Impact of Environmental Enrichment on Adult Hippocampal Neurogenesis in a Preclinical Mouse Model of Fetal Alcohol Spectrum Disorder (FASD)

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Impact of Environmental Enrichment on Adult Hippocampal Neurogenesis in a Preclinical Mouse Model of Fetal Alcohol Spectrum Disorder (FASD)

by

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DISSERTATION

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Impact of Environmental Enrichment on Adult Hippocampal Neurogenesis in a Preclinical Mouse Model of Fetal Alcohol Spectrum Disorder (FASD)

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Abstract

Alcohol is a potent teratogen to the developing CNS, and moderate-heavy alcohol intake during pregnancy causes fetal alcohol syndrome (FAS) and fetal alcohol spectrum disorder (FASD). These patients display CNS symptoms associated with developmental deficits, cognitive impairment and social abnormalities, with structural impairments in several brain regions.

In this dissertation, we investigated the role of FASD in neurogenic functional deficits in the adult hippocampal dentate gyrus. The dentate gyrus is a subregion of the hippocampus that displays unique plasticity, in that new dentate granule neurons are continuously produced throughout life. Adult hippocampal neurogenesis is thought to play key roles in pattern separation and associative learning and in depression-like behaviors. Interestingly, adult neurogenesis is stimulated by physical and social enrichment (enriched environments) and learning. It is becoming increasingly apparent that prenatal alcohol exposure produces long-lasting impairments in postnatal hippocampal neurogenesis and learning impairment.
My dissertation research addressed the hypothesis that prenatal alcohol exposure (FASD) impairs the EE-mediated survival and electrophysiological properties of newborn DGCs in the adult hippocampus. Utilizing a combination of histological, behavioral and electrophysiological approaches, we addressed the following specific aims

**Specific Aim 1:** To determine whether prenatal alcohol (FASD) impairs the neurogenic response to environmental enrichment in adult Nestin:CreER$^{T2}$:YFP bitransgenic mice; and if so, to delineate the stage of neural stem cell lineage most impacted by prenatal alcohol exposure.

**Specific Aim 2:** To determine whether adult-generated DGCs in FASD mice display normal electrophysiological properties after developing under standard and enriched conditions.

**Specific Aim 3:** To determine whether adult-generated DGCs in FASD mice display dendritic morphological plasticity in response to enriched environment.

My overall findings indicate that FASD severely impairs the late-stage survival of adult-generated DGCs and disrupts the electrophysiological response to EE in the existing granule cell layer, and that this is accompanied by heightened excitatory synaptic transmission in surviving DGCs. These findings suggest that FASD imparts resistance to the full benefits of social and physical enrichment therapies through both impairment of neurogenic and GCL circuitry mechanisms.
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1. Introduction

The goal of this dissertation research was to elucidate the electrophysiological underpinnings of impaired hippocampal neurogenesis in a preclinical mouse model of fetal alcohol spectrum disorder (FASD). Prior studies from our research laboratory demonstrated that the hippocampal neurogenic response to enriched environment (EE) is severely impaired in FASD mice (Choi et al., 2008). Utilizing a combination of histological, behavioral and electrophysiological techniques, my research has explored the hypothesis that the impaired neurogenic response to EE in FASD mice is due to impaired maturation and integration of newborn dentate granule cells (DGCs) within the adult hippocampal dentate circuitry. This Introduction provides a background outlining the therapeutic importance of FASD research, the impact of FASD on the hippocampus, as well as the molecular, cellular and environmental factors regulating adult hippocampal neurogenesis.

1.1 Fetal alcohol spectrum disorder (FASD) and hippocampal function

1.1.1 Fetal alcohol spectrum disorder (FASD)

Clinical significance: FASD presents an important public health issue, with estimated prevalence at nearly 2-5% of children in the U.S (May et al., 2009). FASD is associated with many cognitive and behavioral disabilities and very few therapeutic options (Kodituwakku & Kodituwakku, 2011). Identification of new approaches to mitigate the behavioral and learning deficits in clinical FASD would represent an important step toward improving the lives of individuals afflicted with FASD and alleviating the public health burden of this disorder.
Clinical features of FASD: Alcohol consumption during pregnancy results in a broad range of developmental and cognitive deficits in children. Fetal alcohol syndrome (FAS) can be diagnosed with facial abnormalities (small head, widely spaced eyes, flat midface and thin upper lip) and central nervous system impairment. Fetal alcohol spectrum disorder (FASD) includes FAS, alcohol related neurological disorder (ARND) and alcohol related birth defects (ARBD). Individuals afflicted with less severe forms of FASD are difficult to diagnose, as there is no facial dysmorphology; however, physical, mental, behavioral, and social disabilities persist throughout life in individuals afflicted with FASD (Ismail et al., 2010).

FASD patients show lower IQ (Streissguth et al., 1991; May et al., 2011) and difficulties in planning and problem solving (Kodituwakku et al., 1995). In addition, FASD children have communication difficulties, language problems and several cognitive impairments (McGee et al., 2009). It is now well-accepted that prenatal alcohol exposure leads to impairment in a broad range of human brain functions. Neuroimaging studies have shown decreased volume of several brain regions, including cerebral cortex, corpus callosum, cerebellum and hippocampus, in FASD children (Autton-Ramo et al., 2002). Pertinent to this dissertation research, children exposed to alcohol prenatally display deficits in spatial learning and working memory tasks that are linked to hippocampal function (Hamilton et al., 2003; Burden et al., 2005).

1.1.2 Hippocampal dysfunction in preclinical rodent models of FASD

Although many brain regions are affected in FASD patients, the hippocampus is a critical brain region for learning and memory, and represents an important therapeutic
target in FASD. Elucidating the mechanisms underlying the behavioral and learning deficits in humans with clinical FASD is difficult, due to wide variations in the timing and dose of gestational alcohol exposure and the inherent difficulty for elucidating molecular mechanisms in human studies in general. However, preclinical rodent models of FASD have been created that mimic aspects of clinical FASD (Gil-Mohapel et al., 2011), which have provided insight into neurodevelopmental mechanisms and potential therapeutic targets. As previously mentioned, common behavioral problems in clinical FASD include those associated with hippocampal dysfunction, such as impaired memory processing (Hamilton et al., 2003), and emotional problems (Streissguth et al., 2004). Importantly, rodent models of FASD mimic many of these behavioral deficits, including impairments in hippocampal-dependent learning (Brady et al., 2012; An et al., 2013), and increased depressive and anxiety behaviors (Hellemans et al., 2010; Brocardo et al., 2012).

In rodents, exposure to alcohol during prenatal (1st-2nd trimester equivalent for human brain development) or early postnatal periods of brain development (3rd trimester equivalent) can result in long-lasting structural and functional changes within the hippocampus. The parameters of alcohol-induced damage to the hippocampus are dependent on both dose and timing of alcohol exposure during brain development. For example, offspring of pregnant rats provided with 20% alcohol as the only source of drinking water throughout the prenatal and postnatal period (Daily consumption: 41±1.4 ml/ day, Blood alcohol concentration (BAC) is not reported) results in depleted population of CA1 and CA3 pyramidal neuron loss and hippocampal dentate granule cells (DGCs) (Milotova et al., 2008). Exposure to more moderate levels of alcohol (BAC:
80-120 mg/dl) throughout the 1\textsuperscript{st} and 2\textsuperscript{nd} trimester equivalents (as utilized in this dissertation research) leads to long-lasting changes within the hippocampus, including impaired neurotransmitter receptor expression and activation (Samudio-Ruiz \textit{et al}., 2009; Galindo \textit{et al}., 2004), impaired long-term potentiation (LTP) (Sutherland \textit{et al}., 1997; Varaschin \textit{et al}., 2010; Brady \textit{et al}., 2013) impaired learning and memory (Brady \textit{et al}., 2012; Allan \textit{et al}., 2003), and impaired adult hippocampal neurogenesis(Choi \textit{et al}., 2005; Kajimoto \textit{et al}., 2013). Gestational alcohol exposure also leads to decreased expression of GluN1 and GluN2B NMDA receptor subunits (Samudio-Ruiz \textit{et al}., 2010) and receptor function (Savage \textit{et al}., 1992), and impaired $\gamma$-Aminobutyric acid (GABA) receptor expression and activity within the adult hippocampus (Allan \textit{et al}., 1998; Iqbal \textit{et al}., 2004).

1.1.3 Impaired adult hippocampal neurogenesis in preclinical rodent models of FASD

Increasing evidence shows that gestational alcohol exposure has a negative impact on adult hippocampal neurogenesis. Although different in timing, mode of administration and dose, several studies consistently demonstrate impaired neurogenesis in the postnatal brain following prenatal alcohol exposure. The capacity for self renewal and neural differentiation is impaired in neural stem cells isolated from young adult mice exposed to alcohol during gestation (Roitbak \textit{et al}., 2011). In addition, ethanol induces hypermethylation of genes encoding cell cycle regulatory proteins in neural stem cells and alters the cell cycle length (Hicks \textit{et al}., 2010). These studies suggest that long-term intrinsic mechanisms of neural stem cells are altered by alcohol exposure. In rats, intragastric intubation of alcohol throughout all three trimester equivalents was reported to increase the number of immature DGCs, without a net increase in the number of
surviving postmitotic neurons (Gil-Mohapel et al., 2011). On the other hand, binge alcohol exposure restricted to the third trimester equivalent in rats reduced the survival of newborn neurons (Klintsova et al., 2007). Also, a single subcutaneous injection of alcohol at postnatal day 7 was reported to decrease the proliferation and survival of newborn neurons in adulthood (Ieraci & Herrera, 2007a). Proliferation and survival of newborn neurons were both reported to decrease in offspring of pregnant rats fed by a liquid ethanol diet throughout all three trimester equivalents (Redila et al., 2006). These studies suggest that various aspects of adult hippocampal neurogenesis are disrupted by prenatal or early postnatal alcohol exposure, depending on the dose, timing, species and mode of prenatal alcohol administration. Previous studies by our lab demonstrated that continuous exposure to moderate alcohol throughout gestation does not affect adult hippocampal neurogenesis in group housed mice under standard housing conditions, but significantly impairs the neurogenic response to physical and social enrichment (Choi et al, 2005). These findings suggest that behavioral therapy alone may not be effective in reversing all behaviors that depend on hippocampal neurogenesis (e.g. mood and learning).

1.2 Hippocampus

The hippocampus is a critical structure for learning and memory in the mammalian brain, and the hippocampal dentate gyrus (DG) shows a high capacity for structural plasticity (Leuner & Gould, 2010). The hippocampus processes information through a tri-synaptic circuit. The primary neuronal cell type in the DG is the dentate granule cell (DGC). These small neurons are tightly packed within the dentate granule cell layer (GCL), with dendritic arbors that extend into the hippocampal molecular layer (ML)
where they receive afferent information from the entorhinal cortex (EC) (Amaral et al., 2007). In rodents, the production of DGCs begins during late prenatal development, peaks during the first postnatal week (Schlessinger et al., 1975) and continues at a much slower pace throughout adulthood from a neural stem cell population within the dentate subgranular zone (SGZ). DGCs receive a major presynaptic input from the EC via the perforant path, and send axonal efferents to hippocampal CA3 pyramidal neurons via mossy fiber axons. The perforant pathway originates from layer 2 of the EC, and is represented by medial and lateral pathways. The medial portion of the perforant path processes spatial information, whereas non-spatial information is processed through the lateral perforant path (Koehl & Abrous, 2011). DGC activity is tightly regulated by inhibitory GABAergic interneurons with feedback and feedforward inhibition (Mott et al., 1997; Houser, 2007). CA3 pyramidal neurons project axons to synapse on neighboring CA3 pyramidal neurons, (i.e. Recurrent connections), and to CA1 pyramidal cells (Le Duigou et al., 2014). CA1 pyramidal neurons receive information from CA3 pyramidal neurons via Schaffer collaterals and also receive input from the EC via the perforant path. Similar to DGCs, the activity of CA3 and CA1 pyramidal neurons is also regulated by local interneurons (Bartos et al., 2011; Kullmann, 2011). Then, CA1 pyramidal neurons project information back to EC (Amaral et al., 2007) (Figure 1.1).
Figure 1.1

Figure 1.1. Hippocampal DG tri-synaptic circuit. Dentate granule cells (Orange cells) receive input from entorhinal cortex (EC) and project to CA3 pyramidal neurons. CA3 pyramidal neurons (Green cell) project into CA1 pyramidal neurons (Red cells). CA1 pyramidal cells send information back to cortex. ML: molecular layer.

This unique hippocampal circuit plays critical roles in learning and memory, attentional processes, motivation and emotional states (Cahill & McGaugh, 1998; Eichenbaum, 2000; 2001; 2004; Koehl & Abrous, 2011). Previous studies emphasize that the DG is necessary for acquisition of specific learning and memory tasks, such as spatial pattern separation (Kesner, 2013) and contextual fear conditioning (Hernandez-Rabaza et al., 2008). For example, DG-lesioned rats demonstrate deficits in learning on the radial eight-arm maze (Walsh et al., 1986; McLamb et al., 1988). Also, DG-lesioned rats display impairments in learning the Morris water maze task when the starting location is changed on each trial (Xavier et al., 1999; Jeltsch et al., 2001). In addition, DG-lesioned
rats have deficits in the acquisition and retrieval of contextual fear conditioning learning tasks (Lee & Kesner, 2004). Thus, the DG has an important role for encoding and retrieval of memory and is especially required for distinguishing separate, but highly similar environmental stimulations (Lee & Solivan, 2010).

As mentioned previously, new DGCs are continuously produced throughout life. The production of newborn neurons in the adult brain was originally described by Altman & Das in 1965 (Altman & Das, 1965), but was largely ignored in the scientific literature until additional methods for detecting adult neurogenesis became available. Recent studies have demonstrated that the GCL of the adult DG is heterogeneous, such that DGCs born during embryonic and early postnatal periods are found primarily in the outer two thirds of the GCL, whereas most postnatal DGCs migrate only a short distance from the SGZ to populate the inner to middle two thirds of the GCL (Mongiat & Schinder, 2011; Yu et al., 2014). Thus, the GCL represents heterogeneity of DGCs with respect to their birthdate, i.e., an outer shell of developmentally-generated DGCs and an inner shell containing postnatally-generated DGCs. Whether these regions are functionally distinct is currently unresolved. However, recent studies suggest that adult-generated DGCs play a distinct role in pattern separation (Drew & Hen, 2007; Sahay & Hen, 2007; Sahay et al., 2011; Gu et al., 2012; Nakashiba et al., 2012).

1.2.1 Adult hippocampal neurogenesis

Mentioned above, new DGCs are continuously generated in the DG throughout life. This postnatal neurogenesis is conserved in vertebrates, including birds (Barnea & Pravosudov, 2011), rodents (Altman & Das, 1965), non-human primates (Kornack &
Rakic, 1999), and humans (Eriksson et al., 1998; Spalding et al., 2013). The degree of postnatal neurogenesis decreases with age and with increasing evolutionary brain complexity. In mammals, new neurons are continuously produced throughout adulthood in only two restricted regions, the olfactory bulb (OB) and the hippocampal DG (Brill et al., 2009). This dissertation is focused on elucidating the mechanisms by which prenatal alcohol impacts adult hippocampal neurogenesis. Therefore, the following literature review will focus on neurogenesis in the adult hippocampus.

1.2.1.1 Adult hippocampal neurogenesis from the subgranular zone (SGZ)

In the DG of hippocampus, 4000-7000 new cells are generated each day but less than 30% of these new cells survive and integrate into the hippocampal circuit (Kim et al., 2012). In addition, ~6% of the total population of dentate granule cells represent newly generated neurons (Cameron & McKay, 2001).

In hippocampal neurogenesis, radial glia-like stem cells (Type-I cells) exist within the DG-SVZ which is situated between the granule cell layer and hilus. Radial glia-like stem cells give rise to transient amplifying cells (TAPS; Type-II cells) and TAPs give rise to neuroblasts that migrate a short distance into the DG where they undergo differentiation to mature postmitotic DGCs (Lledo et al., 2006; Ming & Song, 2011). In contrast to OB neurogenesis which produces primarily inhibitory interneurons, the majority of neuroblasts in the adult DG become glutamatergic dentate granule cells. A small percentage of progenitors also become astrocytes (Steiner et al., 2004) (Figure 1.2).

Each stage of the neural stem cell lineage within the adult hippocampus can be identified by stage specific cellular markers. Radial glia-like cells express nestin, glial
fibrillary acidic protein (GFAP), brain lipid-binding protein (BLBP) and sex determining region Y-box 2 (Sox2). TAP cells express the proliferation markers mini-chromosome maintenance proteins 2 (MCM2), Ki67 and proliferating cell nuclear antigen (PCNA). Proliferating neuroblasts also express the proliferation markers, but can be distinguished from TAPs by their expression of the cytoskeletal protein doublecortin (DCX). Finally, as immature neurons transition to a postmitotic state, they continue to express DCX, but not the proliferation markers, whereas mature neurons express NeuN and Calbindin, but no longer express DCX (Ming & Song, 2011) (Figure 1.2).

The sequence of synaptic integration that occurs during neurogenesis in the DG is well characterized. The major milestones of electrophysiological maturation from radial glia-like cell to mature DGC are similar to that which occurs within the DG during early postnatal developmental, but occurs over a much longer timeline (Ge et al., 2006) (Ming & Song, 2011). The electrophysiological maturation of DGCs takes approximately 2 weeks during early postnatal formation of the GCL, but requires approximately 8 weeks from stem cell to mature postmitotic DGC the process of adult neurogenesis. It is noteworthy that newborn DGCs must integrate into an existing circuitry during adult neurogenesis whereas the maturation of DGCs born during perinatal development occurs concomitant with circuit formation. In adult neurogenesis, immature neurons first receive tonic GABAergic input and then phasic GABAergic input at 1 week following their birth and begin receiving glutamatergic input by 2 weeks of neuronal age (Ge et al., 2006) (Figure 1.2). These cells continue to undergo further electrophysiological maturation to fully integrate into the circuit during 4-8 weeks neuronal age. The details regarding the
electrophysiological maturation and synaptic integration of DGCs in adult hippocampus are described in further detail below.

1.2.1.2. Function of adult hippocampal neurogenesis

The electrophysiological properties of the inner layer DGCs show increased cell activity and plasticity at the single cell level (Wang et al., 2000; Schmidt-Hieber et al., 2004). This observation is further supported by retroviral birth date labeling technique that has confirmed enhanced electrophysiological plasticity of newly generated DGCs at 4 weeks of neuronal age in adult mice (Ge et al., 2007). In order to detect LTP by field recording within DG, GABAR blockers are commonly used. However, a small percentage of GluN2B dependent LTP (10%) can be induced by high frequency stimulations (HFS) without GABAR blockers, and this LTP component is mediated by newly generated immature neurons (Snyder et al., 2001). Moreover, this neurogenesis-dependent LTP is necessary for contextual fear conditioning (Saxe et al., 2006b). These studies suggest that the unique electrophysiological properties of newborn neurons confer a specialized processing component that is important for hippocampal DG dependent learning.

Functionally, adult hippocampal neurogenesis is not required for all hippocampal dependent behaviors, but is thought to be required for pattern separation (Sahay et al., 2011; Gu et al., 2012; Nakashiba et al., 2012). This neurogenesis function was predicted based on computational analysis (Aimone, 2006; Aimone et al., 2011). Aimone et al. proposed that the sparse activity of DGCs following learning and experience is important for discriminating two similar yet distinct events or environments, and that immature
DGCs specifically play an important role for increasing memory resolution (Aimone et al., 2011).

In addition to pattern separation, adult hippocampal neurogenesis has also been closely linked with antidepressant-like behavior. Antidepressant drugs are known to stimulate hippocampal neurogenesis, and antidepressant like behavioral effects require neurogenesis (Drew & Hen, 2007; Sahay & Hen, 2007). Thus, adult hippocampal neurogenesis is thought to be required for discriminating between different contexts and environments and for mood regulation.
1.2.2 Regulation of adult hippocampal neurogenesis

For the continuous production of new DGCs throughout life, the process of neurogenesis is highly regulated to balance neuronal production and maintenance of the...
neural stem cell pool. This process is regulated through electrophysiological and environmental signaling as discussed below.

1.2.2.1 Electrophysiological activity-dependent regulation of hippocampal neurogenesis

The electrophysiological maturation of newborn neurons has been well characterized. First, newborn neurons start receiving tonic GABAergic input and then phasic GABAergic input at 1 week of neuronal age. Finally, the newly generated neurons receive phasic glutamatergic input at 2 weeks neuronal age (Ge et al., 2006). However, adult generated neurons do not reach their final stage of maturity until 4-6 weeks. At 4 weeks of neuronal age, synaptic plasticity is enhanced via expression of the GluN2B subtype-containing NMDARs (Ge et al., 2007). This enhanced plasticity is marked by higher amplitude of LTP and a lower threshold for induction of LTP (Schmidt-Hieber et al., 2004; Ge et al., 2007; Marin-Burgin et al., 2012). During this period of maturation, newly generated DGCs also send axonal output to the CA3 pyramidal neurons (Gu et al., 2012). Previous studies have demonstrated that the unique electrophysiological maturation and integration process is important for hippocampal neurogenesis and function (Kheirbek et al., 2012; Nakashiba et al., 2012).

Tonic GABA activity is the first input into newborn DGCs. This tonic GABA input is first excitatory and then inhibitory at 3 weeks due to sequential expression of the Na⁺-K⁺-2Cl⁻ transporter (NKCC1) followed by the K⁺-coupled Cl⁻ transporter (KCC2) (Ge et al., 2006). Disruption of the excitatory tonic GABA input by retrovirus-mediated shRNA to block expression of NKCC1, impairs dendritic development of newly generated DGCs (Ge et al., 2006). Recently, optogenetic approaches demonstrated that parvalbumin (PV+)
interneurons within the DG are important for regulation of proliferation and survival of both radial glia-like stem cells and neuroblasts (Song et al., 2012; Song et al., 2013). For radial glia-like stem cells, increased PV+ interneuron-mediated tonic GABA input leads to a quiescent state. On the other hand, radial glia-like stem cells shift to an activated proliferative state when Parvalbumin (PV) + interneuron-mediated tonic GABA input is suppressed (Song et al., 2013). Interestingly, activity of PV+ interneurons affects neuroblasts in an opposite way to radial glia-like cells. Survival of neuroblasts and dendritic development are both enhanced by optogenetic activation of PV+ interneurons expressing channelrhodopsin-2 (ChR2). In contrast, survival of neuroblasts and dendritic development are decreased when PV+ interneuron activity is optogenetically suppressed via expression of halorhodopsin (NpHR) or archaerhodopsin (Arch) (Song et al., 2013). Also, dendritic development is impaired in immature and mature newborn neurons following deletion of the α4 subunit in GABA_A R (Duveau et al., 2011). Therefore, both tonic and phasic GABAergic inputs are required for proper regulation of adult hippocampal neurogenesis.

As previously mentioned, glutamatergic input from the EC to newly generated DGCs occurs subsequent to GABAergic input. Newborn neurons start receiving glutamatergic input at 2 weeks and increased connectivity occurs as the newly generated DGCs mature (Ge et al., 2006; Ge et al., 2007). In addition, by 4 weeks newborn neurons display GluN2B-dependent enhancement of synaptic plasticity (Ge et al., 2007). Interestingly, conditional KO of the GluN2B subunit in newborn DGCs does not impair hippocampal neurogenesis per se, but does impair acquisition of learning tasks that require the presence of newly generated neurons (Kheirbek et al., 2012). Moreover, deletion of the
GluN1-NMDAR subunit impairs the survival of 3 week old newborn neurons (Tashiro et al., 2006a). Furthermore, electrical stimulation of the EC in vivo promotes the proliferation and survival of newborn DGCs (Stone et al., 2011). These previous studies all suggest that the glutamatergic input is required for the maturation of adult generated DGCs and for learning tasks that are dependent upon these cells. The proper timing and sequence of electrophysiological maturation and integration, including both GABAergic and glutamatergic connections, is essential for maintaining adult neurogenesis and neurogenic function.

1.2.2.2 Regulation of hippocampal neurogenesis by Enriched Environment

Environmental enrichment results in profound benefits for many aspects of brain function. In the late 1940s, Hebb first proposed and demonstrated that enriched experienced mice have improved capacity for learning and memory. For experimental environmental enrichment, mice are housed in larger cages and have access to complex toys, including tunnels, hiding objects, ladders and running wheels (van Praag et al., 2000). The mechanisms by which complex environment and voluntary exercise exert positive effects on brain functions and behaviors are multifaceted and include enhanced growth factor molecular signaling (Kobilo et al., 2011; Eckert & Abraham, 2013; Vivar et al., 2013), alteration of neuroimmune processes (Singhal et al., 2014) as well as improved general energy metabolism and balance (Novak et al., 2012). Therefore, combined environmental complexity and voluntary exercise exert peripheral systemic effects on normal physiology and CNS-specific functions.
Hippocampal neurogenesis is stimulated by physical and social enrichment. Hippocampal neurogenesis and hippocampal dependent learning are enhanced by voluntary physical activity (van Praag et al., 1999a; van Praag et al., 1999b) and environmental enriched living conditions (Kempermann et al., 1998). It is important to note that voluntary exercise and environmental complexity have different mechanisms for promoting adult hippocampal neurogenesis (Fabel et al., 2009; Kempermann et al., 2010). Physical activity increases the proliferation of precursor cells and environmental enrichment promotes survival of new born neurons (Fabel et al., 2009). Also, enriched environment promotes survival of newborn neurons (Fabel et al., 2009). Thus, these two different types of environmental stimulations have additive effects on hippocampal neurogenesis. In this dissertation, we utilized a combination of increased environmental complexity and voluntary exercise to achieve a maximal neurogenic effect.

Brain-derived neurotrophic factor (BDNF) has been intensively researched and investigated for its role in hippocampal function and adult neurogenesis. BDNF and its receptor TrkB are expressed in the hippocampus, including the DG (Wetmore et al., 1994). Spatial learning and memory is impaired by blockage of endogenous BDNF activity by BDNF antibody (Mu et al., 1999). Heterozygote BDNF null mice (BDNF+/−) display decreased survival of newborn neurons in DG at 3 weeks after BrdU injection. Also, application of BDNF increases survival and neural process outgrowth in cultured hippocampal neurons (Lowenstein &Arsenault, 1996). Interestingly, BDNF is upregulated in the hippocampus by exercise and by chronic antidepressant drug administration (Ernst et al., 2006). Several studies have reported that the environmental enrichment and voluntary exercise elevate BDNF level in hippocampus (Ickes et al.,
Importantly, BDNF plays a role in mediating the neurogenic response to environmental enrichment (Bekinschtein et al., 2011) (Rossi et al., 2006), and elevation of BDNF is also required for environmental enriched mediated neurogenesis (Rossi et al., 2006).

Vascular endothelial growth factor (VEGF) is also an important regulator of neurogenesis that is involved in neurogenic responses to environmental enrichment. VEGF is well-known for its ability to promote vasculogenesis and angiogenesis (Lee & Son, 2009). The VEGFR1/Flt-1 receptor is expressed within DCX+ immature DGCs and proliferation of neural stem cells is stimulated by intracerebroventricular (i.c.v.) administration of VEGF (Jin et al., 2002). This suggests that VEGF can regulate proliferation of neuroblasts through VEGFR2/Flk-1 receptor signaling directly. Also, the proliferation and survival of newborn neurons within the hippocampus are both stimulated by VEGF overexpression using recombinant adeno-associated virus (rAAV) in vivo (Cao et al., 2004). In addition, VEGF enhances spatial learning, memory and hippocampal angiogenesis (Cao et al., 2004). VEGF activity in the brain is upregulated by behavioral challenge and exercise, which are both components of environmental enrichment (Cao et al., 2004). Similar to BDNF, VEGF is required for exercise and enriched environmental-mediated hippocampal neurogenesis (Fabel et al., 2003; Cao et al., 2004). Thus, environmental enrichment can stimulate hippocampal neurogenesis by promoting both proliferation and survival of precursor cells via enhanced growth factor signaling.
1.3 Hypothesis and Specific Aims

Overarching Hypothesis: Prenatal alcohol (FASD) impairs enrichment-mediated survival and electrophysiological plasticity of newborn DGCs within the adult hippocampus.

Specific Aim 1: To determine whether prenatal alcohol (FASD) impairs the neurogenic response to environmental enrichment in adult Nestin:CreER\textsuperscript{T2}: Yellow fluorescence protein (YFP) bitransgenic mice; and if so, to delineate the stage of neural stem cell lineage most impacted by prenatal alcohol exposure. (Chapter 2)

Rationale: Previous work found that the neurogenic response to environmental enrichment was impaired by moderate levels of fetal alcohol exposure using bromodeoxyuridine (BrdU) labeling (Choi et al., 2005). However, there are several limitations to the use of BrdU as a marker for neurogenesis. BrdU, a thymidine analogue incorporated into proliferating progenitors during S-phase, becomes diluted by continued cell division and is therefore of low efficiency for long-term labeling of newborn postmitotic neurons (Lagace et al., 2007). BrdU can also be incorporated into DNA of damaged postmitotic neurons during DNA repair (Taupin, 2007b) (Taupin, 2007a) and also does not label quiescent neural stem cells (Lugert et al., 2010). Therefore, I utilized Nestin:CreER\textsuperscript{T2}:YFP bitransgenic mice (described below) to visualize adult hippocampal neurogenesis and to analyze the long-term phenotypic fate of newborn DGCs in a well-characterized preclinical mouse model of moderate FASD. This approach allowed us to examine the maturational stage at which prenatal alcohol impacts the adult neural stem cell lineage under conditions of EE.
**General Approach:** Figure 1.3. shows the genetic construction of Nestin:CreER\textsuperscript{T2}:Yellow fluorescence protein (YFP) bitransgenic mouse. This bi-transgenic mouse expresses a tamoxifen-inducible mutated estrogen receptor fused to a bacterial Cre recombinase (CreER\textsuperscript{T2}) under transcriptional control of the nestin promoter. The YFP reporter gene at the Rosa26 locus contains an upstream floxed transcriptional stop sequence. Tamoxifen binds to the mutated estrogen receptors (which do not bind to estrogen at physiological concentrations) to activate Cre-recombinase. Tamoxifen-activated Cre then moves to the nucleus where it acts to excise the floxed stop codon to allow YFP reporter expression in nestin-expressing neural stem cells and their progeny (Lagace *et al.*, 2007).

Nestin:CreER\textsuperscript{T2}:YFP bitransgenic mice were used for long term phenotypic fate analysis of adult hippocampal progenitors in Control vs. FASD mice following exposure to standard and enriched environment housing. Confocal stereology was utilized for quantitative analysis and phenotypic fate mapping of YFP+ cells. Phenotypic fate mapping involved the use of maturation stage-specific markers in the neurogenic lineage to label neural stem cells, transit amplifying cells, neuroblasts, postmitotic immature neurons, and mature DGCs.
Figure 1.3. NestinCreER<sup>T2</sup>:YFP reporter gene construction. Adopted from (Lagace et al., 2007)

Specific Aim 2: To determine whether adult-generated DGCs in FASD mice display normal electrophysiological properties under standard and enriched conditions. (Chapter 3)

Rationale: Previous studies have demonstrated a negative impact of prenatal alcohol on glutamate (Savage et al., 1991; Wijayawardhane et al., 2007; Barbier et al., 2008; ) and GABA (Allan et al., 1998b; Iqbal et al., 2004b; Everett et al., 2012) receptor expression and function in adult hippocampus. However, it is unknown whether prenatal alcohol exposure leads to changes in glutamatergic and GABAergic afferent synaptic connectivity in hippocampal newborn DGCs under standard and enriched conditions. Therefore, in specific aim 2, we investigated whether newborn neurons that survive in FASD mice display normal glutamatergic and GABAergic connectivity and plasticity in response to enriched housing conditions. In addition, we compared the effect of prenatal alcohol and EE effects on the electrophysiological properties of preexisting DGCs in FASD mice.
**General Approach:** Electrophysiological maturation and integration were analyzed by whole cell patch clamp recordings at different time points following retroviral labeling of hippocampal progenitors in control vs. FASD mice under both standard and enriched environment (EE) conditions. Retroviral labeling allowed visualization for patch clamp recordings and birth dating of newborn DGCs.

We utilized a replication-deficient retroviral vector that is based on the Moloney murine leukemia virus, which can only transduce dividing cells. The vector conferred GFP (Green fluorescence protein) expression to adult hippocampal progenitors. Because the vector DNA integrates into the host DNA during S-phase, GFP expression continues in all progeny (Harel et al., 1981; Miller et al., 1990). A volume of 0.5µl of high titer viral stock solution (from Dr. Ge Lab, Stony Brook University, New York) was stereotaxically injected for conferring GFP expression to proliferating cells and their progeny, and patch clamp recordings were performed in acute hippocampal slices 4 and 8 weeks after viral injection.

**Specific Aim 3: To determine whether adult-generated DGCs in FASD mice display dendritic morphological plasticity in response to enriched environment. (Chapter 3)**

**Rational:** Previous studies have reported a reduction of dendritic complexity and spine density in hippocampal regions such as CA1 and CA3 pyramidal neurons in rodents exposed to prenatal alcohol (Berman & Hannigan, 2000) and the shell of the nucleus accumbens in adult rats (Rice et al., 2012). Therefore, prenatal alcohol exposure results in the impairment of dendritic complexity in hippocampus and other brain regions. In contrast to prenatal alcohol effects, some previous studies suggested that voluntary
exercise increases both dendritic complexity and spine density of developmentally preexisting (Redila & Christie, 2006) and adult generated DGCs (Beauquis et al., 2010; Piatti et al., 2011). However, other publications suggested that the morphology of the spines were impacted such that the density of more mature spines with a mushroom morphology was increased without changing total spine density (Zhao et al., 2006; Morgenstern et al., 2008). The impact of prenatal alcohol exposure on the dendritic development in adult generated DGCs under enriched housing is unknown. In this aim, we investigated the impact of FASD on spine density in retrovirally labeled GFP+ adult newborn DGCs as well as the morphological complexity in preexisting DGCs under standard and EE housing conditions.

**General Approach:** Morphological maturation was analyzed by measures of spine density in control and FASD mice at two different time points under EE and standard housing conditions. Newborn neurons were birth dated by GFP retroviral vector labeling and dendritic spine density was assessed using standard histological methods and high resolution confocal microscopy. For developmentally generated DGCs, in order to assess sholl analysis, single DGCs were filled with biocytin and stained to visualize single cell morphology for sholl analysis of dendritic complexity.
2. Fate Analysis of Adult Hippocampal Progenitors in a Murine Model of Fetal Alcohol Spectrum Disorder (FASD)

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2.1 Abstract

Prenatal alcohol exposure can lead to fetal alcohol spectrum disorder (FASD) and associated behavioral impairments that may be linked to disruptions in adult hippocampal neurogenesis. Social and physical enrichment has been proposed as a potential therapeutic approach toward reversing behavioral deficits associated with FASD and is also a potent stimulator of adult hippocampal neurogenesis. In the present study, we utilized a genetic fate mapping approach in nestin-CreER\textsuperscript{T2}/YFP bitransgenic mice to identify the stage-specific impact of prenatal alcohol exposure on the stepwise maturation of adult hippocampal progenitors. Using a limited alcohol access “drinking-in-the-dark” model of FASD, we confirm previous findings that moderate prenatal alcohol exposure has no effect on adult neurogenesis under standard housing conditions, but abolishes the neurogenic response to enriched environment (EE). Furthermore, we demonstrate that this effect is primarily due to failed EE-mediated survival of postmitotic neurons. Finally, we demonstrate that the neurogenic deficit is associated with impaired spatial pattern recognition, as demonstrated by delayed learning of FASD-EE mice in an A-B contextual discrimination task. These results identify a potential maturational stage-specific mechanism(s) underlying impaired neurogenic function in a preclinical model of FASD, and provide a basis for testing regulatory pathways in this model through conditional and inducible manipulation of gene expression in the adult hippocampal progenitor population.
2.2 Introduction

Fetal alcohol spectrum disorder (FASD) encompasses a range of physical, behavioral and cognitive disabilities resulting from prenatal alcohol exposure (Kelly et al., 2000; Streissguth & O’Malley, 2000; Guerri et al., 2009). Neurological complications in FASD range from severe mental retardation as a consequence of high dose alcohol (fetal alcohol syndrome; FAS) to more subtle behavioral abnormalities as a result of moderate levels of alcohol exposure, including learning deficits, increased anxiety and depression. FASD represents a significant public health problem, with the prevalence of FASD estimated to be as high as 2-5% within the United States and some Western European countries (May et al., 2009). Despite this, very few empirically supported interventions are available for mitigating the cognitive and behavioral disabilities associated with this spectrum disorder (Kodituwakku & Kodituwakku, 2011).

The production of new neurons in the postnatal and adult hippocampal dentate gyrus is thought to play an important role in learning, memory and mood (Koehl & Abrous, 2011a; Samuels & Hen, 2011); and may represent a neural substrate for several behavioral manifestations of clinical FASD (Gil-Mohapel et al., 2010). The rate of adult hippocampal neurogenesis has been linked to learning performance, particularly on tasks that require spatial and temporal pattern separation (Clelland et al., 2009; Aimone et al., 2011; Tronel et al., 2012). Potential mechanisms include preferential behavioral activation of newborn dentate granule cells (DGCs) due to their lowered activation threshold and heightened dendritic plasticity (Ge et al., 2007; Tronel et al., 2010), and temporal processing as waves of new neurons are added to the hippocampal network (Aimone et al., 2011). Impaired neurogenesis may also underlie some forms of
depression and anxiety (Perera et al., 2011). For example, chronic stress reduces neurogenesis and results in depressive-like states in rodent models; whereas chronic treatment with multiple classes of antidepressants increases neurogenesis (Perera et al., 2011; Surget et al., 2011).

Preclinical rodent models of FASD mimic many of the behavioral aspects observed in clinical FASD, including impaired learning, increased anxiety and depression (Caldwell et al., 2008b; Brady et al., 2012a; Brocardo et al., 2012). Furthermore, long-lasting impairments in postnatal hippocampal neurogenesis have been documented following prenatal or early postnatal alcohol exposure (reviewed by (Gil-Mohapel et al., 2010b)). For example, high dose alcohol exposure during the prenatal and early postnatal period results in impaired production and maturation of DGCs in adult rats (Ieraci & Herrera, 2007b; Klintsova et al., 2007b; Helfer et al., 2009) (Boehme et al., 2011a). More moderate alcohol exposure throughout gestation in mice has no effect on neurogenesis under standard housing conditions, but abolishes the neurogenic response to social and physical enrichment (Choi et al., 2005). The mechanism(s) by which alcohol exposure during development leads to enduring neurogenic deficits in adulthood remains unknown. Because each maturational stage of the adult neurogenic lineage (progenitor proliferation, neuronal differentiation and functional integration of postmitotic DGCs) can be differentially regulated by behavioral, environmental and genetic factors, we hypothesized that prenatal alcohol exposure targets a specific maturational step in the adult neurogenic lineage. If so, pinpointing the stage of vulnerability might facilitate therapeutic intervention strategies useful in clinical FASD.
In the present study, we characterized the influence of prenatal alcohol exposure on the stepwise maturation of adult hippocampal progenitors using a genetic fate mapping approach. For these studies, we utilized Nestin-CreER<sup>T2</sup>/YFP mice, which harbor a yellow fluorescent protein (YFP) reporter gene at the Rosa 26 locus and a tamoxifen-inducible Cre recombinase (Cre-ER<sup>T2</sup>) under transcriptional control of the nestin promoter (Lagace <i>et al.</i>, 2007). Tamoxifen administration to nestin-CreER<sup>T2</sup>/YFP mice results in restricted and transient activation of Cre recombinase within nestin+ adult hippocampal progenitors, and induction of YFP reporter expression in all subsequent progeny. This approach facilitates detailed phenotypic fate mapping and distribution analysis of progenitors and their progeny following tamoxifen-induced recombination.

Using a limited alcohol access “drinking-in-the-dark” exposure paradigm, we investigated the impact of moderate prenatal alcohol exposure on the adult hippocampal neural progenitor lineage. These studies confirm our previous findings that gestational exposure to moderate levels of alcohol impairs the neurogenic response to enriched environment; and extend those findings to demonstrate impaired survival and integration of postmitotic neurons at late neurogenic stages in FASD mice. We further demonstrate delayed acquisition of contextual discrimination learning in FASD mice, a behavior that is thought to be dependent upon the production of new DGCs. These results shed light on potential stage-specific mechanisms underlying impaired neurogenic responses in preclinical models of moderate FASD, and provide a platform for testing regulatory pathways through conditional and inducible manipulation of gene expression in the adult hippocampal progenitor population.
2.3 Materials and Methods

2.3.1 Animals

Ethics Statement: Animal experiments were approved by the University of New Mexico Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The Nestin-CreER\(^T2\)/YFP bi-transgenic strain used in this study was generously provided by Dr. Amelia Eisch (Department Psychiatry, University of Texas Southwestern Medical Center, Dallas, TX) and previously described (Lagace \textit{et al.}, 2007). These bi-transgenic mice are on a C57Bl/6J background. All mice were housed in reverse 12-hour dark / 12-hour light cycle (lights off at 08:00 hours). Food and water were available \textit{ad libitum} except during the maternal drinking period during which water (but not food) was withheld as described below.

2.3.2 Prenatal Alcohol Exposure

For these studies, we utilized a limited access paradigm of maternal drinking that was previously established for C57Bl/6J mice (Brady \textit{et al.}, 2012). To obtain stable drinking levels across mice, 60 day old nestin-CreER\(^T2\)/YFP female mice were first subjected to a ramp-up period in which the normal drinking water was replaced with 0.066% saccharin containing 0% ethanol (2 days), 5% ethanol (2 days) and finally 10% ethanol, for 4 hrs per day from 10:00-14:00 (2 weeks prior to pregnancy and throughout gestation). Female mice offered 0.066% saccharin without ethanol during the same time periods and throughout pregnancy served as controls. Following one week of 10% ethanol or saccharin consumption post-ramp, individual females were placed into the cage of singly housed males for 2 hours from 14:00-16:00, immediately following the daily drinking
period, for five consecutive days. Water and food were available *ad libitum* in the male cages. Females were then returned to their home cages after each 2 hour mating session, where they continued on the limited access schedule of ethanol or saccharin exposure for 4 hours per day throughout gestation. Ethanol and saccharin concentrations were halved every two days beginning on the day of birth, and returned to drinking only water only on day 5. A consumption volume during the 4 hour access period was determined for each mouse from the onset of the drinking paradigm. Offspring were tail-clipped and genotyped at weaning and subjected to enriched or non-enriched living conditions as outlined below. The limited access maternal drinking paradigm has no impact on litter size, offspring weight or maternal care (Brady *et al.*, 2012).

2.3.3 Blood Ethanol Measurements

Blood ethanol concentrations were determined using an alcohol dehydrogenase enzymatic method as previously described (Brady *et al.*, 2012). Briefly, blood samples obtained from submandibular vein (40 µl) were treated with 2 ml of 3.5% (v/v; 0.58 M) perchloric acid and centrifuged to obtain serum. Forty microliters of serum or ethanol standard (0-400 mg/dl ethanol) were incubated in reaction buffer (10 units alcohol dehydrogenase, 2.0 mM NAD, 0.5 M Tris-HCl, pH 8.8) for 15 minutes at 30°C and optical densities measured at 340 nm using a Beckman DU380 spectrophotometer. Blood ethanol concentrations were calculated from the standard curves using regression analysis.
2.3.4 Environmental Enrichment (EE)

Mice were gender segregated at weaning. Male pups were used for further study to avoid potential gender-specific effects of prenatal alcohol on hippocampal function and neurogenesis. To activate Cre-mediated recombination, male offspring received daily i.p. injections of tamoxifen (TAM; 180 mg/kg dissolved in 10% EtOH/90% sunflower oil; ~150 µl per mouse) for five consecutive days beginning at postnatal day 40. TAM-treated mice were then placed into either the standard housing condition (3 mice per 28cm X 18cm X 13cm mouse cage without running wheels or toys) or were placed in EE housing conditions (6 mice per 48cm X 27cm X 20cm cage with 2 running wheels, one ladder, one tunnel and multiple hanging toys that were changed weekly). All mice were perfused for histological analysis after 10 weeks of standard or EE housing. To control for potential litter effects, each cage contained individual offspring from 2-3 different litters for each experiment. Twelve litters were used for these studies (6 litters for each alcohol treatment group).

To monitor interaction of Sac and FASD mice with their environment, movement within the EE home cage was recorded and analyzed using the Noldus EthoVision 3.0 video tracking system (Noldus, Leesburg, VA). For this analysis, three target zones encompassing the running wheel, ladder and tunnel were outlined with minimal border zones and the duration of time spent in each environmental zone was recorded over a one hour period. It should be noted that cage-mates were removed from the EE during the recording session (n=5 mice per treatment group taken from 2-3 different litters per group).
2.3.5 Histology

Mice were overdosed with sodium pentobarbital (150 mg/kg, i.p.; Fort Dodge Animal health, Fort Dodge, IA), and transcardially perfused with 0.1M phosphate-buffered saline (PBS) containing 0.1% procaine and 2 U/ml heparin, followed by 4% paraformaldehyde (PFA) in PBS. Brains were removed and post-fixed in 4% PFA overnight, followed by cryoprotection with 30% sucrose in PBS. The right hemisphere of each brain was sectioned at 30 µm thickness in the coronal plane using a freezing sliding knife microtome. Sections were stored at -20°C in cryoprotectant (25% glycerol, 25% ethylene glycol and 50% of 0.1M phosphate buffer) until used for immunostaining.

Slide mounted and free floating-sections were subjected to immunofluorescence staining protocols as previously described (Kokovay & Cunningham, 2005; Roitbak et al., 2008). The following antibodies were used: mouse anti-NeuN (1:1000, Millipore, Billerica, MA), rabbit anti-DCX (1:500, Cell Signaling Technology, Danvers, MA), mouse anti-PCNA (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-GFAP (1:1000, Sigma-Aldrich, St. Louis, MO), chicken anti-GFP (1:1000, Invitrogen, Carlsbad, CA), and rabbit anti S-100β (1:1000, DAKO, Carpunteria, CA). Sections were subsequently incubated with donkey secondary antibodies directed against mouse, rabbit or chicken IgG. Secondary antibodies were conjugated to Cy3, Cy5, FITC (1:250) or biotin (1:1500) (Jackson Immunoresearch, West Grove, CA). Preincubation with 10 mM of sodium citrate (pH 6.4, 80°C) for 30 minutes was required for antigen retrieval when staining for PCNA. Briefly, sections were rinsed with 0.1% of Tween-20 in PBS (PBS-T) for 10 minutes, permeabilized with 0.4% of triton X-100 in PBS for 20 minutes, followed by incubation in blocking solution (0.1% BSA and 10% normal donkey serum.
in PBS-T) for 1 hour. Sections were then incubated overnight with primary antibodies in 0.5% BSA/PBS-T at the indicated dilutions. YFP immunofluorescence was visualized using biotinylated secondary antibody and the Tyramide-Plus signal amplification kid (PerkinElmer Life Science, Boston, MA). Sections were counterstained using DAPI nuclear dye and coverslipped using Prolong ® Gold Antifade reagent (Invitrogen). All images were taken using an Olympus DSU spinning disk confocal microscope.

2.3.6 Stereology

The number of YFP+ neurons was estimated within the right dentate gyrus using the optical fractionator method and Stereoinvestigator™ software (Microblightfield, Williston, VT) linked to an Olympus DSU spinning disk confocal microscope. The contour of the dentate gyrus was manually outlined in each section using a 20X objective. YFP+ cells were counted in every 8th section (240 µm apart) through the rostro-caudal extent of the right dentate gyrus. For immunophenotyping the YFP+ cells, 100-200 YFP+ cells were sampled and scored for each phenotypic maker using rapid z-analysis on the confocal microscope. Based on co-localization for the following markers, YFP+ cells were categorized as type-1 stem cells (GFAP+/S100β+) transient amplifying progenitor cells (PCNA+/DCX−), proliferating neuroblasts (PCNA−/DCX+), postmitotic neurons (NeuN+), or astrocytes (GFAP+/S100β+). The number of cells within each phenotypic category was calculated by multiplying the percentage of cells in each category by the total number of YFP+ cells as estimated by stereology.
2.3.7 Contextual fear discrimination learning

Male C57Bl/6J Saccharin controls and FASD mice were assessed for contextual fear discrimination learning. After 4 weeks exposure to EE housing, mice were trained five consecutive days in the A-B contextual fear discrimination learning task, as modified from Sahay et al., (Sahay et al., 2011). Each mouse was placed into either Context A (foot shock) or Context B (no foot shock) for 90s, followed by applied foot shock (0.8 mA) to only Context A for 2 s. Following a second 90s interval, another 1s foot shock was applied only in Context A. Three hours after the first session, mice were exposed to a second identical training session, except that the mouse initially subjected to Context A with foot shock was now subjected to Context B without footshock. Context A was a standard chamber with a stainless steel floor, clear plexiglas front wall, and aluminum side and back walls. Context B was a similar chamber, except the floor was a wire mesh non-shock floor and the side and back walls were covered in striped black and white contact paper. Mice were transported into a holding room located just outside of the context discrimination room for at least one hour before testing. The context chambers were cleaned with 70% isopropyl alcohol between each session. The order of testing in Context A vs. B was altered every day for each mouse. Trials were recorded using a digital camera and mice were scored for time spent freezing during the first 90s of each trial (observer blinded to treatment). A mean discrimination score was calculated as: (freezing score context A – freezing score context B) / (freezing score context A + freezing score context B). Thus, higher discrimination scores indicate better contextual discrimination.
2.3.8 Statistical analysis

Data were analyzed by ANOVA with appropriate post hoc analysis using Prism or SPSS (v.20) software programs. Data are expressed as means + S.E.M., with p<.05 considered statistically significant.

2.4 Results

2.4.1 Drinking behavior and BECs in Nestin-CreER\textsuperscript{T2} / YFP mice

The limited access model of moderate prenatal alcohol exposure used in the current study to generate FASD offspring was previously described (Brady et al., 2012a). Briefly, female mice were offered 10% ethanol in 0.066% saccharin for four hours during their dark cycle (10:00-14:00), beginning one week before pregnancy and continuing throughout gestation. This drinking paradigm results in an average blood ethanol concentration (BEC) of 68 mg/ dl and 88 mg/dl after 2 and 4 hours of drinking, respectively; with ethanol consumption rate predictive of BECs (Brady et al., 2012). As shown in Figure 2.1, the average alcohol consumption rate and BEC values (as assessed after one hour access) were not different comparing wildtype (WT) and transgenic mice, indicating that neither drinking behavior nor alcohol metabolism were altered in the nestin-CreER\textsuperscript{T2}/YFP mice compared to background strain.
FIGURE 2.1

Average daily ethanol consumption over 10 consecutive days

<table>
<thead>
<tr>
<th>Strain</th>
<th>Volume of 10% ethanol consumed during one hour access</th>
<th>BECs after one hour access (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype C57BL/6</td>
<td>2.43 + 0.1</td>
<td>44.72 + 3.1</td>
</tr>
<tr>
<td>Nestin-CreERT2/YFP</td>
<td>2.57 + 0.2</td>
<td>44.08 + 4.0</td>
</tr>
</tbody>
</table>

Figure 2.1 Alcohol Consumption and BECs in Nestin-CreER\textsuperscript{T2}/ YFP mice. (A) Average daily consumption of ethanol (mg/kg/day) over 10 consecutive days in wild type (WT) and transgenic mice (n=5 mice/group) (B) Alcohol consumption (ml) and BEC (mg/dl) after only one hour alcohol access (n=5 mice/group). Data expressed as means ± S.E.M.

2.4.2 FASD mice display impaired enrichment-mediated neurogenesis

To evaluate the impact of FASD on hippocampal neurogenesis in adulthood, we estimated the number of YFP+ cells in Sac nestin-CreER\textsuperscript{T2}/YFP vs. FASD nestin-CreER\textsuperscript{T2}/YFP mice housed under standard or EE living conditions for 10 weeks. YFP reporter expression was induced in nestin\textsuperscript{+} NSCs by tamoxifen (TAM) administration to all mice for 5 consecutive days (180mg/kg i.p.) prior to placement into standard or EE housing for 10 consecutive weeks (Figure 2.2). Therefore, all mice were approximately
3.5 months of age at the time of sacrifice. Previous studies have demonstrated that the TAM dosing regimen used here is optimal for phenotypic fate mapping, resulting in a gradual buildup of YFP+ cells as adult hippocampal progenitors proliferate and give rise to postmitotic DGCs, reaching a steady state by approximately 30 days post TAM treatment (Lagace et al., 2007).

**FIGURE 2.2**

![Experimental Design Diagram](image)

**Figure 2.2 Experimental Design.** Tamoxifen (180 mg/kg, i.p.) was administered once each day for 5 consecutive days to induce YFP expression in adult hippocampal progenitors of 4-6 week old nestin-CreERT2/YFP mice. Tamoxifen-treated mice were exposed to standard or enriched housing conditions for 10 consecutive weeks prior to histological analysis.

As shown in Figure 2.3, the mean number of total YFP+ cells was increased approximately 4-fold by EE compared to standard housing in Sac nestin-CreERT2/YFP mice (1,821 + 406 vs. 7,424 + 1,737 YFP+ cells under Standard housing vs. EE, respectively). In contrast, there was no effect of housing on the number of YFP+ cells in FASD nestin-CreERT2/YFP mice (3,261 + 617 vs. 4,291 + 1,217 YFP+ cells under Standard housing vs. EE, respectively). Two-way ANOVA revealed a significant
housing effect, (F (1, 28) = 8.72, p=.006), and significant FASD x housing interaction, (F (1, 28) =4.15, p = .05). Post hoc analysis confirmed increased YFP+ cell number under conditions of EE in SAC nestin-CreERT2/YFP mice, but not in FASD nestin-CreERT2/YFP mice. There was not a significant difference in the total number of YFP-labeled cells in Sac vs. FASD nestin-CreERT2/YFP mice under standard housing, even though labeling appeared to be slightly higher in FASD mice.

**FIGURE 2.3**

---

**Figure 2.3 The neurogenic response to EE is blunted in FASD mice.** (A) Confocal images of hippocampal dentate: YFP immunofluorescence (green) and DAPI nuclear stain (blue), (B) Total number of YFP positive cell in dentate gyrus 10 weeks after TAM injection. Post hoc analysis revealed a significant increase in the total number YFP+ cells in Sac nestin-CreERT2/YFP mice, but not FASD nestin-CreERT2/YFP mice housed under environmental enrichment (*p<0.05, n=8 mice per group).
2.4.3 FASD abolishes the beneficial effects of EE on the survival of postmitotic adult-generated DGCs

To determine the phenotypic distribution of YFP+ cells across the neurogenic lineage, we surveyed the YFP+ cell population in Sac and FASD nestin-CreER$^{T2}$/YFP mice for co-expression of markers specific for each maturational stage. YFP+ cells were categorized as radial-glia-like type-1 NSCs, transit amplifying progenitor cells (TAPs), neuroblasts, postmitotic immature neurons, mature neurons or astrocytes based on expression of immunohistochemical markers as indicated in Table 2.1 and depicted in Figure 5A (Kempermann et al., 2004; Duan et al., 2008; Ables et al., 2010).

Table 2.1 Phenotypic markers used to identify maturational stages of YFP+ cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Phenotypic Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I NSC</td>
<td>GFAP+/S100β-</td>
</tr>
<tr>
<td>TAP</td>
<td>PCNA+/DCX-</td>
</tr>
<tr>
<td>Neuroblast</td>
<td>PCNA+/DCX+</td>
</tr>
<tr>
<td>Immature neuron</td>
<td>PCNA-/DCX+</td>
</tr>
<tr>
<td>Neuron</td>
<td>DCX-/NeuN+</td>
</tr>
<tr>
<td>Astrocyte</td>
<td>GFAP+/S100β+</td>
</tr>
</tbody>
</table>

Table 2.1 Phenotypic markers used to identify maturational stages of YFP+ cells include GFAP (glial fibrillary acidic protein), S100beta (a calcium binding protein expressed in mature astrocytes), PCNA (proliferating cell nuclear antigen), DCX (doublecortin; a microtubule-associated protein expressed by neuroblasts and immature neurons) and NeuN (neuronal nuclear antigen).

As shown in Figure 2.4, YFP+ cells represented the entire neurogenic lineage in all treatment groups. Two-way ANOVA revealed a significant main effect of housing on YFP+ type-1 stem cells (F(1, 28) = 7.34; p = .011) and neuroblasts (F(1, 28) = 4.94; p =
.035), but FASD x housing interaction was not significant for these cell types. However, FASD significantly impaired EE-mediated increases in the number of postmitotic immature neurons (F (1, 28) = 12.69; FASD x housing interaction, p = .001) and mature neurons (F (1, 28) = 8.69; FASD x housing interaction, p = .006). There was no significant effect of FASD or housing on the number of YFP+ astrocytes, indicating that abolishment of EE-mediated neurogenesis in FASD mice was not due to a progenitor switch towards astrocyte lineage. No significant effects of FASD or housing were observed for the number of YFP+ TAPs.
FIGURE 2.4

Figure 2.4 Fate analysis of YFP+ cells in Sac and FASD mice exposed to EE and standard housing conditions. For each panel, representative confocal images are shown at top and quantitative data at bottom (A) Type-1 stem cells were identified as GFAP+ (green) and S100beta- (red), (B) Transiently amplifying progenitor cells (TAPs) were PCNA positive (white) and DCX negative (C) Neuroblasts were PCNA+ (white) and DCX+, (D) Postmitotic immature neurons were DCX+ (red) and PCNA- (white), (E) Mature neurons were NeuN positive (red), (F) Astrocytes were GFAP+ (green) and S100beta+ (red). Data represent means ± SEM, n=8 mice per group. *p<0.05 post-hoc analysis.
To account for potential differences in the YFP labeling efficiency in Sac vs. FASD nestin-CreER\textsuperscript{T2}/YFP mice, data were normalized to standard housing conditions within each alcohol treatment group. Statistical analysis revealed significant differences between Sac vs. FASD groups in the number of immature (p=0.007) and mature (p=0.01) postmitotic neurons, but not for type I NSCs, TAPs or neuroblasts under EE conditions when data were normalized to standard housing conditions (Figure 2.5B). However, the overall proportion of YFP+ cells comprising the various cell types in the neuronal lineage did not display a significant shift, although there was a tendency for a higher percentage of early progenitors amongst all YFP+ cells in FASD-EE mice (Figure 2.5C). This suggests that fewer postmitotic neurons in FASD-EE mice is not associated with a significant buildup of early progenitors, suggesting that the effects of FASD are not due to stalled differentiation.
Figure 2.5 Fold Change and Distribution of YFP+ Cells in SAC-EE and FASD-EE. 
(A) Depiction of markers used to identify neurogenic cell types. (B) Number of YFP+ cells in EE normalized to Standard housing within each alcohol treatment group. Data are expressed at the mean normalized values + S.E.M. (n=8 mice per group, *p<0.05 unpaired t-test) (C) Percent distribution of cell types among all YFP+ cells in SAC-EE vs. FASD-EE mice. Data are expressed as the mean percentage of all YFP+ cells in each category + S.E.M (n=8 mice per group).
2.4.4 Motion tracking in Sac and FASD mice within EE cage environment

Recent studies have demonstrated that EE-mediated neurogenesis is correlated with the manner in which mice interact with their environment (Freund et al., 2013). To ensure that the impaired EE-mediated neurogenesis in FASD mice was not due to reduced interaction with the environment, we utilized the EthoVision Motion Tracking system to quantify duration spent within cage zones, as described under the methods section. There were no significant differences between Sac-EE and FASD-EE mice in the duration of time spent in the various cage zones. Seconds spent in various cage zones during a one hour tracking session for Sac-EE vs. FASD-EE mice, respectively, were as follows: running wheel (565 + 115 vs. 466 + 236), ladder (266 + 44 vs. 119 + 20), tunnel (248 + 61 vs. 309 +35, n= 5 mice per group). This suggests that the resistance to EE-mediated neurogenesis in FASD mice is unlikely due to failure to interact with environment.

2.4.5 FASD mice display delayed learning in an A-B context discrimination task

We next compared Sac-EE and FASD-EE mice for their ability to learn an A-B contextual discrimination task, which requires mice to discriminate between two similar contexts. Performance on this learning task has previously been shown to depend on survival and integration of adult-generated DGCs (Sahay et al., 2011; Kheirbek et al., 2012a; Nakashiba et al., 2012). The ability to discriminate between similar contexts was assessed by comparing the time spent freezing in the shock context A vs. the non-shock similar context B, expressed as the discrimination ratio: (freezing context A – freezing context B)/(freezing context A + freezing context B). As shown in Figure 2.6, FASD-EE
mice required a longer time to learn the task compared to Sac-EE mice. Repeated two-way ANOVA (training day vs. treatment) revealed significant main effects of training days (F (4, 64) = 21.22; p < .0001) and alcohol treatment (F (4, 16) = 5.064; p = .0118). This suggests that both control and FASD mice learned to discriminate contexts over the five testing days, but FASD mice displayed a significantly slower rate of learning compared to control mice. Post hoc t-test of discrimination scores at each testing day showed that FASD mice displayed significantly lower discrimination scores compared to control mice at testing day 4 (p = .0425); however, the level of discrimination in FASD mice reached the same level as control mice at testing day 5. Thus, Sac-EE mice could discriminate between contexts by training day 4, whereas FASD-EE mice required 5 days of training. These results demonstrate that impaired neurogenesis in FASD-EE mice is reflected by delayed acquisition of a spatial pattern recognition task previously shown to be reliant on the functional integration of newborn DGCs.
Figure 2.6 Delayed learning acquisition in an A-B Context Discrimination task in FASD mice. (A) Experimental design of the contextual discrimination task. (B) Daily discrimination scores for Sac-EE and FASD-EE mice over five consecutive days of training. Data are expressed as the means + S.E.M., n=5 mice per group.

2.5 Discussion

In the present study, we utilized nestin-CreER\textsuperscript{T2}/ YFP mice to elucidate the stage-specific effects of moderate prenatal alcohol exposure on adult hippocampal neurogenesis. The nestin-CreER\textsuperscript{T2}/YFP mice provide the opportunity to survey the full constellation of maturational cell types in the neurogenic lineage, from primitive type I stem cells to mature postmitotic neurons, at a single time point following tamoxifen-induced recombination in a cohort of nestin\textsuperscript{+} cells within the SGZ. Our results confirm
previous findings of an impaired neurogenic response to EE in FASD mice (Choi et al., 2005), and suggest this is due primarily to a failed survival-promoting effect of EE on postmitotic neurons. In addition, we found that FASD-EE mice display delayed learning in a contextual fear conditioning task that is known to rely on adult neurogenesis. Taken together, these findings demonstrate impaired neurogenic function in a preclinical mouse model of FASD and identify postmitotic neurons as the cells most vulnerable to the detrimental effects of moderate prenatal alcohol exposure.

The limited access “drinking in the dark” alcohol exposure paradigm used in the present study is a voluntary consumption paradigm modified from a mouse model originally established by Boehm and colleagues (Boehm et al., 2008). The limited access to 10% ethanol for 4 hours daily results in average BECs in mouse dams of 88mg/dl throughout the gestational period (Brady et al., 2012). While this is considered a moderate exposure paradigm experimentally, it mimics daily drinking to legal limits of intoxication (80 mg/dl BECs legal intoxication) throughout the first and second trimesters of pregnancy in humans, since the third trimester equivalent of CNS development occurs during the first 2 postnatal weeks in rodents (Maier et al., 1999). This paradigm is relevant to human drinking behavior, since most women who drink during pregnancy report greatly decreased alcohol consumption by the third trimester (Muhuri & Gfroerer, 2009). The limited access mouse model of FASD does not result in altered maternal care, pup weight, litter size, food and water consumption or altered locomotor activity in adulthood (Brady et al., 2012). However, these FASD mice exhibit significant deficits in several hippocampal dentate-specific tasks including delay fear conditioning, trace fear conditioning and nonmatching to place radial arm maze (Brady et al., 2012), and long-
lasting deficits in hippocampal NMDA-receptor-dependent long-term potentiation (LTP) in adulthood (Brady et al., 2013). Our study further shows that EE-mediated neurogenesis is abolished in these mice, even though their exploratory behavior within the complex environment is not impaired. Using a continuous gestational exposure paradigm in a mixed strain background (BECs ~121 mg/dl), we previously found no differences in the size of the SGZ progenitor pool during adulthood, but a significant impairment of EE-mediated neurogenesis as assessed by BrdU labeling of progenitors. The current study confirms and extends those findings to include the limited access paradigm and identify late stage neurogenesis to be most vulnerable.

It is important to note that our EE conditions included continuous access to running wheels in addition to increased social and physical environmental complexity. Both voluntary running and environmental complexity stimulate adult hippocampal neurogenesis, but through different mechanisms (van Praag et al., 1999a; van Praag et al., 1999b; Kempermann et al., 1998). Continuous access to a running wheel has transient proliferative effects on early progenitors in mice, peaking within 10 days and returning to baseline by one month (Kronenberg et al., 2006), whereas environmental complexity in the absence of running primarily enhances the survival and functional integration of newly generated postmitotic neurons (Kempermann et al., 2010). Running and environmental complexity have additive effects on neurogenesis when combined (Fabel et al., 2009). Although we did not observe a significant FASD x housing interaction on the number of YFP+ early progenitors, the significant housing effect on type I NSCs and neuroblasts is consistent with previous studies (Kempermann et al., 1998; Kronenberg et al., 2006; Lugert et al., 2010). That a significant FASD x housing
interaction (p = .001) was detected only for the number of postmitotic neurons suggests that the impaired neurogenic response to EE in FASD mice is largely due to failed EE-mediated survival and integration of newly generated neurons, and not due to an inadequate pool of early progenitors.

Several studies have demonstrated a negative impact of prenatal or early postnatal alcohol exposure on adult hippocampal neurogenesis (reviewed in Gil-Mohapel et al., 2010). Pertinent to our study, Hamilton et al., (2012) reported that rats exposed to high dose (binge) alcohol (~300 mg/kg BEC) during the third trimester equivalent (postnatal day 4-9), show impaired neurogenesis under standard housing conditions, which could be rescued by 12 days of wheel running followed by exposure to environmental complexity. Similarly, Boehme et al., (2011) reported that alcohol exposure throughout all three trimester equivalents (~200 mg/dL BEC) resulted in impaired progenitor proliferation in young adult female mice that could also be reversed by voluntary wheel running. In contrast, our FASD mice showed no deficits in baseline neurogenesis under standard housing, but displayed resistance to the beneficial effects of EE. The discrepancy between these studies could be due to species differences, but are more likely due to differences in the dose and timing of alcohol exposure relative to hippocampal development.

Development of the human hippocampus occurs during the second and third trimesters. In rodents, formation of the dentate granule cell layer (GCL) begins during the third week of gestation (second trimester equivalent) and continues postnatally, with the adult SGZ becoming established by the end of the second postnatal week (third trimester equivalent) (Miller, 1995). In rodents, the oldest DGCs are generated during
the late gestational period from progenitors of the subventricular zone that constitute the secondary germinal matrix of the dentate, whereas younger DGCs are generated during postnatal development from a tertiary matrix that gradually gives way to the SGZ by the second postnatal week (Martin et al., 2002; Hodge et al., 2008; Mathews et al., 2010; Hodge et al., 2013). Recent studies suggest that the long-lasting progenitors within the adult SGZ originate from a distinct population of neural stem cells within the ventral hippocampus generated during the perinatal period (Li et al., 2013). Synaptic input from the entorhinal cortex begins during late gestational periods in the rodent and continues to reach adult levels by postnatal day 25 in the rat (Helfer et al., 2012). Clearly, alcohol exposure during late gestation vs. early postnatal periods could influence distinct aspects of dentate development that lead to long-lasting deficits in neurogenesis that may manifest with distinct characteristics. In our FASD model, hippocampal progenitors are exposed to alcohol during the generation of the oldest DGCs, suggesting that it may be the neurogenic niche environment that is primarily altered by alcohol rather than the postnatal SGZ progenitors themselves.

Brain-derived neurotrophic factor (BDNF) is one niche factor affected by prenatal alcohol that is known to play a critical role in EE-mediated neurogenesis (Rossi et al., 2006a; Bekinschtein et al., 2011a). EE and voluntary exercise elevate levels of BDNF and its receptor, TrkB, in adult hippocampus (Ickes et al., 2000; Vaynman et al., 2004; Wu et al., 2008; Boehme et al., 2011) and enhance synaptic transmission and neuronal excitability (Yamada & Nabeshima, 2003b). Furthermore, knockdown of BDNF levels in heterozygous BNDF+/- mouse impairs EE-mediated neurogenesis (Rossi et al., 2006). BDNF levels in adult hippocampus have been reported to decrease (Feng et al., 2005b;
Caldwell et al., 2008) or to remain unchanged (Boehme et al., 2011) following prenatal alcohol exposure. It will be important to determine whether impaired neurogenesis in response to EE in our paradigm is associated with impaired BDNF signaling; if so, it might be possible to utilize conditional and inducible genetic approaches to enhance BDNF signaling in FASD mice in an attempt to restore neurogenic responsiveness to EE.

Another potential mechanism that could underlie impaired neurogenic responses to EE in FASD mice is altered electrophysiological function of the existing hippocampal circuitry. Neurogenesis is tightly linked to neuronal excitation (Deisseroth et al., 2004; Song et al., 2012). Activity-dependent synaptic integration into existing hippocampal circuitry is one of the most important factors in determining long-term survival of early postmitotic DGCs. Early 1-3 week old progenitors and neuroblasts require GABAergic and glutamatergic input for survival (Ge et al., 2006; Duveau et al., 2011), whereas 4-6 week old postmitotic neurons require activity dependent synaptic integration during a critical period of heightened plasticity and lowered threshold for long-term potentiation (LTP) (Ge et al., 2007). Expression of both NR1 (Tashiro et al., 2006b) and NR2B (Kheirbek et al., 2012) NMDA receptor subunits in newborn DGCs is important for survival and neurogenesis-dependent LTP, respectively. Thus disruptions of the existing circuitry or impaired expression of NMDA receptor subunits in newly generated DGCs could abolish activity-dependent integration and survival of postmitotic neurons in response to behavioral challenge.

Gestational alcohol is known to have marked effects on the electrophysiological properties of the adult dentate gyrus. Prenatal alcohol exposure results in decreased dentate LTP (Sutherland et al., 1997; Varaschin et al., 2010; Helfer et al., 2012; Brady et
decreased expression of both NR1 and NR2B NMDA receptor subunits (Samudio-Ruiz et al., 2010), and decreased NMDAR1 expression (Savage et al., 1992; Barkho et al., 2008). GABA receptor expression and activity is also altered by prenatal alcohol exposure in rodents (Allan et al., 1998; Iqbal et al., 2004). It will be important to determine whether these alterations in receptor expression and excitation are restricted to the existing DGC population, or also occur in adult progenitors and their progeny. In the current study, neurogenic deficits in FASD mice were only observed under conditions of behavioral challenge (EE). Given our moderate alcohol exposure paradigm, it is possible that synaptic transmission is sufficient to support basal rates of neurogenesis, but suboptimal and rate-limiting under conditions of behavioral challenge such as EE. A recent study has identified a novel niche mechanism that drives excitation-neurogenesis coupling; regulation of Wnt signaling by local existing DGCs in an activity-dependent manner (Jang et al., 2013). This pathway should also be evaluated in the context of FASD.

The impairment of EE-mediated neurogenesis in our FASD model was associated with delayed learning in a context discrimination task that requires mice to distinguish between two similar contexts as a test of pattern separation. Sahay et al., (2011) previously demonstrated that enhancing the survival of newly generated DGCs in adulthood is sufficient to improve performance on this task (Sahay et al., 2011). Conversely, ablation of neurogenesis by irradiation (Nakashiba et al., 2012) or by conditional induction of apoptosis in adult progenitors (Tronel et al., 2012) impairs performance on this task, as does inducible deletion of the NR2B NMDA receptor subtype in adult progenitors (Kheirbek et al., 2012). Clelland et al., (2009) demonstrated
impaired spatial pattern separation as measured in a two-choice touch screen task and a nonmatching to place radial arm maze task following depletion of adult hippocampal progenitors (Clelland et al., 2009). Furthermore, Saxe et al. (2006) showed that ablation of hippocampal neurogenesis by irradiation leads to impairment of contextual fear conditioning (Saxe et al., 2006). Our observation that FASD-EE mice display impaired learning on a spatial pattern separation task compared to their Sac-EE controls suggests a functional correlate of the neurogenic deficit. However, we cannot rule out the possibility that this learning delay is due to other deficiencies such as impaired dentate LTP in FASD mice.

To conclude, these studies demonstrate that moderate exposure to gestational alcohol can result in resistance to the neurogenic benefits of EE in mice. The relevance of this work to clinical FASD is underscored by a recent report describing robust adult hippocampal neurogenesis in humans (Spalding et al., 2013). The implications are that some FASD individuals may display a resistance to behavioral training and EE intervention. The current studies provide the basis for testing therapeutic strategies that might restore EE-mediated plasticity in our preclinical FASD model, by targeting mechanisms important for activity-dependent integration of postmitotic neurons. These studies also provide the framework for future investigation using inducible, cell-type specific genetic manipulation of adult generated DGCs vs. niche regulators to restore neurogenic function in preclinical models of FASD.
2.6 Acknowledgements

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3. Experience-Induced Plasticity in New vs. Old Hippocampal Dentate Granule Cells in a Mouse Model of Fetal Alcohol Spectrum Disorder (FASD)

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Department of Neurosciences

University of New Mexico School of Medicine

In preparation
3.1 Introduction

Alcohol consumption during gestational periods leads to fetal alcohol spectrum disorder (FASD) which is broad range of developmental, cognitive and social behavior deficits in children (Kelly et al., 2000; Coriale et al., 2013). FASD includes fetal alcohol syndrome (FAS), alcohol-related neurodevelopmental disorder (ARND) and alcohol-related birth defects (ARBD). The prevalence of FASD population in the school children may be 1-5% in the US and some western European countries (Calhoun et al., 2006; May et al., 2009). However, there is no appropriate clinical treatment established for children with FASD (Kodituwakku & Kodituwakku, 2011).

The hippocampus is the critical brain structure of learning and memory function. Rodent models of FASD with different dose and timing of alcohol exposure result in deficits of hippocampal development and functions (Berman & Hannigan, 2000; Gil-Mohapel et al., 2010). Interestingly, exposure to relative low doses of alcohol during gestation results in some deficit in hippocampal function (Choi et al., 2005; Brady et al., 2012; Brady et al., 2013; Kajimoto et al., 2013).

The DG is one of the regions has produces new neurons throughout the life and is associated with a key component of the hippocampal dependent learning and memory (Sahay et al., 2011; Nakashiba et al., 2012). Newborn neurons receive tonic GABAergic, phasic GABAergic and phasic glutamatergic input as sequential maturational steps (Ge et al., 2006; Deshpande et al., 2013). Parvalbumin (PV) -expressing interneuron mediated GABAergic input regulates the activation and proliferation at the earlier stage of the neuronal maturation such as Type-1 stem cells and neuroblasts, (Song et al., 2012; Song
et al., 2013). Also, glutamatergic input is necessary for survival of newborn neurons (Tashiro et al., 2006a). These findings suggest that new born neurons have to receive proper input from the presynaptic neurons and become integrate into local hippocampal circuits.

Hippocampal neurogenesis is positively and negatively regulated by environmental stimulation. Exercise and voluntary running increase the proliferation of newborn neurons and environmental complexity increase the survival of new born neurons (Fabel et al., 2009). In addition to exercise and environmental complexity, hippocampal dependent learning increases in hippocampal neurogenesis (Gould et al., 1999; Epp et al., 2013). On the other hand, stress induces impairment of hippocampal neurogenesis (Yun et al., 2010).

Interestingly, hippocampal neurogenesis is negatively impacted by exposure of alcohol during gestational period (Gil-Mohapel et al., 2010). Although different in timing, mode of administration and dose, several studies consistently demonstrate impaired neurogenesis following prenatal alcohol exposure. The capacity of self renewal and neural differentiation is impaired in isolated prenatal alcohol exposed neural stem cell progenitors (Roitbak et al., 2011). In addition, ethanol induced hypermethylation of the cell cycle associated protein genes of the neural stem cells and resulting in disruption of the length of cell cycle (Hicks et al., 2010). These studies suggest that the intrinsic mechanisms of neural stem cells are altered by alcohol exposure. In a study with rats, there was no significant change in adult hippocampal neurogenesis but an increase in new immature neurons in animals exposed to alcohol via intragastric intubation of alcohol throughout prenatal and early postnatal periods (Gil-Mohapel et al., 2011). Binge alcohol
exposure during the third trimester equivalent reduced the survival of new born neurons in rats (Klintsova et al., 2007). Also, importantly even a single subcutaneous injection of alcohol at postnatal day 7 has been shown to decrease proliferation and survival of newborn neurons in adulthood (Ieraci & Herrera, 2007). Proliferation and survival of newborn neurons are decreased by liquid ethanol diet during the equivalent of all three trimesters (Redila et al., 2006). These previous studies suggest that adult hippocampal neurogenesis is disrupted by prenatal alcohol exposure.

Previously, our lab found that the environmental enriched mediated enhancement of hippocampal neurogenesis is impaired in FASD mice and the stage of postmitotic immature neurons and mature neurons were severely impacted in these mice (Choi et al., 2005; Kajimoto et al., 2013). However, the impact of prenatal alcohol exposure on the electrophysiological properties of newborn dentate granule neurons has yet to be characterized. In the present study, we investigated the impact of prenatal alcohol exposure on the glutamataergic excitatory and GABAergic inhibitory connectivity in newborn neurons (4 and 8 week old of neuronal ages) and existing granule cells. For this study, we utilized the retrovirus-GFP birth dating technique to visualize newborn neurons for electrophysiological measurement and morphological analysis.

3.2 Materials and Methods

3.2.1 Animals

All animal experiments were approved by the University of New Mexico Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of laboratory Animals. C57/BL6J mice were used for all experiments. Mice were housed in reverse

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12-hour dark / 12-hour light cycle (lights off at 08:00 hours). Food and water were available \textit{ad libitum} except during the maternal drinking period, during which water (but not food) was withheld as described below.

3.2.2 Prenatal Alcohol Exposure

Prenatal alcohol exposure was performed using a limited access paradigm of maternal drinking as previously established for C57Bl/6J mice (Brady \textit{et al.}, 2012), and described in detail elsewhere (Kajimoto \textit{et al.}, 2013). Briefly, stable drinking levels were established using a ramp-up period where water was gradually replaced with 0.066% saccharin containing 0% ethanol (2 days), 5% ethanol (2 days) and finally 10% ethanol for 4 hrs per day from 10:00-14:00 hours. Mice were exposed to 0.066% saccharin containing 10% ethanol (4 hrs per day) for 2 weeks prior to pregnancy and throughout gestation. Female mice offered 0.066% saccharin without ethanol for equivalent time periods served as controls. At 10 days post-ramp, individual females were placed into the cage of singly housed males for 2 hours from 14:00-16:00 for five consecutive days, and then returned to their home cages after each 2 hour mating session, where they continued on the limited access schedule of ethanol or saccharin exposure for 4 hours per day throughout gestation. Ethanol and saccharin concentrations were halved every two days beginning on the day of birth, and returned to drinking only water only on day five. A consumption volume during the 4 hour access period was determined for each mouse from the onset of the drinking paradigm. Average alcohol consumption was 6.98 ± 0.76 g ethanol/ kg body weight/ day. We observed no effect of alcohol exposure on litter size or weight gain (Table 3.1), as reported previously (Brady \textit{et al.}, 2012). The limited access paradigm results in average blood ethanol concentrations of 68.5 + 9.2 mg/dl after 2
hours of drinking and 88.3 + 11.5 mg/dl after 4 hours of drinking in C57/Bl6J mice (Brady et al., 2012).

Table 3.1 Basic parameters of SAC and FASD mice

<table>
<thead>
<tr>
<th></th>
<th>SAC</th>
<th>FASD</th>
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<tr>
<td>Averaged number of pups from one</td>
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<tr>
<td>Pregnant mom</td>
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<tr>
<td>Total:</td>
<td>7.09±1.59</td>
<td>6.50±1.55</td>
</tr>
<tr>
<td>Male:</td>
<td>3.57±1.50</td>
<td>3.18±1.28</td>
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<tr>
<td>Female:</td>
<td>3.52±1.53</td>
<td>3.32±1.70</td>
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<tr>
<td>Averaged Alcohol Drinking (g/kg/day)</td>
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<td>6.98±0.76</td>
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<tr>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>SAC Standard</td>
<td>FASD Standard</td>
</tr>
<tr>
<td></td>
<td>Housing</td>
<td>Housing</td>
</tr>
<tr>
<td>Body weight (g) at injection day</td>
<td>21±0.71</td>
<td>22±1.65</td>
</tr>
<tr>
<td>(PND40-50)</td>
<td>21±0.94</td>
<td>22.7±2.00</td>
</tr>
<tr>
<td>Weight gain (7 weeks after housing</td>
<td>8.70±1.80*#</td>
<td>3.26±1.40*#</td>
</tr>
<tr>
<td>exposure) (g)</td>
<td>3.26±1.40*#</td>
<td>7.38±1.93*†</td>
</tr>
<tr>
<td></td>
<td>3.39±1.27*†</td>
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</tbody>
</table>

* Indicates that Two-way ANOVA showed significant housing effect. # and † indicate that post hoc analysis showed significant changed (p<.05)

3.2.3 Retrovirus injection

Replication-deficient murine retrovirus was used to confer expression of green fluorescent protein (GFP) in proliferating cells and their progeny within the hippocampal dentate gyrus, as previously described (Ge et al., 2006). Mice were anesthetized with isoflurane and 0.5 µl of retrovirus was injected into each of 4 sites targeting the dorsal and ventral hippocampal subventricular zone bilaterally, using a 1 µl Hamilton syringe (#7001). Stereotaxic coordinates were measured from bregma as follows: -2 mm AP, +2.0 mm L, and -2 mm DV for sites in dorsal hippocampus and -3 mm AP, +2.6 L, -2.5
DV for sites in ventral hippocampus. Following the injections, burr-holes were filled with Gelfoam and the scalp sutured with Perma-Hand Silk 6-0 (Ethicon). Buprenorphine (1.0 mg/Kg of body weight) analgesic was subcutaneously administrated after surgery.

3.3.4 Housing conditions

Mice were housed in standard or environmental enriched (EE) conditions for 4 weeks or 8 weeks following retroviral labelling as previously described (Kajimoto et al., 2013). Standard housing included 2-3 mice group housed within a standard mouse cage (W: 16.5 x L: 27.5 x H: 13.0 cm), whereas EE included 5 mice group housed within a larger cage (W: 38.0 x L: 48.0 x H: 20.5 cm) that also included two types of running wheels, a ladder, and both hanging and hidden toys (see Figure 3.1), which were changed out weekly.

3.3.5 Electrophysiology

Mice were anesthetized with ketamine (250 mg/kg, i.p.), transcardially perfused with pre-oxygenated (95%O₂ / 5% CO₂) cold cutting solution containing (in mM): 220 sucrose, 2 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 12 MgSO₄, 10 glucose, 0.2 CaCl₂ and immediately decapitated. The brain was removed and incubated for two minutes in pre-oxygenated (95%O₂/5% CO₂) ice-cold cutting solution. The brain was bisected midsagittally, and the right hemisphere was used for electrophysiological recordings. Transverse hippocampal slices (250 μm-thick) were obtained using a vibrating tissue slicer (Pelco 102 Vibratome, Ted Pella, Redding, CA), and transferred to a holding chamber at 32°C containing oxygenated (95%O₂/5% CO₂) HEPES artificial cerebrospinal fluid containing: (in mM) 126 NaCl, 2 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 1
MgSO$_4$, 2 CaCl$_2$, 0.4 ascorbic acid and 5 HEPES. After 40 minutes in the holding chamber, the slices were incubated at room temperature for at least 30 minutes with fresh HEPES ACSF. Individual slices were transferred to the recording chamber and perfused for 5 minutes with regular ACSF without HEPES (2-3 ml/min). Recordings were performed at 32°C. Adult-generated GFP+ dentate gyrus granule cells (DGCs) were detected using an Olympus BX51WI microscope with epifluorescence illumination. GFP mature DGCs were identified based on their position within the outer region of the dentate granule cell layer. All recordings were performed with Axopatch 200B and Clampex 9.2 software (Molecular Devices, Sunnyvale, CA.). Miniature spontaneous inhibitory postsynaptic currents (mIPSCs) were detected using the following internal pipet solution (in mM): 19 K-gluconate, 121 KCl, 5 NaCl, 4 MgCl$_2$, 0.1 EGTA, 10 HEPES, 4 MgATP, 0.3 Na$_3$GTP, 10 Phosphocreatine, 5 QX-314 Cl (pH 7.4, 300-310 mOsm), in the presence of 3 mM of kynurenic acid and 0.5 µM tetrodotoxin (TTX) added into the recording ACSF. Miniature spontaneous excitatory postsynaptic currents (mEPSCs) were detected using an internal pipet solution containing (in mM) 120 K-Gluconate, 15 KCl, 4 MgCl$_2$, 0.1 EGTA, 10 HEPES, 4 MgATP, 0.3 Na$_3$GTP, 7 phosphocreatine, 5 QX-314 Br (pH 7.4, 290-300 mOsm), with 100 µM of picrotoxin and 0.5 µM TTX added into the recording ACSF. The pipet tip resistance was 3-4 MΩ. The access resistance for recording was <35MΩ and was not compensated. Recordings were not analyzed if access resistance changed by ≥20% during the recording period. The event data were analyzed using the Mini Analysis Program (Synaptosoft, Decatur, GA).
3.3.6 Intracellular Biocytin Cell Fill and Staining

Existing mature DGCs located at the outer region of the hippocampal granule cell layer were filled with biocytin for morphological analysis using 0.5% of biocytin (Sigma) added to the internal pipet solution (tip resistance increased to 5-6 MΩ). The slices were then incubated in the recording chamber for 15-20 minutes to allow biocytin to fill distal processes, followed by fixation in 4% paraformaldehyde in 0.1M phosphate-buffered saline (PFA) overnight. Slices were washed with PBS for 30 minutes, permeabilized with 0.4% of Triton-X100 in PBS for 30 minutes and incubated with Cy-3 conjugated Streptavidin (0.125 µg/ml in PBS containing 1% BSA) for 90 minutes. Slices were mounted on the glass slides and coverslipped with Fluoromount-G™ (Electron Microscopy Sciences, Hatfield, PA).

3.3.7 Morphological analysis

All Images were taken using a Zeiss 510 META microscope. Dendritic complexity was analyzed using Sholl analysis of biocytin-filled mature DGCs. Briefly, an image stack was acquired using a 20x objective with 1 µm optical intervals, and then compressed into a single image plane by applying the maximum intensity projection function. All dendrites from a single biocytin-filled neuron were traced using Neurolucida software (MBF Bioscience, Inc. Williston, VT). Dendritic intersections crossing each concentric ring, (beginning 10 µm from the center of the cell soma), were counted manually, with concentric rings spaced at 10 µm apart. For measuring spine density in newborn GFP+ neurons, the left hemisphere of each brain was immediately fixed with 4% PFA overnight, followed by overnight cryoprotection with 30% sucrose in PBS. Transverse
hippocampal slices (60 µm thickness) were obtained using a freezing sliding knife microtome. Sections were stored at -20°C in 25% glycerol, 25% ethylene glycol and 50% of 0.1M phosphate buffer solution. Sections were mounted on glass slides and coverslipped with Fluoromount-G™. Image stacks were taken using a 100x oil objective with 0.2 µm optical section intervals. For each mouse, > 50 µm of distal dendrite segments were sampled. Neurolucida software was used to trace the dendrite as stack images and to quantify spine number.

3.3.8 Statistics

Data were analyzed by two-way ANOVA with post-hoc Multiple comparison (Sidak’s multiple comparison or Tukey's multiple comparison) using Prism (Graph Pad, San Diego, CA). Data are expressed as means ± S.E.M., with p<0.05 considered statistically significant.

3.3 RESULTS

3.3.1 FASD Influences the Electrophysiological Response to EE in Adult-born Hippocampal DGCs

We previously demonstrated that FASD mice display an impaired hippocampal neurogenic response to EE, resulting in a 50% reduction in the number of adult-generated DGCs in FASD mice compared to controls following a 10 week exposure to EE (Kajimoto et al., 2013) (Choi et al., 2005). Impaired neurogenesis in FASD-EE mice was also associated with delayed learning on a context discrimination task, although FASD mice eventually acquired the learning task (Kajimoto et al., 2013). To determine whether
housing and/or prenatal alcohol exposure influences the synaptic integration of surviving adult-generated DGCs, we performed whole-cell patch-clamp recordings of mEPSC and mIPSCs in 4 week old and 8 week old DGCs following exposure to standard vs. EE housing conditions. Hippocampal progenitors from Sac control and FASD mice were labeled by stereotaxic injection with GFP retrovirus, and subsequently placed into standard or EE conditions similar to our previous paradigm (Kajimoto et al., 2013). The mice were then sacrificed at 4 or 8 weeks post-retroviral injection (wpi) for slice electrophysiological recordings of newborn GFP+ DGCs (Figure 3.1).

**Figure 3.1**

**Figure 3.1** Experimental procedure of this study. **A.** Experimental design. PND: Post natal day **B.** GFP fluorescence in newborn neurons at 4 weeks following retrovirus injection. Scale bar: 100µm **C.** Environmental enrichment (5 mice) and standard cages (3 mice).
3.3.1.1 FASD potentiates EE-mediated glutamatergic neurotransmission in newborn DGCs

As shown in Figure 2, spontaneous miniature excitatory postsynaptic currents (mEPSCs) were readily detectable in both 4 week and 8 week old GFP+ DGCs. Miniature EPSCs were blocked by 3 mM kynurenic acid, indicating that these events were mediated by ionotropic glutamate receptors, most likely AMPA receptors (Figure 3.2A and 3.2E). As anticipated, the frequency of mEPSCs increased ~2-fold between 4 and 8 wpi, reflecting maturation and synaptic integration of newborn DGCs as previously described (Ge et al., 2006; Zhao et al., 2006; Bischofberger, 2007).

Miniature EPSC frequency in 4 week old GFP+ DGCs was not influenced by prenatal alcohol or housing (Figure 3.2B), but was increased ~2-fold by EE in 8 week old GFP+ DGCs from FASD mice but not mice housed under standard conditions (Figure 3.2F). Two-way ANOVA indicated a heightened mEPSC frequency response to EE in FASD mice that was not observed in control mice (FASD x housing interaction (F (1, 20) = 5.056 p= 0.036). However, 8 week old GFP+ DGCs from FASD mice also displayed a unique decrease in mEPSC amplitude (F (1, 20) = 4.426; p= 0.048) and an increase in mEPSC decay time (F (1, 20) = 0.009) in response to EE, neither of which were observed in SAC control mice (Figure 3.2G and H; post hoc analysis, p<0.03 for both amplitude and decay, FASD-EE vs. FASD-standard). These observations suggest that the glutamatergic properties of 8 week-old newborn neurons from FASD mice display hyper-responsivity to EE compared to SAC controls in terms of action potential-independent glutamatergic synaptic transmission, although the net effect of increased frequency and
decay time of mEPSCs may be offset by changes in amplitude. Nevertheless, those changes in glutamatergic neurotransmission were only observed in FASD mice, suggesting a compensatory response to EE on a per neuron basis. This may reflect the fact that FASD-EE mice are functioning with approximately 50% fewer adult-generated DGCs compared to SAC-EE mice. (Choi et al., 2005; Kajimoto et al., 2013).
Figure 3.2

Whole cell electrophysiological recordings of AMPA receptor mediated miniature activity (mEPSCs) in newborn GFP+ neurons at 4 weeks (A-D) and 8 weeks (E-H) post retroviral injection (wpi). A and E. Whole cell patch trace recordings from individual GFP+ neurons at 4 (A) or 8 (E) wpi in the absence (top traces) or presence (bottom traces) of 3 mM kynurenic acid (scale bar = 20pA and 1s). B-D. Frequency, amplitude and decay values for mEPSCs recorded from GFP+ newborn neurons at 4 wpi. F-H. Frequency, amplitude and decay values for mEPSCs recorded from GFP+ newborn neurons at 8 wpi. Data represent means ± SEM, n=6 mice per group. * indicates post-hoc Tukey’s multiple comparison F and Sidak’s multiple comparison G-H (p<0.05).
3.3.1.2 FASD impairs EE-mediated disinhibition in newborn DGCs

As shown in Figure 3.3, spontaneous miniature inhibitory postsynaptic currents (mIPSCs) were also readily detectable in GFP+ DGCs, with a slight increase in frequency between 4 and 8 wpi. Miniature IPSCs were blocked in the presence of 100 μM bicuculline (Figure 3.3A and 3.3E), indicating that these events were mediated by GABA<sub>A</sub> receptor-mediated synaptic transmission. In contrast to mEPSCs, neither prenatal alcohol nor housing influenced mIPSC frequency (Figure 3.3 B, F) or decay (Figure 3.3 D, H) in 4 or 8 week old GFP+ DGCs. However, two-way ANOVA indicated a significant influence of EE on the amplitude of mIPSCs in 8 week-old newborn DGCs (F (1, 20) = 11.39; p= 0.003) in SAC (post-hoc, p= 0.005), but not FASD mice compared to standard housing for both groups. These data suggest that EE stimulates a disinhibition of newborn DGCs, which is blunted by FASD. However, this decreased inhibition in SAC-EE mice is not associated with enhanced frequency of glutamatergic activity (see above).
Figure 3.3 Whole cell electrophysiological recordings of GABA<sub>A</sub> receptor mediated miniature activity (mIPSCs) in newborn GFP+ neurons at 4 weeks (A-D) and 8 weeks (E-H) post retroviral injection (wpi). A and E. Whole cell patch trace recordings from individual GFP+ neurons at 4 (A) or 8 (E) wpi in the absence (top traces) or presence (bottom traces) of 100 mM bicuculline (scale bar = 40pA and 2s). B-D. Frequency, amplitude and decay values for mIPSCs recorded from GFP+ newborn neurons at 4 wpi. F-H. Frequency, amplitude and decay values for mIPSCs recorded from GFP+ newborn neurons at 8 wpi. Data represent means + SEM, n=6 mice per group. * indicates post-hoc Sidak’s multiple comparison (p<0.05).
3.3.1.3 Neither FASD nor EE influence dendrite spine density in adult-generated DGCs

The enhanced glutamatergic current frequency in FASD-EE mice would suggest increased numbers of dendritic synapses, presumably accompanied by increased dendrite branching and/or increased dendritic spine density. To determine whether the EE-mediated electrophysiological changes in newborn DGCs were associated with changes in the density of dendritic spines, we utilized high resolution confocal microscopy to quantify spine density in GFP+ cells at 4 and 8 wpi across treatment groups. As shown in Figure 3.4, there were no effects of prenatal alcohol, housing or age on spine density in GFP+ DGCs. However, we could not assess spine morphology due to limitations in imaging resolution, or dendritic branching morphology due to extensive overlap of dendritic arbors in adjacent GFP+ DGCs. Thus, we cannot rule out potential effects of FASD or EE on these other parameters of morphological plasticity.
3.3.2 FASD influences the Electrophysiological Response to EE in Preexisting Hippocampal DGCs

To determine the influence of EE on the electrophysiological properties of preexisting DGCs in the context of FASD, we performed whole-cell patch-clamp recordings from GFP- DGCs situated in the outer third of the dentate granule cell layer. The outer third of the dentate granule cell layer (GCL) contains the oldest DGCs that are born during the perinatal period, whereas adult-generated DGCs are restricted to the innermost layers of
the granule cell layer (Muramatsu et al., 2007; Mathews et al., 2010b). Although the basic electrophysiological properties and synaptic plasticity at 8 week old new born neurons are identical to preexisting DGCs (Ge et al., 2007), morphological modifications occur as new born neurons continue to mature (Zhao et al., 2006; Piatti et al., 2011). Thus, it was of interest to compare the electrophysiological responses of these older DGCs to newborn DGCs in the context of responses to prenatal alcohol and environment.

3.3.2.1 FASD impairs EE-mediated enhancement of glutamatergic transmission in preexisting DGCs

As shown in Figure 3.5, preexisting DGCs displayed an approximate 2-fold increase in glutamatergic synaptic current frequency in SAC mice, but not in FASD mice. Two-way ANOVA revealed that the frequency (F (1, 20) = 16.07; p<0.001) and decay of mEPSCs (F (1, 20) = 5.39; p=0.031) were significantly affected by the housing conditions, but there was no significant effect on amplitude (Figure 5A-D). Post-hoc analysis showed a significant increase in frequency and decay time in SAC EE mice when compared to SAC standard housed mice, but there was still a trend toward an increase in frequency in EE-housed FASD mice (p=0.062). These observations suggest that prenatal ethanol exposure impairs the EE-mediated enhancement of action potential-independent glutamatergic neurotransmission in existing DGCs. This effect is opposite to that of FASD in adult-born neurons, where FASD results in enhanced glutamatergic neurotransmission following EE.
3.3.2.2 FASD promotes EE-mediated enhancement of GABAergic transmission in preexisting DGCs

As shown in Figure 3.5 F and G, preexisting DGCs displayed a significant (~2-fold) increase in mIPSC frequency in response to EE in FASD, but not SAC mice. In addition, two-way ANOVA revealed that the frequency (F (1, 20) = 19.55) and amplitude (F (1, 20) = 4.367), but not decay, of mIPSC were significantly influenced by housing (Figure 3.5 E-H). Post-hoc analysis showed that the frequency of mIPSC in SAC mice was trending to increase with EE (p=0.058) and significantly increased in FASD EE mice (p=0.002) (Figure 3.5 F). In addition, the amplitude of mIPSC was significantly increased by EE only in FASD mice (p=0.018). These data suggests that preexisting DGCs display a trend toward increased GABA_A receptor-mediated synaptic input in both SAC and FASD mice following EE, but more inhibitory connections are formed in EE-housed FASD mice when compared to standard conditions.
Figure 3.5. Effect of mEPSCs and mIPSCs on preexisting dentate granule neurons with EE and FASD treatment. A. Trace of mEPSCs recording from existing dentate granule neurons (Scale bar: 20pA and 1s). Top trace shows mEPSCs event without Kynurenic Acid. Bottom trace shows all mEPSCs events were blocked with 3mM Kynurenic Acid. B. Frequency, C. Amplitude and D. Decay of existing dentate granule neurons. E. Trace of mIPSCs recording from existing dentate granule neurons (Scale bar: 40pA and 2s). Top trace shows mIPSCs event without bicuculline. Bottom trace shows all mIPSCs events were blocked with 100 µM bicuculline. F. Frequency, G. Amplitude and H. Decay of existing dentate granule neurons. * indicates post-hoc Sidak’s multiple comparison (p<0.05). Date represent mean ± SEM, n=6 animals per group.
3.3.2.3 FASD abolishes EE-mediated enhancement of dendritic branching in preexisting DGCs

To determine whether EE-mediated changes in synaptic connectivity in preexisting DGCs revealed by electrophysiological measures were accompanied by changes in dendritic branching, individual DGCs in the outer third of the hippocampal GCL were backfilled with biocytin and processed for Sholl analysis of dendritic branching complexity. As shown in Figure 6, EE increased branching complexity in distal dendrites in preexisting DGCs, and this effect was abolished by prenatal alcohol (FASD-EE). As shown in Figure 6D-E, two-way ANOVA revealed significant housing (F (1, 20) = 7.3; p= 0.014) and FASD (F (1, 20) = 4.86; p= 0.039) effects on distal dendritic segments >170 µm from soma. Post-hoc analysis confirmed that the effect of EE on dendritic branching was only significant in SAC mice (Figure 3.6. E). In addition, total dendritic length was increased by EE treatment only in SAC mice (Figure 3.6. F). Therefore, distal dendritic complexity is enhanced by environmental stimulation in SAC mice, but this environmental effect is blunted in FASD mice. These data suggest that prenatal alcohol (FASD) impairs dendritic plasticity in preexisting DGCs.
3.4 Discussion

In this study, we investigated the effects of prenatal alcohol exposure (FASD) on EE-mediated changes in basal excitatory and inhibitory synaptic transmission of newborn and existing hippocampal DGCs. Surprisingly, we found no effects of EE on basal excitatory synaptic transmission within newborn DGCs recorded from SAC control mice,
but observed a marked *enhancement* of basal synaptic activity in 8 week old DGCs in response to EE only in FASD mice. In contrast, we found that EE significantly enhanced excitatory basal synaptic transmission within existing DGCs from SAC control mice, an effect that was significantly *diminished* in FASD mice. This effect of EE on preexisting DGCs on FASD mice was accompanied by an impairment of EE-mediated branching of distal dendrites from existing DGCs. These observations suggest that the electrophysiological changes in basal synaptic transmission in response to EE are different in newborn vs. existing DGCs and that FASD significantly influences the magnitude and direction of this response. These studies further suggest that both the electrophysiological properties and morphological plasticity of the adult hippocampal GCL may be resistant to the full benefits of social and physical enrichment in FASD.

**Newborn DGCs**

We utilized retroviral GFP labeling to identify adult-generated DGCs, coupled with whole cell patch clamp recordings to assess the electrophysiological responses of newborn DGCs to EE in SAC control and FASD mice. Our results demonstrated that EE markedly enhanced the basal glutamatergic excitatory synaptic transmission in 8 week old DGCs only in FASD and not in SAC control mice. mEPSC frequency in FASD-EE mice was increased approximately 2-fold compared to that of FASD-standard housed mice. The increased mEPSC frequency was accompanied by a significant prolongation of mEPSC current decay. These observations suggest a significant enhancement of basal excitatory neurotransmission in adult-generated DGCs, although this may be somewhat offset by a small but significant reduction in mEPSC amplitude in FASD-EE mice compared to the other groups. Interestingly, EE also stimulated a significant reduction of
mIPSC amplitude in SAC control mice, which was abolished by FASD. Taken together, these observations suggest enhanced excitatory neurotransmission in newborn DGCs from FASD mice. This heightened basal excitatory connectivity in FASD-EE mice may represent a compensatory hyper-responsiveness to EE that could act to partially counteract the previously documented impaired neurogenic response to EE, in which FASD-EE mice harbor 50% fewer newborn DGCs compared to their SAC-EE counterparts (Choi et al., 2005; Kajimoto et al., 2013). Alternatively, FASD may select for survival of only the most electrophysiologically integrated DGCs, and the 50% reduction in the number of surviving DGCs in FASD-EE mice may result in a bias for recording from this subpopulation.

Surprisingly, the increased frequency of mEPSC in FASD-EE mice was not associated with an increase in spine density which would be anticipated if the increased mEPSC frequency were due to increased number of excitatory synapses. Additional morphological correlates of increased synapse number might also include enhanced spine maturation or dendritic branching complexity. Unfortunately, we were unable to obtain the resolution necessary to assess spine morphology and were unable to assess dendritic branching due to overlap of GFP+ dendritic arbors. However, it is important to note that previous studies have reported an increase in the density of mushroom spines, thought to represent sites of enhanced excitatory synaptic transmission, without a change in overall dendrite spine density in adult-generated DGCs in response to physical activity (Zhao et al., 2006). Also, prior studies have demonstrated increased dendritic branching specifically in 4 month old adult-generated DGCs in response to repetitive training on a complex spatial learning task that did not occur in existing perinatally-generated DGCs.
(Tronel et al., 2010; Lemair et al., 2012). To our knowledge, prior studies have not assessed the electrophysiological or morphological changes that occur in newborn DGCs following EE, nor the impact of prenatal alcohol on these parameters in adult-generated DGCs. Thus, it will be important in future studies to elucidate the morphological correlates of enhanced synaptic transmission in response to EE in FASD mice and to further investigate the behavioral functional significance of this enhanced response. The increase in mEPSC frequency in FASD mice in response to EE may also represent a persistent increase of presynaptic glutamate release and/or activation of existing silent synapses.

In addition to increased frequency of mEPSCs in newborn DGCs from FASD-EE mice, we also observed a reversal of EE-mediated mIPSC frequency and small but significant changes in the glutamatergic and GABAergic amplitude and decay. In 8 week old newborn neurons, mEPSC amplitude was decreased and decay was prolonged only in EE-housed FASD mice. There are several possibilities to explain the alteration of current amplitude and decay of mEPSCs. One mechanism could be a change in AMPAR subunit expression. For example, AMPARs have an alternative spliced exon called flip/flop domain, encoding the extracellular M3-M4 loop (Sommer et al., 1990; Partin et al., 1996), which regulates the kinetics of channel function in a cell-type specific and developmental manner (Partin et al., 1996; Koike-Tani et al., 2005). Another possibility is altered expression of the AMPAR GluA1-4 subunits that regulate the kinetics and permeability of AMPAR (Hollmann & Heinemann, 1994; Angulo et al., 1997). Finally, AMPAR trafficking to the membrane regulated by transmembrane AMPAR regulatory protein (TARP) (Rouach et al., 2005), could also account for changes in gating kinetics.
of AMPAR (Tomita et al., 2005). Thus, changes in current amplitude and decay could be caused by enhanced flip-flop splicing, AMPAR subunit composition changes, and/or TARP activation. It is important to note that the AMPA mediated current decay is fastest in the newborn DGCs, and the slowest decay is displayed in older DGCs (Schmidt-Salzmann et al., 2014). This suggests that the properties of AMPAR in newborn DGCs are likely regulated by cell maturation. Therefore, it is possible that this unique sequential property of AMPARs during maturation of DGCs neurons is impacted by prenatal alcohol exposure in response to EE. Indeed, several studies have demonstrated effects of prenatal alcohol on NMDAR-mediated neurotransmission and receptor subunit expression within the adult hippocampus (Savage et al., 1992; Farr et al., 1988; Wijayawardhane et al., 2007; Vaglenova et al., 2008; Samudio-Ruiz et al., 2010), although this has not been studied in newborn DGCs in the context of EE and FASD.

Regardless of the underlying mechanisms, the enhancement of basal excitatory synaptic transmission following EE in FASD mice raises the question of whether this response is physiologically relevant for hippocampal function. In previous studies, we demonstrated that the impaired neurogenic response to EE in FASD mice is associated with delayed learning in an A-B contextual discrimination learning task, a behavior that is thought to be reliant on neurogenic function (Kajimoto et al., 2013). However, in those studies, FASD-EE mice eventually learned the task to perform at the same level as SAC-EE mice. This suggests that the surviving subpopulation of newborn DGCs in FASD-EE mice are functionally competent to mediate this behavioral improvement, albeit over a slower timecourse compared to SAC-EE mice. Alternatively, repeated training on this
task may result in the recruitment of other non-neurogenesis-dependent circuitry for learning.

**Preexisting DGCs**

In contrast to our findings of enhanced excitatory synaptic transmission in adult-generated DGCs from FASD-EE mice, we found electrophysiological evidence for the suppression of EE-mediated excitatory transmission in existing DGCs, as assessed by recording from older DGCs situated in the outer region of the GCL. In SAC-control mice, EE stimulated a >2-fold increase in mEPSC frequency, accompanied by a significant prolonged decay and no change in amplitude. Although mEPSC frequency was also enhanced in existing DGCs from FASD mice, this did not reach statistical significance. In contrast, EE stimulated an increase in mIPSC frequency in existing DGCs that reached statistical significance only in FASD mice, which was coupled with increased mIPSC amplitude in FASD mice and no change in decay. The increase in EE-mediated excitatory synaptic transmission in SAC-control mice was correlated with enhancement of dendritic branching complexity, which was also significantly diminished in FASD mice. While there have been few studies of EE or FASD on the basal synaptic transmission of DGCs within the outer region of the GCL, prior studies have demonstrated that EE stimulates an increase in population spike amplitudes of DGCs (Irvine et al., 2006; Green and Greenough, 1986) and the induction of dendritic branching in DGCs of female rats (Juraska et al., 1985). In addition, EE facilitates LTP, enhances intrinsic excitability and EPSP-spike coupling in hippocampal CA1 neurons (Malik and Chatterji, 2012). Similar to our finding, Berman et al., (1996) also demonstrated EE-mediated enhancement of dendritic spine density in CA1 pyramidal neurons, which was
blunted by prenatal alcohol exposure. Interestingly, several papers have reported previously that FASD animals displayed LTP deficits within DGCs as measured by field excitatory postsynaptic potentiation (Sutherland et al., 1997; Varaschin et al., 2010a; Brady et al., 2013). Our studies add to these findings, to demonstrate impaired morphological plasticity of preexisting DGCs in FASD mice.

FASD not only impaired EE-mediated increases in basal excitatory synaptic transmission in existing DGCs, but also exacerbated EE-mediated enhancement of inhibitory neurotransmission as indicated by an increase in mIPSC frequency that reached significance only in FASD mice. Interestingly, GABA_A receptors have previously been shown to be altered by prenatal alcohol exposure in rodents (Allan et al., 1998; Iqbal et al., 2004). Iqbal et al. (2004) reported that GABA_A receptor subunit β2/3 protein expression is increased by prenatal alcohol exposure in the guinea pig at postnatal day 60. Modulation of GABA_AR is altered in hippocampal neurons by prenatal alcohol exposure (Allan et al., 1998). GABA_ARγ2 is also elevated by EE housing during late prenatal and early postnatal period (Liu et al., 2012). Overall, these findings suggest that prenatal alcohol exposure may induce long term alterations in glutamatergic and GABAergic receptor subunit expression, which could shift the balance of basal synaptic activity from excitatory to inhibitory under EE conditions.

**Conclusion**

The overall findings from this study indicate that FASD disrupts the EE-mediated enhancement of basal excitatory synaptic transmission in existing DGCs, and that this is accompanied by heightened excitatory synaptic transmission in the subpopulation of
newborn DGCs that survive in response to EE in FASD mice. These findings raise the intriguing possibility that it is a disruption of GCL circuitry that impairs the neurogenic response to EE in FASD mice. In addition, these results suggest that FASD may diminish the benefits of certain types of behavioral therapies in clinical FASD that are targeted toward enhancement of hippocampal function and associated behaviors.
4. Discussion

4.1 Summary

This dissertation research addressed the hypothesis that prenatal alcohol exposure (FASD) impairs the EE-mediated survival and electrophysiological plasticity of newborn DGCs in the adult hippocampus. We utilized histological, behavioral and electrophysiological approaches to address this hypothesis. Our overall findings indicate that FASD severely impairs the late-stage survival of newborn DGCs and disrupts the electrophysiological response to EE in the existing GCL, and that this is accompanied by heightened excitatory synaptic transmission in surviving DGCs. These results suggest that FASD may impart resistance to certain types of behavioral therapies targeting hippocampal function. The findings also raise several intriguing questions pertaining to mechanisms and functional significance of these findings, which are outlined in my critical analysis in the next section.

To summarize our findings, we utilized nestin-CreER$^{T2}$/ YFP mice to elucidate the stage-specific effects of moderate prenatal alcohol exposure (FASD) on adult hippocampal neurogenesis, and confirmed previous findings of an impaired neurogenic response to EE in FASD mice (Choi et al., 2005). Our findings also demonstrated that the impaired neurogenic response to EE is due primarily to a failed survival-promoting effect of EE on postmitotic neurons. The functional significance of this impairment is underscored by our findings of delayed learning in a contextual fear conditioning task in FASD-EE mice that is known to rely on adult neurogenesis. The next step in our quest to understand how FASD impacts adult neurogenesis was to determine whether the
newborn DGCs that do survive in FASD-EE mice display impaired electrophysiological properties. If so, future therapies would require targeting not only survival mechanisms for newborn neurons, but also targeting of electrophysiological function.

To determine whether FASD impairs the basal electrophysiological properties of newborn DGCs that survive under EE conditions, we utilized a retrovirus-GFP labeling technique and whole cell patch clamping recordings of newborn and existing DGCs. Surprisingly, we found that basal excitatory synaptic transmission was markedly enhanced in 8 week old DGCs following EE in FASD mice, compared to that of SAC-EE mice, suggesting a compensatory hyper-responsiveness to experience that may partially counteract the impaired neurogenic response to EE (Figure 4.1). Also unexpectedly, we found that FASD significantly altered EE-mediated changes in basal synaptic transmission within developmentally generated preexisting DGCs, as assessed by electrophysiological recordings of older DGCs situated in the outer region of the GCL. The impact of FASD on existing GCL circuitry is also underscored by the severe impairment of EE-mediated distal dendritic branching in FASD mice (Figure 4.2).

An important question is whether these effects of FASD are mechanistically linked; i.e., is the impaired neurogenic response to EE in FASD mice due to disruption of the preexisting circuitry into which these nascent DGCs must functionally integrate? If so, what are the potential mechanisms by which the existing GCL circuitry influences neurogenic responsiveness to environmental experience, and can this impaired plasticity be reversed by targeting these mechanisms? Equally important is to elucidate the mechanisms responsible for FASD-induced electrophysiological hyper-responsiveness of
adult-generated DGCs to EE and to determine whether the heightened excitatory transmission in these newborn DGCs is functionally significant.

**Figure 4.1**

![Diagram](image)

**Figure 4.1.** Summary of Alcohol and EE effects on the number and electrophysiological connectivity of adult-generated DGCs.
Figure 4.2 Summary of Alcohol and EE effects on the morphological plasticity and electrophysiological connectivity of developmentally-generated DGCs.

4.2 Critical Analysis, Unanswered Questions and Future Directions

4.2.1 Is the impaired neurogenic response to EE in FASD mice due to impaired neurotrophic factor and/or neurotransmitter receptor signaling?

4.2.1.1 Role of BDNF Signaling

We utilized nestin-CreER$^{T2}$/ YFP mice to determine the stage-specific effects of moderate prenatal alcohol exposure on the neurogenic response to EE in FASD mice. Our findings demonstrated impaired neurogenic function and identified postmitotic and
matured newborn DGCs as those most vulnerable to the detrimental effects of moderate prenatal alcohol exposure.

It is well known that brain-derived neurotrophic factor (BDNF) plays an important role for EE-mediated neurogenesis (Rossi et al., 2006; Bekinschtein et al., 2011). Several studies have reported that EE and voluntary exercise elevate BDNF levels within the adult hippocampus (Ickes et al., 2000; Vaynman et al., 2004; Boehme et al., 2011). BDNF signaling has been shown to enhance synaptic transmission and neuronal excitability (Yamada & Nabeshima, 2003). Importantly, increased BDNF signaling is required for EE-mediated neurogenesis, as demonstrated using heterozygous BNDF+/− mice (Rossi et al., 2006).

Although we did not measure the expression of BDNF or signaling through its receptor TrkB in our studies, previous reports suggest that BDNF signaling may be impaired by prenatal alcohol exposure. BDNF protein expression level has been reported to decrease within the hippocampus in offspring of rats fed alcohol by intragastric intubation throughout gestation (3 g alcohol/kg/day: no BAC reported) (Feng et al., 2005) and following prenatal and early postnatal alcohol exposure through voluntary drinking (BAC: ~130 mg/dl) (Fiore et al., 2009). Also, the ratio of phosphorylated-TrkB to nonphosphorylated receptor was previously demonstrated to decrease following prenatal alcohol exposure (Feng et al., 2005). In contrast, Heaton et al., (2000) reported no change in BDNF expression following alcohol exposure during all three trimester equivalents (i.e., prenatal liquid diet for rat dams (BAC: ~168 mg/dl) followed by vapor chamber inhalation during early postnatal period (BAC: ~300 mg/dl) (Heaton et al., 2000). Similarly, Caldwell et al. (2008) reported that the BDNF protein level was not affected by
moderate prenatal alcohol exposure, although BDNF mRNA expression was decreased within the DG (Caldwell et al., 2008). These conflicting results may be due to the use of different prenatal alcohol exposure paradigms, timing and dose.

It is important to note that none of the studies mentioned above investigated BDNF signaling under conditions of the interaction of EE and FASD. Our observations that the rate of neurogenesis is unchanged by FASD under standard housing suggests that it is the effect FASD on BDNF signaling under conditions of EE that would be most relevant and functionally significant. While one study demonstrated that BDNF levels are enhanced by running in both control and FASD rats (Boehme et al., 2011), there is no evidence that FASD animals have normal downstream signaling of BDNF under standard housing or under conditions of EE. Thus, it will be imperative in future studies to determine whether impaired EE-mediated neurogenesis in FASD mice is associated with impaired BDNF and TrkB signaling. If so, this mechanism could be targeted experimentally using genetic overexpression of BDNF or its activated receptor, in an attempt to reverse the neurogenic deficit in FASD mice. If successful, those studies would identify a novel therapeutic target of potential use to enhance the benefits of behavioral therapy in clinical FASD.

4.2.1.2 GluN2B-NMDAR signaling

Another possibility for the failed survival response to EE in newborn DGCs in FASD mice may relate to an intrinsic incompetence to respond to activity-dependent integration due to impaired expression of the GluN2B-NMDA subunit in newborn cells. Previous studies have shown a critical time window in the maturation of newborn DGCs during which these cells display lowered threshold and for a heightened LTP. Importantly, that
response is dependent on expression of the GluN2B containing NMDAR subunit. This period of GluN2B-dependent heightened plasticity is required for integration of newborn DGCs into the existing circuitry (Ge *et al.*, 2007), but not for early stem cell proliferation (Kheirbek *et al.*, 2012b). This GluN2B mediated enhanced plasticity is required for neurogenesis dependent behaviors, especially contextual fear discrimination and novelty exploring (Kheirbek *et al.*, 2012b). Our data of contextual fear discrimination on a pattern separation learning task shows that FASD mice displayed a slower rate of learning compared to control mice under enriched conditions, suggesting that newborn neurons have impaired plasticity in FASD mice.

Although we did not test the NR2B-dependent enhancement of LTP in newborn DGCs in our study, a previous report using the same drinking paradigm as our study described FASD impairment of GluN2B dependent LTP in the DG (Brady *et al.*, 2013b). This raises the possibility that the impaired LTP observed by Brady *et al.*, (2013) may be due in part to impaired GluN2B-dependent LTP in newborn neurons. It will be important in future studies to determine whether newborn DGCs in FASD mice display impaired GluN2B dependent LTP during a critical time window for activity-dependent integration. These studies could be performed in electrophysiological slice by measuring the LTP component that remains in the absence of GABA$_A$ receptor inhibition (neurogenesis-dependent LTP). It is noteworthy that despite repeated attempts, we were unsuccessful in detecting this small LTP component, presumably due to subtle technical requirements that we were unable to identify or resolve (please see Appendix).
4.2.2. What is the mechanism and functional significance of heightened excitatory synaptic transmission in newborn DGCs from FASD-EE mice?

Our electrophysiological studies demonstrated that EE enhanced the basal glutamatergic excitatory synaptic transmission in 8 week old newborn neurons only in FASD mice. Therefore, surviving 8 week old newborn neurons from FASD-EE mice display heightened excitatory transmission under the more demanding behavioral challenge of environmental enrichment. This indicates a functional attempt to compensate for the impaired neurogenic response to EE in FASD mice, through enhanced glutamatergic connectivity of newborn cells.

An alternative explanation for heightened basal synaptic transmission in FASD-EE mice is that FASD results in a natural selection for survival of only the most electrophysiologically fit newborn DGCs and that the 50% reduction in the number of surviving cells in FASD-EE mice results in a bias for recording from this subpopulation of surviving DGCs. Regardless of underlying cause, these studies raise the question of whether the selective survival of an “electrophysiologically fit” DGC subpopulation and apparent hyper-responsivity of surviving newborn DGCs to EE is functionally significant. Although we demonstrated that FASD-EE mice display delayed learning in a hippocampal neurogenesis-dependent task, they do eventually learn the task after 5 days of training and perform at the same level as control mice. This suggests that the surviving subpopulation of newborn DGCs in FASD-EE mice are functionally competent to mediate this behavioral improvement, albeit over a slower time course compared to SAC-EE mice. Alternatively, repeated training on this task may result in the recruitment of other non-neurogenesis-dependent circuitry for learning.
Surprisingly, the increased frequency of mEPSC in FASD-EE mice was not associated with an increase in spine density. Unfortunately, we were unable to obtain the resolution necessary to assess dendritic branching or spine morphology of GFP-labeled DGCs in our studies. Presumably, the increased mEPSC frequency in FASD-EE mice is correlated with enhanced morphological complexity, and this will require further study with improved microscopic resolution and sparser GFP labeling to prevent overlap of neighboring dendritic arbors.

Nevertheless, it is important to note that selective enhancement of morphological complexity has been shown to occur in adult-generated hippocampal DGCs in response to environmental stimuli. For example, dendritic length was shown to increase within 10 days of EE housing (Beauquis et al., 2010), and spine density of DGCs within the temporal region of the hippocampus was increased by 24 days of voluntary exercise (Piatti et al., 2011) specifically in adult-generated postmitotic immature neurons. Additionally, training on complex spatial learning tasks resulted in enhanced dendritic branching specifically in newborn neurons, and NMDAR activation was required for training-mediated enhancement of dendritic complexity (Tronel et al., 2010; Lemaire et al., 2012). These previous studies suggest that matured newborn DGCs do display a morphological response to environmental stimulation and learning.

Although our data did not reveal a significant effect of either FASD or EE on spine density of 4 or 8 week old newborn DGCs, previous studies have reported that running does not increase spine density newborn neurons but does stimulate an increase in the density of mushroom spines which are likely to represent sites of enhanced excitatory synaptic transmission (Zhao et al., 2006). Therefore, we anticipate that the enhancement
of basal excitatory transmission in newborn DGCs from FASD-EE mice will be associated with morphological indices of spine maturation and/or dendritic branching. If so, this will raise the question of why FASD would enhance this process in newborn but not developmentally generated preexisting DGCs, where EE-mediated dendritic branching and excitatory transmission is suppressed by FASD.

4.2.3. Is the impaired neurogenic response to EE in FASD mice due to disruption of the existing GCL circuitry?

4.2.3.1 Distinct electrophysiological responses of existing and newborn DGCs to environmental stimuli

In contrast to our findings of enhanced excitatory synaptic transmission in adult-generated DGCs from FASD-EE mice, we found electrophysiological and morphological evidence for suppression of EE-mediated excitatory transmission in existing DGCs. For example in SAC mice, there was no alteration in the frequency of glutamatergic mEPSCs in 8 week old newborn DGCs in response to EE; however, the frequency of mEPSCs was enhanced by EE in existing DGCs from SAC mice and this was suppressed by FASD. This effect was also correlated with suppression of EE-mediated distal dendritic branching in existing DGCs by FASD. That this branching complexity effect of EE and FASD occurred only in distal dendrites also suggests an under-estimation of the impact of FASD on EE-mediated excitatory transmission due to the possibility of space clamping error. Therefore, it is likely that a further suppressive effect of prenatal alcohol exposure on EE-mediated excitatory transmission in existing DGCs, which could be revealed by investigating dendritic evoked potentials.
Our observation of differing electrophysiological responses of existing and newborn DGCs under EE conditions in both SAC and FASD mice represents somewhat of a conundrum, in light of previous studies that have shown equivalent basal electrophysiological properties and plasticity in mature newborn neurons and existing DGCs under standard housing conditions (Ge et al., 2006; Ge et al., 2007). However, as mentioned above, prior studies demonstrated a selective morphological plasticity in response to spatial learning tasks only in matured newborn neuron, and not in existing DGCs (Lemaire et al., 2012). Taken together, these observations may imply that matured newborn neurons and existing DGCs have similar electrophysiological properties under standard housing conditions but respond quite differently to environmental stimuli. Indeed, recent studies have suggested a selective role for newborn neurons in pattern separation, whereas existing neurons are selectively required for recall and pattern completion (Nakashiba et al., 2012). Thus, newborn and existing DGC populations may subserve distinct functions, which may account for the observed differences in their response to EE in SAC and FASD mice.

The impaired morphological and electrophysiological response to EE in existing DGCs from FASD mice suggest that prenatal alcohol suppresses the plasticity and alters the circuitry of the dentate GCL, which is not unexpected in light of prior investigations. Previous studies have shown that the dendritic length and spine density increased in existing DGCs by 2-3 weeks of voluntary exercise in both rats (Redila & Christie, 2006) and mice (Dietrich et al., 2008). Although not studied in the dentate GCL, a previous study demonstrated that prenatal alcohol exposure resulted in a reduction of dendritic complexity and spine density in hippocampal regions such as CA1 and CA3 pyramidal
neurons in rodents (Berman & Hannigan, 2000). Also, both dendritic length and branching were reduced in another brain region, (the shell of the nucleus accumbens) in a rat FASD model (Rice et al., 2012). Therefore, these previous studies suggest that EE enhances and prenatal alcohol exposure impairs the dendritic plasticity in neurons born during development. These observations are supportive of our finding that EE enhances dendritic complexity in existing DGCs in SAC mice, but that this enhancement was blunted by FASD. Thus, existing DGC exhibit impaired dendritic plasticity in FASD mice.

Although there are few studies that have investigated the effects of EE and FASD on basal electrophysiological properties of DGCs, several publications have reported that FASD animals display LTP deficits in DGCs, as assessed by field excitatory postsynaptic potentiation (Sutherland et al., 1997; Varaschin et al., 2010; Brady et al., 2013). Thus, existing DGCs exhibit impairment of both electrophysiological and morphological plasticity in FASD mice and our studies further suggest a resistance to the beneficial effects of environmental stimuli on the existing DGC circuitry. This raises the intriguing question of whether the impaired neurogenic response to EE in FASD mice is due to impaired plasticity of the existing circuitry.
4.2.3.2 Disruption of Presynaptic Circuitry

Figure 4.3

Figure 4.3. Summary of unanswered questions in the existing circuit. EE and/or FASD may alter the function of preexisting microcircuit in DG. Orange arrow: Glutamatergic connection. Black arrow: GABAergic connection. Blue cell: Preexisting DGCs. Green cell: Newborn DGCs.

If the impaired neurogenic response to EE in FASD mice is due to an impaired existing GCL circuitry, this begs the question of the mechanisms by which the existing circuitry can influence the survival and integration of newborn DGCs. One possibility is via disrupted signaling through parvalbumin (PV)-expressing inhibitory interneurons. Interneurons connect with DGCs, providing feedback and feedforward inhibition pathways (Papadopouloules & Soykan, 2011). These interneurons receive input from both the EC and DGCs themselves. It is known that dendritic development is regulated in
immature and matured newborn neurons by GABA$_{\alpha}$R (Duveau et al., 2011). Interestingly, one study suggested that locally synthesized BDNF released by dendrites of existing DGCs promotes the differentiation and maturation of new born neurons specifically through the release of GABA from PV+ GABAergic interneurons (Waterhouse et al., 2012). Therefore, these previous findings suggest that hippocampal interneurons have a critical role for regulating maturation of newborn neurons through direct and indirect GABAergic signaling. This suggests that PV+ GABAergic interneurons provide a functional link between the existing DGCs and newborn DGCs that may regulate the neurogenic response to environmental stimulation (Figure 4.3).

Unfortunately, only a few studies have investigated the effects of prenatal alcohol exposure on hippocampal interneurons. Acute alcohol exposure increases GABA release from CA3 interneurons (Galindo et al., 2005) and chronic prenatal alcohol exposure enhances post-tetanic potentiation of stimulated glutamate release in the hippocampus of the postnatal guinea pig (Hayward et al., 2004). These studies suggested that prenatal exposure of alcohol alters interneuron activity. However, there is no study to demonstrate the effect of prenatal alcohol exposure on DG interneurons. If FASD animals display an LTP deficit in DGCs (Sutherland et al., 1997; Varaschin et al., 2010; Brady et al., 2013), LTP may also be impaired in DG interneurons, such as PV+ interneurons, as well. This consequence may be one of the mechanisms leading to loss of EE-mediated hippocampal neurogenesis in FASD mice. Thus, a fruitful area of future investigation would be to determine whether the number or types of interneurons such as PV+, somatostatin+ (SOM) and vasoactive intestinal polypeptide+ (VIP), within the GCL are
altered by FASD and EE and/or whether FASD impairs the plasticity of interneurons (LTP induction and maintenance) in response to EE (Figure 4.3).

It is possible that the impaired circuit response to EE in FASD mice is initiated upstream from DG as the primary inputs to the dentate GCL are from glutamatergic neurons that lie within the EC. Cortical neurons in medial EC project axons into medial ML, and similarly neurons in lateral EC project axons into lateral ML (Kumamoto et al., 2012). Indeed, pyramidal cells in EC display increased spine density in response to running (Stranahan et al., 2007), but this has not been studied for EE. Pertaining to our data, distal dendritic morphology of existing DGCs was enhanced by EE, and this effect may be caused by more activation of lateral ML from lateral EC. If so, the impairment of EE mediated distal dendritic plasticity may be caused by impairment of lateral EC functions (Figure 4.3).

With regard to the effects of prenatal alcohol on EC afferents to GCL, several studies have shown that the input/output responses to perforant path stimulation of dentate gyrus are not altered by prenatal alcohol exposure (Varaschin et al., 2010a; Brady et al., 2013b), but this has not been assessed under conditions of EE and FASD. Conversely, EC neuronal growth and differentiation was reported to be impaired potentially due to reduced levels of nerve growth factor (NGF) expression following prenatal alcohol exposure (Angelucci et al., 1999). These studies suggest that prenatal alcohol effect on DG may be due to the impairment of EC development or activity.
4.2.4 Receptor expression pattern and subunit composition in existing DGCs

We observed moderate but significant effects of EE and FASD on the amplitude and decay of mEPSC and mIPSC currents within existing DGCs. For example, the amplitude of mEPSC current was increased in existing DGCs from FASD, but not SAC mice under conditions of EE. Also, the decay time but not amplitude was increased for mIPSC current in SAC but not FASD mice. Amplitude effects may be due to altered presynaptic release of neurotransmitters due to changes in the quantal size. Future analysis will be required to distinguish these possibilities using by assessing the coefficients of variation of the recorded current. In contrast alterations in the decay time suggest a postsynaptic effect that involves the kinetics of receptor activation.

Previous studies have demonstrated alterations in GABAergic receptor function in rodents exposed to prenatal alcohol (Allan et al., 1998; Iqbal et al., 2004). Iqbal et al. (2004) also reported that GABA$_A$ receptor subunit β2/3 protein expression was increased by prenatal alcohol exposure in the guinea pig at postnatal day 60 and modulation of GABA$_A$R was found to be altered in hippocampal neurons by prenatal alcohol exposure (Allan et al., 1998). Interestingly, GABA$_A$Rγ2 was found to be elevated by environmental enriched housing during late prenatal and early postnatal period (Liu et al., 2012). These findings suggest that both prenatal alcohol exposure and environmental stimuli can lead to altered glutamatergic and GABAergic receptor expression patterns. However, it remains unknown whether the altered kinetics of the mEPSC or mIPSC current by FASD and EE are due to changed basal glutamatergic and GABAergic receptor expression patterns and/or subunit composition.
4.3 Conclusion

Overall, our findings suggest that FASD imparts resistance to the full benefits of social and physical enrichment therapies for adult hippocampal neurogenesis, both with respect to the survival of newborn DGCs and the electrophysiological properties of basal synaptic transmission in the preexisting GCL circuitry and newborn neurons. However, physical exercise has been shown to rescue spatial memory deficits and to enhance LTP in FASD animals model (Christie et al., 2005) and prior studies have shown that learning enhances adult hippocampal neurogenesis (Gould et al., 1999; Drapeau et al., 2007). In human studies, the behavioral and learning problems were reduced in FAS children by cognitive control therapy, which includes training for more challenging tasks, body movement with environment and organize information (Riley et al., 2003; Coriale et al., 2013). In addition, rehearsal training (Loomes et al., 2008) and socio-cognitive habilitation (Kable et al., 2007) showed a significant benefit on working memory for FASD children. These studies suggest that special behavioral and social training programs have a significant benefit for FASD children. Thus, a combination of physical exercise and practiced learning tasks may maximize the beneficial effect behavioral therapy as a possible therapeutic approach in FASD patients. However, we found preclinical mouse model of FASD displayed resistance to full benefit of environmental enrichment for hippocampal neurogenesis. This result suggests that FASD may diminish the benefits of certain types of behavioral therapies in clinical FASD. Also, our results could imply that FASD children may not receive same degree of benefit from physical and cognitive training programs. Alternatively, once the mechanisms of impaired plasticity are identified, pharmaceutical drug treatment may be developed to reverse loss.
of hippocampal plasticity and restore neurogenic responsiveness to behavioral therapeutics.
Figure A.1 LTP could not be induced by High Frequency Stimulation (HFS).

According to some literatures, neurogenesis dependent small LTP can be detected by HFS without GABAR blockers (Snyder et al., 2001; Saxe et al., 2006b). However, both neurogenesis dependent LTP and normal LTP could not be induced by HFS (4 trains, 100Hz 1s, 15s interval). A. Input-output curve of fEPSP slope. B. Neurogenesis dependent LTP was induced by HFS without Gabazine (10 µM). C. Normal LTP was not maintained. The recording was performed with Gabazine (10 µM). N=1 animal.
Figure A.2

Most of the recorded events are mediated by action potential independent miniature IPSCs and EPSCs.

A. Frequency and B. Amplitude of GABAAR mediated spontaneous and miniature IPSCs in existing DGCs. sIPSCs were recorded without TTX (Sodium channel blocker) and mIPSCs were recorded with TTX (0.5 µM). Both sIPSC and mIPSC events were recorded with Kynurenic Acid (3 µM) to eliminate glutamatergic events. n=3 animals. C. Frequency and D. Amplitude of AMPAR mediated spontaneous and miniature EPSCs in existing DGCs. sEPSCs were recorded without TTX (Sodium channel blocker) and mEPSCs were recorded with TTX (0.5 µM). Both sEPSC and mEPSC events were recorded with Picrotoxin (100 µM) to eliminate GABAergic events. n=2 animals. Data are expressed as means + S.E.M., with p<.05 considered statistically significant.
Figure A.3

Figure A.3 Tonic GABAergic currents were not altered by EE and prenatal alcohol exposure.

Tonic current can be detected with GABA$_A$R blocker (100µM Bicuculline) (Ge et al., 2006; Duveau et al., 2011a), and this could be mediated by extrasynaptic GABARs by spillover of synaptic GABA (Farrant & Nusser, 2005). A. Trace of the tonic current and detected small tonic current. Tonic current of B. Developmental generated preexisting DGCs, C. 4 week old, and D. 8 week old new born neurons. Two-way ANOVA did not show any significant effect of EE and FASD. Data are expressed as means ± S.E.M., with p<.05 considered statistically significant. n=4-6 animals for all groups.

Therefore, extrasynaptic GABA$_A$R may not be altered by both EE and prenatal alcohol exposure.
Figure A.4

The cell was recorded for 15 minutes by whole cell recording (5 min: Baseline + 5 min: Bath application of EtOH + 5 min: Washout). A. Frequency and B. Amplitude of 8 week old new born neurons was not altered by bath application of acute EtOH (50mM) exposure. Similar to 8 week old new born neurons, C and E. Frequency and D and F. Amplitude of existing DGCs did not altered by 50mM and 100mM of acute EtOH exposure on slices. C and E. Acute alcohol exposure may increase the frequency of sIPSCs by around 20%. However, in order to investigate the effect of acute alcohol exposure on the electrophysiological responses of adult-generated and developmentally-generated DGCs, more experiments are required.
Figure A.5 Mouse activity in EE.

Movement within the EE cage was recorded and analyzed using the Noldus EthoVision 3.0 video tracking system (Noldus, Leesburg, VA) 10 weeks after EE housing. For this analysis, three target zones encompassing the running wheel, ladder and tunnel were outlined with minimal border zones and the duration of time spent in each environmental zone was recorded over a one hour period. It should be noted that cage-mates were removed from the EE during the recording session (1 hour). A. Total distance moved and B. Velocity in EE cage were decreased in FASD mice. However, the duration on the C. running wheels, D. tunnel and E. Ladder were not altered by prenatal alcohol exposure. Data are expressed as means + S.E.M., with p<.05 considered statistically significant. n=5 mice per treatment group.

This suggested that FASD mice did not change the interaction with items present in the EE. However, it is important to note that only one mouse could be traced by this recording, so other cage mates were removed during the recording. Therefore, the
distance moved and velocity in EE cage may reflect the different degrees of anxiety. In addition, this EthoVision program cannot measure the total distance run on the wheels.

From this experiment, it can be concluded that FASD mice did not show a significant alteration in their interaction with the EE. However, mouse activity has to be recorded with cage mates. Therefore, the radio frequency identification (RFID) system (Freund et al., 2013) could be the possibility to track the animal activity in EE cage in future experiments.
Abbreviations Used

ARBD: Alcohol related birth defects
Arch: Archaerhodopsin
ARND: Alcohol related neurological disorder
BDNF: Brain-derived neurotrophic factor
BAC: Blood alcohol concentration
BLBP: Brain lipid-binding protein
BMP: Bone morphogenetic protein
BrdU: Bromodeoxyuridine (5-bromo-2’-deoxyuridine)
ChR2: Channelrhodopsin-2
DCX: Doublecortin
DG: Dentate gyrus
Dlx 2: distal-less homeobox 2
EC: Entorhinal cortex
ePSC: Evoked postsynaptic current
EPSC: Excitatory postsynaptic current
ePSP: Evoked postsynaptic potential
EPSP: Excitatory postsynaptic potential
FAS: Fetal alcohol syndrome
FASD: Fetal alcohol spectrum disorder
fEPSP: Field excitatory postsynaptic potential
GABA: γ-Aminobutyric acid
GCL: Granule cell layer
GFAP: Glial fibrillary acidic protein
GFP: Green fluorescence protein
HFS: High Frequency Stimulation
i.c.v: intracerebroventricular
IPSC: Inhibitory postsynaptic current
KCC2: Potassium-chloride transporter
LTP: Long term potentiation
NGF: Nerve growth factor
NKCC1: Sodium, potassium, and chloride cotransporter
MCM2: mini-chromosome maintenance proteins 2
ML: Molecular layer
NMDA: N-methyl-D-aspartate
NMDAR: N-methyl-D-aspartate receptor
NpHR: Halorhodopsin
OB: Olfactory bulb
PCNA: Proliferating cell nuclear antigen
PV: Parvalbumin
rAAV: Recombinant Adeno-associated virus
SGZ: Subgranular zone
sEPSC: Spontaneous excitatory postsynaptic current
sIPSC: Spontaneous inhibitory postsynaptic current
SOM: Somatostatin
Sox2: SRY (sex determining region Y)-box 2

SSH: Sonic hedgehog

SVZ: Subventricular zone

TAP: transient amplifying progenitor

TrkB: Tropomyosin related kinase B

VEGF: Vascular endothelial growth factor

VEGFR: Vascular Endothelial Growth Factor Receptor

VIP: Vasoactive intestinal polypeptide

Wnt: Wingless-type family

YFP: Yellow fluorescence protein


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