Elucidating the Role of Integrin Linked Kinase Pathway in Moderate Drinking Prenatal Alcohol Exposed Rat Model

by

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Abstract

Alcohol and nicotine are well known teratogen which has been reported to have large scale effect on developing fetus. Prenatal alcohol cause many deleterious effect during fetal development which either persist throughout the life of an individual or may disappear as age progresses. Moderate drinking model is considered to be more appropriate model of maternal drinking in human. Time pregnant Sprague Dawley rats were used to generate moderate drinking alcohol model. Integrin Linked Kinase (ILK) is an effector of integrin and growth-factor signaling which regulates various signaling processes. In FASD, a downstream effector of ILK, Glycogen Synthase Kinase 3β (GSK3β) remains highly active (reduced Ser^9 phosphorylation). GSK3β has been known to modulate glutamate receptor trafficking and channel properties. Therefore, we hypothesize that the cognitive deficits accompanying FASD are associated with impairments in the ILK signaling pathway. A significant population of women are present who drinks and smokes simultaneously during pregnancy. Thus, both of these drugs of abuse during pregnancy may have significant neurobehavioral effect on the offspring. In our study, we also investigated the effect of prenatal alcohol and nicotine co-exposure on the behavior and plasticity in adolescent rat. Another part of the study is the use of BDNF receptor TrkB agonist 7,8-DHF to ameliorate the deficiencies observed in prenatal alcohol model.

We reported deficits in contextual fear conditioning behavior, spatial memory and synaptic plasticity in FASD rat model. Prenatal co-exposure to nicotine ameliorate the behavioral deficits, although failed to improve synaptic plasticity. The major finding from this study was the reduced ILK activity, interaction to calcium impermeable GluR2 AMPA receptor and increased synaptic expression of GluR2. The net effect of this has been correlated to synaptic plasticity impairment in the model. Prenatal nicotine also showed increased synaptic expression of GluR2 and therefore reduced plasticity. The neurotrophic growth factor BDNF is also deficient in the model which can reduce the ILK activity. 7,8-DHF can potentially enhance ILK activity through PI3kinase protein. Indeed, 7,8-DHF showed promising result in improvement of behavior in the model with reduced GluR2 at the synapse. This shows that plasticity can be improved using TrkB agonist in the model.

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1. INTRODUCTION

In US, alcohol consumption is very common in social life style. Neurophysical effect of alcoholism has been well studied and researched. Apart from devastating life threatening chronic disorders such as liver cirrhosis, hepatitis, gastritis, and pancreatitis, alcohol also effects the cognition and memory(Heffernan 2008). The effect of alcohol on brain has been a topic of research for decades. The effect of alcohol on brain functioning ranges short duration to long lasting. Apparently alcohol can affect many neuronal signaling pathways effecting memory, speech, vision, consciousness and thought processing (Desmond, Chen et al. 2003; Tapert, Pulido et al. 2004). Drinking during pregnancy leads to a range of mental and physical defects in the child which is known under an umbrella term called Fetal Alcohol Spectrum Disorder (FASD) (Becker, Warr-Leeper et al. 1990). According to Centre of Disease Control and Prevention, FASD is very prevalent in US to a maximum of 1.5-2.0 cases per 1000 births. National Survey on Drug Use and Health (NSDUH) predicted from their survey (2011-2012) that around 8.5% of pregnant women (aged between 15 and 44) consumed alcohol during pregnancy. Drinking any form of alcohol is not permissible at any stage of pregnancy. However, the major cause being unknown pregnancy during drinking (Abuse 2013). There is no safe limit to alcohol drinking during pregnancy. Above data and findings suggest that in future cases of FASD will be a major concern. To date, no known medication or treatment

exists to ameliorate FASD related symptoms. Only way to prevent FASD like problems is to abstain from drinking during pregnancy.

Drinking both acute (binge or moderate) or chronic during pregnancy may result in FASD like conditions in the child (Li, Yang et al. 2007; May, Blankenship et al. 2013). Exposure of fetus to alcohol at any trimester may be harmful to its development. The main reason is that the brain is developing throughout all trimesters. Alcohol can easily cross the blood placental barrier and affects the developing fetus (Rodier 1995). There are prominent features in a child suffering FASD like symptoms. Most distinctive facial features being small eyes, upturned nose with flat nasal bridge, thin upper lip and small head circumference. The growth of the child is slow with many physical deformities (Warren and Foudin 2001). At the same time there is effect on the cognition and learning abilities of the child. Attention deficits, anxiety, hyperactivity, aggression and fear are manifested in FASD patients (Green, Mihic et al. 2009). This is a clear indication that alcohol has prominent effect on the developing brain on cortex, hippocampus and amygdala. The physical deformities suggest the developmental perturbations in the expression of genes responsible for normal physiological development such as homeotic genes. Overall in developing fetus alcohol may have an overhauling effect on cell signaling and genetic environment thus causing FASD like symptoms (Zhou, Zhao et al. 2011).

Clinical studies with FASD children helped in better understanding the neuropsychological impairment. The cognitive tasks may help identify FASD patients as they fail to process complex information. On the basis of a series of general cognitive measures ranged from simple to complex tasks, FASD children performed poorly in

complex tasks. However the simple task paradigm did not find any change between the FASD and control subject (Aragón, Kalberg et al. 2008). Therefore, such cognitive tasks may help in better identification of children with FASD symptoms. Similarly, other clinical studies also showed that FASD children showed significantly reduced intelligence, perceptive intelligence, recognition memory and working to long term memory (Kully-Martens, Denys et al. 2012; May, Blankenship et al. 2013). There is high probability of developing attention deficit and autistic behavior in FASD patient. They both show impaired memory and cognition when considered as two separate diseases (Aronson, Hagberg et al. 1997). However another study found that FASD or FASD with ADHD showed more severe memory impairment than ADHD alone (Glass, Ware et al. 2013). Therefore, it may be argued that ADHD and FASD may be a comorbid disorder and not one responsible for the other. Overall these clinical studies emphasized the use of pharmacological intervention to improve cognition and memory. Hence, it is very much required to study the pathways involved in memory formation and storage and how it is impaired in prenatal alcohol exposure. Since the autism and ADHD are comorbid to FASD patients, the neurochemical signaling pathways and receptors involved in those diseases have to be given emphasis as well.

Recent literature reviews fail to identify a potential signaling mechanism that can be associated to the neurophysiological and anatomical deficits observed in children exposed to alcohol in utero (Tan, Berman et al. 1990; Guerri 1998). Looking at the developing stages of an embryo, the effect of alcohol can be severe at 3-16 weeks on the developing central nervous system. The effect is moderate on 16 week old embryo (Thomas, Goodlett et al. 1998). The effect of alcohol on developing brain varies from

inducing neuronal death or intervention in the normal physiology of the cell. Hence, understanding the mechanism of alcohol induced brain damage during development is critical and at the same time its complex. In utero exposure of alcohol may affect the distribution and functional role of various receptors, proteins and their subunits (Davies 2003; Bird, Candelaria-Cook et al. 2015). The developing cells may become susceptible to cell division arrest, differentiation and neuronal migration. The neuronal ion channel receptors in various regions of the brain are also affected by ethanol exposure. The early development and expression of these receptors make them vulnerable to the teratogenicity of alcohol. The proper channel functioning is key to the maturation and development of the individual parts of the brain. The likely cause of CNS depression during its early developmental period is impaired functioning of the ion channel receptors in FASD. Many studies have shown the extent of damage to the ion channels of the hippocampus by prenatal alcohol exposure (Brady, Diaz et al. 2013). Many attempts were made using ion channel receptor modulators to improve FAS conditions in animal models of FASD. However, we do not have enough insight on the putative signaling mechanism in neurons affected by prenatal alcohol.

Glutamate receptors are the major excitatory ion channel receptors in mammalian brain (Monaghan, Bridges et al. 1989; Boulter, Hollmann et al. 1990). There are three major classifications of ionotropic glutamate receptors: NMDARs (N-Methyl-D-aspartate receptors), AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) and KARs (Kainate receptors); each has unique characteristics and downstream signaling owing to their receptor subtypes (Dingledine, Borges et al. 1999). NMDARs and AMPARs are involved in learning and memory processes in the brain. Prenatal alcohol

can modulate the glutamatergic signaling at the level of neurotransmission and the expression and behavior of the ionotropic glutamate receptors. Such modulation may inhibit maturation of neuronal circuit and affect learning and memory processes. These deficits during development may sustain throughout the lifespan of an individual or may slowly disappear with age (Bird, Candelaria-Cook et al. 2015). However, not much is known how alcohol interacts to these receptors or controls their expression during developmental exposures. Therefore, the signaling mechanisms underlying the trafficking and presentation of these receptors during development need extensive research.

A number of studies reported that animals exposed to prenatal alcohol show deficits in various spatial memory tasks including T-mazes, the Morris water maze, and the radial arm maze (Berman and Hannigan 2000). Such deficits in hippocampal spatial memory have been directly correlated to reduced neuronal density, lowered dendritic spine growth and decreased synaptic plasticity (Gil-Mohapel, Titterness et al. 2014). However, most of these neuroanatomical studies do not talk about the underlying mechanism vulnerable to prenatal alcohol related toxicity. Hence, this study was directed to identify a potential mechanism which may be susceptible to prenatal alcohol and ameliorate the alcohol related behavioral and plasticity deficits through drug targets. Proteins which play significant role in maintaining the dendritic spine growth and synaptic plasticity should be the essential focus of developing therapeutics. Integrin Linked Kinase, ILK is associated to the synaptic integrins which modulate spine density and synaptic plasticity (Mills, Digicaylioglu et al. 2003). ILK has been very deeply studied in cancer metastasis as it helps in cellular differentiation and survival (Hannigan, Troussard et al. 2005). In neurodegenerative disorders such as Alzheimer's disease,

Parkinson's disease, ILK can be a major target to ameliorate neuronal cell death (Shonesy, Thiruchelvam et al. 2012). A few studies have shown that inhibition of ILK can potentially reduce plasticity. Hence, in the present study we focused on the role of ILK in behavioral and plasticity deficits observed in prenatal alcohol rat model.

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2 REVIEW OF LITERATURE

Outline

2.1 Overview of fetal alcohol spectrum disorder (FASD)

Fetal Alcohol Syndrome (FAS) received attention for the first time in 1973 (Jones and Smith 1973). They reported about 11 children born to alcohol-abusing mothers (Jones, Smith et al. 1973). Deficits in growth and physical defects such as deformity of facial structure are hallmarks of FASD (Larkby and Day 1997) and brain structure malformations like microcephaly. FASD charecteristics can be distinguished as primary and secondary deformities which delay social and motor performance (O'Leary 2004). The diagnosis of FASD should include a history of prenatal alcohol exposure , a range of craniofacial anomalies (such as flat midface, thin upper lip, epicanthal folds, smooth or flattened philtrum, underdeveloped jaw, low nasal bridge, small eye openings, and/or a short nose; figure 2.1) and CNS dysfunction. These defects can be temporary which disappear with age or be permanent. FASD can be diagnosed based on the four criteria as described in table 2.1.

Four Diagnoses under the Umbrella of FASD				
Diagnosis		Growth	FAS Face	Brain
1. FAS	Fetal Alcohol Syndrome	growth	face	severe
2. PFAS	Partial FAS		face	severe
3. SE/AE*	Static Encephalopathy / Alc Exposed			severe
4. ND/AE	Neurobehavioral Disorder / Alc Exposed			moderate

* Also referred to as:
- Alcohol Related Neurodevelopmental Disorder (ARND) or
- Neurodevelopmental Disorder Prenatal Alcohol Exposed (ND-PAE)

Table 2.1: Adapted from: Association of Reproductive Health Professionals

The majority of children who were exposed to alcohol during the gestation period however show only a few neurological symptoms without the visible physical deformities as described above (Sampson, Streissguth et al. 1997), and they are referred to as having Alcohol Related Neurodevelopmental Disorders (ARND).

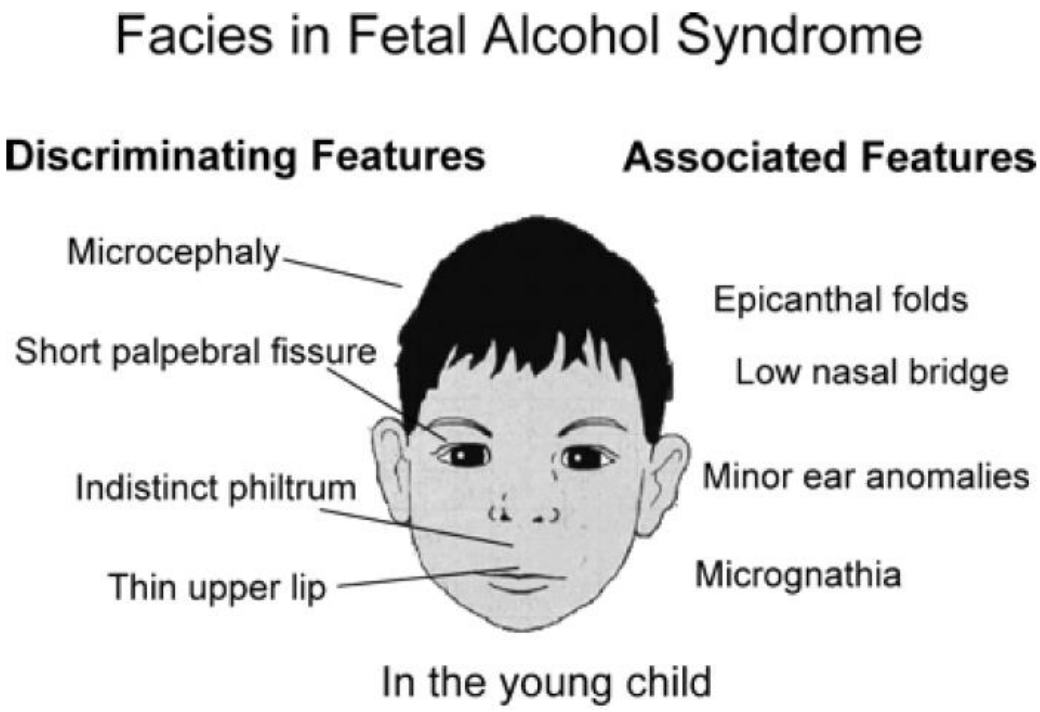

Figure 2.1Facies in Fetal Alcohol Syndrome;modified from http://www.semel.ucla.edu/fas/about/characteristics

CNS development starts from the 3rd week of gestation until the 38th week of full foetal development in humans. The period from 3-16th week is the most vulnerable to teratogenic effect of alcohol. Gestation alcohol exposure can affect normal development of certain body parts based on the time of exposure unlike CNS which is the most vulnerable throughout its development (figure 2.2). Timing of fetal exposure to alcohol during pregnancy is very important determinant for the effect and severity of teratogenic toxicity. First trimester exposure can interfere with the differentiation and maturation of the brain nerve cells (Cook and Moore 1993); (Livy, Miller et al. 2003). The developing brain suffers damages in the cortex, cerebellum and hippocampus which are essential in learning and memory during gestational alcohol consumption (Coles 2001; Livy, Miller et al. 2003).

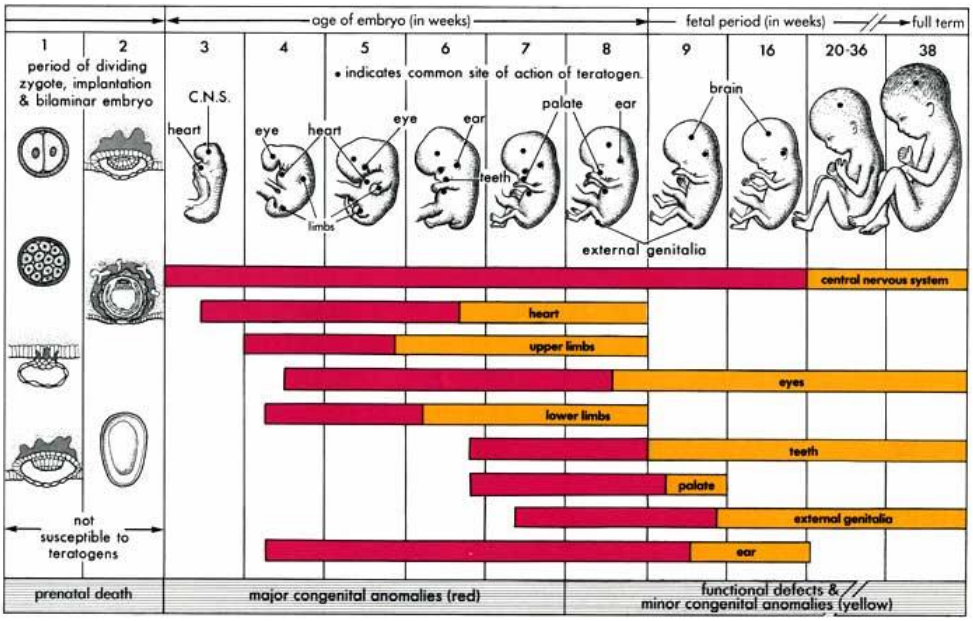

Figure 2.2: Adapted from http://carolguze.com/text/442-13-teratogens.shtml

The Substance Abuse and Mental Health Services Administration has reported that around 1 out of 4 pregnant women have consumed binge alcohol (corresponding to 5 or more drinks at a time). The number of children born with FASD like symptoms in US is around 0.5 to 2 per 1000 newborn births (May and Gossage 2001). At present, the FASD cases in US estimated to around 1000 to 6000 infants (Bertrand, Floyd et al. 2005). Many of these children are also reported to have Attention Deficit Hyperactivity Disorder (ADHD) like symptoms. There is increasing evidence emerging from clinical and animal research, a link between FASD and ADHD symptoms (O'Malley and Nanson 2002). However, the underlying mechanism responsible for the onset of these disorders and cognitive deficits are still not known.

2.2 Neurobehavioral and cognitive deficits in children with FASD

2.2.1 Executive function and attention

Executive dysfunction is common in neurodevelopmental disorders like FASD and ADHD (Barkley 1997; Nigg 2001; Rasmussen 2005). The executive function includes the following abilities: planning, working memory, fluency, response inhibition, and attentional vigilance. Children with FASD have also shown deficiency in non-verbal and verbal fluency (Mattson, Schoenfeld et al. 2001), predominantly generation of words beginning with certain letters under specific conditions (Kodituwakku, Handmaker et al. 1995; Kodituwakku, Coriale et al. 2006). It is still not clear whether the disorder manifests only attentional deficits or higher order executive function (Lee, Mattson et al. 2004; Aragón, Coriale et al. 2008). It is mainly the timing of alcohol exposure during pregnancy or the pattern of exposure (moderate to binge drinking) causing these differences. Research is still at its initial phase to understand the pattern and extent of executive dysfunction

associated with FASD (Kingdon, Cardoso et al. 2015). The comparative study of such deficits in control non-exposed children with or without comorbid ADHD children will shed light on the extent of CNS damage.

2.2.2 Learning and memory

Prenatal alcohol both moderate and binge consumption can affect learning and memory in the offspring. Moderate alcohol exposure can show recall and recognition of verbal memory deficits in the adolescents. Timing and dose of alcohol alone is not responsible for the verbal and spatial memory deficits in these children. The sociodemographic, environmental, and psychosocial conditions of the mother are equally responsible (Willford, Richardson et al. 2004). Animal research has shown that the hippocampus dependent learning and memory is impaired (Berman and Hannigan 2000). Spatial navigation tasks to associate hippocampal damage and learning and memory further demonstrated the deleterious effect of prenatal alcohol (Hamilton et al., 2003). The deleterious effect of prenatal alcohol is also predominant in the cerebellar learning deficits. FASD children showed increased latencies in the eye blink conditioning, localized at the cerebellum (Coffin, Baroody et al. 2005). In summary, prenatal alcohol can affect the learning and memory processes in the CNS and the underlying mechanisms are not completely elucidated.

2.2.3 Language

Prenatal alcohol exposure in children has been associated to poor performance in education. Early development of language and speech is imperative for proper cognitive and behavioral characteristics in an individual. Therefore, any delays in the development

of the language acquiring skills due to teratogen like alcohol may hamper the educational and social behavioral outcome of an individual. Some clinically relevant studies have shown no association between the alcohol exposure and deficits in language development (Greene, Emhart et al. 1990).

2.2.4 Motor abilities

The severity of prenatal alcohol consumption can be understood from the fact that FASD children suffer from significant delay in their fine motor skills compared to non-exposed children in terms of Motor Domain Standard Scores (Motor SS) (Kalberg, Provost et al. 2006). The FASD children perform poorly in maintaining postural balance in the absence of proper somatosensory and visual inputs compared to non-alcohol exposed children. This suggest the cerebellar developmental defects due to prenatal alcohol exposure. Ethanol exposure in first postnatal week in rats (third trimester equivalent of humans) showed that the pyramidal layer 2/3 of the somatosensory cortex lack dendritic spine formation suggesting the developmental defects of alcohol in the brain region (Kalberg, Provost et al. 2006; De Giorgio and Granato 2015). However, most of these studies do not take into account the time of exposure to alcohol or age when the conditions are tested. Future study should utilize a standard approach to assess the gross proficiency in FASD.

2.2.5 Visuospatial abilities

Children with FASD are performed poorly in visual perceptual tasks (Kodituwakku, Coriale et al. 2006). They had reduced efficiency in drawing designs which require planning and visual-motor integration as already described above (Uecker and Nadel 1996).

2.3 FASD associated behavioral phenotype in children

Children with FASD has consistently shown average IQ (mildly retarded to borderline range) and suffer from attention deficit and inability to process information. The most persistent behavioral problems manifested in FASD children are anxiety and depression. HPA (Hypothalamus-pituitary axis) could be the target for developmental alcohol inducing behavioral dysfunction. Animal models of FASD also showed that fetal alcohol mediated dysregulation of the HPA axis (Wieczorek, Fish et al. 2015). The children fail to socialize and lack daily functional skills or adaptive behavior. The children suffering from FASD find it difficult to process complex information. Streissguth, Herman et al. 1978 reported impaired intellectual performance in all age groups of individuals exposed to prenatal alcohol. A number of reports also identified symptoms of mood disorder (O'Connor, Shah et al. 2002) in FASD children. Direct damage of the developing CNS with prenatal ethanol resulted in mutated familial genetic factors and adverse postnatal development.

2.4 Prenatal alcohol changes brain structures in FASD children

Alcohol is a well-known teratogen that can have severe to moderate brain damage. The mass of the brain reduces and shrink in size due to developmental alcohol exposure. The brain region damage during development is responsible for the behavioral phenotypes observed in FASD children. The regions of the brain that are vulnerable to teratogenic alcohol are corpus callosum, cerebellum, basal ganglia, hippocampus, hypothalamus and the frontal lobes.

The damage to corpus callosum in FASD patients results in impaired movement of information between the two hemispheres. This makes the individual impulsive unaware

of the consequence of an act. FASD related impulsive behavior is also attributed to the frontal lobe damage (Venkatasubramanian, Anthony et al. 2007). FASD children and young adults show abnormalities in cerebellar vermis. The shrinkage of the anterior part of the vermis is significantly high compared to the control. There is increased loss of purkinje cells in the region. This results in deficits in motor learning and attention deficits in FASD patients (Sowell, Jernigan et al. 1996). Magnetic Resonance Imaging (MRI) study revealed dysmorphology of the parietal and posterior temporal cortices. Heavy prenatal alcohol exposure increased the frontal cortical thickness in children with FASD. It is correlated to deficits in intellectual functioning, learning, language, attention, visuospatial, and executive functioning (Archibald, Fennema-Notestine et al. 2001). The HPA axis have been shown to be affected due to prenatal alcohol and affects the hormonal secretion in both male and female children with FASD. This make them vulnerable to various diseases as they progress in age (Weinberg, Sliwowska et al. 2008). As discussed earlier, FASD children show impairment in hippocampal dependent verbal learning and spatial memory. Prenatal alcohol reduces the sizes of left and right hippocampus in the FASD children. This has been correlated to poorer verbal learning and spatial recall problem (Livy, Miller et al. 2003).

2.5 Animal models of prenatal alcohol exposure

Rodent models are powerful mechanistic model to study behavior, learning and memory related mechanism (Hannigan and Abel, 1996). To maintain chronic blood concentration of alcohol in rats, several procedures were followed such as administration of alcohol liquid diet (Driscoll, Streissguth et al. 1990). It is required to understand the human equivalent period of brain development in the animal model. The 21 days of

gestation in rats is equivalent to the first two trimester in humans. Hence, to develop a prenatal alcohol model equivalent to the third trimester in human, the rats will be exposed to alcohol during the first postnatal week through the lactating female rat. Apart from rodent models, several other species are also considered in order to identify the effect of prenatal alcohol. The zebra fish larva model is preferred in order to visually assess the developmental effect of alcohol. The simple non-mammalian models provide several advantages including simple nervous system, short generation interval, lack of a placenta, and the ability to understand the genetic changes (Bilotta, Barnett et al. 2004). Avian models are also often use as prenatal alcohol model to understand the effect of alcohol on the embryo development (Su, Debelak et al. 2001). Non-human primates are used as models to understand the complex cognitive function, alcohol preference and dopamine system during developmental exposure of alcohol (Clarren, Astley et al. 1990).

The effect of alcohol as a teratogen can vary on the developing fetus depending on the pattern of exposure. Binge drinking are more harmful as compared to the equivalent amounts of alcohol over long periods (Goodlett, Mahoney et al. 1989). Maternal alcohol abuse slowly through drinking water over the second and third trimester produces more severe effects on behavioral and cognitive development. In summary, animal models and the pattern of alcohol insult have been chosen based on the requirement of the cases of human counterpart. The prenatal alcohol exposure during the first two trimester of human are mostly replicated in animal models to understand the underlying mechanisms and behavior. The intervention studies have also made it possible to understand the probable mechanism and identify drug targets to treat the FASD related symptoms.

2.6 Behavior alterations in animal models of FASD

Prenatal alcohol crosses the blood brain barrier and affect the developing brain (Jeejeebhoy, Phillips et al. 1972). The vulnerable parts of the brain as discussed above include the corpus callosum, hippocampus, cerebellum and cortex. Hence, the effect of alcohol on the developing progeny is seen as behavioral deficits (Guerri, Bazinet et al. 2009). Some of these behavioral deficiencies have been studied in animal models and drug interventions are investigated. Spatial behavioral deficits are studied in animal models to understand the extent of damage of prenatal alcohol on the hippocampus. T-mazes have been used widely to understand the deficits in acquisition and retention of spontaneous and conditional alterations (O'keefe and Nadel 1978). Dose dependent error rates have significantly increased in rats with liquid alcohol diets. Reverse learning acquisition deficits, indicating the effect of prenatal or neonatal binge level alcohol, have been observed in mice model. Overall, prenatal alcohol studies on rodent models indicate impairment in spatial memory in sex dependent manner.

Morris water maze is used to map the spatial locations where the animal has to escape to a hidden platform submerged under opaque water (Morris 1984). Modifications introduced by removing the platform can discriminate the spatial learning deficits (knowing the location of the platform) to motor deficits (learning the pattern to swim to the platform). Prenatal alcohol damage to the hippocampus considerably damage the capacity to learn spatial cues. Prenatal exposed rats take longer time to reach the escape platform due to hippocampal dysfunction. Females do poorly in morris water maze test as compared to the males (Blanchard, Riley et al. 1987). In summary, the spatial deficits observed in rodent models show variable age dependency of prenatal alcohol damage.

The more significant effect is seen in younger animals with delayed testing after the training session. The dose of alcohol and severity in spatial deficits as observed with various maze tests strongly support the hypothesis that prenatal alcohol affects the developing hippocampus.

2.7 Neuroanatomical findings of brain in animal models of FASD

2.7.1 Cell loss

Prenatal ethanol exposure delays maturation and proliferation of neurons in the hippocampus but not cell death. The decrease in hippocampal cell loss is also selective in prenatal alcohol exposed animals. There is almost 20% reduction in dorsal hippocampus cornus ammonis (CA1) pyramidal neurons in 60 Postnatal day (PND) rats and 10% in all CA fields in 36 PND rats (Staubli, Rogers et al. 1994). This reductions range from 31% in CA3 to 46% in CA1 pyramidal neuronal populations (Perez, Villanueva et al. 1991). Binge like alcohol exposure has more dramatic effect on the pyramidal and the granule cell counts in CA1 area at PND 21. However, many studies do not report any change the density of mature granule cells. The significant change is observed in prenatal model of binge alcohol exposure where the cell numbers reduced considerably. With age, the cell counts vary significantly in FASD models (Lobaugh, Wigal et al. 1991).

2.7.2 Neuronal branching and spines

Hippocampal neurite growth and spine density are severely affected due to prenatal alcohol exposure. Binge alcohol exposure has been reported to have caused abnormal branching of mossy fibre afferents to CA3 hippocampal region (West and Pierce 1986). CA1 hippocampal neurons have deficient dendritic spines in FASD model with different dosage of alcohol exposure (Ferrer, Galofre et al. 1988; Tan, Berman et al.

1990). However, another study observed no change in spine density in their prenatal alcohol model at PND 90. This suggests that prenatal alcohol mediated changes sometimes not persistent throughout the age of an individual. The hippocampal pyramidal neurons also show developmental delay as evident from the dense package and less dendritic arborization similar to younger animals. There were changes in mitochondrial and endoplasmic reticulum distribution in CA1(Smith and Davies 1990). Spine numbers, synapses and dendritic arborization change in prenatal alcohol model suggest change in synaptic plasticity. A significant decrease in synapse turnover is observed in electron microscope study (Hoff 1988).

2.8 Electrophysiological alterations of hippocampus in FASD rodents

Behavioral and neuroanatomical studies have been validated with evidence from electrophysiology data in prenatal alcohol exposed animals. Electrophysiological measurements include Echo encephalogram (EEG) studies and *in vitro* slice electrophysiology. In hippocampal Cornus AmmonisCA1 region, stimulation-evoked extracellular field potentials were compared to the control groups at the age of PND 40-60. Paired pulse ratio at intervals less than 50 msec shows paired pulse inhibition (PPI) and over 100 msec intervals shows paired pulse potentiation (PPP). PPI was decreased in prenatal alcohol exposed rats whereas there was enhanced PPP as compared to control rats (Hablitz 1986; Tan, Berman et al. 1990). The basal synaptic transmission measured with input/output curves showed insignificant change compared to the controls. FASD rat models showed reduced Long Term Potentiation (LTP), a cellular correlate of synaptic plasticity in hippocampal CA1 region (Swartzwelder, Farr et al. 1988; Tan, Berman et al. 1990). Most of these models are from binge drinking pregnant rats. Ethanol was

administered at the amount of 6g/kg/day from Gestational day GD 8-21. The models showed significant reduction in maximum evoked population spike in offspring at PND 25-32 (Krahl, Berman et al. 1999). However, at PND 70 no change was observed in input/output profiles or paired pulse responses showing the age dependent profile of alcohol exposure. The effect on behavior and electrophysiology in FASD rat models was also dependent on the route (oral or IP) and pattern (moderate vs binge) of alcohol administration. The alcohol self-administered model produced abnormal LTP and paired pulse responses in the CA1 whereas intragastric gavaging showed no change in those responses (Hablitz 1986; Krahl, Berman et al. 1999). A study on moderate drinking paradigm showed no change in input/output character but reduced LTP whereas binge drink exposure with 350 mg/dl Blood Alcohol Concentration (BAC) showed changes in basal synaptic transmission but no change in LTP (Sutherland, McDonald et al. 1997; Bellinger, Bedi et al. 1999). Moderate alcohol exposure with steady availability through drink or liquid diet is considered to be a more appropriate model for prenatal alcohol in humans. In summary, the above literature studies have clearly demonstrated that the effect of alcohol on the developing fetus and subsequently their behavior, anatomical and electrophysiological characteristics are dependent on the pattern of alcohol consumption. Abnormal hippocampal electrophysiological activity (both *in vivo* and *in vitro*) resulted from prenatal alcohol exposure are due to loss of hippocampal and mossy fibre projections. This was correlated with deficits in spatial learning and behavioral alterations. However, the other brain regions besides hippocampus are equally considered vulnerable and responsible for the learning and memory deficits in FASD animals.

2.9 Theories of FASD

There are profound theories of FASD: 1. Glutamatergic neurotransmission associated synaptic dysfunction 2. Cholinergic mechanism is attributed to FASD related deficiency in learning and memory (Perkins 2014). 3. Genetic and epigenetic factors play a major role in HPA axis dysfunction in FASD patients. All these theories alongwith hyperactivation of $GABA_A$ receptors result in lifelong neurobehavioral deficits associated with FASD. However, the glutamatergic neurotransmission theory is the most researched related to FASD model.

2.10 Glutamatergic synaptic transmission

Glutamate is the major excitatory neurotransmitter in the brain which regulates a vast number of synapses in the Central Nervous System (CNS). The synaptic excitation is related to a differentiation, neuronal cell survival and death, cellular proliferation and also changes in synaptic functions underlying the learning and memory functions (Mayer and Westbrook 1987; Kleckner and Dingledine 1988; Monaghan, Bridges et al. 1989). They play a major role in shaping the developing brain function.

The glutamate receptor system consists of 3 major ionotropic receptors and metabotropic receptors (Seeburg 1993; Nakanishi 1994). The cation specific ionotropic glutamate receptors include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptor channels which conduct fast neurotransmission. Metabotropic receptors are G-protein (GTP-binding proteins) coupled receptors which modulates ion channels directly or indirectly through intracellular effector enzymes.

2.10.1 Ionotropic glutamate receptors

These receptors are permeable to Na^+ and K^+ ions down their electrochemical gradients when bound to gluatamate neurotransmitter. The influx of positive ions into the neuron depolarizes the cell causing excitatory postsynaptic potential (EPSP). It depolarizes the neuron from the resting membrane potential of -70 mV to positive potential values. A series of such depolarization causes generation of action potential that propagates down the dendrites and axons of the neuron.

The gluatamate receptor structurally consists of an extracellular amino terminal domain and a pore forming membrane residing domain separated by a transmembrane domain (Figure 2.3). A large extracellular loop connects the second and third transmembrane domains. An intracellular carboxy-terminus follows the third transmembrane domain (Dingledine, Borges et al. 1999; Mayer and Armstrong 2004). The agonist binds to a pocket formed between the extracellular N-terminal region (S1 region) and the extracellular loop between transmembrane domains 3 and 4 (S2 region).

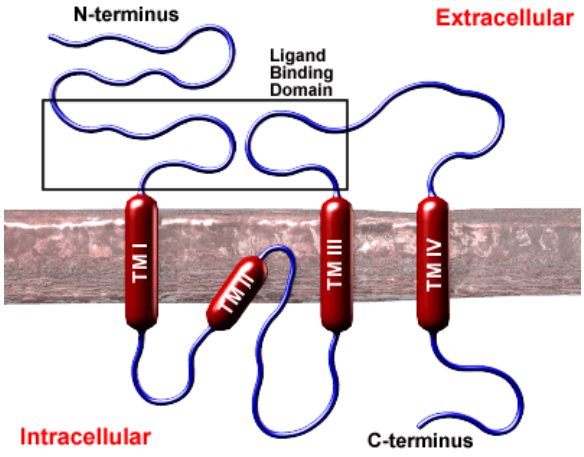

Figure 2..3 General structure of an ionotropic glutamate receptor subunit. The large N-terminal domain is extracellular while the shorter C-terminal domain is intracellular

2.10.1.1 AMPA receptors

AMPARs consist of four main subunits (GluR1-4) synthesized from four separate genes and assembled as tetramer (Rosenmund, Stern-Bach et al. 1998). The AMPAR subunit has a molecular weight of ~105 kDa with ~900 amino acid residues (Rogers, Andrews et al. 1994). Although the AMPAR genes have around 70% sequence homology, AMPAR transcripts undergo alternative splicing at two distinct sites. Due to alternative splicing, the subunits have long (GluR1/4, alternative spliced form GluR2) or short (GluR2/3, alternative splice form of GluR4) C terminal domain and flip or flop variants in the extracellular domain (Black and Grabowski, 2003). The C-terminal tail play an essential role in protein-protein interaction with PDZ domain containing proteins (Dingledine, Borges et al. 1999). GluR1/2 are the most predominant heteromers in the hippocampal pyramidal neurons compared to GluR3/4. GluR2 containing heteromers are impermeable to calcium (Ca^{2+}) ions unlike other subunits (monomeric or hetermomeric forms) and show low single channel conductance. This is attributed to the presence of arginine (R) in place of Glutamine (G) within the M2 domain. Adjacent to this site is the 38 amino acid residues (flip flop cassettes) introduced by alternative splicing. RNA editing and splicing introduces the R/G and flip-flop conversions respectively with postnatal development. The variants have effects on the recovery rates and extent of desensitization of GluR2 containing AMPARs and also influence their sensitivity to allosteric modulators. Unlike GluR2 containing receptors, the GluR2 lacking receptors show polyamine blockade dependent channel rectification. At positive voltage, the GluR2 lacking receptors are blocked by polyamines and show inward current only at negative potentials suggesting inward rectification (Pellegrini-Giampietro, Gorter et al. 1997).

The flux of ions is gated through channel pored formed by the AMPAR reentrant loop of the sununit. Ligand gated AMPARs are permeable to Sodium (Na^+) and Ca^{2+} (Tikhonov, Mellor et al. 2002). AMPAR permeability causes generation of fast excitatory response in the postsynaptic neuron depolarizing the cell.

Glycosylation and phosphorylation are two major forms of post-translational modification in AMPARs which produce a functional effect to the subunits influencing plasticity at the synapse. The mature glysosylated forms of AMPARs are synaptically expressed (Weaver and Woosley 1993; Standley, Tocco et al. 1998). Kinases and phosphatases activity influence the phosphorylation state of the AMPARs determining its synaptic availability, stability and trafficking (Swope, Moss et al. 1999). Protein expression of AMPAR subunits peak gradually in the third postnatal week. GluR1 containing AMPAR incorporation occurs during synaptogenesis (Morissette, Le Saux et al. 2008).

The distribution of the AMPARs subtypes vary region dependent manner in the brain. Hippocampus, cortical lamination, lateral septum, basal ganglia and amygdala reported to have wide distribution of GluR1, GluR2 and GluR3 subtypes (Keinanen, Wisden et al. 1990). GluR4 is not very much expressed in CNS as from rat studies except in the thalamic nuclei and the cerebellum (Petralia and Wenthold 1992). The AMPARs traffic to the synaptic surface from the intracellular receptor pool and interact to the postsynaptic density proteins (PSD) (Rogers, Gillis et al. 1991). The regulation of AMPARs insertion and removal at the synapse is important to maintain the plasticity. AMPARs can be transported to the synapse directly by exocytosis or by diffusion from the extrasynaptic space to the PSD. GluR2/3 AMPARs are continuously cycled from the

synapse. Activity dependent loss of synaptic AMPARs is necessary to maintain the plastic nature of the synapse (Lee and Lucey 2004). Silent synapses are devoid of AMPARs. Stimulation of such synapses induce recruitment of AMPARs by activation of NMDARs making them active synapse (Durand, Kovalchuk et al. 1996).

Prenatal alcohol mediated hippocampal changes in function and structure leading to cognitive and behavioral deficits in part dependent on NMDAR based mechanism. NMDARs blockage during development resulted in neurodegeneration in CNS. However, prenatal alcohol can also effect the AMPA and/or kainite receptors which play a pivotal role in CNS maturation (Ozawa, Kamiya et al. 1998). It can be hypothesized that prenatal alcohol can modify the synaptic expression pattern of the AMPARs and glutamate neurotransmission during embryonic stage. Therefore, any target protein pathway affecting the trafficking or regulation of surface AMPARs could have a potential therapeutic importance.

2.10.1.2 NMDA receptors

NMDA glutamate receptors consist of seven subunits NR1, NR2A-D and NR3A/B transcribed from different genes. Expression of NR2B is predominant in the fetus whereas NR2A and NR2C expression pick during the early postnatal days (Zukin and Bennett 1995). NMDA tetrameric receptors are composed of NR1 subunit and NR2/NR3 subunits. NR1/NR2 subunit composition forms functional channels. They are coincident detectors i.e they require ligand binding and membrane depolarization to get activated. The NMDARs require two obligatory ligands, glycine and glutamate binding to sites at NR1(Kuryatov, Laube et al. 1994; Wafford, Kathoria et al. 1995) and NR2

subunits (Laube, Kuhse et al. 1998). Depolarization of the membrane removes the Mg^{2+} blockade of the channel pore allowing the flow of both monovalent and divalent ions (Monyer, Burnashev et al. 1994).

Functional NMDARs are critical for normal neurodevelopment including cell growth, proliferation, differentiation, migration and plasticity. Prenatal alcohol has been reported to decrease expression of NR2B subunits in the postnatal FASD model (Samudio-Ruiz, Allan et al. 2010). Another study looked into the dentate gyrus where the NR2B receptors decreased and NR1 and NR3A receptors increased in expression (Brady, Diaz et al. 2013). Therefore this study did not focus on the investigation of expression and function of NMDARs.

2.10.1.3 Kainate receptors

These receptors mainly are from two related subunit families, GluR5-7 and KA1-2. KA-2 requires the GluR5-7 subunits to be expressed at the synapse or else it undergoes protesomal degradation or retained in the endoplasmic reticulum (Gallyas, Ball et al. 2003). GluR6 is the predominant kainate receptors in the hippocampus dentate gyrus and CA3 pyramidal neurons (Contractor, Sailer et al. 2003). They desensitize faster upon agonist binding. Slowly, the physiological role of kainate receptors are being established.

2.11 Prenatal alcohol exposure and glutamatergic transmitter system

Ethanol disrupts neurogenesis in prenatal alcohol model through NMDAR and GABAA receptors. The period of prenatal alcohol exposure and the blood alcohol concentration determines the extent of damage. The effect of glutamatergic neurotransmitter system is not the same in all brain regions.

2.11.1 Prenatal ethanol exposure and NMDA receptors

Neonatal NMDA receptor blockade by ethanol causes apoptotic neurodegeneration in brain including cortex, hippocampus, hypothalamus, thalamus and caudate nucleus (Ikonomidou, Bittigau et al. 2000). In FASD model there is decrease in NMDA sensitive 3H-glutamate binding sites in the dendate gyrus and the dorsal hippocampus (Staples, Porch et al. 2014). In a guinea pig model, (Abdollah and Brien 1995) showed decreased glutamate and NMDA receptor number (Bmax) without any alteration of K_d suggest reduced NMDAR expression. Reduced NMDA receptor sensitivity in the hippocampus to NMDA and increased inhibition to Mg^{2+} ions observed with *in utero* ethanol exposure (Morrisett, Mott et al. 1990). There is considerable reduction of intracellular Ca^{2+} levels in prenatal alcohol model (Gruol, Ryabinin et al. 1998) suggesting many underlying protein signaling pathways are compromised.

2.11.2 Prenatal ethanol exposure and non-NMDA glutamate receptors

Prenatal ethanol exposure affects the AMPA and/or kainate receptors in the hippocampus. A study has shown decreased ^{3}H-vinylidene kainate in the CA1 region of the hippocampus (Farr, Montano et al. 1988). However, similar study on AMPA receptors show no change in ^{3}H-AMPA binding sites in the hippocampus (Martin and Dombrowski 2008). Hence, most studies have concluded the effect of prenatal alcohol on NMDARs compared to non-NMDARs. Bellinger, Bedi et al. 1999 showed ethanol vapor exposure reduced the GluR1 AMPA expression. Prenatal alcohol modulates the regulatory protein responsible for the proper functioning of AMPARs. The AMPAR mediated mEPSCs in the hippocampal CA1 were reduced in prenatal alcohol model.

Aniracetam treatment has shown reversal of behavioral deficits in prenatal alcohol with increased frequency and amplitude of mEPSCs in CA1 pyramidal cells (Vaglenova, Pandiella et al. 2008).

2.12 Integrin linked kinase

Integrin linked kinase acts as an adaptor protein remained bound to beta 1 subunit of intergrins (Dedhar, Williams et al. 1999; Hannigan, O'Leary-Moore et al. 2007). It controls various downstream signal transduction mechanism including cell survival, differentiation and gene expression (McDonald, Hicks et al. 2014). Structurally, ILK contains three main domains: N-terminal domain, a central pleckestrin homology (PH)-like domain and a C-terminal domain, each interacting with specific proteins. N-terminal domain associates with proteins like PINCH (Particularly interesting new cysteine-histidine 25 protein), a LIM domain-adaptor protein facilitating the position of ILK at the focal adhesion sites (Zhang, Turton et al. 2011). The PH-like domain connects the growth factor receptor to phosphatidyl inositol-3,4,5- triphosphate (Ptd Ins(3,4,5)P3) and mediate Phosphoinositol 3 kinase (PI3K) dependent ILK activation (Hannigan, Troussard et al. 2005). ILK is endogenously regulated by ILK-associated protein serine/threonine phosphatase of the PP2 family (ILKAP), or phosphatase and tensin homolog (PTEN) (Wu and Dedhar 2001). ILK phosphorylates both AKT on serine 473 and glycogen-synathase kinase-3β (GSK-3β) at serine 9 (Dedhar, Williams et al. 1999). C-terminal domain has the kinase activity and interacts to the integrins and the actin-binding adaptor proteins, paxillin (Nikolopoulos and Turner 2001; Nikolopoulos and Turner 2002).

The complex interplay among the molecules GSK3β, BDNF, ILK and Akt strengthen the notion that ILK is critically involved in LTP. Some preliminary studies in

our lab suggests, increase in ILK levels with LTP production and the impairment in LTP associated with ILK inhibition by Kp-27389, that ILK plays an important role in LTP in the hippocampal Shaffer collateral CA3-CA1 synapses. Analysis of basal synaptic transmission illustrates that ILK inhibition attenuated fEPSPs. These findings highlight a functional link between ILK, basal synaptic transmission and LTP in the hippocampus. Interestingly ILK is also implicated in the regulation of neural plasticity, in Nucleus Accumbens (NAc) in response to cocaine sensitization (Chen, Zhu et al. 2010). Alcohol toxicity and prenatal alcohol have been shown to associate to PTEN activity (Shearn, Smathers et al. 2013). Ethanol has also been linked to PI3K pathway stimulation in hypertension (El-Mas, Fan et al. 2009). In this study, we tested whether prenatal alcohol has any role in ILK signaling modulation and synaptic plasticity.

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3. IMPAIRED ILK FUNCTION IS ASSOCIATED WITH DEFICITS IN HIPPOCAMPAL BASED MEMORY AND SYNAPTIC PLASTICITY IN A FASD RAT MODEL.

3.1 Abstract

Fetal Alcohol Spectrum Disorder (FASD) is an umbrella term that encompasses a wide range of anatomical and behavioral problems in children who are exposed to alcohol during the prenatal period. There is no effective treatment for FASD, because of lack of complete characterization of the cellular and molecular mechanisms underlying this condition. Alcohol has been previously characterized to affect integrins and growth factor signaling receptors. Integrin Linked Kinase (ILK) is an effector of integrin and growth-factor signaling which regulates various signaling processes. In FASD, a downstream effector of ILK, Glycogen Synthase Kinase 3β (GSK3β) remains highly active (reduced Ser^{9} phosphorylation). GSK3β has been known to modulate glutamate receptor trafficking and channel properties. Therefore, we hypothesize that the cognitive deficits accompanying FASD are associated with impairments in the ILK signaling pathway.

Pregnant Sprague Dawley rats consumed a "moderate" amount of alcohol throughout gestation, or a calorie-equivalent sucrose solution. Contextual fear conditioning was used to evaluate memory performance in 32-33-day-old pups. Synaptic

plasticity was assessed in the Schaffer Collateral pathway, and hippocampal protein lysates were used to evaluate ILK signaling. Alcohol exposed pups showed impaired contextual fear conditioning, as compared to control pups. This reduced memory performance was consistent with decrease in LTP as compared to controls. Hippocampal ILK activity and GSK3β $Ser^{21/9}$ phosphorylation were significantly lower in alcohol-exposed pups than controls. Increased synaptic expression of GluR2 AMPA receptors was observed with immunoprecipitation of post-synaptic density protein 95 (PSD95). Furthermore, immunoprecipitation of ILK revealed a decreased interaction with GluR2.

The ILK pathway appears to play a significant role in memory and synaptic plasticity impairments in FASD rats. These impairments appear to be mediated by reduced GSK3β regulation and increased synaptic stabilization of the calcium-impermeable GluR2 AMPA receptors.

3.2 Introduction

Alcohol is probably the most commonly used and socially accepted psychoactive substance. However, alcohol consumption is not recommended during any stage of pregnancy; indeed, alcohol use during pregnancy can lead to a range of cognitive and physical consequences in the developing fetus (Jones and Smith 1973). Indeed, FASD are the leading cause of mental retardation in the United States. According to the United States Centers for Disease Control and Prevention, the prevalence of FASD in the U.S is relatively high (1.5-2.0 cases/1000 births), and exceeds that of other countries around the world (samhsa).

Up to 94% of FASD children report mental health problems, and 79% have difficulties maintaining employment. The lifetime cost of each individual with FASD is estimated at $2 million (Lupton, Burd et al. 2004) . Despite this great cost and the fact

that the neuroanatomical and neurochemical effects of chronic alcoholism have been well elucidated, to date there are no therapeutic interventions available to treat FASD-induced cognitive deficits. FASD is fully preventable by abstaining from drinking during pregnancy; however, 100% compliance with this preventative measure may be difficult considering that the majority of FASD cases are the result of drinking prior to detecting the pregnancy. Furthermore, an estimated 9% of women will continue to drink despite the fact they know they are pregnant, some of them heavily (up to 0.3%) or in binges (up to 3%) (2014). Of great concern are "moderate" drinkers (women consuming a maximum of 7 drinks per week) since there is some debate as to whether moderate alcohol consumption is safe during pregnancy (Humphriss, Hall et al. 2013). Hence, it is of extreme relevance to determine the mechanisms underlying FASD to develop optimal therapeutic interventions to address and potentially reverse the deleterious effects of alcohol exposure during gestation.

The two major cognitive consequences of FASD are reduced intelligence and memory impairments (Kully-Martens, Pei et al. 2012; Kalberg, May et al. 2013). FASD children are also at an increased risk of developing attention deficit hyperactive disorder (ADHD) and autism spectrum disorders (ASD), both of which are associated to significant learning disabilities and memory impairments (Aronson, Hagberg et al. 1997). At the root of these impairments may be the profound detrimental effect that prenatal alcohol exposure can have on the development of various brain regions associated with short- and long-term memory storage. Indeed, FASD can result in anatomical, biochemical and electrophysiological changes in the regions of the brain involved in memory formation and storage, namely the hippocampus and cortex (Klintsova, Helfer et al. 2007).

Prenatal alcohol exposure also disrupts development of a variety of cellular processes, including insulin resistance and reduced neurotrophic factor expression. Insulin signaling is linked to ILK signaling through Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), downstream Protein Kinase B (Akt), and GSK3β phosphorylation (Yao and Nyomba 2008). Prenatal alcohol increases expression of the Phosphatase and tensin homolog (PTEN), a negative regulator of the PI3K pathway (Xu, Yeon et al. 2003). PTEN endogenously suppresses ILK activity, which is a downstream effector of integrins and insulin signaling. However, the role of ILK in prenatal alcohol-related deficits has not been investigated to date.

ILK appears to be localized with β1 integrins in humans and rodents (Xu, Zheng et al. 2015). ILK facilitates various cellular functions such as survival, cytoskeletal dynamics, and proliferation; thus, it has played a vital role in cancer research (Hannigan, Troussard et al. 2005). ILK activity is required to promote neurite growth factor (NGF) mediated neurite growth in rat pheochromocytoma cells; indeed, inhibition of ILK abolishes NGF-mediated neurite growth. Thus, ILK appears to play an important role in neurogenesis (Mills, Digicaylioglu et al. 2003). ILK appears to be involved in cocaine-induced synaptic plasticity, possibly via an interaction with subunits of the α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptors (AMPAR) and PSD95 proteins. Such interaction proves crucial for addiction-related modulation of synaptic plasticity and memory (Chen, Zhu et al. 2010). In neurodegenerative diseases (e.g., Alzheimer's disease and Parkinson's disease) characterized by learning and memory deficits, ILK-related mechanisms are compromised (Benitez-King, Ramirez-Rodriguez et al. 2004; Müller, Meyer et al. 2008). Inhibitors of ILK have been found to increase Tau protein hyperphosphorylation (a hallmark of neurodegenerative disorders) through

activation of GSK3β (Mills, Digicaylioglu et al. 2003), highlighting the important role of ILK in the etiology of neurodegenerative disease. Despite the apparent relevance of ILK in conditions associated to memory deficits, its actual role in memory formation and synaptic plasticity has not been well elucidated. However, a few studies have provided evidence for a strong role of ILK function in learning and memory. For example, we have previously reported that ILK signaling through GSK3β is impaired in a diabetic model of insulin-resistant brain, with accompanying impairments in memory and synaptic plasticity (Shonesy, Thiruchelvam et al. 2012).

The observations described above suggest that the ILK pathway could be the focus of a novel therapeutic target in FASD. Indeed, synaptic plasticity and cognitive function can be enhanced through different manipulations of ILK activity. For example, in aged rats, PI3K-ILK signaling can be potentiated with Brain derived neurotropic factor (BDNF) treatment to obtain enhanced synaptic plasticity and cognitive function (Li, Dai et al. 2012), and ILK activation inhibits GSK3β activity and helps restore synaptic plasticity (Li, Dai et al. 2012).

In the present study, we hypothesized that cognitive deficits accompanying moderate prenatal alcohol exposure is associated with impairments of the ILK pathway. We considered a continuous exposure of low level alcohol dose in rats as an appropriate model to study FAS like memory impairments. This model can indeed produce functional deficits in learning and memory (Patten, Fontaine et al. 2014). The functional effects of this modulation were assessed using a contextual fear conditioning preparation, in which an environment is associated to the delivery of an electrical stimulus. Memory of the conditioning episode was correlated to ILK-GSK3β signaling and synaptic plasticity.

3.3 Materials and Methods

Subjects:

The animal protocol and experiments were pre-approved by Auburn University IACUC committee (PRN# 2013-2265). We strictly adhered to the guidelines and directions mentioned in the protocol. Eight time-pregnant albino rats (200-250 g) (Sprague Dawley strain, Harlan Laboratories) consumed 10% (v/v) alcohol prepared with 95% ethyl alcohol and tap water throughout the gestation period, starting from the second day of gestation. The alcohol solution was sweetened with 3% glucose and 0.125% saccharin (Sigma Aldrich, USA) (Briones and Woods 2013). Six Non-exposed time pregnant rats received an equivalent solution lacking the ethyl alcohol. Animals were housed in a vivarium maintained on a 12 h:12 h light:dark cycle (lights on at 6:00 am) and at a temperature of 22-24°C. Bottles filled with the alcohol solution were offered at 6:00 pm and fluid consumption was measured after 15 hours of free access to the solution (access to food was *ad lib*). Differences in liquid volume were converted to volume intakes after accounting for the ethanol solution density (weight in grams/0.9868). There were no differences in consumption or weight gain between dams receiving alcohol and sweetened water. This volume of alcohol is considered to be animal model of moderate alcohol consumption: The dams consumed around 20ml of ethanol solution (equivalent to 6g/kg/day) which is comparable to 1 to 2 drinks per day for an adult human (Åberg, Hofstetter et al. 2005). The day after parturition was considered postnatal Day 1 (PND 1) and all litters were culled to ten pups per dam.

Contextual fear conditioning: Contextual fear conditioning is mainly dependent on hippocampal function. Dorsal hippocampus has a significant role in conditioned

contextual freezing (Fanselow and Dong 2010, Phillips and LeDoux 1994) . Twenty-six animals served as subjects in this experiment. They were assigned to two groups, Alcohol (Eth, n = 16) or Control (Ctrl, n = 10). All animals were approximately 33 days old at the initiation of the behavioral portion of the study (average weight was 200 g). Each group was further randomly divided into shock and no shock subgroups. Animals were first trained to lick from a water spout in a novel environment, Context B. Conditioning was conducted in a different context, Context A, which was paired with a shock (Figure 3.1a). Memory of the association between Context A and the shock was assessed by placing the animals back in Context A, allowing them to begin licking from the spout, and recording the number of licks. The testing session was terminated at 10 min; thus, 600 s represent ceiling latency. Subjects' access to water was gradually restricted to one hour per day over the week prior to initiation of the study, provided approximately one hour after completion of each day's session. All conditioning procedures occurred in standard MedAssociates rat operant chambers and the experiments were scored by individuals who were blinded to the condition. Two modifications of these chambers served to create Context A (chambers with no additional cues), and Context B (a different set of chambers, modified using a striped pattern to cover the otherwise clear walls). Behavioral training and assessment occurred over 4 days, as follows. On Days 1 and 2 (lick training), subjects were trained to lick for water in Context B during two 30-min sessions. On Day 3 (conditioning), subjects were placed in Context A for 270 s. A 0.65-mA electric foot shock was delivered at 180 s for a duration of 2 s. Water bottles were not available during the conditioning session. On Day 4 (memory assessment), subjects were exposed to Context A for a 10 min period with water bottles available; this test evaluated recall of the association between Context A and the foot shock. Timing at which each lick was

produced was recorded. Rats 'freeze' in anticipation of shock; thus, longer latencies to drink were assumed to reflect higher expectation of shock to be delivered in Context A (i.e., stronger memory of the conditioning experience). Latencies are skewed to the right; thus, a log (base 10) transformation was applied to the data to meet the normality criterion of parametric statistics. A 2 (treatment: alcohol vs. control) x 2 (condition: shock vs. no shock) analysis of variance (ANOVA) was conducted on the latency to complete 50 licks, assessed from the moment the first lick was produced until the 50th lick was produced.

Electrophysiology recording: Rats were euthanized by decapitation and the brains rapidly removed and placed in ice-cold cutting solution containing (in mM) 120 NaCl, 11 D-Glucose, 26 $NaHCO_3$, 6 $MgCl_2$, 3 KCl, 0.5 $CaCl_2$, 5 HEPES and 0.3 kynurenic acid. Coronal slices (300 mM) were cut with a Leica VT-1200S in the cutting solution. The slices were then transferred to artificial cerebrospinal fluid (aCSF, in mM 124 NaCl, 3 KCl, 1.5 $MgSO_47H_20$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 10 D-Glucose bubbled with 95%CO_2/5%O_2) for at least 1 h and then maintained at room temperature until use.

Measurement of Basal Synaptic Transmission and Long Term Potentiation: Following incubation, electrophysiological recordings were performed in a submerged recording chamber with continuous perfusion with aCSF (2-3ml/min) bubbled with 95%CO_2/5%O_2 carbogen, maintained at room temperature. fEPSPs were recorded from Schaffer collateral pathway SC-CA1 synapses with a glass pipette filled with aCSF (2-4MΩ). Stimulating pulses were applied at Schaffer collaterals via a bipolar stimulating electrode positioned 300 μm closer to CA3 subfield than the recording electrode. After placing

stimulating and recording electrodes in the CA3 and CA1 regions respectively, stimulus intensity was lowered to the point where the fEPSP disappeared completely leaving the stimulus artifact intact. For stimulus response curves, current intensity was altered from 0-120μA. For LTP experiments, baseline was recorded at 50% of amplitude at which the initial population spike appeared. LTP was induced after 15 mins of stable baseline recording using a Theta Burst Stimulation protocol (TBS), and recording was continued for 60 mins post TBS. All electrophysiological data are presented as means ± SEM. For plasticity experiments, significance was determined using one-way ANOVA, followed by comparisons using a Bonferroni correction.

Immunoprecipitation (IP) assay: PSD95 was immunoprecipitated from pooled hippocampal tissue lysate using 1:10 anti-PSD95 (Santa-Cruz) antibody coated on Pure-Proteome A/G magnetic beads (Millipore), and using vendor-supplied direct IP protocol. The immunoprecipitated fraction was purified through several washing steps with 1X IMP buffer, pH=7.4. Finally, beads were boiled in 50ul of Laemmle Buffer at 70°C and separated on SDS PAGE, which was followed by western blot analysis.

Western Blot analysis: The PSD95 immunoprecipitation assay was probed with PSD-95 rabbit primary antibody (1:1000, Cell Signaling) to check for presence of PSD-95 pulled down. Equal amounts of sample were loaded to probe interaction of PSD-95 with GluR2 (Millipore), GluR1, and ILK with rabbit primary antibodies (1:1000, Cell Signaling). Whole hippocampal protein lysates were probed for BDNF or proBDNF, and GSK3β to total GSK3β, using their respective primary antibodies at 1:1000 (Cell Signaling). All blots were probed with Dy-Light 660 anti-rabbit secondary antibody (1:10000, Thermo

Scientific) using a Fuji FLA 5100 scanner. They are presented as means ± SEM. Significance was determined using a two-tailed Student's t-test.

ILK activity assay: ILK activity was determined in hippocampal tissue homogenates using an immune complex kinase assay. Briefly, tissue lysates were pooled and incubated with 1:50 anti-ILK mAb (cell signaling). The resulting immune complexes were washed three times in kinase reaction buffer, followed by incubation with 1 μg inactive Akt and ATP (final concentration: 200 μM) in 50ul kinase reaction buffer for 1 h at 30 °C. The reaction products (supernatant) were resolved on SDS/PAGE. The beads were then processed as described in IP assay for ILK immunoblot. Membranes were probed with antiphospho-Akt (ser^{473}) mAb (Cell Signaling Technology). The blot was developed using Dy-Light 660 anti-rabbit secondary antibody (1:10000, Thermo Scientific) using a Fuji FLA 5100 scanner. The data are presented as means ± SEM. Significance was determined using a two-tailed Student's t-test.

3.4 Results

Effect of prenatal alcohol on litter size and body weight:

There was no difference in the number of pups per litter (approximately 4-5 male pups per dam) and body weight observed between the prenatal alcohol exposed and nonexposed rats.

Moderate prenatal exposure to alcohol resulted in impairments in hippocampal-based contextual fear memory:

Previous studies on the behavioral outcomes of prenatal exposure to alcohol suggest that moderate drinking impairs spatial memory (Gianoulakis 1990; Brady, Allan

et al. 2012; Cullen, Burne et al. 2014). However, more recent studies suggest such impairments occur even after moderate to low dose prenatal exposure (Cullen, Burne et al. 2014). This controversy is not easily addressed because these reports either included different cognitive tasks or the age of the models used was different; thus, further studies are warranted. In the present study we assessed the effects of prenatal exposure to ethanol on memory, using a contextual fear conditioning preparation, assessed as disruption of licking behavior. The effects of treatment (alcohol *vs.* control) and condition (shock *vs.* no shock) did not reach statistical significance, $Fs_{1,51}$ = 1/74 and 3.16, *p*s = 0.19 and 0.08, respectively. However, the interaction of these two factors was significant, $F_{1,51} = 6.85$, $p < 0.05$ (Figure 3.1b). Thus, level of fear observed in the shock vs. no shock animals was dependent on whether they had been exposed to alcohol during the prenatal period. This conclusion was confirmed with pairwise planned comparisons, which revealed that the shock and no shock conditions differed in the control, $F_{1, 51} = 7.59$, $p < .01$, but not the alcohol condition, $F_{1, 51} < 1$ (Figure 3.1c).

Moderate prenatal alcohol impaired synaptic plasticity in the hippocampus:

Deficits in hippocampus-dependent associative learning and memory have been supported with long-term potentiation (LTP) in the Schaffer collateral pathway. We measured the changes in field potential in one-month old rat brains. In animals exposed to alcohol during the prenatal period, both moderate and binge drinking have been shown to result in deficits in cognitive function and neurogenesis (Bird, Candelaria-Cook et al. 2015; Smith, Guévremont et al. 2015). However, some studies questioned the amount of alcohol intake that can influence the deficits in memory impairment (Canales and Ferrer-Donato 2013; Graham, Crocker et al. 2013; Tyler and Allan 2014). The differences in the

pattern of administration of alcohol could also be a reason for the differences seen in behavioral studies. In this study, we looked into moderate continuous drinking, in which the animal has access to an alcohol solution as their only source of fluid. Following this administration regime, we initially determined the overall CA3-CA1 synaptic neurotransmission in the brain of animals prenatally exposed to alcohol, as well as in control rats. We monitored input-output (I/O) curves as a measure of overall basal synaptic transmission. Prenatal exposure to alcohol had no impact on I/O (p = .96; Figure 3.2a; also see (Brady, Diaz et al. 2013)), which appears to be affected in acute or binge alcohol consumption models (Wijayawardhane, Shonesy et al. 2007). LTP was recorded from the Schaffer collateral pyramidal cell region at CA1 using theta burst stimulation (TBS) applied at CA3. Moderate prenatal alcohol decreased LTP measured as percentage change of fEPSP from baseline as compared to the unexposed controls (Figure 3.2b, 3.2c; 121.453 ± 8.2% vs 167.188± 16.5% , $p < 0.05$). LTP induction was lowered in exposed animals, which suggests a possible modulation of glutamate receptors at the synapse.

Moderate Prenatal alcohol exposure reduced ILK activity but not expression**:**

Prenatal alcohol increases GSK3β activation (Goggin, Caldwell et al. 2014), and GSK3β activity impairs learning and memory by modulating expression of surface receptor proteins (as well as affecting other physiological neuronal functions (Hernández, de Barreda et al. 2009)). ILK phosphorylates both GSK3β and Akt (which is, in turn, the primary regulator of GSK3β phosphorylation) as downstream signaling molecules. Phosphorylation of GSK3β inhibits its activity (Hannigan, Troussard et al. 2005); thus, deficits in ILK should result in enhanced GSK3β and, consequently, impairments in learning and memory. However, the role of ILK in learning and memory after prenatal

alcohol exposure has not yet been investigated. In the present study, phosphorylation of GSK3β in the hippocampus of rats prenatally exposed to alcohol was reduced by approx. 20% as compared to nonexposed controls (Figure 3.3a, $p < 0.05$). Interestingly, expression of ILK in the hippocampus was equivalent in both exposed and nonexposed animals (Figure 3.3b). Nonetheless, despite equivalent expression, changes in ILK activity may impair downstream phosphorylation. An ILK kinase assay revealed that ILK activity was indeed reduced by approximately 60% in the hippocampus of exposed animals, as compared to nonexposed controls (Figure 3.3c). These observations appear to confirm our hypothesis that decreased ILK activity reduces GSK3β phosphorylation, and that this reduced phosphorylation may be responsible for the memory and LTP impairments observed in the exposed animals.

Moderate Prenatal alcohol exposure altered synaptic AMPAR expression:

ILK is known to interact with PSD95, an important scaffolding protein present at the synapse that interacts with several surface receptors. To understand the changes in AMPAR expression at the synaptic surface, we performed protein immunoprecipitation assays using anti-PSD95 antibody (Bats, Groc et al. 2007), and synaptic expression of GluR1, GluR2 and ILK was measured (Figure 3.4a). Synaptic expression of GluR2 was increased while synaptic expression of GluR1 was unchanged in animals exposed to alcohol prenatally. Increased GluR2 expression suggest an increase in calcium impermeable receptors at the synapse (GluR2 containing), which reduces the probability of action potential generation and, consequently, affect the NMDA-dependent LTP generation unlike calcium permeable (GluR2 lacking) receptors. Furthermore, calcium

permeable receptors may also help in maintaining the availability of calcium ions necessary for LTP maintenance (Liu and Savtchouk 2012).

Reduced ILK activity in prenatal alcohol exposure impaired ILK-AMPA receptor interaction:

LTP induction requires GluR2-lacking inwardly rectifying AMPA receptor incorporation at synapse followed by replacement of GluR2-containing receptors (i.e., a GluR1 to GluR2 switch) (Plant, Pelkey et al. 2006). Therefore, regulation of GluR2 receptors at the synapse is essential for LTP maintenance (Jia, Agopyan et al. 1996). ILK interacts with surface glutamate receptors and proteins at the post-synaptic density, and it may act as a scaffolding protein by mediating phosphorylation and modulating trafficking of glutamate receptors to the surface. ILK immunoprecipitation assays used to assess GluR2 binding to ILK revealed a reduced interaction of GluR2 with ILK in animals exposed to alcohol in the prenatal period (Figure 3.4b). This impaired interaction suggests that ILK may play a crucial role in GluR2 stability at the synapse, resulting in reduced LTP.

Moderate prenatal alcohol exposure resulted in reduced proBDNF/BDNF ratio:

Expression of the mature form of BDNF is associated to increased plasticity and cell survival mechanisms in neurons (Brigadski and Leßmann 2014). Reduced mature BDNF can significantly impair ILK activity and downstream signaling processes [17, 41]. Contrarily, expression of proBDNF is associated to decreased neuronal survival and reduced synaptic plasticity (Yang, Harte-Hargrove et al. 2014). Thus, the ratio of BDNF/proBDNF can provide a justification for reduced ILK activity at hippocampal glutamatergic synapses in the prenatal alcohol exposed rats. In our prenatal alcohol

exposure model, we observed increased proBDNF and reduced BDNF; hence, the ratio of BDNF/proBDNF was significantly lower ($p<0.05$) than in nonexposed animals (Figure 3.5). This finding is consistent with earlier studies (Feng, Yan et al. 2005), and suggests that alcohol-induced reductions in the BDNF/proBDNF ratio contribute to the cognitive deficits observed in FASD animals.

3.5 Discussion

Exposure to teratogens such as alcohol, nicotine, and cocaine during fetal development can modulate glutamate receptor and downstream signaling. Gestational exposure to alcohol results in reductions in brain size, with the cerebellum and corpus callosum being gravely affected by this exposure (Lebel, Roussotte et al. 2011; Cardenas, Price et al. 2014). Prenatal exposure to alcohol can also produce severe to moderate damage to hippocampal neurons (Burd, Klug et al. 2003). The hippocampus is considered to be the 'sorting region' of the brain, categorizing inputs from other parts of the brain and beginning the process of storage of information into long- and short-term memory (Kryukov 2008). The Schaffer collateral pathway (CA3 to CA1) of the hippocampus plays an integral role in memory formation; therefore, any change in protein expression or interaction at the CA1 region could lead to memory impairments (Bartsch, Döhring et al. 2011). The hippocampus is essential for acquisition and performance of spatial and contextual memory tasks; thus, hippocampal function can be assessed behaviorally with procedures that require spatial or contextual information. In the present study, we used contextual fear conditioning to assess hippocampal memory in a rodent model of FASD, and observed marked deficits in short-term memory formation in exposed animals as compared to nonexposed controls. Behavioral assessment of hippocampal function was

followed by field potential recording from the Schaffer collateral pathway. Consistent with the behavioral memory assessment, we observed LTP deficits that suggest that function of the hippocampus was dramatically affected by prenatal alcohol exposure.

AMPARs (fast excitatory glutamate receptors) play a crucial role in LTP induction and maintenance. AMPAR regulation at the synaptic surface controls NMDAR-dependent synaptic plasticity at the CA3-CA1 Schaffer collateral pathway (Anggono and Huganir 2012; Paoletti, Bellone et al. 2013). Some preliminary studies from our laboratory suggest that the interaction of ILK with glutamate receptors is of relevance in neurodegenerative diseases (Chen, Zhu et al. 2010; Shonesy, Thiruchelvam et al. 2012) because changes in ILK expression can produce changes in downstream signaling molecules as well as changes in its scaffolding property. Interestingly, we observed no effects of prenatal alcohol exposure on ILK expression. However, there was reduced activity of ILK in exposed animals as compared to nonexposed controls. This suggests that ILK activity could be of high importance in synaptic plasticity after prenatal alcohol exposure. ILK is also present at the synaptic surface; therefore, changes in its activity may lead to changes in synaptic receptor phosphorylation. Surface AMPAR composition (measured by PSD95 immunoprecipitation assays) was altered by prenatal alcohol exposure, with increased expression of calcium-impermeable GluR2 receptors, and no change in expression of calcium-permeable GluR1 receptors. LTP maintenance requires a calcium permeable GluR1 to calcium impermeable GluR2 switch after LTP induction (Jonas and Burnashev 1995; Malinow and Malenka 2002). Due to calcium impermeability, increased GluR2 containing receptors could impair LTP induction and maintenance, as appears to be the case in our exposed animals. Furthermore, we observed reduced GluR2 interaction with ILK in the hippocampus. Such reduced interaction may

influence GluR2 stabilization through reduced phosphorylation of target amino acid residues because such reduced phosphorylation may help stabilize GluR2 receptors on the synaptic surface. A potential target residue is Ser^{880} on GluR2 (Chung, Xia et al. 2000). Thus, the present results identify ILK as an important molecule, whose activity warrants further research in models of FASD-induced cognitive deficits.

3.6 Conclusion

The present study constitutes the first report highlighting the involvement of ILK in FASD-related memory impairments and synaptic plasticity. From this study, we conclude a close association of impaired ILK pathway and synaptic plasticity deficits in prenatal alcohol exposed rat model. Reduced ILK activity could be due to reduced BDNF to proBDNF ratio. The reduced kinase activity and diminished interaction to GluR2 AMPAR could be responsible for increased stabilization of GluR2 containing receptors at the synapse. The increased calcium impermeable AMPAR is responsible for impaired LTP induction and maintenance. Reduced LTP can also be due to increased GSK3β activation which could affect receptor trafficking and protein expression required for LTP maintenance. These findings from this research work have been summarized in Figure 3.6. Furthermore, our study suggests that FASD-related memory impairments can be due to impaired ILK pathway.

3.7 References

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3.8 Figure Legends

Figure 3.1: Prenatal alcohol impairs hippocampal-based contextual fear memory. **a**: four day lick suppression behavioral assay on control (n = 10) and alcohol groups (n = 16). Days 1 and 2 constituted training of the licking response in Context B, followed by conditioning in Context A on Day 3. A 0.65-mA scrambled footshock was delivered after 180^{th} s of context exposure during the conditioning day. The latency to complete 50 licks in Context A were used as a measure of memory of the conditioning experience. **b**: Time to complete 50 licks was calculated for the shock and no shock alcohol groups, compared to their nonexposed counterparts. **c**: cumulative latencies to complete 50 licks are shown

in 10 lick intervals for control and alcohol groups for shock and no shock category respectively. Differences were taken as statistically significant if $p < 0.05$.

Figure 3.2: Prenatal alcohol impairs synaptic plasticity. a: Input-output curves showing hippocampal basal synaptic transmission did not change in rats prenatally exposed to alcohol compared to controls. The graph shows f-EPSP amplitudes (mean ± SEM) as a function of stimulus intensity in the CA1 stratum radiatum. b: TBS-induced LTP was recorded in hippocampal brain slices from prenatal alcohol exposed and nonexposed control rats (n= 5). The figure presents the time-course of percentage change in field EPSP slopes (mv/ms) with representative traces. The arrow indicates the time at which TBS protocol was delivered and 5 minute baseline is shown for clarity. Inset, representative fEPSP traces taken before the TBS (1,3) and after stabilization of LTP expression (2,4) for control and alcohol groups accordingly. c: LTP was reduced in animals exposed to prenatal alcohol as compared to the nonexposed controls; [average for exposed animals was $162.2 \pm 19.0\%$, and for nonexposed animals was $129.6 \pm 20\%$, $F = 6.217$; $p < 0.05$].

Figure 3.3: Prenatal alcohol impairs ILK activity. a: Western blot analysis of total GSK3β and its Ser9 phosphorylation state was performed in hippocampal lysates from rats prenatally exposed to alcohol and nonexposed controls (n = 4). There was a significant decrease in the pGSK3β/GSK3β ratio in exposed rats, suggesting increased GSK3β activity as a result of alcohol exposure ($p < 0.05$). b: Western blot analysis of expression of ILK in brain hippocampal protein lysates from exposed and nonexposed rats (n = 4). There were no differences in densitometric evaluation shows in expression of ILK as a result of alcohol exposure. c: ILK activity assay was performed with pooled hippocampal protein lysates from control and alcohol rats (n = 4) and Akt ser473 phosphorylation

assessed with western blot analysis. The quantitation of band density analysis shows reduced ILK activity in alcohol-exposed animals ($p < 0.05$).

Figure 3.4: Prenatal alcohol may control GluR2 protein at the synapse through ILK. a. Immunoprecipitation (IP) with anti–PSD-95 from pooled hippocampal protein lysates of rats prenatally-exposed to alcohol and nonexposed controls ($n = 5$). In exposed rats, precipitate of GluR2 increased as compared to controls, while precipitates of GluR1and ILK did not change. The same blotting membrane was reprobed with anti–PSD-95 as a control for PSD95 pull down. The quantitation is shown adjacent to the blot image ($p < 0.05$). b. Immunoprecipitation (IP) with anti–ILK coprecipitates GluR2 from hippocampal protein lysates of exposed and nonexposed animals ($n = 5$). There was reduced interaction in the prenatal exposed rats as compared to the nonexposed controls. The western blot analysis of immunoprecipitated ILK with anti–ILK antibody was used to confirm equal ILK immunoprecipitation. The quantitation is shown adjacent to the blot image ($p < 0.05$).

Figure 3.5: Prenatal alcohol exposure reduces mature BDNF expression. Western blot analysis of expression of mature BDNF and proBDNF proteins in hippocampal lysates from prenatal alcohol exposed and nonexposed rats ($n = 4$). Densitometry analysis shows that BDNF to proBDNF ratio decreased in prenatal alcohol exposed as compared to nonexposed rats ($p < 0.05$).

Figure 3.6: Schematic diagram showing the probable mechanism through which prenatal alcohol modulates ILK and affects plasticity. ILK may affect an unknown amino acid phosphorylation which may help in GluR2 receptor internalization. Reduced ILK activity may reduce the target phosphorylation and stabilize GluR2 at the surface thereby increase GluR2 at the synapse. Increased GluR2 and downstream active GSK3β may

reduce LTP induction and maintenance. The probable cause of reduced ILK activity could be less mature BDNF availability in the brain of animals prenatally exposed to alcohol.

3.9 Figures

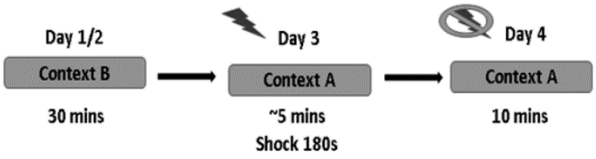

Figure 3.1a Lick suppression behavioral assay protocol

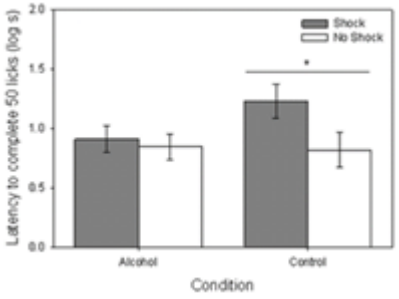

Figure 3.1b Latency to complete 50th lick

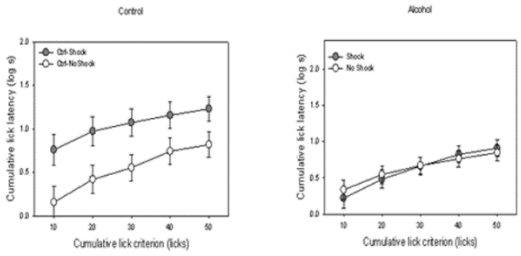

Figure 3.1c Latency to complete the counted licks

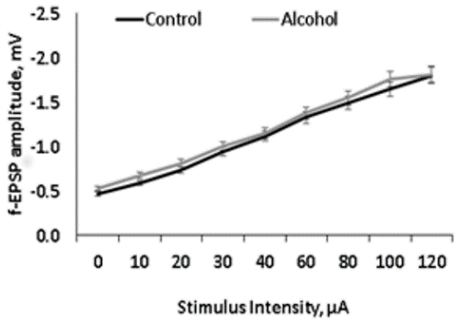

Figure 3.2a Input-output curve

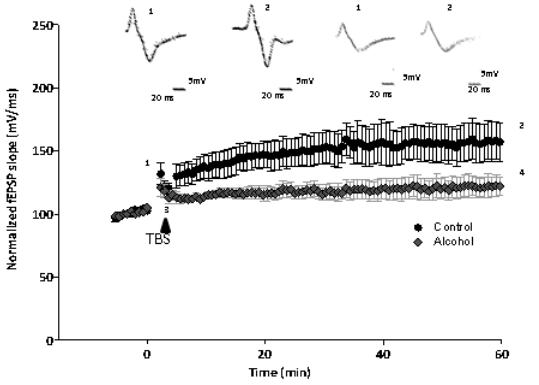

Figure 3.2b Long Term Potentiation with Theta Burst Stimulation

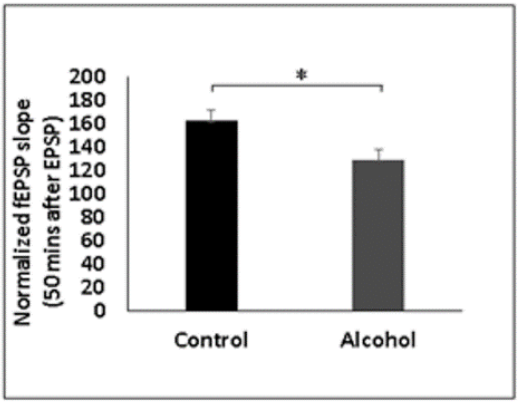

Figure 3.2c Quantification of LTP maintenance

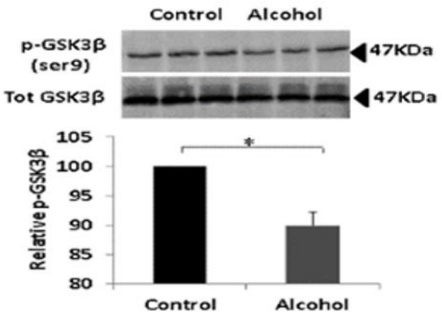

Figure 3.3a Western blot analysis of total GSK3β and its Ser9 phosphorylation

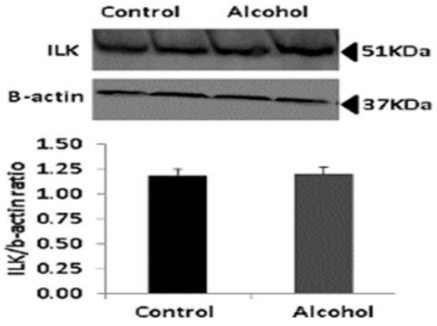

Figure 3.3b Western blot analysis of expression of ILK

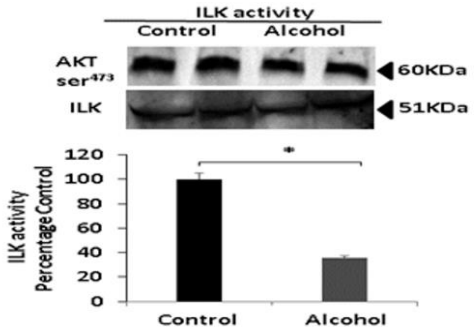

Figure 3.3c ILK activity assay

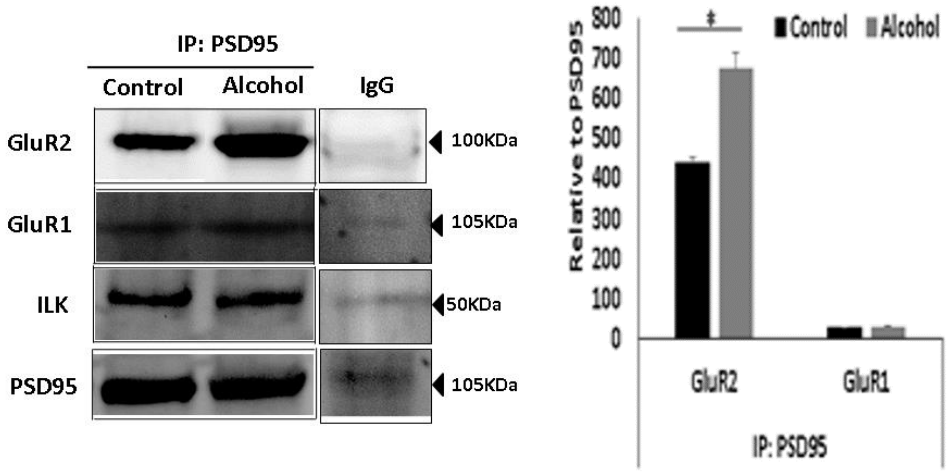

Figure 3.4a Immunoprecipitation (IP) with anti–PSD-95

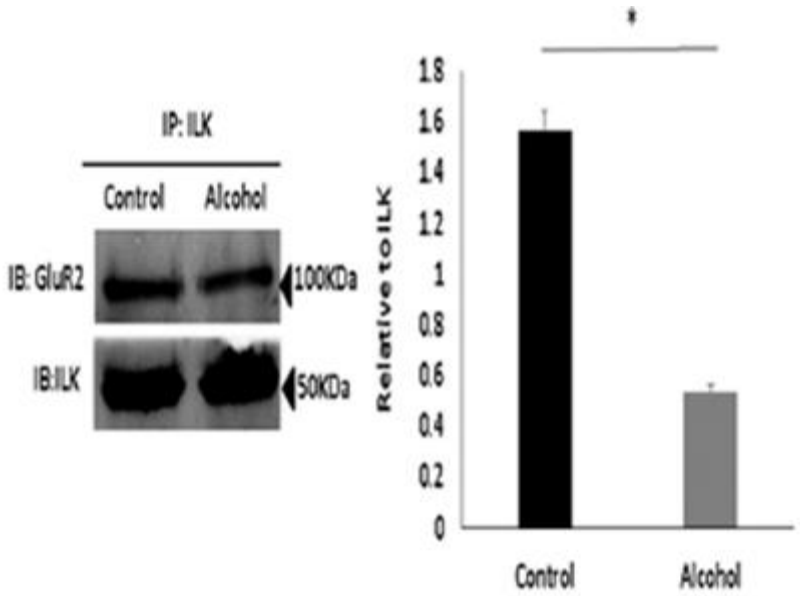

Figure 3.4b Immunoprecipitation (IP) with anti–ILK

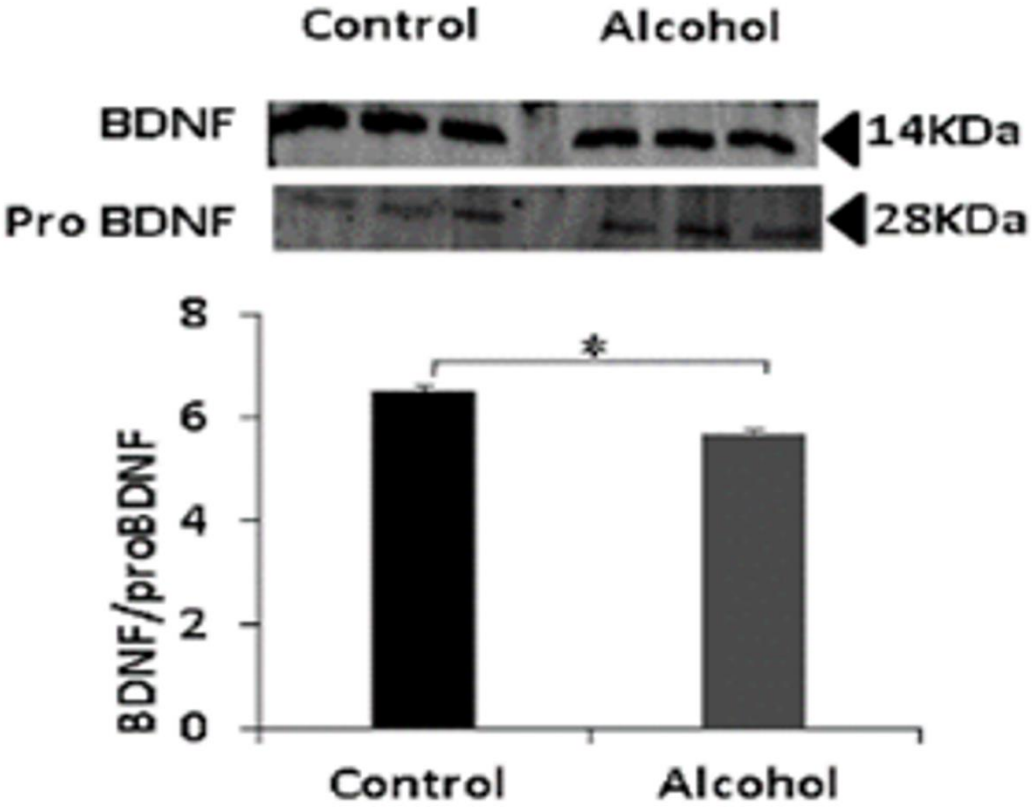

Figure 3.5 Western blot analysis of expression of mature BDNF and proBDNF proteins

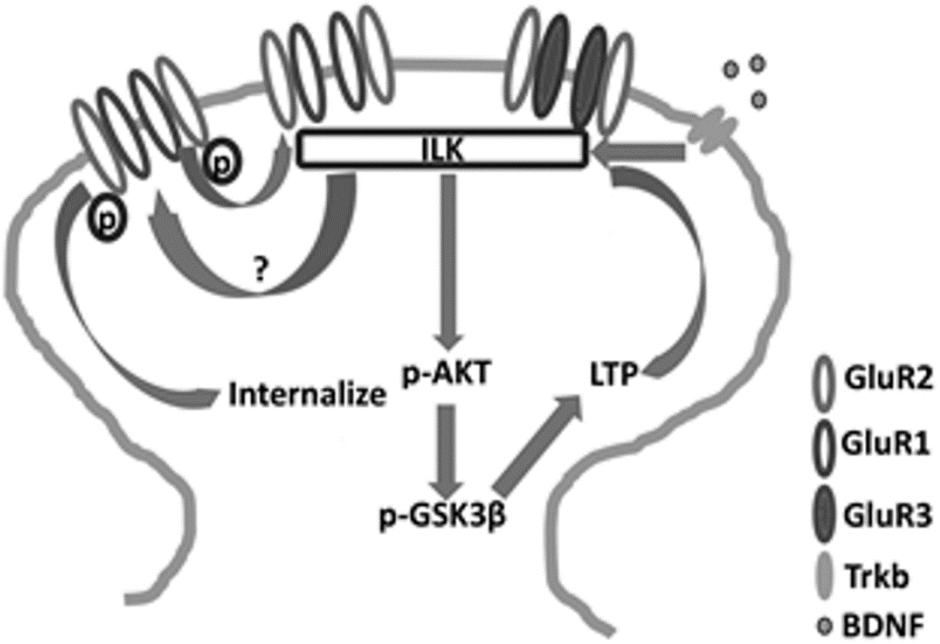

Figure 3.6 Schematic diagram showing the probable mechanism through which prenatal alcohol modulates ILK and affects plasticity

4. EFFECT OF PRENATAL ALCOHOL AND NICOTINE ON LEARNING AND MEMORY

4.1 Abstract

Prenatal alcohol exposure has been correlated to multitude of dose dependent effect on structure and function of the central nervous system (CNS) in humans and nonhuman animals. The hippocampus has been found to be very sensitive to the pattern and chronicity of the prenatal alcohol exposure (Jia, Agopyan et al. 1996). About 10-20% of women have been found to be heavy drinkers during pregnancy (Waterson and Murray-Lyon 1989). At the same time, the percentage of women who smoke during pregnancy is estimated to be 20% to 25% (Ernster 2001). Therefore, a significant population of women are present who drinks and smokes simultaneously during pregnancy. Thus, both of these drugs of abuse during pregnancy may have significant neurobehavioral effect on the offspring (Mateja, Nelson et al. 2012). In our study, we used a Sprague Dawley rat model having access to alcohol water during gestation and also received subcutaneous dose of nicotine (6mg/kg/day) through the mini-osmotic pump. We analyzed the Y-maze behavioral task in the offspring through Y-maze test. Our study showed significant deficits in spatial memory task in alcohol group compared to the control group. The offspring exposed to prenatal nicotine and alcohol showed significant improvement in spatial task compared to alcohol group. However, the improvement in spatial learning deficit is not supported by Long Term Potentiation study in these animals. Alcohol and nicotine exposed animals showed significant deficit in LTP

compared to the control group. Similarly, we also found no change in ILK expression in the model as compared to alcohol and control groups. The improved spatial learning could be due to the increased anxiety level in the offspring. The reduced plasticity suggest impairments in these synaptic functions may underlie the cognitive deficits observed in children exposed to in utero to alcohol and tobacco smoke.

4.2 Introduction

Young adults are addicted to concurrent use of alcohol and tobacco as reported in the National Household Survey on Drug Abuse (Substance Abuse and Mental Health Services Administration, 1998). Alcohol and nicotine addiction produce a cross cue reactivity among its users. The alcoholics exhibited their craving for smoke to the same extent as a smoker exhibit craving for alcohol. The effect of both at the CNS could be some unique mechanism with no anticipated phenotype. The major concern is the polydrug abuse among pregnant women. Very few clinical data exists on the effect of alcohol and tobacco on the developing offspring mainly due to limitations in gathering the information (Cornelius, Leech et al. 2000). Therefore, the more suitable alternative is the use of animal models to study the effect of prenatal alcohol and nicotine exposure.

Our study and some previous literature has shown that prenatal alcohol mediated synaptic dysfunction and behavioral deficits in the offspring (Roebuck, Mattson et al. 1998; Wijayawardhane, Shonesy et al. 2008; Riley, Infante et al. 2011). The behavioral deficits include the impairment in spatial learning and contextual fear conditioning as seen in previous chapter. Many studies including ours have shown clearly the effect is persistent in reducing the LTP in prenatal alcohol rats as compared to the control rats (non-alcohol exposed during gestation). Unlike alcohol, very few prenatal studies have

dissected the neurobehavioral, cognitive, neuroanatomical, and neurochemical characteristics in prenatal nicotine model. Some clinical studies and few studies in animal models have shown impairment in hippocampus structure and function with significant deficiency in synaptic plasticity (Roy, Seidler et al. 2002; Jacobsen, Pugh et al. 2006). Parameshwaran, Buabeid et al. 2013 has shown in prenatal nicotine model, the animals are deficient in hippocampal based spatial memory tasks. They also observed impairments in AMPA receptor function and reduced LTP in hippocampal CA3-CA1 schaffer collateral pathway.

The interactive role of prenatal alcohol and nicotine on fetal brain development is not well understood. It has been found that prenatal alcohol and nicotine exposure has interfered with developing brain growth spurt and neuroanatomical organization of the brain (Chen, Parnell et al. 1999). The interactive effect of developmental co-exposure of these teratogens have not shown additive effect on depletion of Purkinje cells (a class of GABAergic neuron located in the cerebellum) as hypothesized (West, Goodlett et al. 1990). The co-administered animal models showed reduced BAC (Blood Alcohol Concentration) as compared to alcohol administered animal under the same observed conditions. This has been considered a critical factor for reduced additive effect of these teratogens. In other words, the prenatal nicotine administration would reduce the extent of damage due to prenatal alcohol on the developing fetus. It is attributed to the delay in gastric emptying of alcohol by nicotine. Therefore, alcohol is metabolized by the gastric alcohol dehydrogenase enzyme and reduce the BAC. In support to this theory of the protective effect of prenatal nicotine on the deleterious effect of prenatal alcohol on the offspring, a study has shown improvement in long term memory in rats exposed to both the teratogens. However, co-administration reduced significantly the cell proliferation in

the hippocampus of these rats (Gomez, Schneider et al. 2015). In summary, the effect of prenatal alcohol with nicotine may have a differential effect on behavior and the underlying cellular organization and signaling mechanisms. Therefore in our present study we hypothesized that teratogenic effect of alcohol on the developing fetus can be modulated if nicotine is co-administered during pregnancy. This study has for the first time tapped into the effect of co-administered teratogens on the behavior and neurophysiology of the developing offspring.

4.3 Materials and Methods

Animals and Chemicals: Time pregnant Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and osmotic mini pumps (Alzet, Cupertino, CA) were implanted, under isoflurane anesthesia, beneath the skin to deliver subcutaneous dose of nicotine at a rate of 6 mg/kg/day. After 2 full days of recovery, 10% (v/v) alcohol prepared with 95% ethyl alcohol and tap water was administered until the pups are born. Osmotic mini pumps were removed once the pups were delivered so that prenatal nicotine exposure was limited from ~ day3 of pregnancy to birth. Unless specified, all the chemicals were purchased from Sigma (St. Louis, MO).

Y-maze task: Thirty five Sprague-Dawley rats served as subjects in this experiment. They were assigned to three groups based on exposure treatment during the prenatal period, nonexposed Control (n = 9), Alcohol exposure (n = 9), and Alcohol+Nicotine exposure (n = 10). All animals were approximately 4 weeks old at the time of initiation of the study (average weight was 57.8 g). Subjects were released into the Start arm and allowed to explore the Start and Other arm for 15 min, upon which time they were removed from the

maze and returned to their home cages. Three hours later, all animals were returned to the maze and allowed to explore all three arms for 6 min. All scores were obtained from at least two independent observers. The following variables were recorded: (1) number of entries into each arm, and (2) dwell time into each arm. Measures for the Start and Other arm were averaged for all variables, and they will be referred as the Familiar arms. Dwell time was analyzed by contrasting the proportion of the total time spent in the maze's arms that subjects spent in the Novel and Familiar arms; these measures reflect exploratory behavior.

Measurement of Basal Synaptic Transmission and Long Term Potentiation: Following incubation, electrophysiological recordings were performed in a submerged recording chamber with continuous perfusion with aCSF (2-3ml/min) bubbled with 5%CO2/5%O2 carbogen, maintained at room temperature. fEPSPs were recorded from Schaffer collateral pathway SC-CA1 synapses with a glass pipette filled with aCSF (2-4MΩ). Stimulating pulses were applied at Schaffer collaterals via a bipolar stimulating electrode positioned 300 μm closer to CA3 subfield than the recording electrode. After placing stimulating and recording electrodes in the CA3 and CA1 regions respectively, stimulus intensity was lowered to the point where the fEPSP disappeared completely leaving the stimulus artifact intact. For stimulus response curves, current intensity was altered from 0-120μA. For LTP experiments, baseline was recorded at 50% of amplitude at which the initial population spike appeared. LTP was induced after 15 mins of stable baseline recording using a Theta Burst Stimulation protocol (TBS), and recording was continued for 60 mins post TBS. All electrophysiological data are presented as means ± SEM. For plasticity experiments, significance was determined using one-way ANOVA.

Immunoprecipitation (IP) assay: PSD95 was immunoprecipitated from pooled hippocampal tissue lysate using 1:10 anti-PSD95 (Santa-Cruz) antibody coated on Pure-Proteome A/G magnetic beads (Millipore), and using vendor-supplied direct IP protocol. The immunoprecipitated fraction was purified through several washing steps with 1X IMP buffer, pH=7.4. Finally, beads were boiled in 50ul of Laemmle Buffer at 70°C and separated on SDS PAGE, which was followed by western blot analysis.

Western Blot analysis: The PSD95 immunoprecipitation assay was probed with PSD-95 rabbit primary antibody (1:1000, Cell Signaling) to check for presence of PSD-95 pulled down. Equal amounts of sample were loaded to probe interaction of PSD-95 with GluR2 (Millipore), GluR1, and ILK with rabbit primary antibodies (1:1000, Cell Signaling). Whole hippocampal protein lysates were probed for BDNF or proBDNF, and GSK3β to total GSK3β, using their respective primary antibodies at 1:1000 (Cell Signaling). All blots were probed with Dy-Light 660 anti-rabbit secondary antibody (1:10000, Thermo Scientific) using a Fuji FLA 5100 scanner. They are presented as means ± SEM. Significance was determined using a two-tailed Student's t-test.

4.4 Results

Prenatal nicotine/alcohol ameliorates the spatial memory deficits observed in prenatal alcohol exposed rats:

Spontaneous alterations in Y-maze was done to measure the willingness of rodents to explore the new environment. They prefer to explore and spend more time in the novel unexplored arm compared to the already visited arm. The task involves

hippocampus, septum, basal forebrain, and prefrontal cortex of the brain. We compared the prenatal alcohol and nicotine exposed group to prenatal alcohol or nicotine group alone. The number of alternations was analyzed with a 2(sex: male vs. female) x 3 groups (control vs. alcohol vs. alcohol and nicotine exposed) analysis of variance (ANOVA), which revealed a main effect of group, $F(2, 20) = 4.17$, $MSE = 36.88$, $p < .05$ (Figure 4.1a). Neither the effect of sex nor the interaction were significant, $Fs < 1$. Alcohol group produced less alternations than the Control, $F(1, 20) = 8.31$, $ps < .01$, whereas no significant change was observed in Alcohol/Nicotine group, $F(1, 20) = 1.32$, $p > 0.26$. Thus, Alcohol exposed animals appeared to exhibit reduced general activity in comparison with the Control animals, and nicotine exposure concurrent with alcohol appeared to have ameliorated this effect. Number of entries into the Novel arm and Familiar arms were also analyzed with a 3 (group) x 2 (arm: novel vs. other) x 2 (sex) ANOVA, which revealed main effects of group, $F(2, 10) = 4.15$, $MSE = 8.92$, $p < .05$, and arm, $F(1, 20) = 47.69$, $MSE = 0.98$, $p = .001$. No other main effect or interaction were significant, largest $F = 2.24$ (Figure 4.1b). This analysis was followed by a post-hoc (Tukey's) test, which revealed that animals in the Control and Alcohol/Nicotine groups produced more entries into the Novel than the Familiar arms, $ps < .01$, reflecting an increased response to novelty in these groups that was not observed in the Alcohol group, $p > .05$.

There were no between-group or sex differences in time spent in the arms (as opposed to dwelling in the center of the apparatus), all $Fs < 1.87$, all $ps > .18$. Dwell time into each arm was calculated as a proportion of total time spent inside the arms. A 2(group) x 2(sex) ANOVA conducted on the proportion of time spent in the Novel arm revealed a main effect of group, $F(2, 20) = 4.08$, $MSE = 261.31$, $p < .05$, and no effect of

sex nor an interaction, Fs < 1.37, ps > .27. A series of planned comparisons (collapsing across sex) revealed that animals in the Alcohol group dwelled in the Novel arm significantly less than subjects in the Control group, $F(1, 20) = 4.84$, $p < .05$, whereas animals in the Alcohol/Nicotine group were equivalent to Controls, $F(1, 20) < 1$ (Figure 4.1c). Animals in the Control group spent more time in the Novel than Familiar arms, $t(8) = 2.93$, $p < .05$, as was the case for animals in the Alcohol+Nicotine grous, $t(7) = 3.68$, $p < .01$. In contrast, animals in the Alcohol group spent equivalent amounts of time in the Novel and Familiar arms, $t(8) = 0.63$, $p > .5$.

No improvement in synaptic plasticity with prenatal nicotine interaction with alcohol in the rat model:

LTP was measured to determine if amelioration of behavioral deficits observed with prenatal nicotine and alcohol exposed rats are correlating to electrophysiological measurement of learning and memory. Input/output curve gives a measure of overall viability of the brain tissue slice. Overall, the basal synaptic transmission in nicotine/alcohol group reduced as evident from input/output curve (Figure 4.2a) suggesting diminished hippocampal neurotransmission in the model. LTP was measured for 50-60 mins after induction of LTP using high frequency theta burst stimulation. The average fEPSP slope, as a percentage of baseline, was measured for the three groups Control, Alcohol and Nicotine/Alcohol. The induction and maintenance of LTP was significantly reduced in alcohol groups as compared to control which correlates well with behavior (162% ±16.2 vs 132±9, $p<0.05$; figure 4.2b). We expected amelioration of synaptic plasticity deficit in nicotine/alcohol compared to the alcohol group. However,

we did not find any significant recovery in LTP deficits observed in prenatal nicotine/alcohol animals (132.19% ±8.71, $p<0.05$; figure 4.2c).

Increased synaptic GluR2 expression in prenatal nicotine/alcohol exposed rats:

In prenatal alcohol exposed rats, we found increased synaptic expression of GluR2 with immuno-precipitated PSD95 explaining in part the reduced synaptic plasticity in this model. In nicotine/alcohol model however we saw amelioration of behavioral deficits associated to prenatal alcohol but LTP showed no significant improvement as compared to the prenatal alcohol model. Hence, we anticipate increased GluR2 expression in the rats exposed to nicotine/alcohol in utero. Immuno-precipitated PSD95 showed significant increase in GluR2 at the synapse of this model compared to the control ($p<0.05$, figure 4.3). We found no change in synaptic expression of GluR1 and PSD95 within the three groups.

No change in ILK expression:

Previously, we found that prenatal alcohol exposure reduced ILK activity in the hippocampus significantly as compared to the control rats (described in Chapter 2). We found increased GluR2 in the synapse of nicotine/alcohol exposed rats similar to alcohol exposed rats. Therefore, we anticipated no change in ILK activity compared to prenatal alcohol. Indeed, we found no change in ILK expression in all the three groups (figure 4.4).

4.5 Discussion

Prenatal nicotine has been associated with long term neurobehavioral deficits in children exposed to smoking *in utero* (Ernst, Moolchan et al. 2001; Batty, Deary et al. 2006). The

children exposed prenatally to nicotine, exhibited hyperactivity and attention deficits. However, many other studies claim the deficits are overstated. Prenatal nicotine exposure has shown increased anxiety and depressive behavior in rats. The study also reported reduced synaptic plasticity and AMPAR mediated synaptic current (Parameshwaran, Buabeid et al. 2012). In prenatal alcohol model, we also showed reduced behavior and synaptic plasticity. There are reports suggesting that cholinergic stimulation may ameliorate the behavioral deficits seen in the FASD model. Choline supplementation helped ameliorate the memory and learning deficits partially in FASD diagnosed children (Wozniak, Fuglestad et al. 2013). Choline stimulates the nicotinic acetylcholine receptors and may promote secretion of presynaptic neurotransmitters (Holz and Senter 1981). Evidence suggest that α7-nicotinic stimulation by choline may promote release of glutamate at the synaptic cleft as evidence suggest glutamatergic nerve terminals contain these receptors in rat and human brains (Marchi, Risso et al. 2002). However, till date not much has been studied on the interaction of nicotine through cigarette smoke with alcohol in development. Therefore, this study wanted to test the hypothesis that prenatal nicotine may ameliorate the developmental defects seen in prenatal alcohol exposed children through cholinergic stimulation.

In our study we found that prenatal nicotine improved the spatial memory deficits in prenatal alcohol exposed rats. Surprisingly, the prenatal nicotine only animals from our lab (data not shown) showed deficits in spatial learning tasks. This suggests that interaction of alcohol and nicotine at the level of development produce an entirely different effect. This is unlikely due to the lack of BAC in the pregnant rats co-administered with alcohol and nicotine. However, the prenatal nicotine/alcohol animals fail to induce and maintain LTP as expected from the spatial memory improvement. It

can be assumed that a common mechanism that manifests learning and memory defects can also impair LTP. But, a number of studies have shown LTP impairment without any spatial memory defects. For example, reduced potassium channel Kv1.4 (Meiri, Sun et al. 1998) expression, AMPA receptor GluR1 knockout model (Zamanillo, Sprengel et al. 1999), Thy-1 knockout (Nosten-Bertrand, Errington et al. 1996), overexpression of NR2D, a predominantly embryonic NMDA receptor subunit (Okabe, Collin et al. 1998), and brain-derived neurotrophic factor (BDNF) heterozygotes (Montkowski and Holsboer 1997) all showed LTP impairment but did not register a change in spatial memory. All the above examples including ours suggest a separate mechanistic pathway affecting the learning and memory, and LTP.

We postulated that reduced synaptic plasticity in prenatal alcohol is associated to increase in GluR2 expression at the synapse. Prenatal nicotine/alcohol group failed to improve the synaptic plasticity deficits, hence we looked into the synaptic expression of GluR2 by immune-precipitating PSD95, the scaffolding protein at the synapse. The GluR2 expression is not significantly different to the prenatal alcohol exposed animals. Increased expression of calcium impermeable AMPARs GluR2, would reduce the extent of depolarization at the synapse and therefore, would also con tribute less to calcium influx thereby reducing LTP generation and maintenance (Jia, Agopyan et al. 1996). Also, increased synaptic GluR2 would reduce the probability of GluR1 to GluR2 containing receptor switch responsible for LTP maintenance (Jonas and Burnashev 1995). This suggest that reduced plasticity in both alcohol and nicotine/alcohol group is apparently dependent on increased synaptic GluR2 expression. Plasticity in the hippocampus is NMDAR dependent and it has been shown that prenatal alcohol decreased NR2B expression at the synapse resulting in impaired LTP (Samudio-Ruiz,

Allan et al. 2010). Overall, increased GluR2 alongwith NR2B expression inhibit LTP at the schaffer collateral synapses. To test whether reduced plasticity in nicotine/alcohol group is through ILK pathway, we compared ILK expression in the three groups. We found no change in ILK expression in our nicotine/alcohol model. However, ILK activity may still be low in this model hence reducing synaptic plasticity.

In conclusion, the current study disprove the theory that in utero nicotine exposure alongwith alcohol improve neurodevelopmental deficits although an improvement in spatial learning and memory. Further studies have to establish the mechanism behind the improved behavior with nicotine.

4.6 References

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4.7 Figure Legends

Figure 4.1: Effect of prenatal nicotine /alcohol on spatial memory task in rats **a**. Prenatal alcohol group showed significantly less number of spontaneous alterations as compared to the control. No significant change in number of alterations was seen in nicotine/alcohol group. **b**. The number of entries into the novel and familiar arms. Control animals

showed significantly more number of entries into novel arm than the familiar arm similar to nicotine/alcohol group. However, prenatal alcohol group do not show significant exploring behavior. **c**. Nicotine/alcohol and control group showed more dwell time in the novel arm than the familiar arm. The alcohol only group showed less dwell time in the novel arm.

Figure 4.2: Effect of prenatal Nicotine/alcohol exposure on the synaptic plasticity in the rat model. (a) Input-output curves showing hippocampal basal synaptic transmission did not change in rats prenatally exposed to alcohol compared to controls. The graph shows f-EPSP amplitudes (mean ± SEM) as a function of stimulus intensity in the CA1 stratum radiatum. (b) TBS-induced LTP was recorded in hippocampal brain slices from prenatal alcohol, nicotine/alcohol exposed and non-exposed control rats (n = 4). The figure presents the time-course of percentage change in field EPSP slopes (mv/ms) with representative traces. The arrow indicates the time at which TBS protocol was delivered and 5 minute baseline is shown for clarity. Inset, representative fEPSP traces taken before the TBS (1,3,5) and after stabilization of LTP expression (2,4,6) for control, alcohol and nicotine/alcohol groups accordingly. (c) LTP was reduced in animals exposed to prenatal alcohol (average 132.9 ± 9.0%) and nicotine/alcohol (average 132.1 ± 8%) as compared to the non-exposed controls (average 162.4 ± 8.0%).

Figure 4.3. Immunoprecipitation (IP) with anti–PSD-95 from pooled hippocampal protein lysates of rats prenatally-exposed to alcohol and/or nicotine and non-exposed controls (n = 5). In exposed rats, precipitate of GluR2 increased as compared to controls. The same blotting membrane was reprobed with anti–PSD-95 as a control for PSD95 pull down. The quantitation is shown below the blot image ($p < 0.05$).

Figure 4.4. ILK expression. Western blot analysis of expression of ILK in brain hippocampal protein lysates from alcohol and/or nicotine exposed and non-exposed rats (n = 4). There were no differences in densitometric evaluation of ILK expression as a result of alcohol exposure.

4.8 Figures

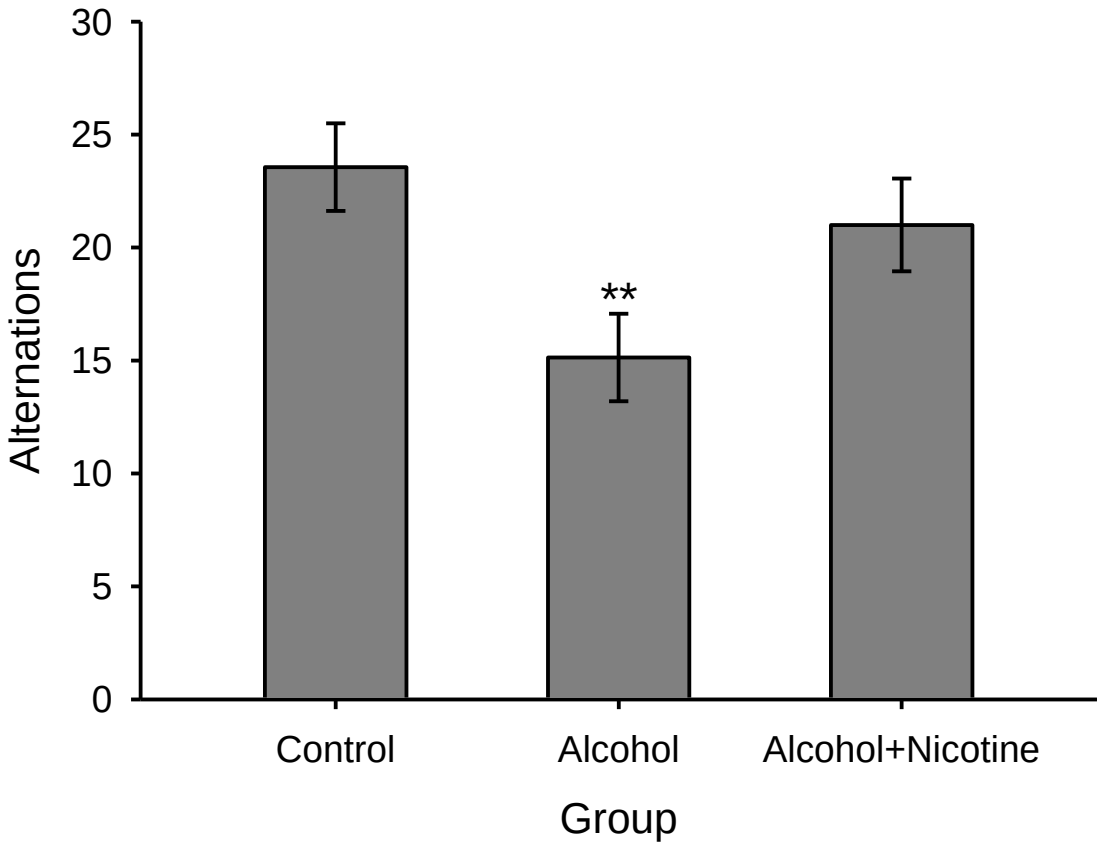

Figure 4.1a Number of alternations

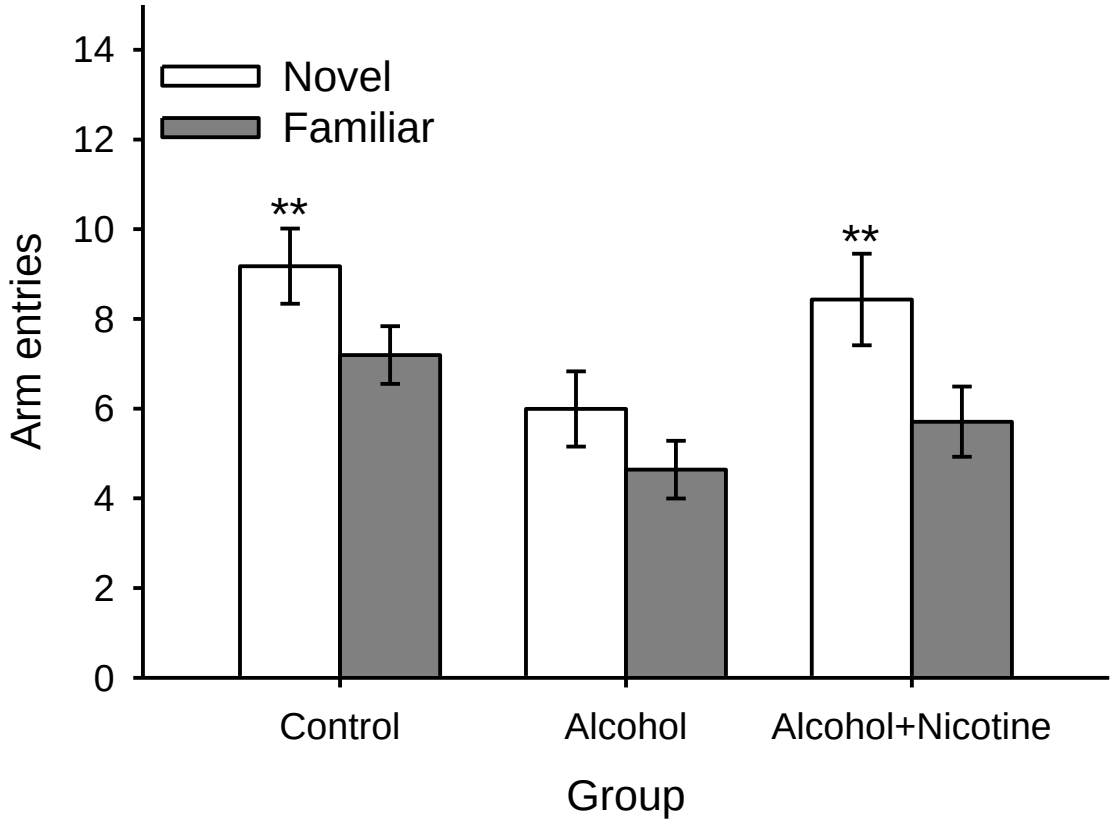

Figure 4.1b Number of entries into the Novel and Familiar arms

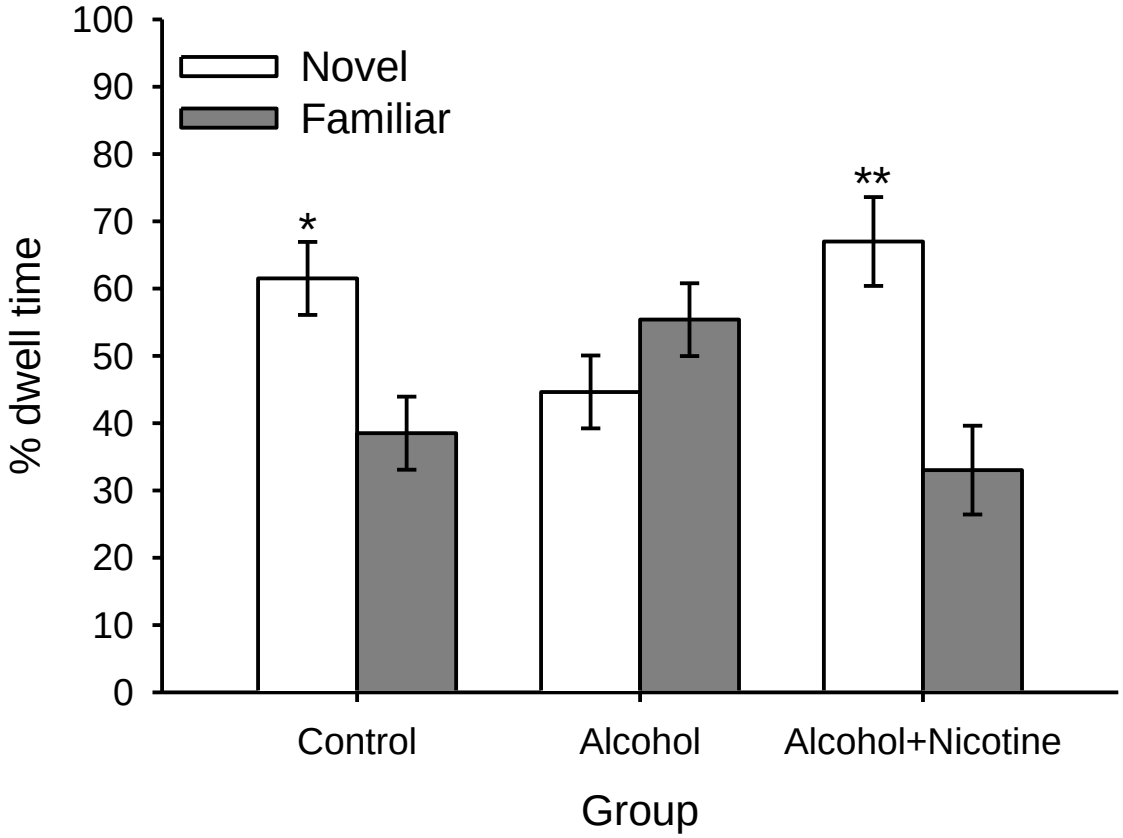

Figure 4.1c Proportion of time spent inside the Novel and Familiar arms

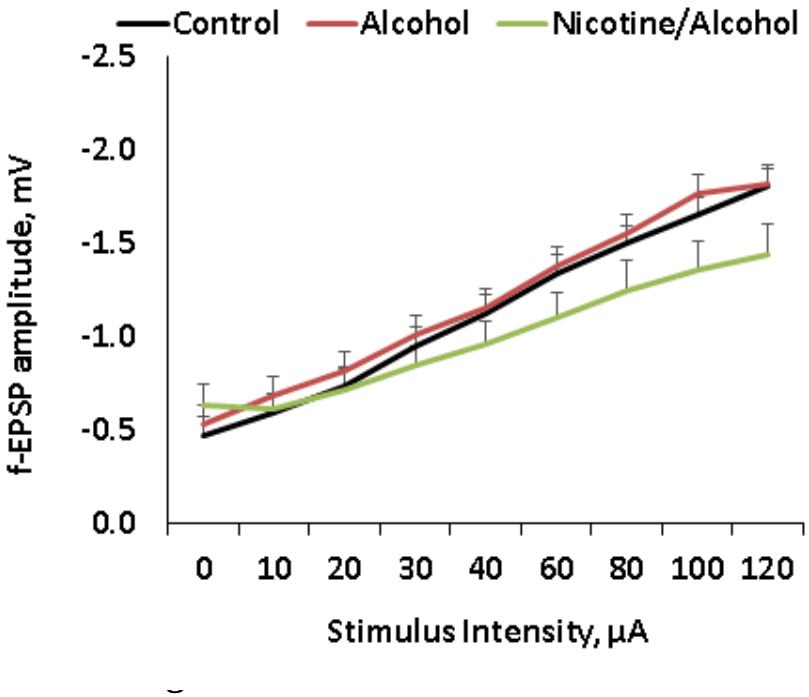

Figure 4.2a Input-output curve

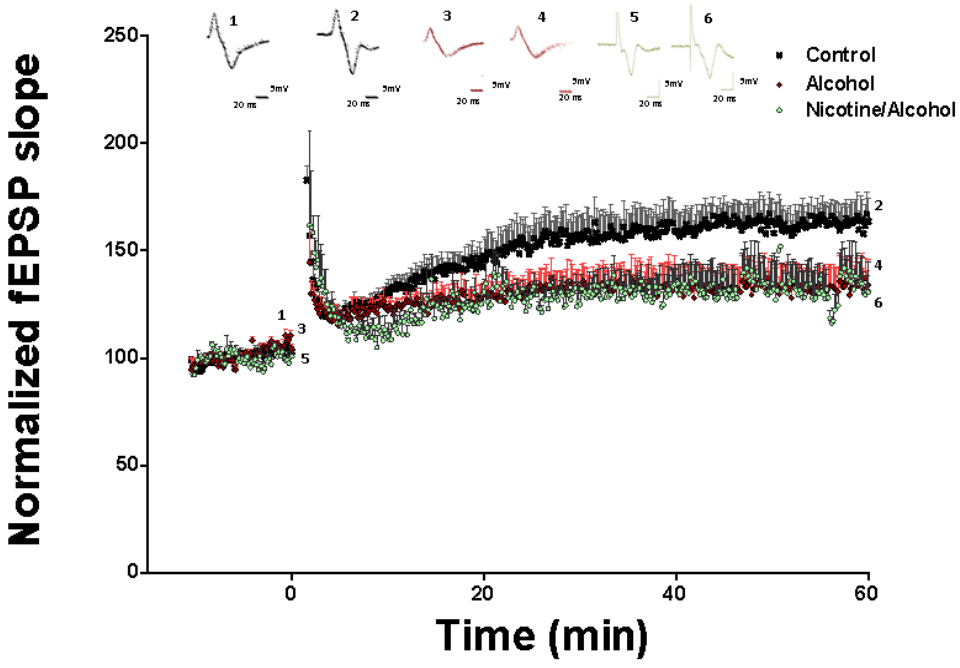

Figure 4.2b TBS generated LTP induction and maintainance

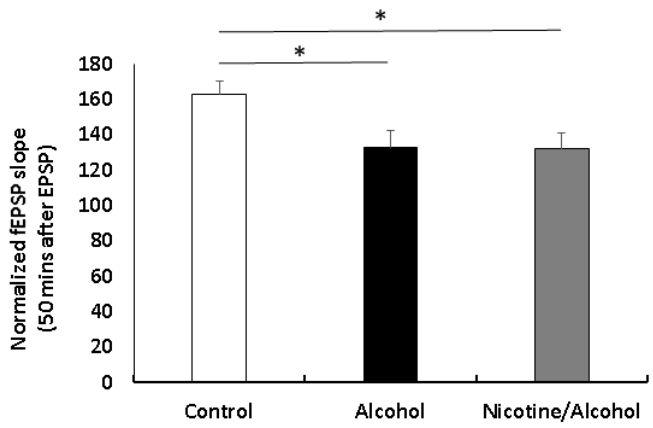

Figure 4.2c Quantification of LTP maintainance

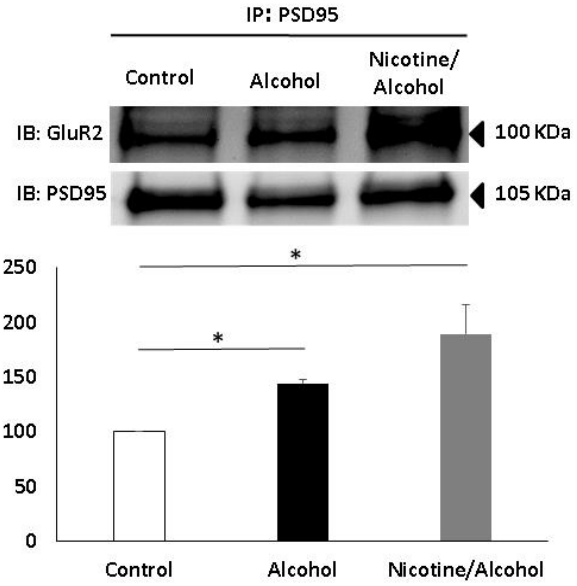

Figure 4.3 Immunoprecipitation (IP) with anti–PSD-95

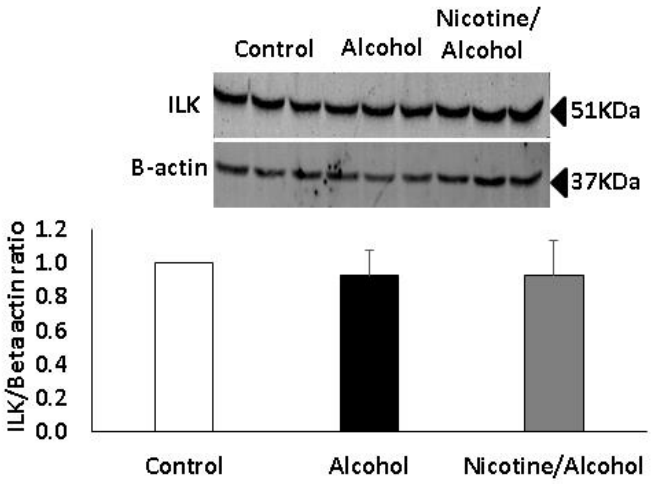

Figure 4.4 ILK expression

5. AMELIORATION OF MECHANISTIC DEFICITS IN ILK SIGNALING THROUGH ADMINISTRATION OF 7, 8 DHF IN PRENATAL ALCOHOL EXPOSED RATS

5.1 Abstract

Moderate prenatal alcohol exposure in rats has shown significant deficits in learning and memory tasks and reduced synaptic plasticity (FASD model). Our previous studies have shown reduced ILK activity and increased GluR2 AMPA receptors at synapse are associated to the plasticity deficits. We hypothesized that enhancing the ILK activity may ameliorate the behavioral and plasticity deficits observed in the FASD model. BDNF infused aged rat model has shown improvement in learning and memory tasks and recovery in ILK activity. 7,8-dihydroxyflavone (7,8-DHF) is a potent agonist for the Brain Derived Neurotrophic Factor (BDNF) receptor Tyrosine receptor kinase B (TrkB). Hence, intraperitoneal administration of 7, 8-DHF in FASD rats, during their early postnatal days, may ameliorate the alcohol associated memory deficits through increased ILK activity and improve synaptic plasticity.

5.2 Introduction

Alcohol consumption during pregnancy can lead to moderate to severe damage to the developing fetus resulting in phenotypes that may persist throughout the lifetime of an individual. The CNS is the major organ of the body which suffers severe to moderate

neurological dysfunction due to the teratogenic effect of ethanol. Even consuming a glass of wine daily has shown startling effect in the fetus as evident from ultrasound images (Hepper, Dornan et al. 2012). Ethanol affect many cellular and molecular pathways in the developing brain, thus impair memory and learning events. The severity and persistence of the phenotype depend largely on the amount, timing and duration of alcohol exposure during pregnancy thus affecting specific regions in the brain (Breese, D'Costa et al. 1993; West, Chen et al. 1994). The hippocampus, corpus callosum and cerebellum are the major brain parts affected due to the prenatal alcohol. Several evidence suggest that prenatal exposure to alcohol affect the glutamatergic pathway in the brain (Barnes and Walker 1981; Farr, Montano et al. 1988). With relation to learning and memory many investigators were interested in impairment of AMPAR-mediated synaptic transmission and time courses of synaptic AMPAR currents (Wijayawardhane, et al. 2008).

Research on prenatal alcohol is very limited to understanding some basic behavioral and synaptic plasticity deficits in animal models. Till date no specific pharmacological treatments available for children or pregnant women exposed to alcohol (Riley 1988). Psychostimulants were used to counteract some of the behavioral deficiencies observed in FASD children. Some of these drugs include methylphenidate (Ritalin), d-amphetamine (Dextrine), premoline (Cylert), and also caffeine were used to treat the attention deficiency and hyperactivity in children with FASD. Choline, the precursor of acetylcholine, supplementation in diet has shown to improve learning and memory capacity in rat models and appears to prevent age related decline in memory and attention (Thomas, Garrison et al. 2004; Wozniak, Fuglestad et al. 2013). However, no

definite pharmacological target related to prenatal alcohol was identified since not much is known about the mechanisms involved in FASD phenotype.

Until recently some underlying mechanisms to the learning and memory impairment have been studied in FASD animal models. Some studies showed that postnatal treatment of FASD rat model with aniracetam, an ampakine, improved learning and memory with increased synaptic plasticity (Vaglenova, Pandiella et al. 2008; Wijayawardhane, Shonesy et al. 2008). It has shown to improve the deficits related to binge alcohol model by increasing AMPA receptor function. Administration of docosahexanoic acid, a omega 3-fatty acid has been reported to partially ameliorate FASD like conditions. However, no underlying mechanisms were studied (Wellmann, George et al. 2015). In our study, we observed significant reduction in behavior and plasticity in moderate drinking alcohol model. There is increased association with reduced ILK activity and increased GluR2 synaptic expression in our model. Hence, we anticipate amelioration of FASD related deficiency in our model through enhancement of ILK activity. Here we hypothesize postnatal administration of BDNF receptor agonist 7,8-DHF can enhance ILK activity and improve memory function in prenatal alcohol model.

5.3 Material and Methods

Subjects:

The animal protocol and experiments were pre-approved by Auburn University IACUC committee (PRN# 2015-2667). We strictly adhered to the guidelines and directions mentioned in the protocol. Two albino rats (200-250 g) (Sprague Dawley strain, Harlan

Laboratories) consumed 10% (v/v) alcohol prepared with 95% ethyl alcohol and tap water throughout the gestation period, starting from the second day of gestation. The alcohol solution was sweetened with 3% glucose and 0.125% saccharin (Sigma Aldrich, USA) (Briones and Woods 2013). Two Non-exposed time pregnant rats received an equivalent solution lacking the ethyl alcohol. Animals were housed in a vivarium maintained on a 12 h:12 h light:dark cycle (lights on at 6:00 am) and at a temperature of 22-24°C. Bottles filled with the alcohol solution were offered at 6:00 pm and fluid consumption was measured after 15 hours of free access to the solution (access to food was ad lib). Differences in liquid volume were converted to volume intakes after accounting for the ethanol solution density (weight in grams/0.9868). There were no differences in consumption or weight gain between dams receiving alcohol and sweetened water. This volume of alcohol is considered to be animal model of moderate alcohol consumption: The dams consumed around 20ml of ethanol solution (equivalent to 6g/kg/day) which is comparable to 1 to 2 drinks per day for an adult human (Åberg, Hofstetter et al. 2005). The day after parturition was considered postnatal Day 1 (PND 1) and all litters were culled to ten pups per dam.

7,8-DHF treatment: The prenatal alcohol exposed rats received intraperitoneal injection of either saline (N=5) or 7,8-DHF (N=5) from PND18 to PND 30. The dosage of 7,8-DHF treatment was 5 mg/kg (1 ml/kg) in phosphate-buffered saline containing 17% dimethylsulfoxide (Andero, Choi et al. 2014). The control rats received either equal volume (1mg/kg) of saline (N=5) or saline containing 17% dimethylsulfoxide (N=5) from PND18 to PND30.

Contextual fear conditioning: Contextual fear conditioning is mainly dependent on hippocampal function. Dorsal hippocampus has a significant role in conditioned contextual freezing (Phillips and LeDoux 1994; Fanselow and Dong 2010). All animals were approximately 33 days old at the initiation of the behavioral portion of the study (average weight was 200 g). Each group was further randomly divided into shock and no shock subgroups. Animals were first trained to lick from a water spout in a novel environment, Context B. Conditioning was conducted in a different context, Context A, which was paired with a shock. Memory of the association between Context A and the shock was assessed by placing the animals back in Context A, allowing them to begin licking from the spout, and recording the number of licks. The testing session was terminated at 10 min; thus, 600 s represent ceiling latency. Subjects' access to water was gradually restricted to one hour per day over the week prior to initiation of the study, provided approximately one hour after completion of each day's session. All conditioning procedures occurred in standard MedAssociates rat operant chambers and the experiments were scored by individuals who were blinded to the condition. Two modifications of these chambers served to create Context A (chambers with no additional cues), and Context B (a different set of chambers, modified using a striped pattern to cover the otherwise clear walls). Behavioral training and assessment occurred over 4 days, as follows. On Days 1 and 2 (lick training), subjects were trained to lick for water in Context B during two 30-min sessions. On Day 3 (conditioning), subjects were placed in Context A for 270 s. A 0.65-mA electric foot shock was delivered at 180 s for a duration of 2 s. Water bottles were not available during the conditioning session. On Day

4 (memory assessment), subjects were exposed to Context A for a 10 min period with water bottles available; this test evaluated recall of the association between Context A and the foot shock. Timing at which each lick was produced was recorded. Rats 'freeze' in anticipation of shock; thus, longer latencies to drink were assumed to reflect higher expectation of shock to be delivered in Context A (i.e., stronger memory of the conditioning experience). In this study, we compared the rats in each group based on their latencies to complete 10^{th} lick. Statistical analysis was not possible as the power of the study was low due to less number of subjects.

Immunoprecipitation (IP) assay: PSD95 was immunoprecipitated from pooled hippocampal tissue lysate using 1:10 anti-PSD95 (Santa-Cruz) antibody coated on Pure-Proteome A/G magnetic beads (Millipore), and using vendor-supplied direct IP protocol. The immunoprecipitated fraction was purified through several washing steps with 1X IMP buffer, pH=7.4. Finally, beads were boiled in 50ul of Laemmle Buffer at 70°C and separated on SDS PAGE, which was followed by western blot analysis.

Western Blot analysis: The PSD95 immunoprecipitation assay was probed with PSD-95 rabbit primary antibody (1:1000, Cell Signaling) to check for presence of PSD-95 pulled down. Equal amounts of sample were loaded to probe interaction of PSD-95 with GluR2 (Millipore), GluR1, and ILK with rabbit primary antibodies (1:1000, Cell Signaling). Whole hippocampal protein lysates were probed for BDNF or proBDNF, and GSK3β to total GSK3β, using their respective primary antibodies at 1:1000 (Cell Signaling). All blots were probed with Dy-Light 660 anti-rabbit secondary antibody (1:10000, Thermo

Scientific) using a Fuji FLA 5100 scanner. They are presented as means ± SEM. Significance was determined using a two-tailed Student's t-test.

ILK activity assay: ILK activity was determined in hippocampal tissue homogenates using an immune complex kinase assay. Briefly, tissue lysates were pooled and incubated with 1:50 anti-ILK mAb (cell signaling). The resulting immune complexes were washed three times in kinase reaction buffer, followed by incubation with 1 μg inactive Akt and ATP (final concentration: 200 μM) in 50ul kinase reaction buffer for 1 h at 30 °C. The reaction products (supernatant) were resolved on SDS/PAGE. The beads were then processed as described in IP assay for ILK immunoblot. Membranes were probed with antiphospho-Akt (ser473) mAb (Cell Signaling Technology). The blot was developed using Dy-Light 660 anti-rabbit secondary antibody (1:10000, Thermo Scientific) using a Fuji FLA 5100 scanner. The data are presented as means ± SEM. Significance was determined using a two-tailed Student's t-test.

5.4 Results

7,8-DHF ameliorates deficits in prenatal alcohol mediated hippocampal-based contextual fear memory:

We here tested the effect of postnatal administration of 7,8-DHF in FASD alcohol model. 7,8-DHF is expected to improve learning and memory since it affects the TrkB receptor which has BDNF as endogenous ligand. Intracranial BDNF administration has been shown to improve learning and memory in rats. The rats were tested for lick suppression test around two week after administration of 7,8-DHF intraperitoneal. Since

we have low number of subjects we compared the latency to complete 10th lick after encountering the aversive context on the test day (table 5.1). We found increased latency to complete 10th lick among shock exposed control rats as compared to the no shock control. However, prenatal alcohol didn't show increased latency to complete the 50th lick compared to no shock alcohol exposed group suggesting deficiency in associating the context to aversive stimuli. 7,8-DHF improved the latency in prenatal alcohol exposed rats which received shock as compared to no-shock counterbalanced control. This data indicated 7,8-DHF as a promising candidate in improving prenatal alcohol associated learning and memory deficiencies. 7,8-DHF on control rats do not show vast change in latencies compared to no drug treated controls.

Table 5.1: 7,8-DHF improved prenatal alcohol related behavioral deficiency:

Substance	NoShock	Shock
Alcohol + 7,8 DHF	8.59	21.975
Alcohol alone	11.23667	9.715
Control + 7,8 DHF	10.15	17.17
Control alone	7.205	25.645

Latency to 50th Lick

7,8-DHF increased ILK activity in prenatal alcohol model:

As described in chapter 3, moderate prenatal alcohol reduced ILK activity in the rats. Reduced ILK activity has been associated to increased GluR2 AMPA receptor and reduced plasticity in the model. Hence, we tested the effect of postnatal administration of 7,8-DHF on ILK activity in the hippocampus protein lysate. We found significant increase in ILK activity in prenatal alcohol exposed rats which received 7,8-DHF

injections (Figure 5.1). Control rats didn't show any significant effect of the drug on ILK activity.

7,8-DHF regulated GluR2 synaptic expression in prenatal alcohol model:

We discussed in chapter 3 that prenatal alcohol increased GluR2 AMPA receptor presence at the synapse thus inhibiting the net rise in synaptic depolarization thereby impairing LTP. Here we tested whether TrkB agonist 7,8-DHF improved synaptic regulation of GluR2 since ILK activity improved. We compared synaptic GluR2 expression through immunoprecipitation of PSD95 in drug treated control and alcohol exposed rats. Drug treated FASD and control rats do not show significant difference in synaptic GluR2 expression when normalized to PSD95 (Figure5.2).

5.5 Discussion

Previously, many studies indicated the role of prenatal alcohol in modulating AMPA receptor function and kinetics. Deficiency in learning, memory and synaptic plasticity was predominantly observed in all the models of prenatal alcohol including binge administration or moderate exposure. The improvement in prenatal binge alcohol mediated impairment in learning and memory was observed with adolescent treatment with aniracetam. Aniracetam can abrogate AMPA receptor channel neurotransmission problems. More insights into the mechanistic reason for observed behavioral and plastic deficiency are required.

In our FASD model, we found problems in associating aversive stimuli with the context as compared to non-exposed control rats. The FASD model showed reduced

plasticity and increased GluR2 AMPA receptor expression at the synapse. Integrin plays an important role in memory and plasticity. Alcohol has been previously characterized to affect integrin and growth factor signaling receptors. Alcohol also affects PTEN and downstream signaling cascade to growth factor receptors. Hence, we were interested in ILK which remain associated to integrin β-subunit and controls the downstream growth factor signaling. In the FASD model, we found reduced ILK activity and hypothesized that reduced ILK activity and association to GluR2 is responsible in modulating GluR2 expression at the synapse. 7,8-DHF is a known agonist to BDNF receptor TrkB. We anticipated that 7,8-DHF administration can improve synaptic plasticity and memory in prenatal alcohol model by influencing proper GluR2 synaptic expression. To further support our hypothesis, a study has shown that 7,8-DHF administration affects memory through increased GluR1 and reduced GluR2 expression at the synapse (Tian, Zeng et al. 2015). In our study, we found 7,8-DHF can improve the behavioral deficit observed in prenatal alcohol model through increased ILK activity and controlling synaptic AMPA GluR2 expression. We required to further investigate the effect of the drug on AMPA receptor signaling and effect on plasticity in the model.

5.6 References

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5.7 Figure legends

Figure 5.1 Improved ILK activity with 7,8-DHF: ILK activity assay was performed with pooled hippocampal protein lysates from prenatal alcohol rats with/without postnatal 7,8-DHF treatment (n = 4) and control rats with/without 7,8-DHF. Akt ser^{473} phosphorylation assessed with western blot analysis. The quantitation of band density analysis shows

reduced ILK activity in alcohol-exposed animals compared to control ($p < 0.05$). Treatment with 7,8-DHF improved ILK activity in prenatal alcohol rats.

Figure 5.2: Immunoprecipitation (IP) with anti–PSD-95 from pooled hippocampal protein lysates of rats prenatally-exposed to alcohol a with/without postnatal 7,8-DHF treatment ($n = 4$) and control rats with/without 7,8-DHF ($n = 4$). In alcohol exposed rats, precipitate of GluR2 increased as compared to controls. 7,8-DHF treatment reduced the synaptic GluR2 to the control level. The same blotting membrane was reprobed with anti–PSD-95 as a control for PSD95 pull down. The quantitation is shown below the blot image ($p < 0.05$).

5.8 Figures

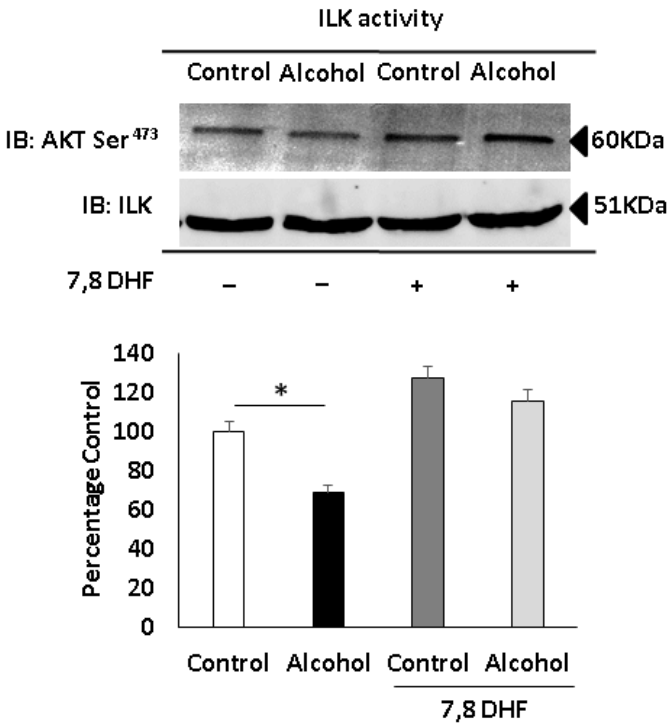

Figure 5.1ILK activity assay

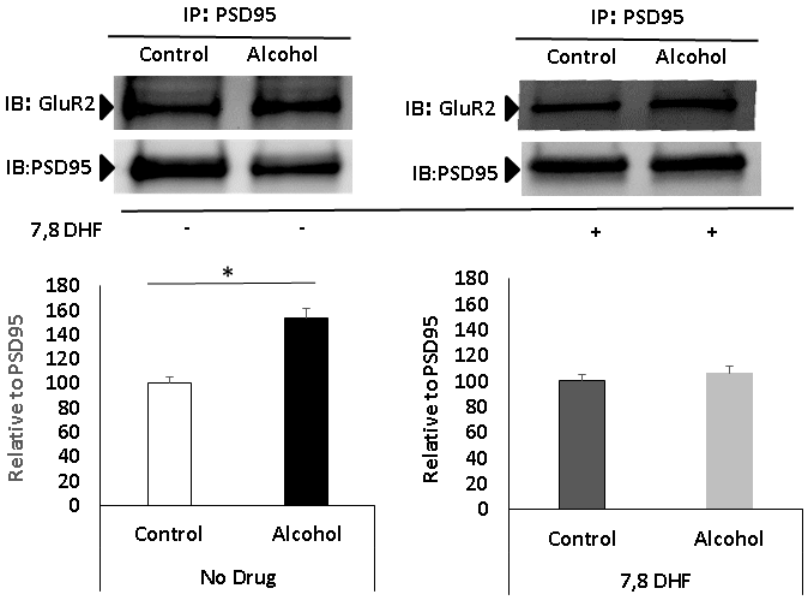

Figure 55.2Immunoprecipitation (IP) with anti–PSD-95

6. Conclusion and Summary

CNS is the part of the brain which develops throughout the period of fetal development. Among all body parts, the danger of teratogens during fetal development is mostly on the CNS. In US, the percentage of women consuming alcohol during pregnancy remain high. The most obvious reason being unplanned pregnancy. Maternal drinking causes severe to permanent damage to the CNS. The regions which are mostly vulnerable to the maternal consumption of alcohol are the hippocampus, cerebellum and the corpus callosum. Damage to the hippocampus is very prominent among children suffering from FASD (Tran and Kelly 2003). Very few studies have tried to delineate the underlying mechanism in the hippocampal formation which affect learning and memory (Guerri, Bazinet et al. 2009).

In our study we put emphasis on the administration of alcohol. We created a moderate drinking model using time pregnant rats starting from the 2nd day of pregnancy until the end of gestation period. We looked into the adolescent age of the rats PND 30-33, when they are more vulnerable to the effect of the teratogen. In humans, FASD individuals show signs of mental retardation during their adolescent age affecting studies and all round development. We measured the extent of damage to the hippocampus, using a fear conditioning paradigm with complex contextual appearance. We found reduced ability in the alcohol exposed rats to associate the aversive stimuli to a particular context as compared to the age matched control rats. The reduced hippocampal deficiency is correlated well with reduced plasticity measured in terms of

LTP in the FASD rat model. Integrin Linked Kinase (ILK) is an effector of integrin and growth-factor signaling which regulates cell survival signaling processes. In our model, we observed that alcohol exposure reduced ILK activity and also its interaction to GluR2 AMPA receptors. Increased stability of GluR2 at the surface could be the result of reduced phosphorylation of certain residues by inhibited ILK activity. The increased calcium impermeable GluR2 is one of the causal factor for decreased hippocampal plasticity.

There are no know treatment regime for FASD related conditions. To identify novel target molecules it is necessary to understand the mechanism underlying the condition. Choline supplementation in the diet ameliorate the memory and learning deficits partially in FASD diagnosed children (Wozniak, Fuglestad et al. 2013). Choline stimulates the nicotinic acetylcholine receptors and may promote secretion of presynaptic neurotransmitters (Holz and Senter 1981). In this study we wanted to see if prenatal nicotine has any remedial effect on the FASD related deficiencies observed in our model. A dose of (6mg/kg/day) through the mini-osmotic pump during gestation indeed ameliorate the deficiency in the spatial learning task associated with prenatal alcohol model. However, we didn't see any change in LTP and GluR2 expression compared to the alcohol model. The improved spatial learning could be due to the increased anxiety level in the offspring or a separate pathway that affected the learning behavior only.

We further considered to look into a drug that can potentiate the ILK activity and reduce the extent of damage observed in the FASD condition. ILK activity can be linked to reduced mature BDNF in hippocampus in our model. ILK can be activated by intracranial BDNF administration. Hence, we used a drug which is an

agonist to the TrkB receptor of the BDNF. We found increased hippocampal based memory association in alcohol exposed rats and increased ILK activity downstream to it. The synaptic expression of GluR2 also came down to the level of control. It could be promising drug to treat FASD like conditions.

6.1 Future Studies

The increased stability of GluR2 at the surface indicate decreased S880 phosphorylation site. S880 phosphorylation decreases interaction with Glutamate receptor interacting protein (GRIP)/ Androgen binding (ABP) proteins which helps in stabilizing GluR2 at the synapse. Hence, decreased ILK interaction and reduced phosphorylation capacity may suggest ILK targets the S880 residue on GluR2. Further, we also have to investigate the effect of the drug 7,8-DHF on GluR2 stability in the alcohol model. The recovery in behavior and subsequently the bidirectional plasticity (LTP and Long Term Depression, LTD) have to be investigated thoroughly.

6.2 References

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