A RAT MODEL OF FETAL ALCOHOL SYNDROME:

MOLECULAR AND BEHAVIORAL ANALYSIS

by

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ABSTRACT OF THESIS

A Rat Model of Fetal Alcohol Syndrome: Molecular and Behavioral Analysis

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The maternal consumption of alcohol during pregnancy produces a wide range of abnormalities in the offspring. The main purpose of this thesis is to investigate the (1) effects of prenatal ethanol exposure in two types of animal behavioral models and (2) effects on gene expression patterns produced by prenatal ethanol exposure. Sprague-Dawley rats from fetal alcohol exposure (FAE) and pair-fed (PF) treatment groups were tested as young adults.

The purpose of the first study is to assess the effects of prenatal ethanol exposure on anxiety and social behaviors. The elevated plus maze model is used to measure anxiety and the social interaction model is used to study social activity in an open-field. Based on previous literature, we hypothesize that prenatal ethanol exposure will result in a significant difference in activity on the elevated plus maze and open-field. However, our data from the behavioral tests do not show a robust difference between the prenatally exposed and the pair-fed animals. There are no significant effects of prenatal ethanol exposure on the open versus closed arms of the plus maze. There are also no significant effects of prenatal ethanol exposure on social interaction with a companion rat. A reason for the subtle differences between the two behavioral tests is likely due to handling prior to the testing manipulations.

The purpose of the second study is to see the effects of prenatal ethanol exposure on gene expression. We hypothesize that FAE significantly affects gene expression, and using gene profiling techniques, we examine the patterns of gene expression in control and treated populations. In our study, we analyze gene expression profiles with pathway analysis as the approach. We identify specific molecular pathways that are significantly impacted by prenatal alcohol exposure. We further extend this study by focusing on the long-term potentiation (LTP) pathway and examine multiple molecular components in this pathway for their gene expression levels. LTP has long been known to be the mechanism by which memories are formed and stored. According to our study, it is compelling to say that FAE can cause profound and long-lasting alterations in the cellular signaling mechanisms associated with activity-dependent synaptic plasticity and memory formation. Our gene expression data also indicate an opposing pattern of ethanol effect on LTP pathway in the hippocampus and hypothalamus.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AC	adenyl cyclases
ANOVA	analysis of variance
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid
BAL	blood alcohol level
Camk2a	calcium/calmodulin-dependent protein kinase II alpha
Camk2d	calcium/calmodulin-dependent protein kinase II delta
cAMP	cyclic adenosine monophosphate
CaN	calcineurins
CR	companion rat
Ct	cycle threshold
CV	coefficient of variation
DAG	diacylglyceride
EPM	elevated plus maze
ERK	extracellular signal-regulated kinase
FAE	fetal alcohol exposed
FAS	fetal alcohol syndrome
Gapdh	glyceraldehydes-3-phosphate dehydrogenase
Gq	G-proteins
Gria2	glutamate receptor, ionotropic, AMPA2
Grin2d	glutamate receptor, ionotropic, N-methyl D-aspartate 2D
Grm5	glutamate receptor, metabotropic 5
HC	hippocampus
HY	hypothalamus

IP3	inositol trisphosphate
IP3R	inositital 1,4,5-triphosphate receptor
Itrp1	inositol 1,4,5-triphosphate receptor, type 1
LTP	long-term potentiation
MAPK	the mitogen-activated protein kinase
Mapk1	mitogen activated protein kinase 1
mGluR	metabotropic glutamate receptor
NMDA	N-methyl-D-aspartate
OF	open-field
PF	pair-fed
PKC	protein kinase C
PKA	protein kinase A
Plcb4	phospholipase C, beta 4
PLC	phospholipase
PP1	protein phosphatase 1
Ppp3cb	protein phosphatase 3, catalytic subunit, beta isoform
Ppp3r1	protein phosphatase 3, regulatory subunit B, alpha isoform
Prkca	protein kinase C, alpha
qRT-PCR	quantitative real-time polymerase chain reaction
RAP	ras-related proteins
SEM	standard error of mean
Stdev	standard deviation
ZT	Zeitgeber time

I. INTRODUCTION

A. Diagnosis of Fetal Alcohol Syndrome

The deleterious effects of fetal alcohol exposure for the developing fetus are well documented. Drinking alcohol during pregnancy results in clinical abnormalities that can be observed in children with fetal alcohol syndrome (FAS). The diagnostic criteria for FAS includes the following: (1) a characteristic pattern of facial anomalies such as short palpebral fissures, flattened philtrum, thin upper lip and flat maxillary area; (2) pre and/or postnatal growth retardation; and (3) central nervous system neurodevelopmental anomalies such as decreased cranial size, structural brain abnormalities and neurological problems (Archilbald, Fennena-Notestine, Gamst, Riley, Mattson, & Jernigen, 2001). More severe cases may include congenital hearing defects and mental retardation. Brain imaging studies have identified structural changes in various brain regions of these children including the basal ganglia, cerebellum, corpus collosum and hippocampus that may account for cognitive deficits (Mattson, Schoenfeld, & Riley, 2001). In addition, brain growth continues to be adversely affected long after the prenatal ethanol exposure, and brain regions most affected may be consistent with the neurochemical deficits characteristic of children prenatally exposed to alcohol (Riley, McGee, & Sowell, 2004). The term fetal alcohol exposure (FAE) has been used to describe cases where all three diagnostic criteria are not met, yet there are still symptoms of ethanol exposure. In the present thesis, the term FAE will be used to denote collectively the individuals in which there are symptoms of the prenatal ethanol exposure but not all criteria of FAS are met.

B. Clinical Features

Alcohol is a dangerous teratogen to the developing fetus because it produces structural and functional abnormalities of both brain and body. Prenatal exposure to alcohol has been shown to produce abnormalities in virtually every system of the organism (Abel & Dintcheff, 1978). The debilitating condition of individuals with FAS is well recognized. Some individuals diagnosed with FAE have normal scores on standard intelligence tests, yet are often unable to function in society, primarily because of their maladaptive behaviors such as hyperactivity, distractibility, response preservation, impaired habituation and poor attention span (Streissguth, 1986). FAS/FAE are not just childhood disorders; there is a predictable long-term progression of the disorders into adulthood. In a comprehensive follow-up examination of 61 individuals diagnosed with FAS/FAE, now 12-40 years of age, it was found that they continued to suffer many adverse effects, making them unable to function in society (Streissguth, Aase, Clarren, Randels, LaDue, & Smith, 1991). In fact, none were independent in terms of both housing and income (Streissguth et al., 1991). Although the characteristic facial anomalies became more subtle, short stature and microcephaly were still evident (Streissguth et al., 1991). More importantly, cognitive deficits and behavioral abnormalities were among the most detrimental long-term effects (Streissguth et al., 1991). Intellectual performance measured IQ scores were shown to be decreased: 42% of individuals scored below 70; the cut-off for the mental retardation classification (Streissguth et al., 1991). Furthermore, these individuals displayed many maladaptive behaviors; the most commonly reported being increased distractibility, poor judgment,

difficulty perceiving social cues, and problems with comprehension (Streissguth et al., 1991).

C. Incidence and Epidemiology

FAS/FAE have been identified in children from all ethnic groups and socio-economic classes. However, it appears that certain ethnic groups (Native and African Americans) and individuals from lower socio-economic classes have higher rates of alcohol abuse, and thus an increased number of diagnosed cases. Overall estimates of FAS incidence range between 0.33 - 4.7 per 1000 live births (Abel & Sokol, 1991). Prenatal ethanol exposure is now recognized as the leading known cause of mental retardation in the Western world, surpassing Down's syndrome and spina bifida (Abel & Sokol, 1991). It may account for 2.3% of all institutionalized mentally retarded patients in the United States (Abel & Sokol, 1991). The resulting effects of prenatal ethanol exposure produce enormous social and financial costs, with conservative estimates of $75-321 million annually in the United States for FAS alone (Abel & Sokol, 1991).

D. Pharmacokinetics of Alcohol

Alcohol is lipid soluble and thus rapidly absorbed from the stomach and gastrointestinal tract following ingestion, and evenly distributed throughout the fluids and tissues of the body and brain (Julien, 1981). During pregnancy, ethanol readily crosses the placental barrier producing approximately equal maternal and fetal blood alcohol levels, BALs (Waltman & Iniquez, 1972). The fetus is limited in its ability to metabolize alcohol due to a lack of hepatic alcohol dehydrogenase, the major metabolizing enzyme

for alcohol. Therefore, the elimination of alcohol from the fetus is through passive diffusion of alcohol across the placenta and then maternal elimination (Waltman & Iniquez, 1972). In addition, the rate of alcohol elimination from amniotic fluid is approximately half that from maternal blood, resulting in relatively high alcohol concentrations in amniotic fluid. Thus, amniotic fluid may act as a reservoir for alcohol, such that the fetus can be exposed for a longer period than would be predicted on the basis of maternal alcohol concentration (Brien, Loomis, Tranmer, & McGath, 1983).

E. Teratogenic Effects of Alcohol

Fetal alcohol syndrome does not occur in all infants exposed prenatally to alcohol. It occurs in approximately one-third of infants born to women with chronic alcoholism; the remaining two-thirds may show partial FAE or no apparent symptoms (Streissguth et al., 1991). Therefore, many factors seem to be involved in the teratogenesis of in utero alcohol exposure. The dose/duration of alcohol exposure has been identified as an important factor in alcohol teratogenesis (Coles, 1992). The minimum dose required to produce deficits or the amount of alcohol that can be "safely" consumed during pregnancy have not been established. Clinical studies have shown that as little as 1 oz. of absolute alcohol per day was associated with intellectual deficits, and two drinks per day produced a seven point decrease in IQ scores (Streissguth et al., 1991). Importantly, what is more critical than the absolute amount and duration of alcohol exposure is the BALs (blood alcohol level) achieved. Binge drinking, condensed exposure producing high BALs, is more harmful to the fetus than the same amount of alcohol consumed at a steady rate over an extended period of time (Pierce & West, 1986). In addition, a host of

factors may contribute to and interact with the impact of alcohol on the fetus: physiological variables affecting uptake and metabolism of alcohol; alcohol's effects on uptake, utilization and metabolism of nutrients; maternal nutritional status, health and age; genetic vulnerability; and the use of other substances such as cocaine, narcotics, marijuana, and nicotines (Coles, 1992).

F. Effects of Alcohol on Brain

Alcohol is a central nervous system depressant, slowing down the body's functions in a matter similar to that of a general anesthetic. When alcohol reaches the brain, it affects millions of nerve cells and changes communication patterns. Alcohol affects vision, distorts hearing, muddles speech, impairs judgment, dulls senses, reduces spatial and/or long-term memory and disturbs motor skills (Mattson & Riley, 2001). This occurs because alcohol inhibits blood from transporting oxygen to brain cells. When brain cells are deprived of oxygen, they become impaired or die. Autopsies of the brains of children with FAS have demonstrated widespread and severe damage, including the following: (1) malformations of the brain tissue, both in the gray matter and white matter regions; (2) failure of certain brain regions (e.g. the corpus collosum) to develop; (3) failure of certain cells to migrate to their appropriate locations during embryonic brain development (Zahr & Sullivan, 2008). Because the brain matures more slowly than other organs of the body, young adults may be even more susceptible to certain permanent and irreversible effects of alcohol. These deficits are particularly serious, because they are pervasive and persist throughout the person's life. In fact, some deficits, such as problems

with social functioning, appear to worsen as the sufferer reaches adolescence and adulthood, possibly leading to an increased rate of mental health disorders.

G. Rodent Models of Prenatal Ethanol Exposure

Human studies have intrinsic limitation in that they cannot control for the many confounding variables (as discussed above) that can affect fetal development independent of ethanol and they cannot address issues of the underlying mechanisms of the teratogenesis. For this reason, animal models of prenatal ethanol exposure have been developed, using a variety of animal species and variety of methods of delivery. Many animal species have been used to study the effects of prenatal ethanol exposure including the monkey, sheep, dog, chick, guinea pig, rabbit, mouse and rat (Colangelo & Jones, 1982). However, rodent models (particularly the rat) are by far the most commonly used because of the ease of handling, short gestation periods, and relatively low cost to purchase, house and feed (Keane & Leonard, 1989). The metabolism of ethanol is similar in humans and rats, although rats metabolize ethanol more quickly. In this thesis, the rat ethanol exposure model was used; this models humans' first and second trimester of ethanol exposure. This rat model is appropriate for the study of cognition and behavior since the neuronal network for these functions begins to develop in utero.

Many different ways of administering ethanol to the developing fetus have been utilized, including injection, inhalation, gastric intubation, and via drinking fluid or liquid diet. Of the listed methods of administering ethanol, each has its advantages and drawbacks. Injection, inhalation and intubation methods allow for controlled doses of ethanol to be administered and high BALs to be achieved, but they use multiple handling

and/or confinement, both of which are stressors. Ethanol in the drinking fluid is nonstressful, but because the taste of ethanol is aversive, animals reduce their liquid intake; this leads not only to low BALs but reduced water and food intake (Weiner, 1980).

The liquid diet method of delivering ethanol provides all the required nutrients and fluids as well as the ethanol. It is a nonstressful way to achieve high BALs with adequate nutritional intake (Weinberg, 1985). Typically in these models, in addition to prenatal ethanol, there is a nutritional control condition in which the nutritional intake is matched with that of the FAE condition. The pair-fed, PF, condition is necessary because food intake and therefore nutrient intake is typically reduced in ethanol consuming animals. This results because ethanol derived "empty" calories displace nutrition rich regular foods, and because ethanol consumption leads to less overall food intake compared to consumption of ethanol free diet (Weinberg, 1985). Thus, primary malnutrition, the simple reduction of nutrient intake resulting from ethanol intake, is controlled by the PF condition. However, it does not control for secondary malnutrition, resulting from ethanol altering the uptake, utilization, metabolism and placental transport of nutrients (Weinberg, 1985). In this thesis, the liquid diet method was used to deliver ethanol to pregnant rat dams.

H. Effects of Prenatal Ethanol Exposure in Rodent Models

In animal studies, rodents prenatally exposed to ethanol demonstrate many of the effects seen in children exposed to alcohol in utero, including growth deficiencies (Abel & Dintcheff, 1978), changes in brain morphology (Meyer, Kotch, & Riley, 1990), and

soft tissue and skeletal abnormalities (Abel, 1978), as well as cognitive (Abel, 1979) and behavioral deficits. The ethanol effects on the developing brain are believed to cause the behavioral abnormalities in the ethanol-exposed offspring (Meyer & Riley, 1986). Many of the behavioral changes observed in fetal ethanol exposed offspring appear to reflect hyperactivity and hyperresponsiveness and/or deficits in response inhibition (Meyer & Riley, 1986). Increased open field activity (Bond & DiGiusto, 1976), increased startle reactions (Anandam, Felegi, & Stern,1980), increased wheel running (Martin, Sigmam, & Radow, 1978) and increased exploratory behavior (Bond & DiGiusto, 1976), and as well as deficits in passive avoidance learning (Bond & Di Giusto, 1978), taste aversion learning (Riley, Barron, Driscoll, & Chen, 1984), reversal learning (Lochry & Riley, 1980), and nose-poking behavior (Riley, Shapiro, & Lochry, 1979) have all been demonstrated in the FAE offspring.

I. Elevated Plus Maze Model

Clinical as well as experimental evidence have demonstrated that ethanol is a teratogenic drug, and its consumption during pregnancy induces harmful effects on the developing fetus. Ethanol is an anxiolytic agent that is known to reduce anxiety. The current study investigates the behavioral responses in FAE animals on the elevated plus maze. The EPM proves a valid and reliable measure of anxiety as measured by behavioral responses (Listar, 1987). The task is based on spontaneous behavior and does not require training of the animal, exposure to noxious stimuli, or manipulation of appetitive behaviors such as food deprivation. The EPM is comprised of an elevated cross-maze with two open arms and two arms enclosed by walls. It can be considered an

aversive or stressful task in that it generates a conflict situation by simultaneously activating two natural tendencies: exploration of novel environment and avoidance of open spaces (Falter, Gower, & Gobert, 1992). It has been shown that control or undrugged animals prefer the closed arms of the maze, demonstrating fewer entries onto the open arms and less time spent on the open arms, compared with the closed arms (Listar, 1987).

J. Social Interaction Model Using the Open-Field Box

Adolescents and adults with FAS show unresponsiveness to social cues, lack of reciprocal friendships, lack of tact, and difficulty in cooperating with peers, as measured by the Vineland Adaptive Behavioral Scale (LaDue, Streissguth, & Randels, 1992). Social behavior in rats has been shown to follow similar principles as in humans and is also a function of genetics, teratogenic influences, and social learning. A rodent model of FAS can be used as means to examine the effects of alcohol exposure on the development of social behavior. In the social interaction test, the open-field box is used to study spontaneous exploration and social behavior. The social interaction test was developed over thirty years ago (File & Hyde, 1978) as the first animal test that used a natural form of behavior as the independent measure. The dependent variable is the time spent by pairs of male rats in social interaction (e.g. sniffing or approaching the companion). Because the behavior of one rat influences that of the other, it is important that the pair of rats is treated as unit, and only one score for the pair is used (File & Seth, 2003). Thus, if only one rat is treated, then only the scores of the treated rat should be used.

K. Fetal Alcohol Exposure Affects Gene Expression Level

Previous studies have shown that prenatal ethanol exposure can cause "epigenetic" modifications to the fetus's DNA (Kaminen-Ahola, Ahola, Maga, Mallitt, Fahey, Cox, Whitelaw, & Chong, 2010). These do not alter the genetic code itself but might switch certain genes on or off, or increase or decrease their expression. Gene expression profiling is the measurement of the activity (the expression) of thousands of genes at once, to create a global picture of cellular function. To detect possible changes in gene expression in prenatal ethanol exposure versus pair-fed, we used Affymatrix's GeneChip® Rat Expression Set 230. The gene expression data from the microarray was analyzed using Biometric Research Branch Array Tools 3.8.0. Pathway Express was an additional tool used to identify pathways relating to fetal alcohol exposure. The long-term potentiation pathway was the most statistically significant pathway relating to ethanol response.

L. Long-Term Potentiation

Long-term potentiation (LTP) of chemical synaptic transmission is the most widely studied physiological model of learning and memory formation in the rodent brain. Research has shown that LTP occurs in the hippocampus and the hypothalamus (Lynch, 2004; Panatier et al., 2006) and other brain regions- midbrain, brainstem, and peripheral ganglia (Blundon & Zakharenko, 2009). In the early 1970s, it was shown that repetitive activation of excitatory synapses in the hippocampus, a brain region long known to be essential for learning and memory, caused an increase in synaptic strength that could last for hours or even days (Bliss & Lobo, 1973). This long-lasting

enhancement in signal transmission between two neurons results from stimulating them synchronously. This improves the ability of the two neurons, one presynaptic and the other postsynaptic, to communicate with one another across a synapse (Malenka & Bear, 2004). In the most well understood form of LTP, enhanced communication is predominantly carried out by improving the postsynaptic cell's sensitivity to signals received from the presynaptic cell (Malenka & Bear, 2004). These signals, in the form of neurotransmitter molecules, are received by neurotransmitter receptors present on the surface of the postsynaptic cell (Malenka & Bear, 2004). LTP improves the postsynaptic cell's sensitivity to neurotransmitters in partly by increasing the activity of existing receptors and by increasing the number of receptors on the postsynaptic cell surface (Malenka & Bear, 2004). Over the past 25 to 30 years, this long-lasting synaptic enhancement has been the object of intense investigation because it is widely believed that LTP provides an important key to understanding the cellular and molecular mechanisms by which memories are formed and stored (Malenka & Nicoll, 1999).

M. General Rationale and Purpose

Ethanol is a teratogenic drug, and its consumption during pregnancy induces harmful effects on the developing fetus, leading to Fetal Alcohol Syndrome. Experimental evidence demonstrates that alcohol interferes with many molecular, neurochemical and cellular events during the normal development of the brain (Guerri, 2002). The impairment of several neurotransmitter systems and their receptors are important factors involved in the neurodevelopmental liabilities observed after in utero alcohol exposure (Guerri, 2002).

Thus, the objectives of the present work are to study behavioral (anxiety and social interaction) as well as neurochemical (long-term potentiation expression) effects presented by rat offspring from dams exposed during their gestation periods to daily oral administration of ethanol. We hypothesize that prenatal ethanol exposure results in a significant difference in anxiety and social interaction in the EPM and OF, respectively. A microarray study was used to examine the effects of ethanol on gene expression. The differential expression of eleven genes from LTP is confirmed using quantitative real-time polymerase chain reaction (qRT-PCR) analysis. We hypothesize that the long-term potentiation pathway is significantly affected during fetal ethanol exposure.

II. MATERIALS AND METHOD

A. Animals and Treatments

Pregnant Sprague-Dawley rats (outbred strain), obtained from Charles River Laboratories (Wilmington, MA, USA), were individually housed in 12 h light/12 h dark cycles (lights on at 07:00 hours, defined as Zeitgeber time, ZT = 0). The pregnant female rats were randomly assigned to one of two groups: (1) FAE, an ethanol-containing liquid diet (Bio-Serv Liquid Rat Diet L/Di82) in which 35% of the calories were derived from ethanol or (2) PF, pair-fed a liquid diet balanced isocalorically to the ethanol diet. Pair-fed dams received the same volume of diet as their FAE partners each day. To avoid stress associated with blood sampling, blood alcohol levels were not measured, but in previous experiments (measured 1 hour into the light cycle) blood alcohol levels ranged 80–100 mg/dL (Ripley, O'Shea, & Stephens, 2003). The daily consumption of ethanol by the pregnant dams leads to the developing fetus being exposed to a chronic level of alcohol. Diets were prepared by Bio-Serv, Inc. (Frenchtown, NJ, USA) and were formulated to provide adequate nutrition to pregnant females regardless of ethanol intake (Weinberg, 1985). Between days 11 and 21 of gestation, fresh diet was provided daily just 1hour prior to lights off. This feeding schedule is designed to minimize shifts in the corticosterone circadian rhythm that typically occur in animals on a restricted diet such as PF females (Gallo & Weinberg, 1981). Experimental diets were replaced with ad libitum access to rat chow and water on Day 22 of gestation.

At birth, designated Day 1 of lactation, pups remained with their natural mothers, and the litters were culled to 9 males per group (FAE: n=9, PF: n=9), Female pups were

not used in the experiments to avoid fluctuations in mnemonic capacity related to estrus cycle and changing estrogen levels. Weaning occurred on Day 22, after which the offspring were group-housed by litter in clear polycarbonate cages (46 x 24 x 20 cm) until testing at 60 days of age. The eighteen male rats were grouped according to their ethanol exposure status. Animals were housed in groups of three per cage prior to experimentation in plastic cages measuring 59.5 x 38 x 22 cm. They were kept under reversed day-night schedule (lights off at 10:00h, lights on at 22:00h) in a temperature and humidity controlled environment with free access to laboratory rat food (5012 PMI) and tap water. The animals were handled for a week by the researchers (Days 55-59). The purpose of handling is to familiarize the rats to their surroundings and to the researchers. On days 60 through 62, the young adult rats were then subjected to two behavioral models, Elevated Plus Maze and Social Interaction. Additional rats were used in the Social Interaction Model. These companion rats (CR), also Sprague-Dawley rats, were obtained from Charles River Laboratories (Wilmington, MA, USA). Eighteen companion rats were used in this study. They received no treatment and were of the same age bracket as the treatment group. Throughout the study, animal care and treatment used for each group were in accordance with institutional guidelines, approved by the Rutgers Animal Care and Facilities Committee, and complied with NIH policy.

B. Apparatus and Procedure

1. Elevated Plus Maze Model:

The elevated plus maze is made of black Plexiglas and consisted of two opposite open arms (50 x 10 cm) and two enclosed arms (50 x 10 x 40 cm), elevated to a height of 80 cm above the floor (Fig. 1). The junction area of the four arms (central area) measured 10 x 10 cm. The closed arms and the center platform were considered "protected" areas while open arms were the "unprotected" areas of the EPM. At the time of testing, animals were taken from the colony room to an adjacent test room and placed on the center of the maze facing an open arm. All testing was conducted during the early hours of the dark phase (between 13:00h and 17:00h) under dim red illumination to minimize test environment generated anxiety. In each series, the testing apparatus was cleaned thoroughly between subjects with 70% ethanol and dry towels. At the end of the 5-min test, animals were placed in a holding chamber until all testing was completed, at which time they were returned to the colony room.

Behavior was video-recorded by an overhead light sensitive camera placed 2 meters above the testing apparatus. The sessions were scored live from a video signal relayed to a computer. The ANYmaze program was used to aid in the scoring process. ANYmaze is a flexible video tracking system designed to automate recordings. The ANYmaze program recorded all of the rat's activity while on the maze. Both "conventional" spatio-temporal and ethological analysis were carried out. The spatio-temporal behavioral parameters (recorded automatically) were the time on open arms, time on closed arms, % open arm entries [(open/total) x 100], % time spent in open arms

of the maze [(time open/session duration) x 100], distance traveled, and total time mobile. An animal was considered to enter into an arm when at least three paws entered it. The ethological manual recordings of the animal's behavior were recorded by using the keys on the laptop. The left hand is held in standard QWERTY position. The count of each activity was recorded by pressing down on the corresponding key. The letter Z key kept track of rearing, the X key kept track of grooming, the C key kept track of stretching, and the V key kept track of head dipping. Rearing is a vertical movement in which the animal stands on its hind legs and/or is leaning against the walls of the maze with its forelegs. Grooming includes licking, scratching, and washing of the head and body. Stretching involves the extension of the rat's muscles, causing the rat's body to be at its longest state and then retracting to the original position without moving forward. Head dipping is an exploratory movement in which the animal's head is protruding over the edge of the open arm and own towards the floor.

Figure 1. Image of the elevated plus maze.

2. Social Interaction Model:

The open-field box is a hexagonal wooden area (perimeter 156 cm) surrounded by a clear Plexiglas wall (40 cm) (Fig. 2). The OF box was divided into four equal areas by radial lines drawn on a cardboard sheet. All testing was conducted during the early hours of the dark phase under dim red illumination (low light). A conventional test of duration of 5 min was employed. In each series, the testing apparatus was cleaned thoroughly between subjects with tap water and dry towels. Cardboard sheets were also changed in between subjects. Behavior was video-recorded by an overhead light sensitive camera placed 2 meters above the testing apparatus. Videos were digitized and transferred to a computer for analysis using custom software developed in our laboratory.

This experiment was a two-day interaction. The first day of the experiment tested the social interaction between a treatment rat with a companion rat in a low light, unfamiliar arena. Two pairs were set up: PF & CR and FAE & CR. The second day of the experiment, the rats were subjected to the same social situation as the first day. However, the conditions are now low light, familiar arena.

The taped sessions were then scored according to eight different behaviors. The purpose of scoring is to count the number of times a behavior is exhibited or how long the duration is. The behaviors that were selected to provide measurements of social behavior directed toward the companion rat were: approaching, crawling under or over, sitting in contact, anogenital sniffing and facial sniffing. The self-directed behaviors included: crossing squares, rearing, and self grooming. Each of the behavior has a certain criteria that it needs to fulfill in order for the behavior to be documented. Crossing behavior, a measure of locomotion, was counted when the rat crossed with all four paws

from one quadrant to another. A rear was counted when a rat stood on its hind limbs, with its forelimbs completely off the floor. The rat may place its forelimbs on the hexagonal Plexiglas or its paws must be in the air. If the experimental rat places its paw on the control rat, then the behavior is not scored as rearing. Approaching occurs only when the experimental rat makes an advance towards the companion rat. Crawling under or over occurs only when the treatment rat crawls directly under or over the control rat. Sitting in contact with each other means that both pairs are idly sitting together. Self grooming is scored when the experimental rat licks his paws or coats to wash himself. Anogenital sniffing is scored only when the treatment rat sniffs the genital and/or anal region of the companion rat. Facial sniffing is scored only when the experimental rat sniffs the facial area of the companion rat.

Behaviors were scored by highly trained observers using an analysis software. All behavior scoring was performed by a rater blind to prenatal diet condition. The scoring program that was used was GW Basic. Each behavior was scored by six independent people; a mean of the six scores was used in the analyses. The scoring session for each of the eighteen trials was five minutes. The behaviors that were measured as count were crossing, approaching CR, and facial sniffing of CR. The behaviors that were measured as duration (seconds) were rearing, crawling under or over CR, sitting together with CR, anogenital sniffing of CR, and self grooming. The data for all the scoring sessions were saved and documented into excel files.

Figure 2. Image of the open-field box.

C. Tissue Collection

Immediately following testing, subjects were transported into the surgery room where they were sacrificed by rapid decapitation. The head caps were removed and the brain was excised from the cranial cavity. Tissue samples from two brain regions, hippocampus (HC) and hypothalamus (HY), were harvested for gene expression profiling by microarray analysis. The microdissected tissues were snap frozen on dry ice and stored at -80 °C until RNA isolation.

D. Affymetric GeneChip Hybridization

Hippocampal and hypothalamus RNA from PF control and FAE animals were sent to Affymetrix (Santa Clara, CA, USA) for hybridization to oligonucleotide microarrays. Affymatrix's GeneChip® Rat Expression Set 230 was used in our analysis of global rat brain gene expression. This array is composed of 31,042 oligonucleotide probe sets, corresponding to ~28,000 annotated rat genes (Affymetrix, 2003)

E. Analysis of GeneChip Results

The Biometric Research Branch Array Tools 3.8.0 by National Cancer Institute (Bethesda, Maryland, USA) is an integrated software system for the comprehensive analysis of DNA microarray experiments. It was used in this study to generate comparison analyses of pair fed control animals and fetal ethanol exposed animals. The BRB-ArrayTools installer loads the package as an add-in to Microsoft Excel under the Microsoft Windows family of operating systems. The array tools package utilizes Microsoft Excel to input expression values and phenotypes. The computing tool for BRB

Array is launched by menu selections written in the R statistical programming language. The Affymetrix expression data is imported as raw CEL files. The class comparison tool of BRB-ArrayTools was carried out in this study to identify the genes that are differentially expressed among groups of animals (PF and FAE) collected from different types of tissues (hippocampus and hypothalamus). This tool allows the control of the number or proportions of false discoveries. In this comparison, 95% confidence was specified that resulted in a gene list that contains no more than 5% false discoveries. The output of the class comparison tool is a list of significant genes, with numerous annotations for the genes and links to websites containing additional information.

Pathway Express by Intelligent Systems and Bioinformatics Laboratory (Detroit, MI, USA) was an additional tool used to identify pathways to better understand a biological phenomenon. The list of significant genes is imported into the program and the system performs a search and builds a list of all associated pathways. An impact factor and p-value is calculated for each pathway by incorporating parameters such as the normalized fold change of the differentially expressed genes, the statistical significance of the set of pathway genes, and the topology of the signaling pathway. The most statistically significant pathways are listed in Table 1. The long-term potentiation pathway was chosen with respect to the magnitude of its ethanol response. A graphical representation of the genes involved in the long-term potentiation pathway is shown in Figure 3. The input genes associated with long-term potentiation is listed in Table 2. These 11 input genes were selected for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis to confirm their differential expression.

Table 1. Pathway analysis by impact factor and p-value

Input Genes/ Pathway Genes	p-value	Impact Factor	Pathway
11/62	2.293E-4	10.853	Long-term potentiation
11/55	5.139E-3	7.399	Phosphatidylinositol signaling system
22/164	6.291E-3	7.169	Calcium signaling pathway
15/113	1.569E-2	6.117	Wnt signaling pathway
12/86	2.709E-2	5.477	Melanogenesis

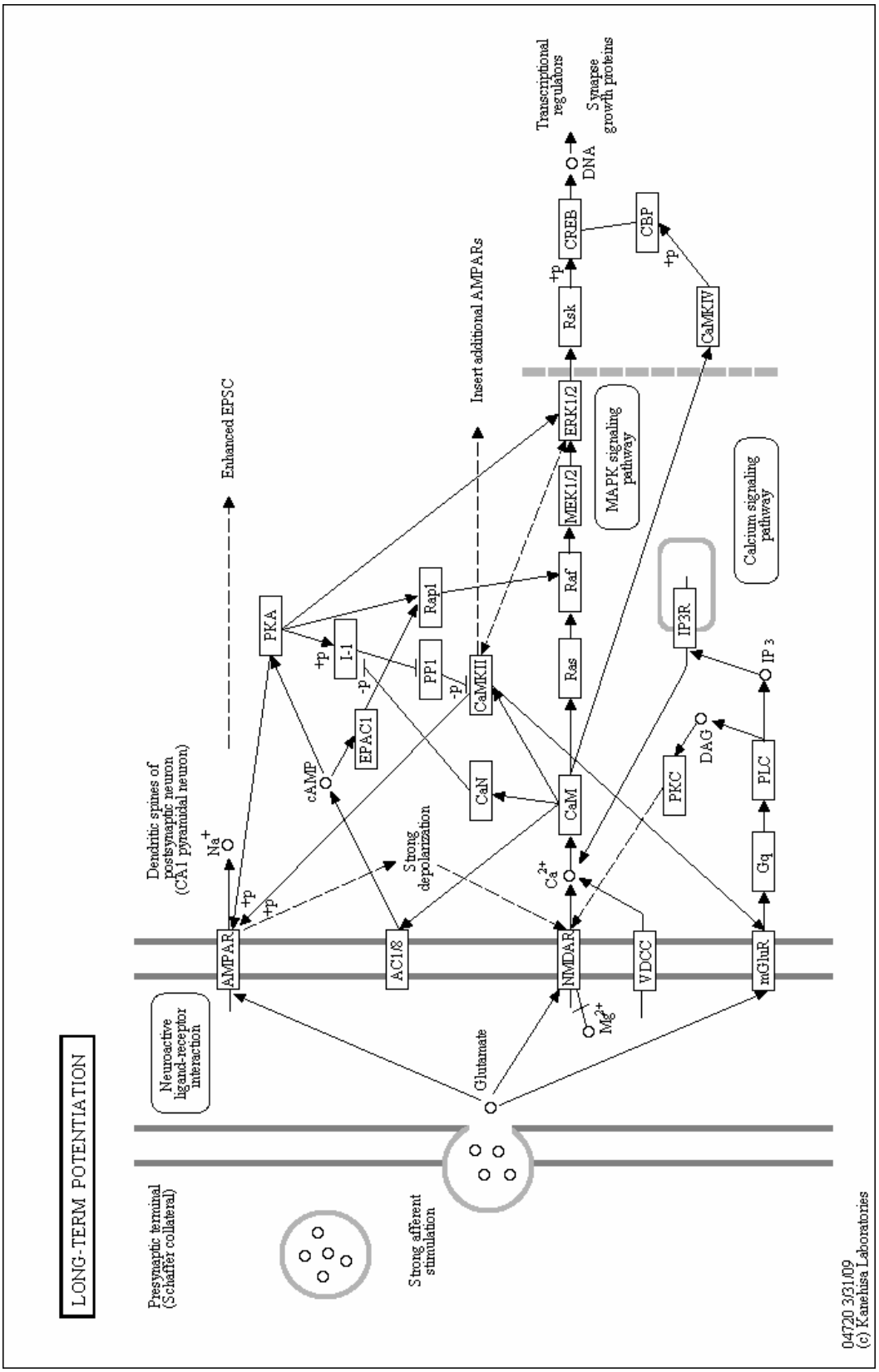

Figure 3. Long-term potentiation pathway (Kanehisa, 2009)

Table 2. Input genes from long-term potentiation pathway

Symbol	Gene Name	Fold Change
Camk2a	calcium/calmodulin-dependent protein kinase II alpha	1.03
Camk2d	calcium/calmodulin-dependent protein kinase II delta	0.75
Gria2	glutamate receptor, ionotropic, AMPA2	0.81
Grin2d	glutamate receptor, ionotropic, N-methyl D-aspartate 2D	1.04
Grm5	glutamate receptor, metabotropic 5	1.06
Itpr1	inositol 1,4,5-triphosphate receptor, type 1	0.99
Mapk1	mitogen activated protein kinase 1	1.05
Plcb4	phospholipase C, beta 4	0.85
Ppp3cb	protein phosphatase 3, catalytic subunit, beta isoform	0.97
Ppp3r1	protein phosphatase 3, regulatory subunit B, alpha isoform	1.04
Prkca	protein kinase C, alpha	1.48

F. Primer Design

The primers were designed using application-based primer design software Primer Express ^{TM} (version 3.0, PE Applied Biosystems, Foster City, CA). Oligonucleotides were purchased from Integrated DNA Technologies (San Diego, California) at 25 nM scale with standard desalting. The oligonucleotides were resuspended to 100 μM in sterile ddH2O and an aliquot is diluted to 5 μM. The forward and reverse 5 μM primers are then mixed at a 1:1 ratio for use in subsequent assays. Table 3 lists the primer sequences for the eleven target genes and one reference (Gapdh).

Table 3. Primer sequences

Gene Symbol	Strand	Primer sequence 5' → 3'
Camk2a	Sense	GCATCTGCCGCTTGTTGAA
Camk2a	Antisense	CTCGGAGATGCTGTCATGGA
Camk2d	Sense	CTCTTGAAGCACCCCAATATTGT
Camk2d	Antisense	CACCAAGTAATGGAAGCCCTCTT
Gria2	Sense	TTTTCCTTTCTTGATCCTTTAGCCTAT
Gria2	Antisense	TCTGCTGACCAGGAATAAAACTACA
Grin2d	Sense	AGTTGCACGTTCTATGCGTTTAAG
Grin2d	Antisense	GCCATATGATGCAGGTTGTGTAC
Grm5	Sense	CCCTGGGTTGCATGTTTGTC
Grm5	Antisense	CGCACATTTCTCTCCGGTTT
Itpr1	Sense	CCGGCTGACGGAAGATAAGA
Itpr1	Antisense	CCCAAATCGCTGGTGTTCAC
Mapk1	Sense	GCGCTACACCAACCTCTCGT
Mapk1	Antisense	CACGGTGCAGAACGTTAGCTG
Plcb4	Sense	CAGGCATGGATGGCATCTTATA
Plcb4	Antisense	CGTGGACAAGTACGGATGGAT
Ppp3cb	Sense	CCCTGAACACCGCACATACC
Ppp3cb	Antisense	TGGTCACTGGGCACTATGGTT
Ppp3r1	Sense	CACCGCTGTCCCCTCAACT
Ppp3r1	Antisense	CAAGCACCGGGATTTTTCTC
Prkca	Sense	CGGACGACACGGAATGACTT
Prkca	Antisense	TGGCATCTTCATCAGCTCTGA
Gapdh	Sense	CTCCCATTCTTCCACCTTTGAT
Gapdh	Antisense	ACCAGGAAATGAGCTTCACAAAGT

G. Extraction of Total RNA and Reverse Transcription

Total RNA in the hippocampus and hypothalamus tissues were extracted using the RNeasy Midi Kit (Qiagen) according to the manufacturer's protocol. The isolated RNA was resuspended in diethyl-pyrocarbonate-treated water, treated with DNase I (Qiagen) and stored at -80 °C. The extracted RNA was reverse-transcribed using the TaqMan Reverse-Transcription Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA). The cDNA synthesis was carried out by following the manufacturer's protocol. Each reverse transcriptase reaction contained 1000 ng of extracted total RNA template, taq man RT buffer, $MgCl_2$, dNTP, random hexamer, oligo $d(T)_{16}$, RNase inhibitor, multiscribe reverse transcriptase, and RNase free H_2O. The 50 µl reactions were incubated at 25°C for 10 min, 48°C for 30 min, and then 95°C for 5 min.

H. Primer Set Validation

To validate the primers, a template titration assay was performed. The assay consists of a 3-fold dilution series of cDNA reverse-transcribed from the standard RNA (.002 µg, .00025 µg, .00003125 µg) and 1 control sample: a no template control (NTC) of RNase free water. Amplification of the NTC sample indicates the presence of primer-dimers. After the template titration assay run is complete, the ABI 7900 instrument software, SDS 2.1, plots a dissociation curve and a standard curve for each target gene. A valid primer set should have a dissociation curve with a single "stacked" peak at the amplicon Tmc (temperature of melting curve). In addition, the standard curve should have a slope between -3.1 and -3.6 and a correlation coefficient (R^2-value) >0.95 for the standard curve (Pfaffl, 2006). The slope of the standard curve is indicative of the PCR

efficiency (Efficiency Percentage = $-1+10^{(-1/slope)}$ x 100). A slope of -3.32 indicates the PCR is 100% efficient; meaning the amount of template is doubled after each cycle. Reaction efficiencies between 90% and 110% are acceptable (Pfaffl, 2006).

I. Quantitative Real-time Polymerase Chain Reaction Preparation

The relative mRNA expression levels of PF and FAE rats were determined in the hippocampus and hypothalamus by quantitative real-time PCR (qRT-PCR). The cDNA was subjected to qRT-PCR using SYBR Green 1 double-stranded DNA binding dye chemistry on an ABI Prism 7900 Sequence Detector (Perkin Elmer Applied Biosystems, Foster City, CA). The cycles for SYBR Green PCR were conducted at 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 sec, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min for primer annealing and elongation. This was followed by a dissociation stage of for 15 s at 95°C to ensure the presence of a single amplicon. The PCR reactions contained 5 μM of each primer, 5 μl of SYBR Green master mix (from Applied Biosystems, Foster City, CA), and 1 μl of the diluted cDNA sample in a final volume of 10 μl. Reaction components are pre-mixed in 8-well PCR tube-strips and then pipetted with a multi-channel pipette into the reaction plate. Premixing the components decreases the amount of user-introduced variation by ensuring a homogenous mix of template and primers. Each sample is run in triplicate for each gene to be assayed. A no template control (RNase-free water) was included for every PCR run. The house keeping gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh) serves as an internal control and simultaneously assessed in separate reaction tubes. The 384-well Optical Reaction Plate

(from Applied Biosystems, Foster City, CA) is then covered with an optical adhesive cover and centrifuged to bring the liquid to the bottom of the wells of the plate.

J. Quantitative Real-Time PCR Analysis Using the Comparative ΔΔCt Method

The instrument software, SDS 2.1, calculates the quantity of transcript in each unknown and the data are exported as a tab-delimited text file. Further data analysis is done using Microsoft Excel. Relative quantification was performed using the comparative threshold method after determining the Ct, cycle threshold, values for reference (Gapdh) and target genes in each sample sets according to the $2^{-\Delta\Delta Ct}$ method (Bookout & Mangelsdorf, 2003). The cycle threshold is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample). Cts < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample. Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid. Cts of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent a contamination. A Ct value of 38 or higher means no amplification and this value cannot be included in the calculations.

For each of the three replicates of a sample, the average Ct, the coefficient of variation (CV) (Formula 1, Table 4), and the standard deviation ($stdev_{Ct}$) is calculated (Formulas 1 and 2, Table 4). For each sample, the average Ct of the target gene is normalized to the average Ct of the reference gene for the same sample to calculate the normalized Ct for the target gene (Formula 4, Table 4). The standard deviation of the ΔCt

is calculated using Formula 5, Table 4. The calibrator is chosen to be the sample, or the control group to which the others will be compared. The cDNA from the pair fed control is chosen as the calibrator sample. The ΔΔCt, or calibrated value, for each sample is given by Formula 6, Table 4. The standard deviation of the ΔΔCt is the same as the standard deviation of the ΔCt. The fold-induction for each sample relative to the calibrator = $2^{-\Delta\Delta Ct}$ (Formula 7, Table 4).The relative gene expression is plotted as a bar graph. The measure of experimental error (SEM) is the standard deviation of the mean, given by Formula 8 and 9, Table 4. t-Tests were performed to detect significant differences in expression between PF and FAE samples. A two-way analysis of variance (ANOVA) test was performed to see the factors of variation between treatment and brain area. The repeated measure parameters were the fold-induction ($2^{-\Delta\Delta Ct}$) values.

Table 4. Formulas for calculating the $2^{-\Delta\Delta Ct}$ values

Formula	Equation
Formula 1	$CV = stdev / avg$
Formula 2	normalized value = target qty avg / reference qty avg
Formula 3	$stdev_{Ct} = (\text{normalized value}) \times \sqrt{(CV_{reference}^2 + CV_{target}^2)}$
Formula 4	$\Delta Ct = avgCt_{target} - avgCt_{reference}$
Formula 5	$stdev_{\Delta Ct} = \sqrt{(stdev_{reference}^2 + stdev_{target}^2)}$
Formula 6	$\Delta\Delta Ct = \Delta Ct$ sample - ΔCt calibrator
Formula 7	Gene expression level = $2^{-\Delta\Delta Ct}$
Formula 8	$stdev_{\text{fold change}} = (\ln 2)(stdev_{\Delta\Delta Ct})(2^{-\Delta\Delta Ct})$
Formula 9	$SEM = stdev_{\text{fold change}} / \sqrt{(\#\text{ of samples in group})}$

K. Statistical Analyses

Normally distributed data were analyzed using a two-tailed t-test for independent samples (two-sample assuming unequal variance) to compare means of the ethanol-exposed rats with the control rats. Outlier values that fell above or below mean ± 2stdev were not included in the analysis. All graphical data are presented as means ± SEM. In all tests, a probability of less than 0.05 was considered statistically significant. An asterisk is used to denote significance. The analysis of the real-time PCR data was performed using two-way analysis of variance (ANOVA) with repeated measures. The factors of variation were prenatal treatment and brain area as independent variables. The repeated measure parameters were the fold-induction ($2^{-\Delta\Delta Ct}$) values.

III. RESULTS

A. Elevated Plus Maze Behavior of Animals Tested Directly from the Homecage.

Behavioral data from the elevated plus maze is listed in Table 5. Analysis revealed that fetal alcohol exposure caused no significant difference in conventional spatio-temporal parameters, such as % open arm entries (Fig. 4A) or % open arm time (Fig. 4B) among PF or FAE males. Closed/open arms ratio showed a tendency for FAE to spend more time on the open arms of the maze than PF (Fig. 5), There were no significant effects in locomotion; however, FAE did show a tendency for more mobility (Fig. 6A) and traveling distance (Fig. 6B) than PF.

Analysis of the ethological parameter of rearing showed no significant treatment effects (Fig. 7A). However, FAE showed a tendency for more head dipping than PF (Fig. 7B). In addition, there was a statistically non-significant tendency in stretching (Fig. 8A) and grooming (Fig. 8B) in PF compared to FAE.

Table 5. Behavioral data from the elevated plus maze. Data represent mean ± SEM. P-values determined using student t-test (two-tailed)

Behavior	PF	FAE	p-value
% Open arm entries	51.01 ± 0.16	51.22 ± 0.17	0.39
% Open arm time	42.71 ± 1.66	44.34 ± 1.23	0.44
Closed/open arms ratio	4.50 ± 1.17	4.85 ± 0.86	0.81
Total time mobile (sec)	137.55 ± 5.41	148.00 ± 11.79	0.44
Total distance traveled (m)	0.23 ± 0.01	0.25 ± 0.02	0.47
Total rearings	15.44 ± 1.33	16.89 ± 4.07	0.74
Total head dippings	12.12 ± 1.39	14.44 ± 2.56	0.44
Total stretchings	7.00 ± 1.20	6.33 ± 1.86	0.77
Total groomings	0.65 ± 0.21	0.33 ± 0.17	0.25

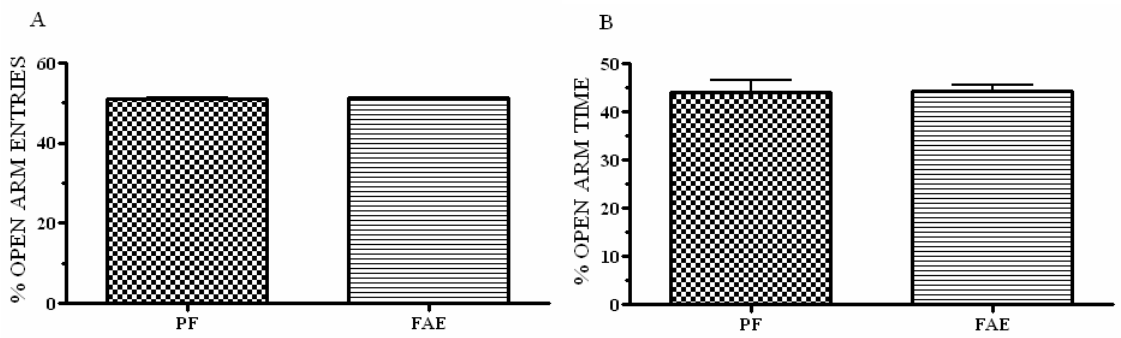

Figure 4. Spatio-temporal behaviors on elevated plus maze. (A) Percent open arm entries. PF: n=8; FAE: n=9 (B) Percent open arm time PF: n=9; FAE: n=8.

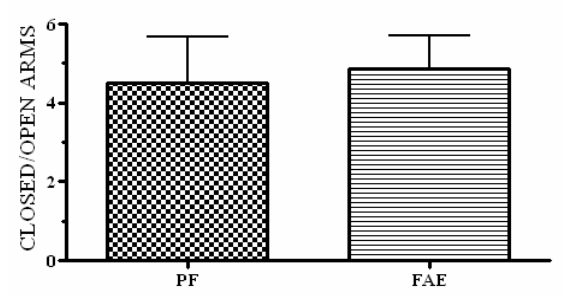

Figure 5. Spatio-temporal behaviors on elevated plus maze. Closed/open arms ratio PF: n=9; FAE: n=9.

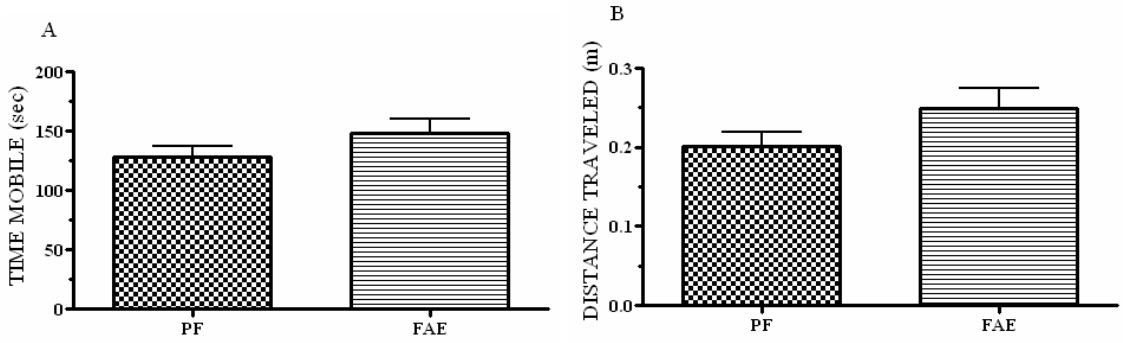

Figure 6. Spatio-temporal behaviors on elevated plus maze. (A) Total time mobile PF: n=9; FAE: n=9. (B) Total distance traveled PF: n=9; FAE: n=9.

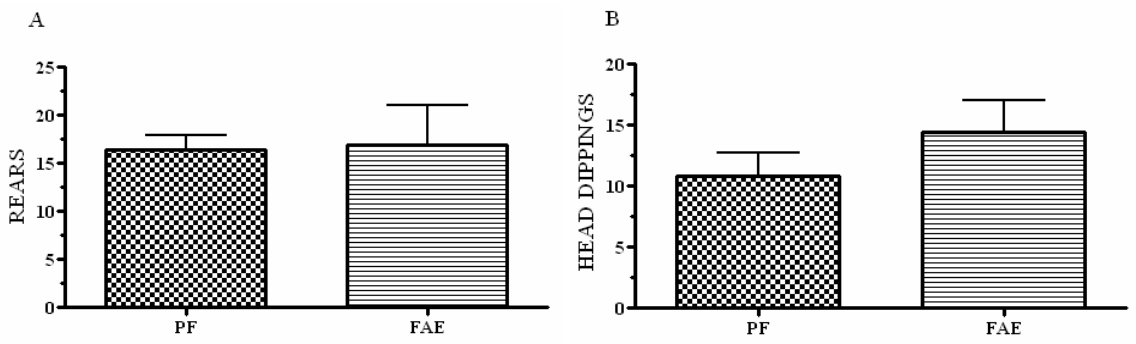

Figure 7. Ethological behaviors on elevated plus maze. (A) Rearings PF: n=9; FAE: n=9. (B) Head dippings PF: n=9; FAE: n=9.

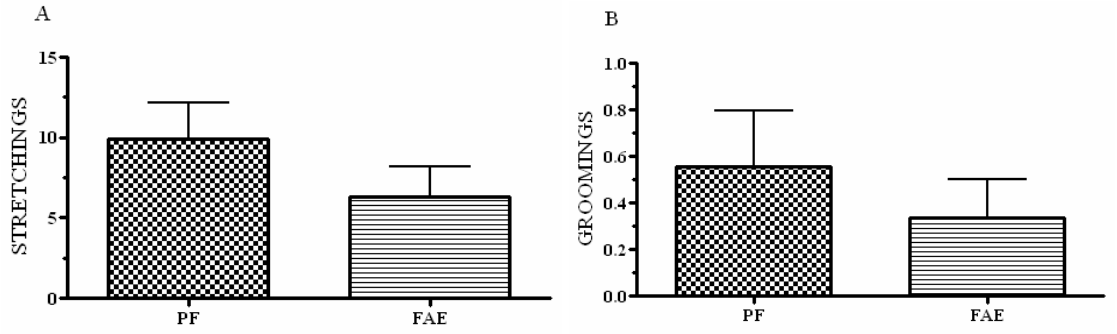

Figure 8. Ethological behaviors on elevated plus maze. (A) Stretchings PF: n=9; FAE: n=9. (B) Groomings PF: n=9; FAE: n=9.

B. Open-field Behavior of Animals' Encounter with Companion for Two Consecutive Days of Testing.

Behavioral analysis showed that prenatal ethanol exposure appears to be correlated with subtle behavior changes, especially over repeated encounters with peers in a social situation. The data for Day1 and Day2 were not significant and a ratio of Day2 and Day1 was then used to look at the social interaction. Day2/Day1 ratio behavioral data from the social interaction test is listed in Table 6. Analyses of open-field self-directed behaviors demonstrated a tendency for square crossing (Fig. 9A) and rearing (Fig. 9B) in FAE groups. Another self-directed behavior showed a non-significant tendency for PF to have a longer duration of self grooming (Fig. 9C). Analysis of companion-directed behavior of the PF group showed a tendency for social investigation, crawling under or over companion rat (Fig. 10A), than FAE. There were no significant differences in the duration of being together with companion rat in PF and FAE (Fig. 10B). Both PF and FAE showed an equal level of anogenital sniffing (Fig. 10C). In addition, analysis of companion-directed behavior demonstrated a tendency for approaching (Fig. 10D) and facial sniffing (Fig. 10E) of companion rat in FAE compared to PF.

Table 6. Day2/Day1 behavioral data from the social interaction test. Data represent mean ± SEM. P-values determined using student t-test (two-tailed)

Behavior	PF	FAE	p-value
Square crosses	0.64 ± 0.21	2.68 ± 0.89	0.13
Rearing	1.02 ± 0.34	2.35 ± 0.78	0.21
Self grooming	1.74 ± 0.58	1.27 ± 0.42	0.48
Crawling over or under CR	1.77 ± 0.59	1.65 ± 0.55	0.87
Being together with CR	1.12 ± 0.37	1.18 ± 0.39	0.63
Anogenital sniffing of CR	0.57 ± 0.19	0.49 ± 0.16	0.52
Approaching CR	0.71 ± 0.24	2.70 ± 0.90	0.21
Facial sniffings of CR	0.97 ± 0.32	1.16 ± 0.39	0.49

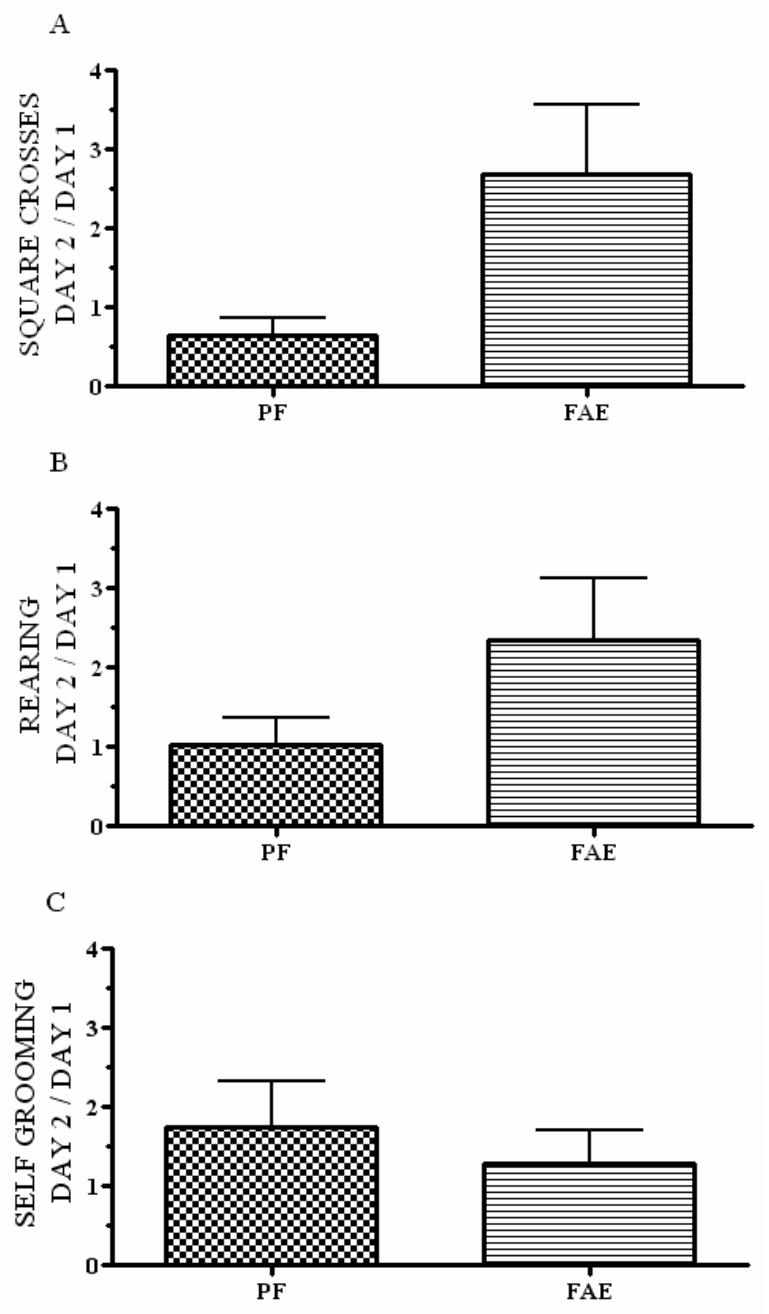

Figure 9. Self-directed behaviors on open-field. Ratio Day 2 vs Day 1. (A) Crossing squares PF: n=9; FAE: n=9. (B) Rearing PF: n=9; FAE: n=9. (C) Self grooming PF: n=8; FAE: n=8.

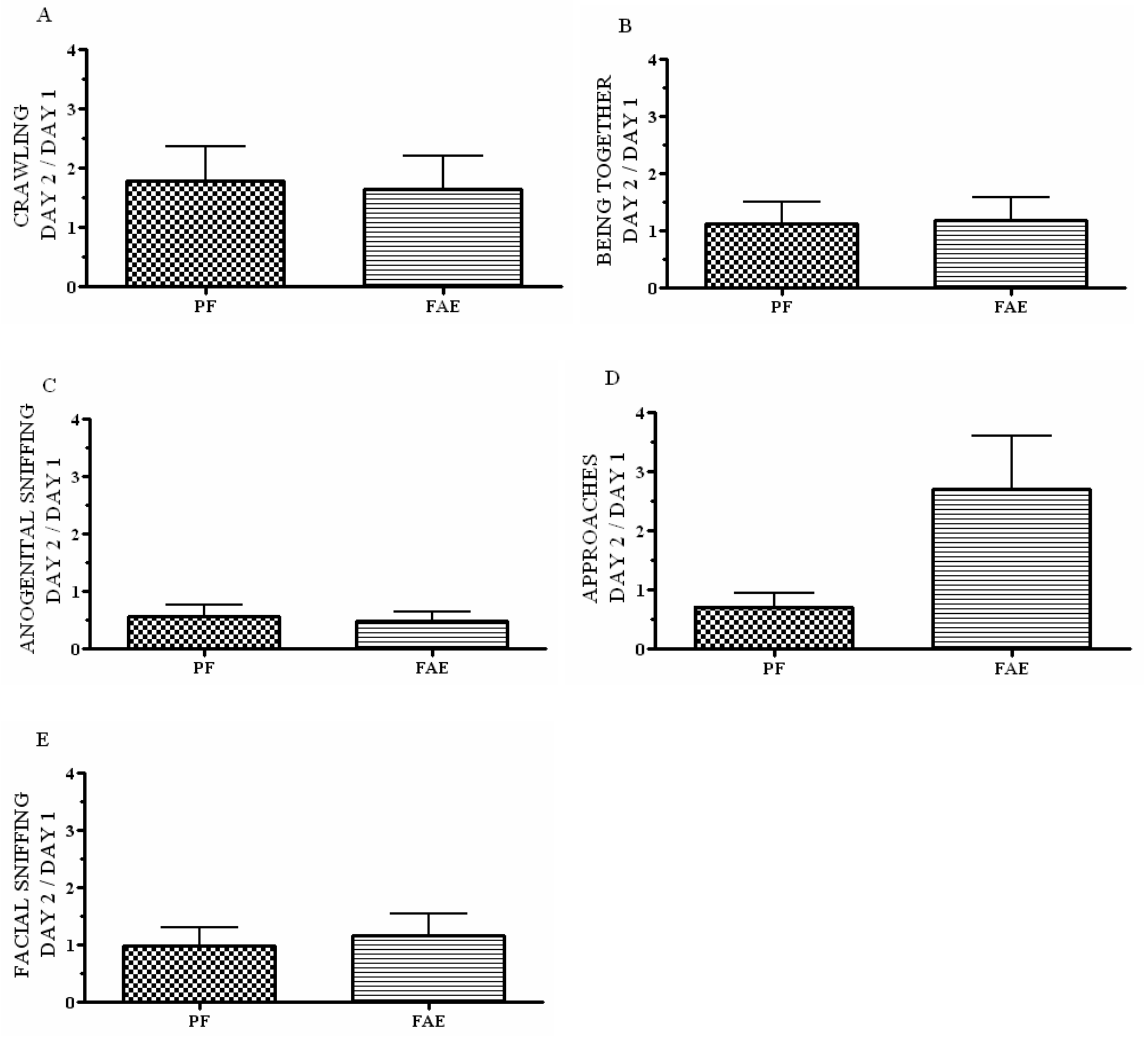

Figure 10. Companion-directed behaviors on open-field. Ratio Day2 vs Day1. (A) Crawling over or under companion rat PF: n=9; FAE: n=9. (B) Being together with companion rat PF: n=9; FAE: n=9. (C) Anogenital sniffing of companion rat PF: n=9; FAE: n=9. (D) Approaching companion rat PF: n=9; FAE: n=9. (E) Facial sniffing of companion rat PF: n=9; FAE: n=9.

C. Efficiency of Amplification for Each Primer Set Using Serial Dilutions of Input DNA

The melting curves revealed a single product for each primer set (Fig. 11) The slope of the standard curves were within -3.1 and -3.6 and the R^2-values were all above 0.99 (Table 7). The reaction efficiencies of most of the primer sets were between 90% and 110%, except for Grin2d, with a reaction efficiency of 88.1 %. However, since Grin2d had an R2 value of 0.998, it was accepted as an efficient primer set. The standard curve for the target genes are represented as a semi-log regression line plot of Ct value versus log of input of RNA (μg) (Figure 12).

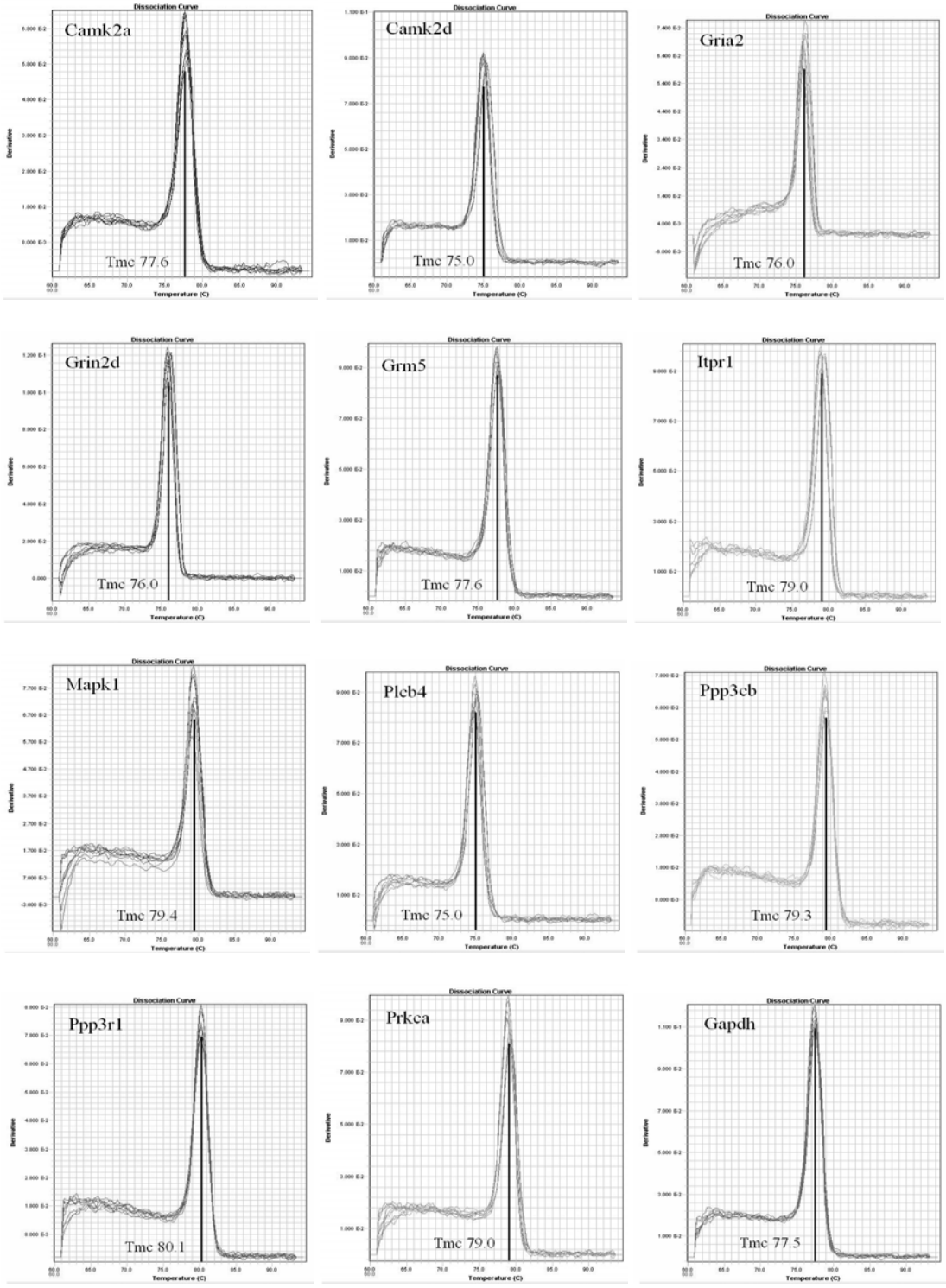

Figure 11. Temperature of melting curve (Tmc). Melting curves revealing a single product for each primer sets.

Table 7. Validation of primer sets for qRT-PCR using standard curve

Target	Slope ± SEM	R^2	Efficiency (%)
Camk2a	-3.145 ± 0.1312	0.998	107.9
Camk2d	-3.330 ± 0.0276	0.999	99.7
Gria2	-3.577 ± 0.0819	0.999	90.4
Grin2d	-3.646 ± 0.1754	0.998	88.1
Grm5	-3.206 ± 0.1505	0.998	105.1
Itpr1	-3.184 ± 0.2211	0.995	106.1
Mapk1	-3.332 ± 0.3861	0.987	99.6
Plcb4	-3.454 ± 0.0527	0.999	94.8
Ppp3cb	-3.145 ± 0.0986	0.999	107.5
Ppp3r1	-3.266 ± 0.1756	0.997	102.4
Prkca	-3.188 ± 0.1465	0.998	105.9
Gapdh	-3.228 ± 0.1165	0.999	104.1

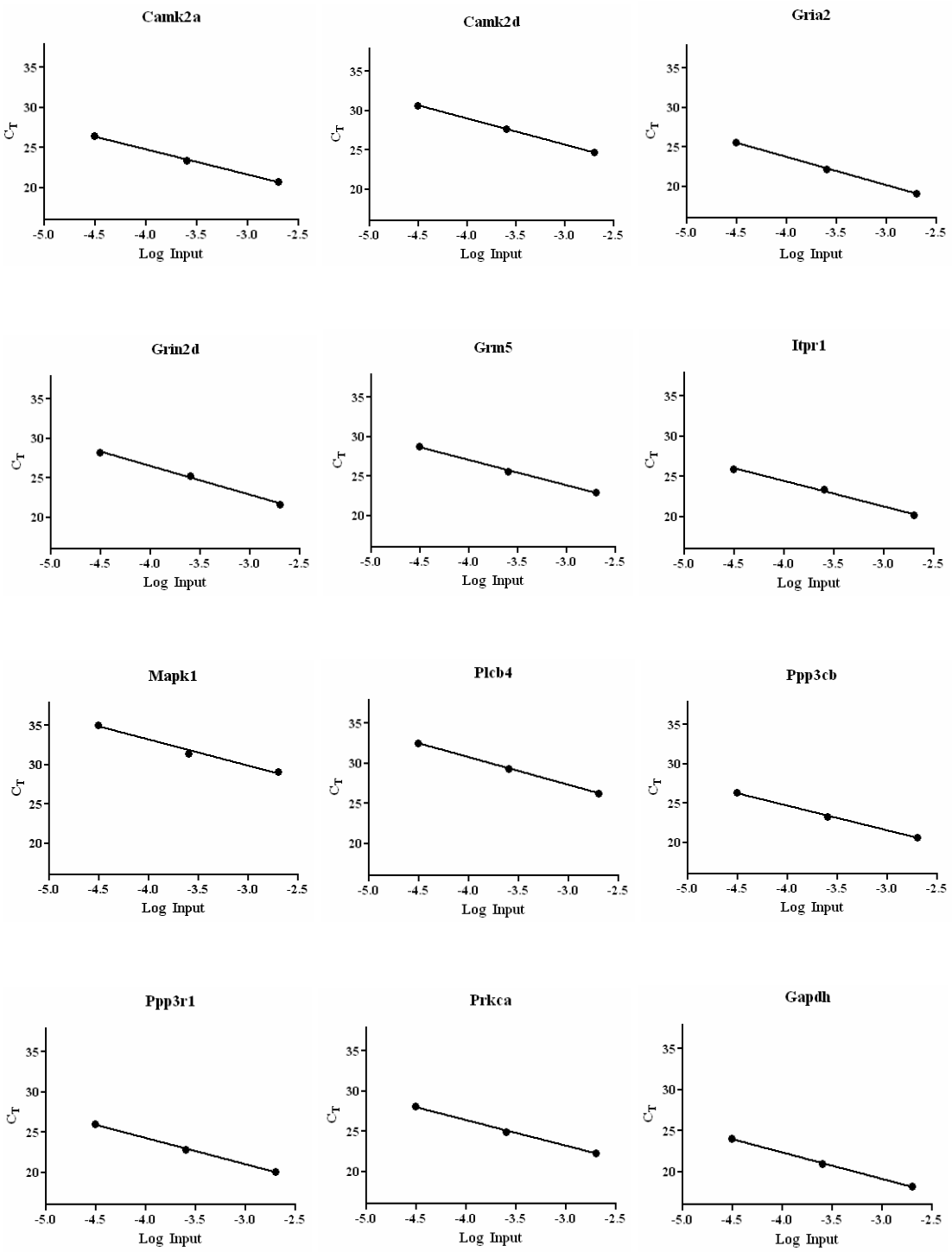

Figure 12. Quantitative Real-Time PCR standard curve. Data represented as semi-log regression line plot of Ct value vs log of input of RNA (μg).

D. Effects of Prenatal Ethanol Consumption on Long-Term Potentiation Gene Expression Level in the Hippocampus and Hypothalamus

In this study, we applied the comparative quantification (ΔΔCt method) of qRT-PCR for comparing changes in gene expression of Camk2a, Camk2d, Gria2, Grind2d, Grm5, Itpr1, Mapk1, Plcb4, Ppp3cb, Ppp3r1, Prkca following prenatal ethanol exposure. Relative quantification was performed using Gapdh as the endogenous control gene. Our two-tailed t-test analysis revealed a significant change in expression in all the target genes (Table 8). Camk2a revealed a 6.26-fold down regulation in the hippocampus (HC) and a 15.14-fold up regulation in the hypothalamus (HY) (Fig. 13). Camk2d has a 3.37-fold up regulation in HC and 4.55-fold down regulation in HY (Fig. 14). Gria2 showed a 4.76-fold down expression in HC tissue and a 5.31-fold up expression in HY (Fig. 15). Grin2d revealed a similar pattern as Gria2 in that it has a 2.50-fold down regulation in HC and a 2.18-fold up regulation in HY (Fig. 16). Grm5 showed a 3.23-fold down expression in HC and 2.50-fold up expression in HY (Fig. 17). Itpr1 revealed a similar pattern as Grm5 in that in has a 3.57-fold down regulation in HC and a 2.09-fold up regulation in HY (Fig. 18). Mapk1 and a Plcb4 showed a fold increase in HC, 4.22 and 4.99 respectively; and a fold decrease in HY, 4.76 and 9.09 respectively (Fig. 19, 20). Ppp3cb, Ppp3r1 and Prkca showed a fold decrease in HC, 3.23, 4.76 and 4.75 respectively; and a fold increase in HY, 2.30, 2.46, and 13.70, respectively (Fig. 21, 22, 23). The divergent gene expression of the eleven isoforms is summarized in Figure 24. The two-way ANOVA analyses (treatment x brain area) with repeated measures revealed a highly significant interaction effect between the treatment groups and brain regions (Table 9).

Table 8. $2^{-\Delta\Delta Ct}$ relative expression of long-term potentiation genes. Data represent mean ± SEM. P-values determined using student t-test (two-tailed)

Gene	Treatment	Hippocampus	Hypothalamus
Camk2a	PF	1.00 ± 0.04	1.00 ± 0.02
	FAE	0.16 ± 0.01 ($p < .001$)	15.14 ± 0.53 ($p < .001$)
Camk2d	PF	1.00 ± 0.04	1.00 ± 0.02
	FAE	3.37 ± 0.05 ($p < .0001$)	0.22 ± 0.01 ($p < .0001$)
Gria2	PF	1.00 ± 0.04	1.00 ± 0.02
	FAE	0.21 ± 0.01 ($p < .0001$)	5.31 ± 0.19 ($p < .05$)
Grin2d	PF	1.00 ± 0.04	1.00 ± 0.02
	FAE	0.40 ± 0.01 ($p < .05$)	2.18 ± 0.08 ($p < .01$)
Grm5	PF	1.00 ± 0.04	1.00 ± 0.02
	FAE	0.31 ± 0.01 ($p < .01$)	2.50 ± 0.09 ($p < .001$)
Itpr1	PF	1.00 ± 0.04	1.00 ± 0.02
	FAE	0.28 ± 0.01 ($p < .0001$)	2.08 ± 0.07 ($p < .01$)
Mapk1	PF	1.00 ± 0.04	1.00 ± 0.02
	FAE	4.22 ± 0.07 ($p < .0001$)	0.21 ± 0.01 ($p < .01$)
Plcb4	PF	1.00 ± 0.04	1.00 ± 0.02
	FAE	4.99 ± 0.08 ($p < .01$)	0.11 ± 0.01 ($p < .01$)
Ppp3cb	PF	1.00 ± 0.04	1.00 ± 0.02
	FAE	0.31 ± 0.01 ($p < .0001$)	2.30 ± 0.08 ($p < .01$)
Ppp3r1	PF	1.00 ± 0.04	1.00 ± 0.02
	FAE	0.21 ± 0.01 ($p < .01$)	2.46 ± 0.09 ($p < .01$)
Prkca	PF	1.00 ± 0.04	1.00 ± 0.02
	FAE	0.21 ± 0.01 ($p < .0001$)	13.70 ± 0.49 ($p < .01$)

Table 9. ANOVA two-factor (treatment versus brain area) with replication (triplicate Ct).

Gene	Source of Variation	F	P-value
Camk2a	Main Effect of Treatment	F (1,8) = 2802.52	$p < 10^{-12}$
	Main Effect of Brain Area	F (1,8) = 3552.76	$p < 10^{-11}$
	Interaction Effect	F (1,8) = 3552.93	$p < 10^{-12}$
Camk2d	Main Effect of Treatment	F (1,8) = 541.59	$p < 10^{-11}$
	Main Effect of Brain Area	F (1,8) = 2109.43	$p < 10^{-8}$
	Interaction Effect	F (1,8) = 2107.64	$p < 10^{-11}$
Gria2	Main Effect of Treatment	F (1,8) = 44.83	$p < 10^{-5}$
	Main Effect of Brain Area	F (1,8) = 94.00	$p < 10^{-4}$
	Interaction Effect	F (1,8) = 93.97	$p < 10^{-5}$
Grin2d	Main Effect of Treatment	F (1,8) = 24.34	$p < 10^{-7}$
	Main Effect of Brain Area	F (1,8) = 239.44	$p < 10^{-3}$
	Interaction Effect	F (1,8) = 243.82	$p < 10^{-7}$
Grm5	Main Effect of Treatment	F (1,8) = 42.58	$p < 10^{-7}$
	Main Effect of Brain Area	F (1,8) = 316.31	$p < 10^{-4}$
	Interaction Effect	F (1,8) = 318.26	$p < 10^{-8}$
Itpr1	Main Effect of Treatment	F (1,8) = 9.58	$p < 10^{-7}$
	Main Effect of Brain Area	F (1,8) = 238.79	$p < 10^{-2}$
	Interaction Effect	F (1,8) = 238.57	$p < 10^{-7}$
Mapk1	Main Effect of Treatment	F (1,8) = 363.65	$p < 10^{-9}$
	Main Effect of Brain Area	F (1,8) = 1001.00	$p < 10^{-8}$
	Interaction Effect	F (1,8) = 996.57	$p < 10^{-9}$
Plcb4	Main Effect of Treatment	F (1,8) = 226.64	$p < 10^{-8}$
	Main Effect of Brain Area	F (1,8) = 561.72	$p < 10^{-7}$
	Interaction Effect	F (1,8) = 562.02	$p < 10^{-8}$
Ppp3cb	Main Effect of Treatment	F (1,8) = 72.57	$p < 10^{-9}$
	Main Effect of Brain Area	F (1,8) = 774.65	$p < 10^{-5}$
	Interaction Effect	F (1,8) = 774.68	$p < 10^{-9}$
Ppp3r1	Main Effect of Treatment	F (1,8) = 41.51	$p < 10^{-8}$
	Main Effect of Brain Area	F (1,8) = 477.22	$p < 10^{-4}$
	Interaction Effect	F (1,8) = 478.70	$p < 10^{-8}$
Prkca	Main Effect of Treatment	F (1,8) = 186.80	$p < 10^{-7}$
	Main Effect of Brain Area	F (1,8) = 239.78	$p < 10^{-7}$
	Interaction Effect	F (1,8) = 239.68	$p < 10^{-7}$

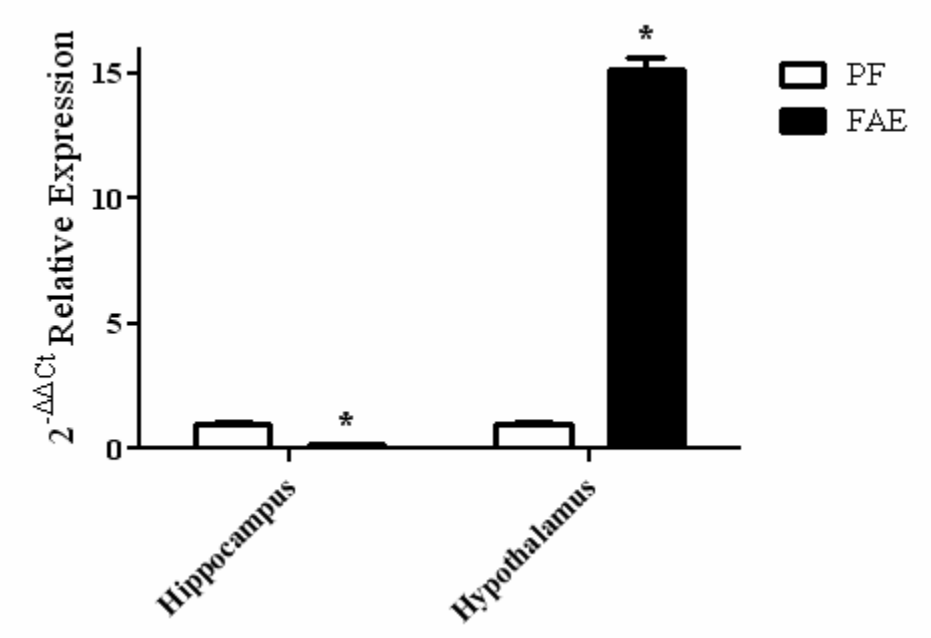

Figure 13. Gene expression of Camk2a in hippocampus and hypothalamus. Data represent mean ± SEM; asterisk (*) denotes significance.

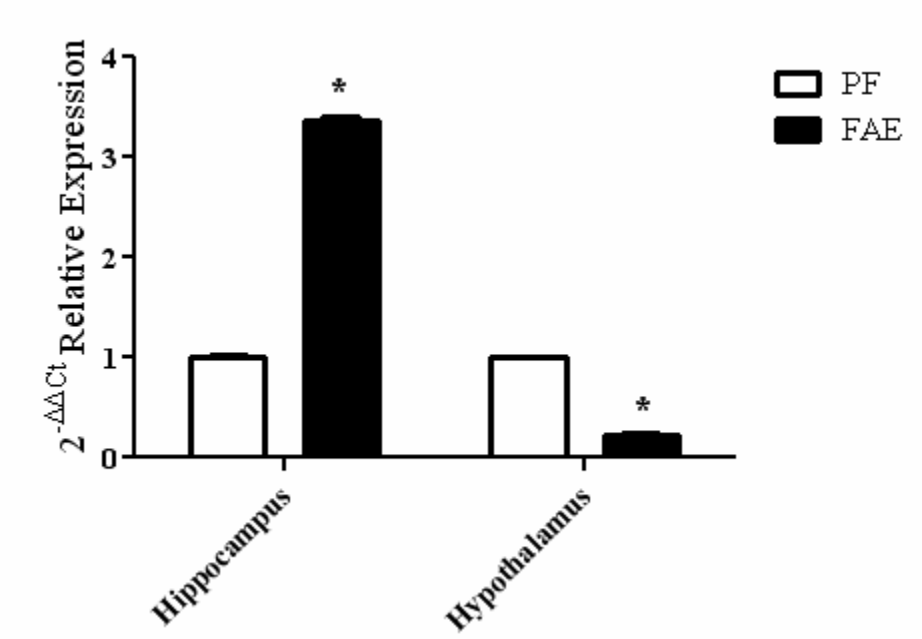

Figure 14. Gene expression of Camk2d in hippocampus and hypothalamus. Data represent mean ± SEM; asterisk (*) denotes significance.

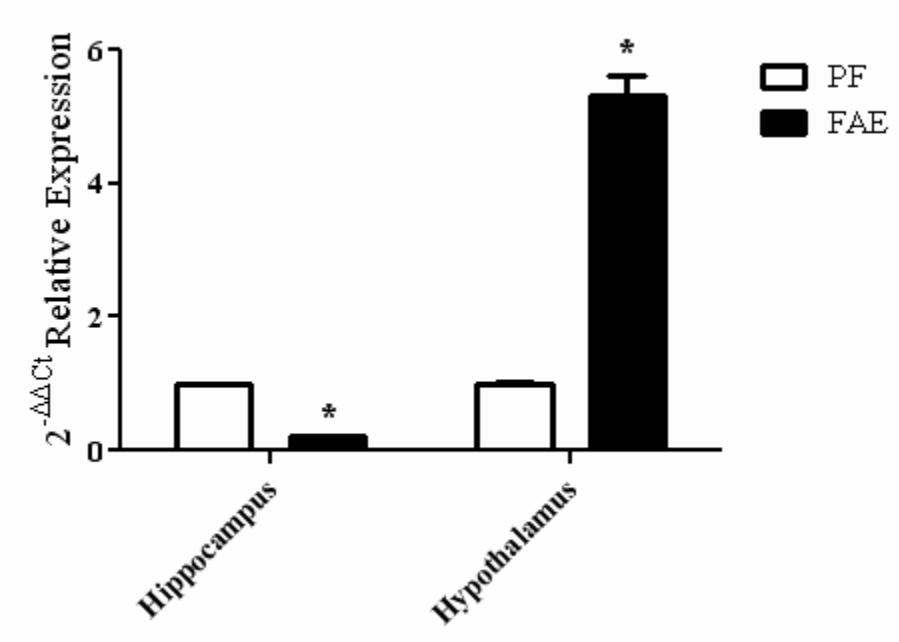

Figure 15. Gene expression of Gria2 in hippocampus and hypothalamus. Data represent mean ± SEM; asterisk (*) denotes significance.

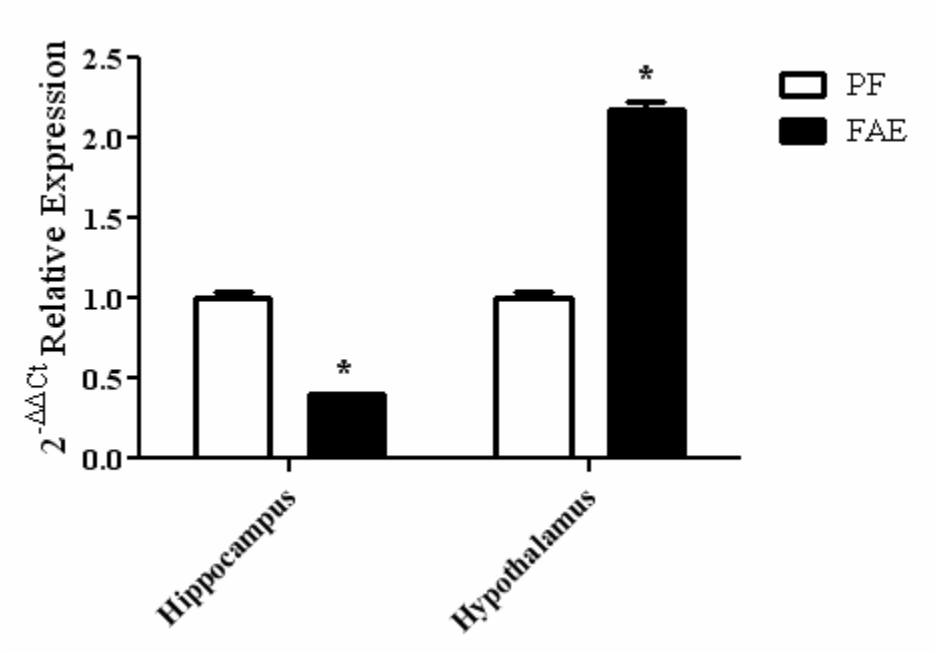

Figure 16. Gene expression of Grin2d in hippocampus and hypothalamus. Data represent mean ± SEM; asterisk (*) denotes significance.

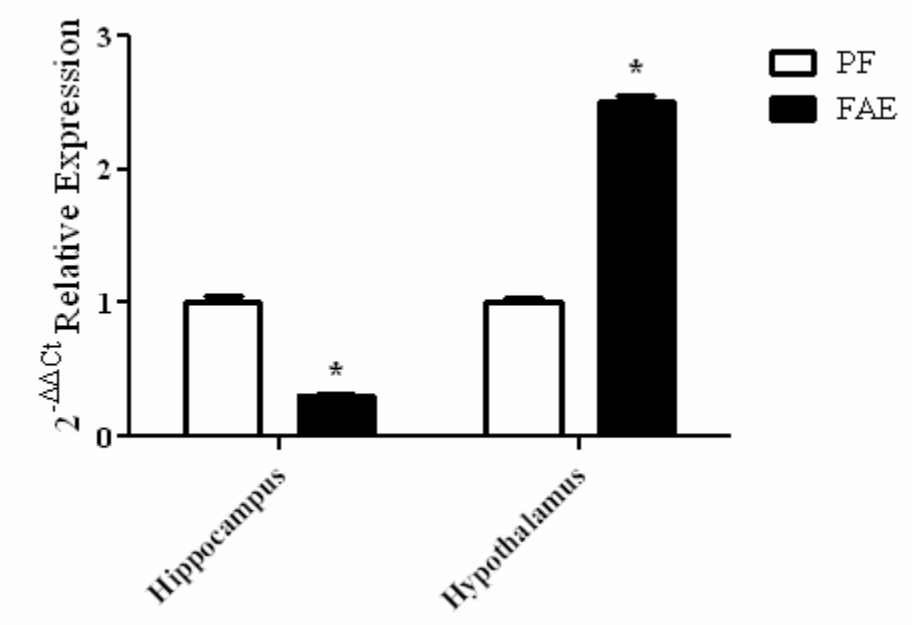

Figure 17. Gene expression of Grm5 in hippocampus and hypothalamus. Data represent mean ± SEM; asterisk (*) denotes significance.

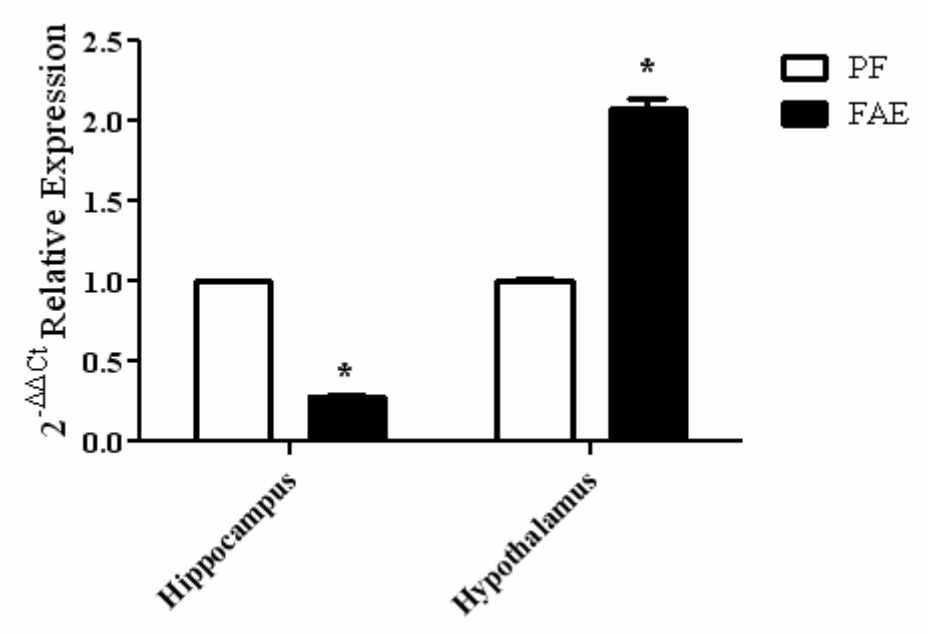

Figure 18. Gene expression of Itpr1 in hippocampus and hypothalamus. Data represent mean ± SEM; asterisk (*) denotes significance.

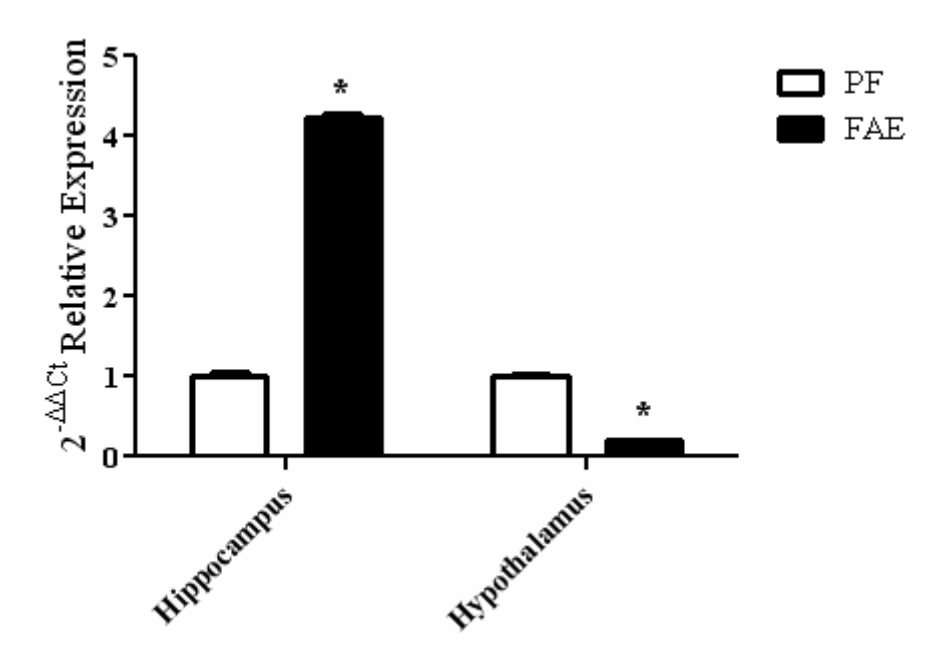

Figure 19. Gene expression of Mapk1 in hippocampus and hypothalamus. Data represent mean ± SEM; asterisk (*) denotes significance.

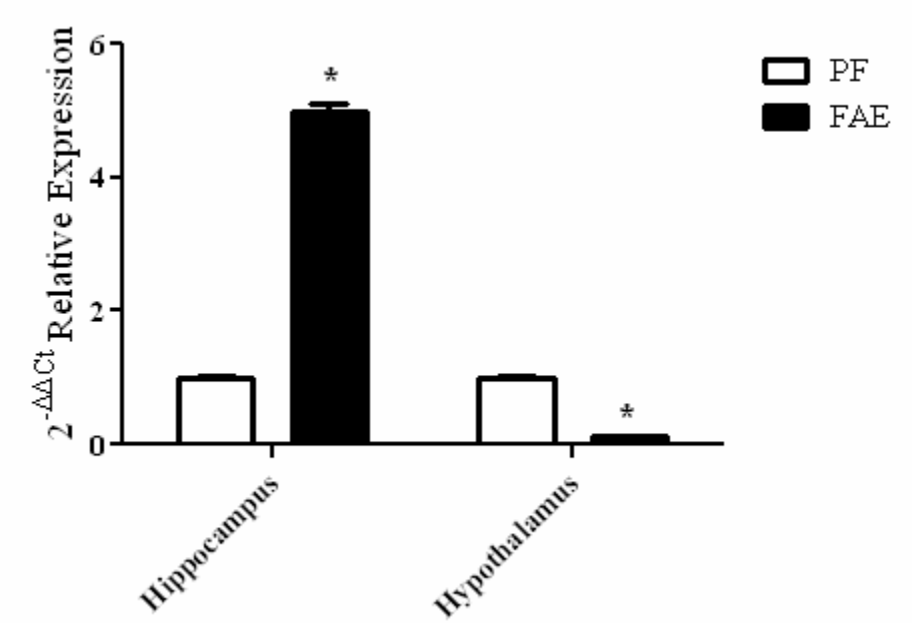

Figure 20. Gene expression of Plcb4 in hippocampus and hypothalamus. Data represent mean ± SEM; asterisk (*) denotes significance.

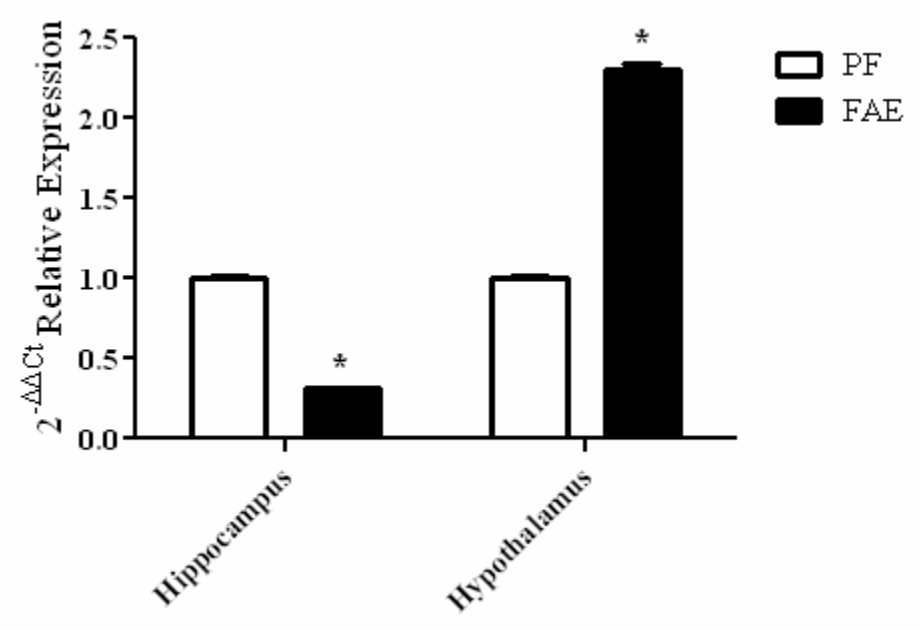

Figure 21. Gene expression of Ppp3cb in hippocampus and hypothalamus. Data represent mean ± SEM; asterisk (*) denotes significance.

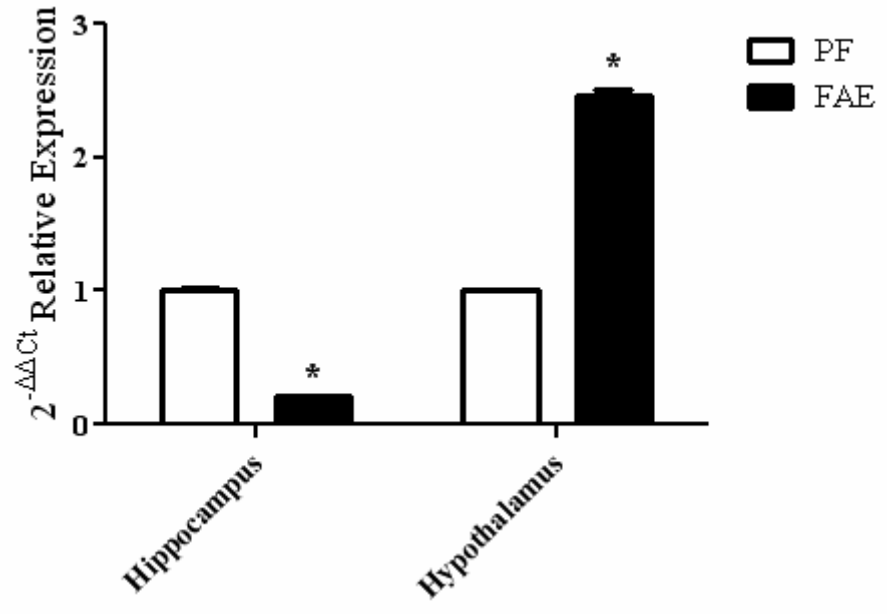

Figure 22. Gene expression of Ppp3r1 in hippocampus and hypothalamus. Data represent mean ± SEM; asterisk (*) denotes significance.

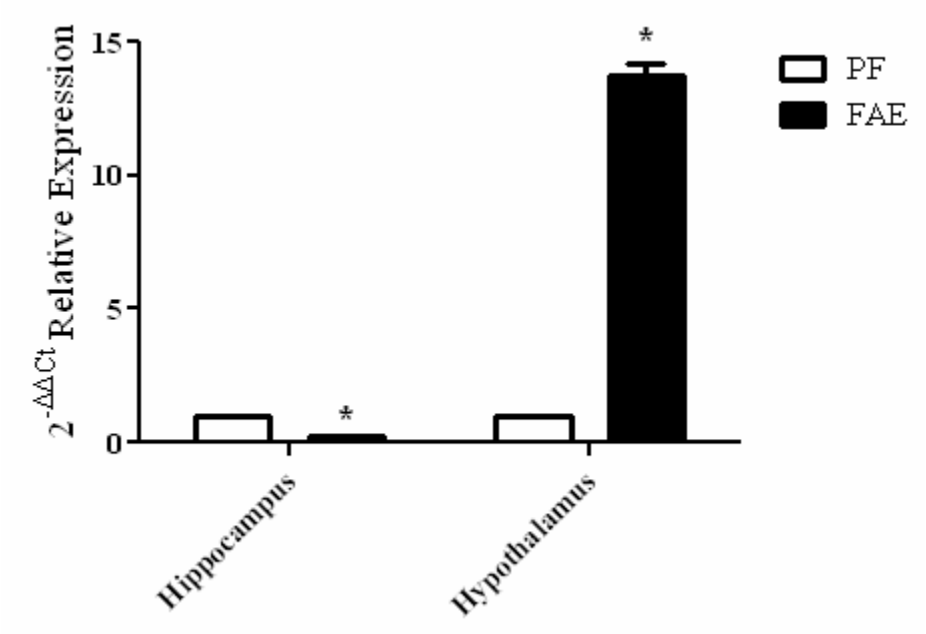

Figure 23. Gene expression of Prkca in hippocampus and hypothalamus. Data represent mean ± SEM; asterisk (*) denotes significance.

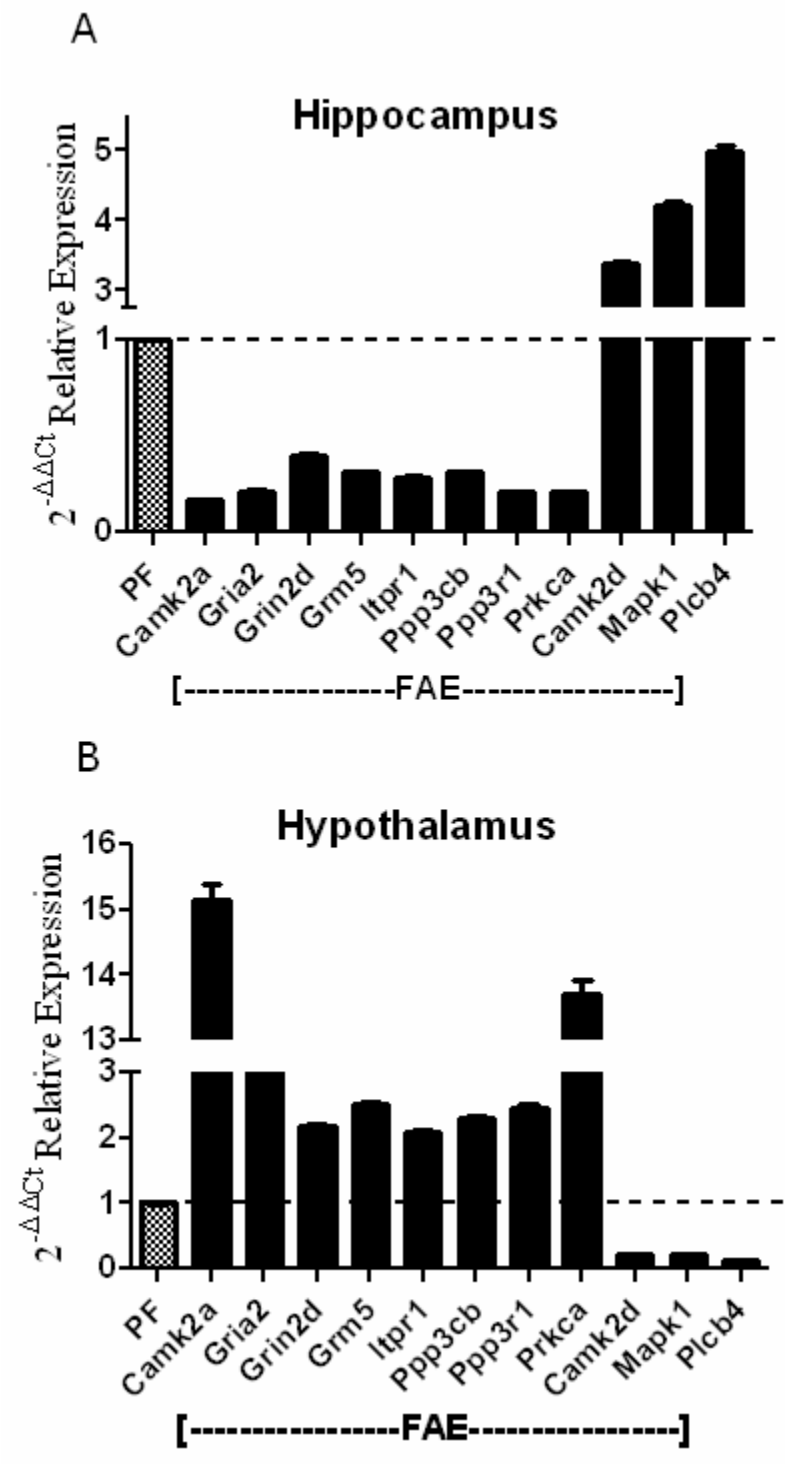

Figure 24. Divergent gene expression of LTP isoforms in (A) hippocampus and (B) hypothalamus. Data represent mean ± SEM.

IV. DISCUSSION

A. Behavioral Effects of FAE on Elevated Plus Maze

Prenatal ethanol exposure induces a wide range of behavioral alterations, including hyperactivity and increased exploratory behavior. The elevated plus maze provides a test of anxiety-like behavior which utilizes the rodent's innate avoidance of open, brightly lit, and elevated places. The number of entries onto the open arms versus the number of total arm entries, and the time spent on the open arms versus closed arms, provide the measures of anxiety-like behavior. In this task, decreased time on and/or entries into the open, "aversive" arms of the plus maze is interpreted as reflecting increased anxiety-like behavior and conversely, increased open arm time or entries is interpreted as reflecting decreased anxiety-like behavior (Listar, 1987). It is widely known that, in rodents, ethanol at moderate doses typically causes motor incoordination, hyperactivity, and increased exploratory behavior. In order to detect the effects of ethanol, an anxiolytic, the elevated plus maze apparatus was designed to be as anxiety provoking as possible. It has been shown that control or undrugged animals prefer the closed arms of the maze, while ethanol exposed rodents tend to explore the open arms (the anxiolytic effects of alcohol reduces the natural aversion of the animal to open arms). In a recent study by Carneiro et al., the results showed that rat offspring prenatally exposed to 10 and 20% v/v ethanol presents a significant increase in the number of entrances and time in the open arms in the elevated plus maze (Carneiro, Diogenes, Vasconcelos, Aragao, Noronha, Gomes, & Viana, 2005). In another study, rat offspring prenatally exposed to a diet of 36% ethanol-derived calories showed more time on the

open arms, more open arm entries, and an increase in rearing compared to control (Osborn, Steiger, & Weinberg, 1998). Gabriel et al. showed that rats exposed to 36% ethanol-derived calories in utero had more open arms entries and total rears than PF (Gabriel, Yu, Osborn, & Weinberg, 2006).

We hypothesize that prenatal ethanol exposure differentially alters behaviors on the elevated plus maze. Based on previous literature, FAE animals showed an increase in the number of entrances and time in the open arms. However, our results did not reflect this behavior. Our results did not show a significant increase in the % open arm time, % open arm entries, or closed/open arm ratio in FAE compared to PF. Ethanol, classified as a central nervous system depressant, is expected to cause a decrease in the spontaneous locomotor activity. Nonetheless, our results did not show this effect; there was no significant decrease in mobility time or traveling distance time in FAE compared to PF. In addition, our results demonstrated no significant changes in ethological behaviors such as rearing, head dipping, stretching, and grooming.

B. Behavioral Effects of FAE on Social Interaction in the Open-Field

Social behavior deficits have been associated with prenatal alcohol exposure in adolescents and adults (Streissguth, 1991). The changes in social behavior observed in adults with FAS could be a function of many factors including their genetic background and alcohol-induced alterations in brain structures. Rodent models of FAS can be used to examine the effect of alcohol exposure during development on social behavior in a simpler and easily controlled system. The open-field test is used to measure the rodent's social interaction and exploratory behavior. In a recent study, Hamilton et al.,

demonstrated a reduction in social investigation (anogenital sniffing and crawling under/over) and self-directed behaviors (rearing) in rats prenatally exposed to 5% v/v ethanol (Hamilton, Akers, Rice, Johnson, Candelaria-Cook, Maes, Rosenberg, Valenzuela, & Savage, 2010). In another study, rat offspring prenatally exposed to 10 and 20% v/v ethanol had a significant reduction in the number of crossings, indicating a decreased locomotor activity (Carneiro et al., 2005). There was also a significant decrease in self-grooming and rearing behavior (Carneiro et al., 2005).

We hypothesize that prenatal ethanol exposure differentially alters social interaction on the open-field. On the basis of previous literature, where FAE animals showed a decrease in social-directed behaviors and self-directed behaviors, one might expect to see a decrease in companion interaction in rodent prenatally exposed to ethanol compared to pair-fed control. However, our data demonstrated no significant social interaction on companion-directed behaviors: crawling over or under companion rat, being together with companion rat, anogenital body sniffing of companion, approaching companion, or facial sniffing of companion rat. Our data also showed no sign of significant self-directed behaviors: square crossing, rearing, or self-grooming.

C. Handling Can Attenuate Adverse Effects of Fetal Ethanol Exposure

The data from the elevated plus maze and the social interaction test did not show a robust difference in anxiety and social behavior between the prenatally exposed and the pair-fed animals. A reason for the subtle differences between the two anxiety tests could be due to daily handling. In the typical handling procedure, postnatal rodents are removed from the homecage into separate holding compartments for a short period (e.g., 3-5 min)

once daily. Handled animals may be less emotional or fearful than nonhandled animals and show more adaptive or appropriate behavioral and physiological responses to novel/stressful stimuli (Levine, 1966). The daily handling reduces the "jitteriness" of animals, making them calmer and more amenable to behavior testing manipulation. In a recent study by Weinberg et al., the researchers examined the role of postnatal experience in modulating or attenuating the long-term adverse effects of prenatal ethanol exposure on the offspring (Weinberg, Kim, & Yu, 1995). The data demonstrate that early experience in the form of postnatal handling can attenuate the adverse effects of fetal ethanol exposure on offspring growth and development as well as on physiological responsiveness to stressors (Weinberg et al., 1995). Handling has been shown to decrease emotionality and anxiety in open-field and elevated plus maze tasks (Weinberg et al., 1995). As a consequence of handling, the robust difference in anxiety and social interaction reported by other investigators has been diminished, further confirming the rationale of common practice for pre-testing handling.

D. Effects of FAE on Gene Expression Molecules

The hippocampus and hypothalamus of the pair-fed and fetal alcohol exposed rats were used in our analysis of global rat brain gene expression. Using the BRB Array tools, we were able to identify a list of genes that were differentially expressed among PF and FAE. The list of significant genes was imported into Pathway Express to build a list of associated pathways. Long-term potentiation was the most associated pathway with respect to the magnitude of its ethanol response (i.e. most significant p-value and impact factor). The eleven input genes from the LTP were selected for qRT-PCR analysis to

confirm their differential expression. Our study has shown that the eleven gene expression molecules of the long-term potentiation pathway are clearly affected by prenatal ethanol exposure. Our data indicate that most of the LTP molecules were down-regulated in the hippocampal brain region of the FAE rodents. The genes that showed a significant down-regulation compared to the PF controls were Camk2a, Gria2, Grin2d, Grm5, Itpr1, Ppp3cb, Ppp3r1, and Prkca. The genes that showed a significant up-regulation were Camk2d, Mapk1, and Plcb4. However, these results were reversed in the hypothalamus of the FAE rodents. The genes that showed a significant down-regulation were Camk2d, Mapk1, and Plcb4, while the genes that showed a significant up-regulation were Camk2a, Gria2, Grin2d, Grm5, Itpr1, Ppp3cb, Ppp3r1, and Prkca.

E. Long-Term Potentiation and the Link to Memory

One of the most significant challenges in neuroscience is to identify the cellular and molecular processes that underlie learning and memory formation. The past decade has seen remarkable progress in understanding changes that accompany certain forms of acquisition and recall, particularly those forms which require activation of afferent pathways in the hippocampus. This progress can be attributed to a number of factors, including well-characterized animal models, well-defined probes for analysis of cell signaling events and changes in gene transcription, and technology which has allowed gene knockout and overexpression in cells and animals (Lynch, 2004). Of the several animal models used in identifying the changes which accompany plasticity in synaptic connections, long-term potentiation has received the most attention, and although it is not yet clear whether the changes that underlie maintenance of LTP also underlie memory

consolidation, significant advances have been made in understanding cell signaling events that contribute to this form of synaptic plasticity (Lynch, 2004).

Activity-dependent synaptic plasticity plays a vital role in sculpting synaptic connections during development and has been identified in several synaptic pathways. For example, it is widely accepted that memory formation is dependent on changes in synaptic efficiency that permit strengthening of associations between neurons. Therefore, activity-dependent synaptic plasticity at appropriate synapses during memory formation is believed to be both necessary and sufficient for storage of information. In 1913, Cajal originally hypothesized that information storage relies on changes in synaptic connections between neurons that are active (Cajal, 1913). Hebb supported this hypothesis and proposed the "Hebbian" idea: if two neurons are active at the same time, the synaptic efficiency of the appropriate synapses will be strengthened (Hebb, 1949). The first full description of LTP by Bliss and Lobo reported that trains of high-frequency stimulation to the rabbit perforant path caused a sustained increase in efficiency of synaptic transmission in the granule cells of the dentate gyrus, one of the two interlocking gyri composing the hippocampus (Bliss & Lobo, 1973). This report, and others followed during the 1970s, confirmed the Hebbian nature of synaptic plasticity and it was immediately recognized that the synaptic changes that underpin certain forms of learning and memory may be similar to those induced upon LTP activation.

F. Long-Term Potentiation Mechanism in the Hippocampus

The vast majority of experimental work aimed at understanding the mechanisms of LTP has been performed on excitatory synapses in the hippocampus, specifically on

the synapses between the presynaptic Schaffer collateral and postsynaptic dendritic spines of CA1 pyramidal neurons (Malenka & Nicoll, 1999) (see Fig. 3 for a more detailed view of the long-term potentiation pathway). LTP molecules and their corresponding input gene/ isoforms are listed in Table 10. These neurons communicate via receptors for the transmitter glutamate, of which there are two predominant types: the AMPA receptor (α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid receptor, composed of subunits GluR1-4, Gria2 isoform) and the NMDA receptor (N-methyl-D-aspartate receptor, Grin2d isoform) (Blitzer, Iyengar, & Landau, 2005). The glutamate receptors, like other ligand-gated receptors, have an ion channel that opens up activation (Blitzer et. al, 2005). The regular excitatory postsynaptic potential is mediated by the AMPA receptors, whereas the NMDA receptors have a much higher affinity for glutamate but yield much smaller and longer-lasting signals (Blitzer et. al, 2005). The NMDA receptor channel is normally blocked by extracellular Mg^{2+} (Malenka & Nicoll, 1999). However, during the induction of LTP, when the postsynaptic cell is depolarized, Mg^{2+}dissociates from its binding sites within the NMDA receptor channel, allowing Ca^{2+}as well to Na^{+} to enter the dendritic spine (Malenka and Nicoll, 1999). The consequent rise of intracellular Ca^{2+} is the critical trigger for LTP (Malenka and Nicoll, 1999).

A major target for Ca^{2+} is Ca^{2+} / calmodulin-dependent kinase type II (CaMKII), a major component of the postsynaptic density (Blitzer et. al, 2005). CaMKII is a holoenzyme with 14 subunits, each comprising of catalytic, autoinhibitory, and anchoring domains (Hoelz, Nairn, & Kuriyan, 2003). CaMKII contains the isoforms Camk2a and Camk2d (Kanehisa, 2009). CaMKII becomes activated when it binds to Ca^{2+} / calmodulin

(CaM), thus removing the enzyme's autoinhibition (Lisman, Schulman, & Cline, 2002). The activity of CaMKII is enhanced by binding to the cytoplasmic carboxy terminus of the NMDA receptor 2B subunit (Lisman et. al, 2002). This binding permits autonomous kinase activity and it anchors CaMKII in the active conformation to the membrane (Lisman et. al, 2002). It is the level of CaMKII activation- and more important its duration- that determines whether a synaptic stimulus will induce LTP. The threshold event here is the rapid phosphorylation of all subunits of the holoenzyme, which creates a stable active kinase (Lisman et. al, 2002).

The phosphorylation of CaMKII is countered by a specific protein phosphatase, PP1, which is also embedded in the postsynaptic density (Blitzer et. al, 2005). PP1, in turn, is controlled by the opposing effects of the cyclic adenosine monophosphate (cAMP)-dependent kinase PKA, protein kinase A (Blitzer et. al, 2005). These opposing effects are mediated via the regulatory protein I-1 (Blitzer et. al, 2005). When PKA phoshorylates I-1, PPI is inhibited and CaMKII is free to become phosphorylated, thus leading to LTP (Blitzer et. al, 2005).

Increases in presynaptic Ca^{2+} are also thought to release bound calmodulin (CaM) from neuromodulin, an abundant presynaptic protein in the brain. CaM activates calcineurins CaN (Ppp3cb, Ppp3r1 isoforms), a protein phosphatase (Xia & Storm, 2005). CaM also activates the CaM-stimulated adenylyl cyclases AC1 and AC8 (Xia & Storm, 2005). This activation of AC1 and AC8 leads to increased PKA activity and the activation of Ras-related proteins RAP1 and RAP2 (Xia & Storm, 2005). Stimulation of RAP1 through cAMP activates the extracellular signal-regulated kinase ERK (Mapk1

isoform) and the mitogen-activated protein kinase MAPK signal transduction pathway, which is important for CA1 long-term potentiation (Xia & Storm, 2005)

After NMDA-receptor stimulation and subsequent Ca^{2+} influx in hippocampal neurons, CaMKII phosphorylates the GluR1 subunit (at serine 831) of the AMPA receptor (Soderling & Derkach, 2000). CaMKII also phosphorylates the metabotropic glutamate receptor mGluR (Grm5 isoform) (Gebremedhin, Yamaura, Zhang, Bylund, Koehler, & Harder, 2003). Glutamate can also increase the postsynaptic activity of mGluR (Kanehisa, 2003). mGluR are coupled through G-proteins (Gq) to activate phospholipase PLC (Plcb4 isoform) (Kelly, MacKinnon, Dietz, Maher, & Wang, 2004). The upregulation of PLC activity can increase levels of inositol trisphosphate IP3 and diacylglyceride DAG (Kelly et. al, 2004). Increased levels of IP3 leads to an upregulation of inosital 1,4,5-triphosphate receptor IP3R (Itrp1 isoform) activity, which stimulate Ca^{2+} release (Kelly et. al, 2004). Increased levels of DAG stimulate protein kinase C, PKC, (Prkca) activity (Kelly et. al, 2004).

Taken together, these results indicate that all of the LTP molecules play important roles in increasing synaptic transmission for long-term potentiation in the hippocampus. The LTP enzymes and molecules have been studied extensively, but their isoforms have not. By studying the isoforms of some of the key enzymes, the individual activity of each isoform can be determined. This may give a better insight into the activity of the long-term potentiation molecules.

Table 10. LTP molecules and their corresponding input gene/isoform

LTP Molecule	Input Gene/Isoform
AMPA receptor	Gria2
NMDA receptor	Grin2d
CaMKII	Camk2a, Camk2d
CaN	Ppp3cb, Ppp3r1
ERK	Mapk1
mGlu receptor	Grm5
PLC	Plcb4
IP3 receptor	Itpr1
PKC	Prkca

G. Long-Term Potentiation Mechanism in the Hypothalamus

In addition to the principal afferent pathways in the hippocampus, several other afferent pathways have been shown to sustain LTP: amygdala, visual cortex, somatosensory cortex, prefrontal cortex and the subiculum. In a recent paper by Panatier et al., it was found that long-term potentiation also occurs in the supraoptic nucleus, SON, of the rat hypothalamus (Panatier, Gentles, Bourque, & Oliet, 2006). The results obtained with coronal hypothalamic slices, using microelectrode recordings, indicated that synapses expression in both AMPA receptors and NMDA receptors can clearly exhibit activity-dependent plastic changes during focal stimulation to the supraoptic nucleus of the hypothalamus (Panatier et al., 2006). Thus, the LTP molecules that play a role in the hypothalamus are AMPA (Gria2 isoform) and NMDA (Grin2d isoform) receptors. There is not much known about the other LTP enzymes/receptors in the hypothalamus.

H. Ethanol Exposure Differentially Affects Induction of LTP

Ethanol exposure during development can produce severe and long-lasting deficits in many organs, including the brain (Warren & Foudin, 2001). Among the consequences of ethanol-induced brain damage are behavioral disorders, mental retardation, and learning and memory deficits (Warren & Foudin, 2001). Electrophysical, structural, and behavioral studies performed with human and animal subjects suggest that learning and memory deficits are, in part, a consequence of damage to the hippocampal formation (Berman & Hannigan, 2000; Costa, Savage, & Valenzuela, 2000). Spatial memory tests have demonstrated abnormalities in hippocampal function in children with

fetal alcohol syndrome and in animal models of this condition (Hamilton, Kodituiwakku, Sutherland, & Savage, 2003). However, the mechanisms by which ethanol exerts its deleterious effects on the hippocampal formation are not well understood.

Studies from several laboratories suggest that the mechanism of action of ethanol on hippocampal development involves alterations in glutamatergic synaptic transmission (Berman & Hannigan, 2000). One study suggests that inhibition of NMDA receptors induced by acute ethanol administration during the neonatal period of development can trigger widespread apoptotic neurodegeneration in many brain regions, including the CA1 hippocampal region (Ikonomidou, Bittigau, Ishimaru, Wozniak, Koch, Genz, Price, Stefovska, Horster, Tenkova, Dikranian, & Olney, 2000). Another study proposes that ethanol acutely inhibits NMDA and AMPA mediated responses, thus affecting long-term potentiation in the developing CA1 hippocampus (Puglia & Valenzuela, 2010). Allan et al. suggest that deficits in PLC activity in the FAE rats would lead to a decrease in the production of IP3 and DAG in the hippocampus, thus leading a compromised regulation of multiple cellular processes that are involved in the induction and maintenance of LTP (Allan, Felegi, & Stern, 1997). Perrone-Bizzozero et al. propose that PKC activity (analysis using Western blot) is reduced in the hippocampus of FAE rats, which could depress long-term potentiation (Perrone-Bizzozero, Isaacson, Keidan, Eriqat, Meiri, Savage, & Allan, 1998). These findings support the model that fetal alcohol exposure leads to persistent alterations in synaptic plasticity mechanisms (i.e. long-term potentiation). Prenatal ethanol exposure produces a significant alteration in the levels and function of specific functions in hippocampus synapse. As these systems participate in

the formation of learning and memory processes, these defects, in turn, may underlie the subtle cognitive deficits in offspring whose mothers consumed alcohol during gestation.

I. Fetal Alcohol Exposure Affects LTP Molecules

According to our study, it is compelling to say that fetal ethanol exposure can cause profound and long-lasting deficits in the cellular signaling mechanisms associated with activity-dependent synaptic plasticity and memory formation. In our hippocampal gene expression data, the down-regulation of Camk2a, Gria2, Grin2d, Grm5, Itpr1, Ppp3cb, Ppp3r1, and Prkca genes could be due to effects of ethanol exposure and its inhibition of long-term potentiation. The inhibition of long-term potentiation may result in a learning and memory deficiency in ethanol-exposed rats. In the hypothalamus, these eight genes are up-regulated. Because there is no literature that discusses all of the long-term potentiation molecules in the hypothalamus, there is nothing that can be deduced from our hypothalamus gene expression data relating to LTP.

J. Isoforms of LTP Components

Activation of the LTP components is essential for induction and maintenance of LTP. Yet, it is not clear which isoforms of the enzyme are involved in this process. For instance, CaMKII is a 14-subunit catalytic enzyme and is known to be activated to induce LTP (Lisman et. al, 2002). However, the activity of each isoform is unknown. In our study, Camk2a showed a significant decrease in expression in the hippocampus, while Camk2d showed a significant increase. Both of these isoforms are part of CaMKII, yet they exhibit different levels of activity. There is no literature that supports this divergence

in activity. Thus, our laboratory has two assumptions for this activity. One reason for this could be that the activity of Camk2a may be opposite of Camk2da. This is highly unlikely because of the summation of their activity counteract each other. Another reason could be that they form similar activity. Therefore, their regulation is coordinated, meaning that the summation of their activity leads to CaMKII activation. This second idea seems to be more reasonable. In another example, CaN has Ppp3cb and Ppp3r1 isoforms. In the hippocampus, these two isoforms both have a significant down-regulation in gene expression. This also seems to be coordinated regulation of both isoforms. In previous studies (refer to section F. in the Discussion), researchers studied the LTP components as a whole (i.e. the enzyme or receptor), not as isoforms. Thus, the individual activities of the isoforms are not known. In our study, we were able to study individual isoforms of the LTP molecules. This may give a better insight into the activity of the isoforms relating to the key LTP enzymes and receptors.

K. LTP Indicates a Divergent Regulation in the Hippocampus and Hypothalamus

It is clear that long-term potentiation is the top candidate that is affected by prenatal ethanol exposure. LTP is a well established molecular mechanism that contributes to brain function (i.e. long-term memory). In our study, it is unambiguous that LTP can be a conduit to mediate the long-term effects of in utero alcohol exposure. The signal of this LTP effect is very strong, suggesting that the molecular level impact by fetal alcohol is much more pronounced and long-lasting than the behavior measurement. This molecular change in gene expression is a much stronger and definitive signature of fetal alcohol impact. When looking at the coordinated regulation of LTP pathway

components, our study was able to present a very distinct molecular profile. In the literature, it is never clear of which individual isoform is being regulated. By looking at the FAS model as a whole system and by analyzing individual LTP isoforms in parallel, we get the molecular signature of system-wide response and this response is a coordinated profile in which individual components are of opposing directions in the hippocampus versus the hypothalamus. Neuroanatomically, the hippocampus and hypothalamus are known to be interrelated brain sites performing different functions. Our data showed that with fetal alcohol exposure, the LTP system-wide regulation is going opposite in the two brain sites. Thus, the LTP pathway not only serves as a strongly impacted neurobiological system by FAE, it also indicates a divergent regulation in the hippocampus and hypothalamus.

L. Ethanol Effect on Gene Expression in the Hippocampus and Hypothalamus

Our relative gene expression data indicate an opposing pattern of ethanol effect on LTP pathway regulation of gene expression in the hippocampus and hypothalamus (i.e. Camk2a is down-regulated in the hippocampus, yet up-regulated in the hypothalamus). In Wistar-Kyoto rats, alcohol consumption increased dopamine transporter sites in the CA1 region of the hippocampus, basolateral and decreased dopamine transporter sites in the lateral and ventromedial hypothalamus (Jiao, Pare, & Tajani-Butt, 2005). In C57BL/6J mice, alcohol consumption suppressed inducible transcription factors in the hippocampus and induced inducible transcription factors in the hypothalamus (Bachtell, Wang, Freeman, Rising, & Ryabinin, 1999). In Sprague-Dawley rats, fetal alcohol exposure increased serotonin transporters sites in hippocampus and decreased serotonin

transporters sites in hypothalamus (Zafar, Shelat, Redei, & Tejani-Butt, 2000). This clear pattern of opposing direction of gene expression regulation in the hippocampus versus hypothalamus corroborates with past literature.

M. Conclusions and Recommendations

In summary, data from the behavioral experiments indicated that fetal alcohol exposure exerts a profound effect on gene expression in the brain of rats, lasting well into their adult life. The molecular changes at the gene expression level are not readily reflected at behavioral levels assessed in anxiety and social interaction paradigms. This is likely due to the handling prior to the testing manipulation, which is known to reduce stress in animals. The handling of young adult rats may attenuate the adverse effects of fetal ethanol exposure on anxiety.

At the cellular level, the phenomenon of long-term potentiation has been studied extensively, especially in the hippocampus. The underlying mechanism for LTP is also well delineated, involving specific enzymes and other molecules in the LTP pathway. However, system-wide regulation of LTP pathway molecules has not been examined. In this study, we quantified the gene expression of LTP pathway molecules, including isoforms of some of the key enzymes. Results from qRT-PCR experiments indicated that fetal alcohol exposure changes the level of gene expression in the hippocampus and hypothalamus across the LTP pathway, in region-specific patterns. Our data showed that with fetal alcohol exposure, the LTP system-wide regulation is divergent in these two brain regions. These changes in gene expression suggest that prenatal exposure to ethanol causes profound and long-lasting effects in the cellular signaling mechanism associated

with long-term potentiation. Future studies are needed to examine the effects of ethanol LTP activity at cellular level, so as to correlate pathway-wide gene expression pattern changes of the long-term potentiation with neuronal activity alterations, and to explore the role of divergent LTP pathway regulation in the hippocampus and hypothalamus in functional context.

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