3 sub-region of the hippocampus. It is believed that this ethanol-induced potentiation of GABAergic activity is the result of increased GABA release at interneuronal synapses and not the result of increased neuronal excitability (Galindo et al., 2005; Yan et al., 2009). Based on these findings it is reasonable to assume that in the immature CNS ethanol can be a potent stimulant of developing neuronal circuits, which might contribute to the abnormal hippocampal development associated with FASD (Galindo et al., 2005). However, other studies have suggested that similarly to what is observed in the mature hippocampus (Galindo et al., 2005; Proctor et al., 2006; Yan et al., 2009), ethanol may act predominantly as a depressant in the developing CNS by suppressing glutamate release and causing the consequent enhancement of the activity of inhibitory GABA synapses. As a result of this diminished neuronal excitation there is a reduction in neurotrophin release, which in turn can lead to cell death through apoptosis (Ikonomidou et al., 2000). Whether this is a mechanism by which ethanol can reduce the DG progenitor/stem cell pool or decrease the rate of neuronal survival still has to be determined. Nevertheless, this hypothesis would result in an impairment of the hippocampal neurogenic function, which in turn will influence the plasticity of this brain region. In fact, it has been suggested that ethanol exerts its deleterious effects by decreasing neurotrophic signaling between neurons and thus disrupting the establishment of normal neuronal pathways. In the case of new neurons, this is particularly important as they have to establish synapses and to integrate into the existing network in order to survive (Tsuji et al., 2008).

In agreement, alcohol exposure during a narrow window of brain growth spurt (i.e., from PNDs 6–8) was enough to induce a significant reduction in mRNA expression of the trophic factors BDNF and glial cell-derived neurotrophic factor (GDNF) and to decrease the levels of phospho-mitogen-activated protein kinase (pMAPK) and phospho-protein kinase B (PKB/Akt) (Tsuji et al., 2008). Akt promotes cell survival by inhibiting apoptosis through phosphorylation and inactivation of several pro-apoptotic proteins such as Bad (Brunet et al., 1999) and caspase-9 (Cardone et al., 1998). Thus, ethanol-induced reduction in trophic support associated with its inhibition of Akt phosphorylation may account for a decrease in the survival of newly born cells (and a consequent decrease in the pool of neural stem cells) in the hippocampus. Moreover, a reduction in trophic support might also potentially compromise the ability of progenitor cells to divide later on in life. However, a different study that used a liquid diet model throughout gestation and lactation has shown an up-regulation of BDNF mRNA expression at PND60 and unchanged levels of this trophic factor at PNDs 14, 30, and 90 (Barbier et al., 2008). It is likely that these discrepancies are a result of the different FASD models used (intragastric intubation during PNDs 6–8 versus liquid diet throughout gestation and lactation). Nevertheless, the regulation of certain neurotrophic pathways such as the ones leading to the expression of BDNF and GDNF is likely to play a critical role during the vulnerable period of brain growth spurt, when glial proliferation, neuronal differentiation, and synaptogenesis occur (Dobbing, 1974). Thus, further studies are warranted in order to clarify the role of BDNF (and other trophic factors) on the dysregulation of adult hippocampal neurogenesis in animals that were exposed to ethanol during the period of brain development.

4.4. *Environmental enrichment and physical exercise as therapeutic strategies for the FASD-induced reduction in adult hippocampal neurogenesis*

Environmental enrichment has long been used as a means to enhance brain plasticity, and early studies have shown that exposure to an enriched environment (i.e., a combination of complex inanimate and social stimulation) can cause morphological and functional changes in the adult hippocampal formation that include: increases in hippocampal thickness (Rosenzweig, 1966; Walsh et al., 1969), dendritic arborization (Fiala et al., 1978), gliogenesis (Walsh et al., 1969), well as neurogenesis and an increase in cognitive performance (Kempermann et al., 1997). On the other hand, rodents 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- to 12-day period, this can result in the DG generating up to three times the number of newborn cells normally observed in the SGZ of sedentary controls (Farmer et al., 2004; Kronenberg et al., 2003; van Praag et al., 1999a). Although the exact molecular mechanisms underlying the environmental enrichment or exercise-induced up-regulation of hippocampal neurogenesis are still under debate, the levels of many trophic factors known to stimulate neurogenesis such as BDNF have been shown to increase upon exposure to an enriched environment (Gobbo and O'Mara, 2004; Rossi et al., 2006) or after physical exercise (Adlard and Cotman, 2004; Adlard et al., 2005; Farmer et al., 2004; Johnson and Mitchell, 2003; Rasmussen et al., 2009). Thus, the potential to bolster adult hippocampal neurogenesis and rescue hippocampal deficits in rodents that were exposed to alcohol during brain development is promising.

In agreement with this hypothesis, our group has found that voluntary physical exercise is able to attenuate the long-

lasting hippocampal neurogenic deficits in a rat model of FASD (where females received 36.5% ethanol through a liquid diet during gestation, which resulted in BAC of approximately 184 mg/dl) (Redila et al., 2006). Moreover, voluntary exercise was also able to completely reverse the deficits in DG LTP and spatial memory that are typically observed at PND60 in this model of FASD (Christie et al., 2005). In a more recent study, Helfer and colleagues (2009) found that a regime of 12 days of running increased cell proliferation and neurogenesis in adolescent (PND42) rats that were exposed to ethanol during the third trimester equivalent (PNDs 4–9) to the same extent as the increase observed in their age-matched controls. However, the long-term survival (assessed at PND72) of those newly generated cells is impaired relative to the control animals. This inability to sustain the exercise-induced increase in neurogenesis may reflect a decrease in BDNF levels that is expected when animals stop exercising after the 12-day period of running. Thus, in order to observe beneficial effects that are long-lasting, it might be necessary to implement a continuous program of physical exercise.

Several studies have also examined the effects of environmental enrichment on different aspects of hippocampal plasticity in rodent models of FASD. Interestingly, in a mouse model of fetal alcohol exposure (where females received 10% ethanol throughout pregnancy resulting in BAC of approximately 120 mg/dl), the offspring failed to up-regulate hippocampal neurogenesis when housed under enriched conditions from 8 to 12 weeks post-weaning (Choi et al., 2005). Thus, at least in this model, this behavioral stimulation was ineffective in reversing the long-term defect in hippocampal neurogenesis induced by prenatal ethanol exposure. Furthermore, rats that were exposed to ethanol during gestation (via intragastric intubation of the pregnant dams with 6 g/kg/day of ethanol between GDs 8 and 19) and reared for 10 weeks in enriched conditions did not show differences in hippocampal spine densities when compared with ethanol-exposed rats that were housed in standard caging (Berman et al., 1996). However, in a different study, 6 weeks of environmental enrichment were enough to reverse some behavioral deficits (ataxia and performance on the Morris Water Maze test) in rats that were exposed to ethanol prenatally (via intragastric intubation of the pregnant dams with 4 g/kg/day of ethanol) (Hannigan et al., 1993). Taken together, these results suggest that although environmental enrichment might not be sufficient to reverse the reductions in adult hippocampal plasticity that are observed in FASD models (i.e., deficits in adult neurogenesis and spine density), it might still be beneficial in the mitigation of some behavioral deficits in these models.

Thus, although there might be some overlap between the mechanisms responsible for the modulation of adult hippocampal neurogenesis (and hippocampal plasticity) by physical exercise and environmental enrichment (such as an increase in the levels of BDNF and other trophic factors), these two strategies might also activate distinct pathways (for review see Gil-Mohapel et al., 2010; Olson et al., 2006). Therefore, it is possible that ethanol might differentially affect the pathways that are modulated by either physical exercise or environmental enrichment, and consequently the outcomes of these two therapeutic strategies. Thus, it is crucial to increase our understanding of the mechanisms that underlie long-term hippocampal plasticity and how these can be affected by prenatal ethanol exposure in order to better design potential therapeutic interventions. Nevertheless, the promising results obtained with physical exercise suggest that this strategy might be used as a non-invasive therapeutic approach for the treatment of FASD related hippocampal deficits. Moreover, it is possible that combining physical exercise with other means of environmental stimulation might have additional beneficial effects for individuals affected with FASD.

5. Conclusions

The consumption of alcohol during pregnancy impairs the development of the unborn offspring and can lead to a plethora of anatomical, cognitive and behavioral abnormalities. The hippocampal formation is particularly sensitive to the effects of prenatal/neonatal exposure to ethanol and a variety of structural changes in this brain region have been observed in the offspring. These deficits have been shown to persist into adulthood and include a loss of hippocampal neurons (Section 3.1) as well as a decrease in synaptic density and the complexity of hippocampal dendrites (Section 3.2). Furthermore, recent studies have indicated that adult hippocampal neurogenesis is also compromised in several rodent models of FASD (Section 4.2), and this deficit might be caused by a decrease in trophic support (e.g., reduction in BDNF levels) induced by the teratogenicity of ethanol (Section 4.3). However, the deficits observed are strongly dependent on multiple experimental factors such as the rodent model used and the mode and route of ethanol administration (Section 2; liquid diet, intragastric intubation, vapor inhalation, artificial rearing, or acute i.p. or s.c. injections), the timing of exposure during the period of brain development (gestation and/or early postnatal period), the dosage of ethanol administered and the peak BAC achieved (low, moderate, or high), the age of the offspring at the time of analysis (juvenile period, adolescence, or adulthood) as well as the cell quantification methods and the cell proliferation and survival markers employed. Nevertheless, despite the discrepancies between studies, we conclude that the hippocampus is structurally affected by ethanol exposure during the period of brain development. Since this brain region is known to be involved in the processes of learning and memory, it is reasonable to speculate that cell loss, ultrastructural neuronal changes as well as a reduction in neurogenesis might be responsible, at least in part, for the learning and memory deficits that are observed in rodent models of FASD as well as children affected with these disorders. Within this scenario, therapeutic strategies that restore some of these hippocampal deficits (e.g., neurogenesis) such as physical exercise and environmental enrichment (Section 4.4) might be promising for the treatment, or at least the improvement of the quality of life, of individuals affected with FASD.

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